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Patterns and causes of population subdivision in the marine environment

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Patterns and causes of population subdivision in the marine environment

Abstract

An accurate assessment of population genetic subdivision is crucial in making informed decisions for the management and conservation of marine resources. However, obtaining a precise assessment is particularly challenging since population subdivision results from the interaction of many biological and environmental variables. Here, the patterns of population subdivision that can be attributable to gene flow in 8 marine fish species from 11 different locations in the Mediterranean Sea and the Eastern Atlantic Ocean are analysed. The species considered are *Mullus barbatus*, *Mullus surmuletus, Tripterygion delaisi, Apogon imberbis, Symphodus tinca, Diplodus vulgaris, Serranus cabrilla* and *Oblada melanura.* These species differ significantly in their dispersal capabilities as presumed by their diverse early-life stage characteristics, and were sampled over multiple habitats at two geographical scales. A total of 1640 individuals (mean 168/species) were surveyed at 63 species-specific and 10 cross-amplified microsatellite loci (mean 9.1/species). The overall results show that genetic structure patterns cannot be predicted confidently by the presumed dispersal capabilities of the species. Species such as *S.tinca, M.barbatus* and *O.melanura*, showed highly incongruent population structuring patterns to those expected. Furthermore, the results also suggest that species with extreme variations between their life history traits such as *D.vulgaris* and *A.imberbis*, can display similar patterns of genetic structuring in the presence of common oceanographic variables, whereas species with slight differences such as *M.barbatus* and *M.surmuletus* can display contrasting patterns of genetic partitioning. These results highlight the importance understanding the role of environmental features and life history traits in establishing ecologically meaningful connections among marine fish species. Furthermore, this study provides evidence of the usefulness of multi-species approaches in planning to incorporate population genetic information into conservation and management of marine fish resources.

CHAPTER I

General Introduction

1.1 GENERAL ASPECTS OF GENE FLOW

Identification of population structure is of primary importance when planning management and conservation strategies. This is particularly true when dealing with exploited populations of marine species, such as fish. Absence or imprecise information of population structuring could lead to overexploitation and subsequent resource collapse. Traditionally, fishery management has largely relied on the collection of fishery-dependent data or catch estimates as the main tool for the resource's management. These can be obtained by a variety of means including landings, fishery observers, logbooks, dockside and shoreside monitoring (FAO 2005). This information is then used by managers to define stocks or populations, determine their geographic boundaries, estimate their abundance and establish quota systems. However, it must be recognized that if used in the absence of complementary information such as genetic data, there is a higher threat of overfishing and subsequent resource collapse. Genetic data can provide valuable information towards conservation-management strategies especially in determining the number of breeding units and genetically distinct stocks present in the area as well as in determining the levels of interdependence between them via gene flow.

The study of gene flow (i.e. interchange of alleles among populations) has been a vital topic in evolutionary biology. The loss or addition of genes through the movement of individuals between populations can easily change gene frequencies even if there are no other evolutionary mechanisms operating. When individuals move into a population they may bring in alleles not already present in that population or in different frequencies, increasing genetic variation on which evolution may act upon. Therefore, gene flow rates have a direct on effect population divergence, extinction rates, species' ranges, local adaptation and effective population sizes (Slatkin 1985; Wright 1943; Wright 1951). Most theoretical models of gene flow stem from concepts developed by Sewall Wright that are based either

on continuous populations using an isolation by distance approach or on populations as islands that become differentiated through mutation and genetic drift (Wright 1943; Wright 1946).

Recently, ecologists, conservation biologists, and ecosystem managers have become particularly interested in gene flow on an ecological time scale. Using molecular genetic markers, many researchers have adopted a genetic structure approach to estimate gene flow. Such estimation has relied mainly on those methods based on Wright's parameter of population differentiation F_{ST}. Wright showed that under equilibrium $F_{ST} = 1/(4Nm + 1)$, where N is the effective population size and m the migration rate (Wright 1951; Wright 1969). Thus, the effective number of migrants Nm is usually derived form this equation as Nm = (1-Fst)/4.Fst) (Wright 1969).

Since its introduction, F_{ST} has been adapted to analyse various types of genetic data, i.e. N_{ST} (Lynch & Crease 1990), G_{ST}, (Nei 1973) θ, (Weir & Cockerham 1984) R_{ST} (Slatkin 1995), Φ_{ST} (Michalakis & Excoffier 1996). However, all F_{ST}-like methods share the common conceptual approach of calculating summary statistics based on the variance in allele frequencies within and among populations and most rely on Wright's infinite island model (Wright 1951) as the basis for gene flow estimation (Neigel 1997). The model assumes an infinite number of populations, no selection, no mutation and gene flow that is unaffected by the geographic distance between populations. These clearly unrealistic assumptions for natural populations have led to criticisms about the use of F_{ST} in evaluating population structure and gene flow (Bossart & Prowell 1998; Whitlock & McCauley 1999). Common arguments against the use of F_{ST} when estimating gene flow between populations state that i) F_{ST} cannot be estimated precisely in populations with large effective sizes, ii) the parameters N and m are assumed to be the same in every population, iii) the effects of variance in mutation rates on F_{ST} are underestimated, iv) populations must be defined *a*

priori, v) and that vi) much of the genetic information is lost by this single summary statistic (Balloux & Lugon-Moulin 2002; Crow & Aoki 1984; Waples 1989; Whitlock & McCauley 1999). In line with the increasing ability to obtain genetic data, recent statistical methods have been developed to circumvent F_{ST} limitations. Bayesian inference approaches are expanding the analytical power available to population and conservation geneticists (Evanno *et al.* 2005; Excoffier *et al.* 2005; Flaush *et al.* 2003; Guillot *et al.* 2005; Pritchard *et al.* 2000; Wilson & Rannala 2003). However, these approaches include their own limitations such as varying assumptions on recombination rates, Hardy-Weinberg equilibrium, over-sensitivity to priors and lack of convergence (Beaumont & Rannala 2004; Beaumont *et al.* 2002). Improved methods based on the Coalescent theory (Kingman 1982) have also been introduced into the population and conservation genetics field (Bahlo & Griffiths 2000; Beerli & Felsenstein 2001; Nielsen & Wakeley 2001; Rozas *et al.* 2003; Stephens 2001). In essence, the coalescent theory is a probabilistic description of the genealogical process for samples of chromosomes in populations, which considers only those events in the genes that are ancestral to those in the sample (Tavare 1984). This allows coalescent-based methods to utilize most of the information contained in the data to infer demographic events based on the evolutionary history of the populations rather than on the current allele frequency distributions as inferred by F_{ST} (Beerli 2006; Beerli & Felsenstein 2001; Nielsen & Wakeley 2001).

Perhaps the most notable difference between F_{ST}-based, Bayesian and coalescent-based methods, is their parameter inference framework. The simplest way to estimate a parameter is to equate the expectation of a statistic with its observed value. This approach is commonly known as moment-estimation and it is utilized by F_{ST}-based approaches (but see (Weir & Hill 2002) for a maximum likelihood estimator of F_{ST}). Bayesian inference, in turn, uses prior information to influence parameter estimation (Beaumont & Rannala 2004). Coalescent methods rely on likelihood to calculate the probability of observing data given

different values of parameters (Beerli 2006). Thus, current coalescent-based methods can estimate multiple parameters simultaneously such as asymmetrical migration rates, effective population sizes and average divergence between pairs of population which can be comparable to $F_{ST.}$ However, these new methods rely on increasingly large data sets, intensive computational power and are still sensitive to assumptions on the evolution of the marker being used (i.e. mutation rates) (Niegel 2002). Furthermore, their performance under varying conditions has been scarcely evaluated (Abdo *et al.* 2004). Consequently, in spite of its known limitations, the use of F_{ST} as an average level of gene flow among a group of populations provides a robust analysis that may be used in conjunction with more sophisticated methods when more precise estimates are needed (Pearse & Crandall 2004).

1.2 GENE FLOW AND LARVAL DISPERSAL IN THE MARINE ENVIRONMENT

Knowledge of the dispersal patterns of organisms among marine populations is of primarily importance in our understanding of the populations' genetics, dynamics, evolution, range expansions as well as for the development of management and conservation efforts (Bostford *et al.* 2001; Kinlan & Gaines 2003; Palumbi 2003; Patterson & Swearer 2007). The scale and rate at which individuals successfully disperse and establish among populations determines genetic connectivity and outlines gene flow patterns between marine populations (Slatkin 1985; Waples 2002). At present, we have only limited understanding of the extent to which gene flow is mediated through adults of highly migratory of species such as Tuna, Squid and Marine Turtles, (Carlsson *et al.* 2004; Chiang *et al.* 2006; Roberts *et al.* 2004; Shaw *et al.* 1999a) and even less about the magnitude of gene flow that may occur via adult straying and rare long-distance dispersal events. Empirical data on this field is lacking and warrants further attention.

Another mayor challenge for marine ecologists is to obtain accurate estimates of gene flow levels that are induced primarily by ocean circulation and the dispersal of pelagic larvae.

This is mainly due to both, the minute size of the dispersing organisms and because dispersal is driven by a combination of complex bio-physical factors (Mora & Sale 2002). Our current understanding of the dispersal patterns of pelagic stages derives from a variety of sources such as oceanographic modelling, larval tagging, resource management, population genetics, as well as from recent approaches coupling oceanographic and genetic methods.

1.2.1 Oceanographic Modelling

Oceanographic models are often used in trying to determine the fate of marine larvae and are constantly increasing in their complexity and development. Models have been built starting from the simple assumption that larvae are dispersed passively by currents, and thereby, current trajectories should reveal routes of larval transport, to the incorporation of multiple life history traits of the target species into the model. (Roberts 1997), analysed basic currents' speeds and directionalities to determine coral reef areas where larvae spawned elsewhere could potentially arrived and to which larvae spawned locally could potentially be transported. The results identified a number of sites as potential sources and recipients with a variability of an order of magnitude in transported larvae. A latter model was developed by (Cowen *et al.* 2000) under the recognition that larvae are not simple drifting particles. This model incorporated the effect of horizontal diffusion and larval mortality in the transport processes of bicolour damselfish (*Stegastes partitus*) larvae across the Caribbean archipelago. The results showed that larval exchange rates may be overestimated by simple flow trajectories and that larval concentrations may be reduced up to nine orders of magnitude as a result of diffusion and mortality processes. (Epifanio & Garvine 2001), evaluated the effects that multiple physical processes (wind stress, tides propagating from the deep ocean and water density) had on larval transport of three marine species (Blue crab *Callinectes sapidus*, Menhaden *Brevoortia tyrannus*, Bluefish

Pomatomus saltatrix) in the Middle Atlantic and South Atlantic Bights of north America. The results displayed that alongshore wind stress and density differences causing buoyancy and current flow, are the primary agents of larval transport in the region. A qualitatively similar result was found in abundance estimates of Capelin larvae (*Mallotus villosus*), which showed evidence that wind-generated currents on the upper layers of the water column can potentially modulate larval survival and recruitment at Trinity Bay, Canada (Dalley *et al.* 2002). (Hinrichsen *et al.* 2003) developed a more advance numerical 3–D hydrodynamic model to investigate the potential drift of cod (*Gadus morhua*) larvae away from their spawning area at Bornholm Basin in the Baltic Sea. In this study, cod larvae were represented as Lagrangian drifters and larval transport was simulated for over a decade. As a result, larval transport yielded a clear dependency on wind-induced surface currents, which in turn, are controlled by local atmospheric conditions. Recently, a complex model was introduced that coupled near-realtime climatological data and biological information into a stochastic model of larval dispersal for the scleractinian coral *A. palmata* (Baums *et al.* 2006). In this model, flow dynamics were determined by physical forcing derivates, whereas biological information included parameters such as maximum pelagic larval duration, precompetent period (period of passive dispersal before larvae becomes active) and spawning strategies. The model was evaluated at different spatial and temporal scales revealing the occurrence of a seasonal filter to gene flow for populations of *A. palmata*. Similarly, (Cowen *et al.* 2006) utilized a high-resolution ocean circulation model to generate an individual based (IBM) model of larval dispersal for a range of reef fish species. The model included a number of biological parameters such as pelagic larval duration, larval behaviour (vertical and horizontal swimming capabilities) and adult spawning strategies (season and fecundity). The results allowed to identify regions of population isolation based on larval dispersal that also correspond to observed genetic and morphological clines across the Caribbean.

1.2.2 Larval tagging

More direct approaches such as larval tagging have also proved useful in elucidating the paths of dispersing larvae. This approach differs from genetic approaches in the way that it can identify demographic links or connectivity between populations, whereas genetic approaches provide information on the success of such links over time. Tagging approaches can broadly be subdivided into natural and artificial tagging. Natural tagging makes use of chemicals signatures recorded in hard parts of marine organisms (i.e. otoliths and shells) to be used as geographic tags (Swearer *et al.* 2002). The rationale is that elements such as barium, strontium, copper and zinc are incorporated into these calcified structures according with environmental availability. Therefore, organisms that have developed from different environments (i.e. different water masses) can be differentiated through this elemental signature. Artificial tagging, in turn, deals with the incorporation of synthesized chemicals to early development stages (i.e. embryos) to allow subsequent identification of the organism through a mark-recapture strategy (Campana 1999). A pioneer study by (Jones *et al.* 1999) utilized tetracycline to tag millions of embryos of damselfish (*Pomacentrus amboinensis*) in the Great Barrier Reef, Australia. After a 30-day period, otoliths from a total of 5000 settlement-stage fish were examined for a fluorescent mark indicating the presence of tetracycline. From the results, the authors were able to conclude that larvae of damselfish return to their natal reef and that this may contribute substantially to local recruitment. Other studies, however, have made use microchemistry techniques to analyse natural tags. For example, (Swearer *et al.* 1999) analysed otolith growth and elemental composition of bluehead wrasse (*Thalassoma bifasciatum*) under the hypothesis that larvae developing in nutrient-rich coastal waters have different signatures compared to larvae developing in less productive oceanic waters. Approximately 70% of bluehead wrasse larvae presented signals of coastal development. This result was interpreted as evidence that larvae remain close to their spawning site and that they are recruited back to

their natal population. A recent study employed a novel technique of *in situ* larvae culturing and elemental fingerprinting to determine larval exchange from two closely related Mussel species (*Mytilus californianus*) and (*Mytilus galloprovincialis*) in the south-western United States (Becker *et al.* 2007). This approach consisted in generating location-specific reference chemical signatures by raising larvae *in situ* at 13 different sites and analysing resulting larval shells for trace element composition. Subsequently, shell chemistry of wild-caught settling recruits was compared against these location-specific signatures to infer natal origin of juvenile Mussels. The results revealed that larvae of these species can be retained close to natal origin. Furthermore, the authors showed that *M. californianus* populations follow a single source model of larval replenishment. Whereas *M. galloprovincialis* populations appear to have multiple larval sources. Similarly, (Patterson & Swearer 2007) analysed trace elements recorded in otoliths as evidence of larval origin of both an endemic (*Coris bulbifrons*) and a widespread (*Coris picta*) coral reef fish species in Lord Howe Island, Australia. The inclusion of an endemic species in the analysis allow them to confirm that both long distance dispersal and local retention are important sources of recruitment to populations of *C. picta* within this region.

1.2.3 Resource management

Another indirect approach to infer larval dispersal patterns stems from conservation-management strategies of marine resources, particularly those from protected areas. The underlying principle of this approach is to compare abundance and/or biomass of the target species from inside and outside protected areas. Accordingly, populations inside protected areas potentially produce larger numbers of eggs and larvae than populations in non-protected areas (Roberts *et al.* 2001). Once a critical level is reached within the protected area, a biomass enhancement to adjacent non-protected areas may occur through an export of larvae commonly known as larval spillover (Palumbi 2004). Therefore, by measuring

this spillover effect, inferences can potentially be made about the scale of larval movement. There are however, a limited number of studies that have successfully obtained quantitative estimates of larval spillover. A locally managed marine area established in Fiji to re-growth populations of clams (*Andara spp*.), showed that neighbouring non-protected populations had increase about 5-fold in abundance of small post-settlement clams (Tawake *et al.* 2001). Due to the sedentary condition of adult clams and the planktonic development of their larvae, this increase was attributed to an export of larvae from the protected area. Another example from a marine reserve in the Bahamas showed that densities of Queen conch (*Strombus gigas*) larvae were 15 times greater inside than outside the reserve. Offshore sampling also showed that larval densities increased by approximately 12-fold within 2 km away from the reserve and from fishing grounds (Stoner & Ray 1996). On a larger scale, larval densities of scallop (*Placopecten magellanicius*) have also showed a marked increase in areas closed to bottom trawling compared to trawling-operating grounds in the Gulf of Maine (Murawski *et al.* 2000).

1.2.4 Population genetics

Recently, an increasing number of studies have employed different genetic markers such as allozymes, mitochondrial DNA (mtDNA), microsatellites and Random Amplified Fragment Length Polymorphism (AFLP) to infer hydrographical-influenced larval dispersal on a variety of marine species. Allozyme studies have examined the effects of the East Australian Current on the genetic structure of muricid snail (*Beveda hanleyi)* populations (Hoskin 2000). The results suggest that this current is an important determinant of genetic structure of muricid snail populations due to the change of directionality that it experiences at different latitudes. Similarly, allozyme data has also revealed significant discontinuities among mussel (*Mytilus galloprovincialis*) populations from the South Atlantic and the Mediterranean Sea, presumably due to a surface current direction change from Cap Ghir

towards the Canary archipelago (Hassane & Touria 2002). Mitochondrial DNA markers have also been implemented in similar ecological studies. (Barber *et al.* 2002), for example, employed mtDNA in trying to elucidate the patterns of connectivity between reserve populations of the mantis shrimp (*Haptosquilla pulchela*) as a result of larval dispersal in the Indonesian current system. Contrary to predictions of high population connectivity based on strong ocean currents facilitating larval dispersal, the results displayed a marked genetic differentiation among local populations of this species. Current trajectories and larval traits of three marine invertebrates (*Ophiothrix lineata, Leucothoe kensleyi* and *Leucothoe ashleyae*) have been recently invoked to explain divergent gene flow patterns revealed by cytochrome oxidase I gene sequence analysis (Richards *et al.* 2007). Similarly, control region sequence variation suggest that spawning season and ocean currents have a direct influence in migration patterns among fourline wrasse (*Larabicus quadrilineatus*) populations in the Red Sea (Froukh & Kochzius 2007).

Microsatellite DNA markers have been used extensively in studies of marine pelagic larvae and its relation to current flows. (Ball *et al.* 2000), found that temperature profiles and current patterns throughout the southern oceans apparently prevent gene flow between South Pacific and Brazilian populations of wreck fish (*Polyprion americanus*). Likewise, restricted gene flow as a result of larval retention by the California Current and Alaska Gyre, has also been reported for populations of Pacific ocean perch (*Sebastes alutus*) in British Columbia as revealed by microsatellite data (Withler *et al.* 2001). In resolving a breakdown of gene flow among populations of squid (*Loligo forbesi*), (Shaw *et al.* 1999b) suggested that hydrogeographic (depth) and hydrodynamic (isolating current regimes) factors are possible barriers to migration between Northeast Atlantic populations. Furthermore, low levels of population structure of highly mobile organisms such as Atlantic cod (*Gadus morhua*) have also been directly related to passive transport of eggs and larvae by surface currents rather than to adult dispersal (Knutsen *et al.* 2003). (Purcell *et al.* 2006)

analysed micorsatellite data from two coral reef fish species, the French grunt (*Haemulon flavolineatum*) and bluehead wrasse (*Thalassoma bifasciatum*). The authors pointed at differences in oceanographic regimes between the eastern and western Caribbean to explain the patterns of genetic differentiation observed. In the western Caribbean, currents may be responsible for rapid advection of larvae homogenizing the genetic signal in this region. Whereas in the eastern region, slower moving and much less clearly advective currents could gradually move a number of larvae offshore, thus promoting a gradual buildup of genetic differentiation. Similarly, microsatellite data revealed a significant genetic structuring among turbot (*Psetta maxima*) populations within the Baltic Sea (Florin & Hoglund 2007). This finding was explained by a strong inflow of water to the Baltic Sea, which may have admixed settling larvae among the locally and relative sedentary populations. Microsatellite markers have also been successful to infer larval dispersal for the brooding coral (*Seriatopora hystrix*) in North-Western Australia (Underwood *et al.* 2007). In this study, input of larvae to local recruitment was observed as a result of long-distance larval dispersal from hydro-dynamically connected spawning sites.

Recently, (Shank & Halanych 2007) employed a genomic DNA fingerprinting technique, based on amplified fragment length polymorphisms (AFLPs) to examine genetic structure and elucidate alternative larval dispersal mechanisms of the hydrothermal vent tubeworm (*Riftia pachyptila*). Genomic fingerprints were recovered from five vent fields spanning a distance of up to 5000 km along the East Pacific Rise. In contrast to previous population genetic studies that found little or no genetic structure using allozymes and mitochondrial DNA (Black *et al.* 1998; Shank *et al.* 1999), the results showed that mechanisms of larval dispersal act to retain cohort fidelity between tubeworms separated by as little as 400 m on a vent region.

1.2.5 Coupled approaches

Approaches combining two or more disciplines are now being developed with the goal of obtaining more realistic insights about the patterns and the mechanisms that outline larval dispersal patterns in the marine environment. Such approaches usually rely on simulation models that make use of theoretical and empirical data. For example, (Hohenlohe 2004), developed a simulation model to examined the process of gene flow via planktonic larvae of two intertidal gastropods (*Littorina scutulata* and *L. plena*) around Point Conception in southern California. Such model utilized data from ocean currents, planktonic period, spawning season as well as from simulated genotypes. The results suggested that ocean currents can create an effective barrier to gene flow and at the same time, extended spawning seasons can eliminate such gene flow barriers when currents vary seasonally. (Galindo *et al.* 2006) developed an Oceanographic-Genetic model to predict genetic patterns resulting from larval dispersal of staghorn corals, (*Acropora cervicornis*) in the Caribbean. Such model uses oceanographic circulation data, simulated larvae as well as genetic data resulting from neutral markers simulations. Model runs were then compared to empirical genetic data showing that this coupled model can predict many of the genetic patterns observed in this threatened species such as isolation of the Bahamas and east-west divergence near Puerto Rico.

1.3 OBJECTIVES

Most marine species possess life histories that include at least one potentially widely dispersive stage (Goldson *et al.* 2001). In fish populations, pelagic stages often serve as the principal means of dispersal (Strathmann 1980). However, pelagic modes can vary greatly among marine fish species. Some species may spawn pelagic eggs from which pelagic larvae hatch, others may hatch pelagic larvae directly from benthic eggs, whereas others may bypass the pelagic larval stage altogether by bearing live young (Bonhomme & Planes 2000; Riginos & Victor 2001). It seems evident that such differences in pelagic modes should be reflected in dispersal capabilities. Being those species with extended pelagic phases more apt to disperse over greater distances than those with restricted or absent pelagic phases. However, the relationship between dispersal capabilities and realized gene flow is not always straightforward. While comparisons between species that differ greatly in their dispersal capabilities, namely direct development vs. planktonic larvae have generally showed greater gene flow levels in the planktonic larvae species (Hedgecock 1986; Riginos & Victor 2001; Shulman 1998; Shulman & Bermingham 1995), in intermediate comparisons such as benthic vs. pelagic eggs, inshore vs. offshore larvae, extended vs. short pelagic duration, such relationship is not as apparent (Doherty *et al.* 1995; Kinlan & Gaines 2003; Waples 1987). Therefore, the identification of gene flow trends across species with varying dispersal capabilities is fundamental for implementing appropriate conservation and management strategies within an ecosystem where multiple species co-occur. Consequently, the overall objective of this study is to identify gene flow patterns in a variety of marine fish species from the Eastern Atlantic Ocean and the Mediterranean Sea for management-conservation purposes and to consider how these can be explained by reference to pre-existing knowledge of their early-life history characteristics and in the presence of geophysical features along the sampling region. To achieve this objective, two sampling scales are considered; meso-scale and regional-scale.

The former includes samples form two highly commercially exploited species (*M. barbatus* and *M. surmuletus*) across the Mediterranean Sea and the Eastern Atlantic Ocean. Whereas the later includes six non-commercial species (*D. vulgaris*, *S. cabrilla*, *A. imberbis*, *S. tinca, T. delaisi* and *O. melanura*) sampled at the same locations within the Mediterranean coast of Spain and the Balearic Islands. The specific objectives and research questions are identify as follows in each chapter:

1.3.1 Chapter II: Molecular tools

- The Objective of this chapter is to describe the isolation and characterisation of polymorphic species-specific microsatellite loci from the subject species of this study.

1.3.2 Chapter III: Discordant patterns of population structure between two congeneric Mediterranean demersal species; the Red mullet (Mullus barbatus) and the Striped red mullet (Mullus surmuletus) from the Atlantic Ocean and the Mediterranean Sea.

- Are the patterns of population structure the same between these two congeneric species?
- How are the differences in population structure correlated with their distinct pelagic life stages?
- How do the observed patterns of genetic subdivision differ from those of other heavily exploited Mediterranean demersal species?
- Are the Mediterranean's reported bio-geographical boundaries also true for these demersal species?
- What are the implications of the genetic structure patterns in terms of conservation-management for the Mediterranean's current shared-stocks of these species?

1.3.3 Chapter IV: A multi-species approach to determine the role of early-life history traits and oceanographic boundaries on population genetic structure and its implications in the design of marine protected areas.

- What is the relative influence that hydrodynamic processes, habitat discontinuities and life history traits have in shaping population genetic structure among these marine fish species?
- Is there a common trend in dispersal trajectories of the species?
- Are gene flow breaks common to all species?
- Which is the most appropriate management-conservation strategy (i.e. single large reserve, network of small reserves) for marine fish species with distinct planktonic stage characteristics?

1.4 TARGET SPECIES

The first two species selected for this study belong to the Mullidae family. The Red mullet (*Mullus barbatus*) and the Striped red mullet (*Mullus surmuletus*) are distributed in the eastern Atlantic, from the British Isles in the north, to Senegal in the south, as well as throughout the Mediterranean and Black Seas (Hureau 1986). Both are bottom-dwelling species inhabiting sandy and muddy substratum and characterized by a pair of chemoreceptor-rich hyoid barbels which they use in order to detect surface and subsurface organisms (Golani & Galil 1991), (Fig. 1, 2).

Fig.1. Red mullet (*Mullus barbatus*)

Fig.2. Striped red mullet (*Mullus surmuletus*)

These demersal fishes rank among the most commercially important species of the Mediterranean Sea and are two of the main target species of the bottom trawl multi-species fishery (Stergiou *et al.* 1997). Considerable research has been devoted to the study of their fishery, biology, population dynamics (Arneri & Jukié 1985; Karlou-Riga & Vrantzas 1989), age and growth (Andaloro & Prestipino 1985), feeding habits (Papaconstantinou & Caragitsou 1987; Vassilopoulou & Papaconstantinou 1993), reproduction (Charbi & Ktari 1981; Haidar 1970; Livadas 1989; Wirszubski 1953), spatial segregation aspects (Golani 1994; Lombarte *et al.* 2000; Machias & Labropoulou 2002) and general ecology (Ananiadis 1950). However, despite their high economic value, genetic population structure is poorly investigated. A few studies based on allozyme data (Mamuris *et al.* 1998a; Mamuris *et al.*

1998b) and only one micorsatellite-based study (Garoia *et al.* 2004) suggest the existence of discrete stocks of the species within the Aegean and the Adriatic Seas.

The Peacock wrasse (*Symphodus tinca*) is the third species chosen for this study (Fig. 3). Belonging to the Labridae (Wrasses) family, this species occurs in the northern, western and central Atlantic as well as in the Mediterranean and Black Seas (Quignard & Pras 1986).

Fig. 3. Peacock wrasse (*Symphodus tinca*)

It is not subject to targeted food fisheries. However, it is not rare to find it as a by-catch of fisheries focussed on other species and its catches contribute significantly to recreational angling and spear-fishing activities (Gordoa *et al.* 2000). This species is found mainly on reefs and shallow coastal areas. Males are territorial and conspicuous male nuptial coloration and courtship has been observed (Costello 1991). Basic aspects have been investigated such as growth parameters (Gordoa *et al.* 2000), reproduction period and sexual maturity (Ghorbel *et al.* 2002), recruitment patterns and substrate preferences (Garcia-Rubies & Macpherson 1995) s well as general biology (Costello 1991). Genetic studies are scarce and have relied mainly on mtDNA to infer phylogenetic relationship and speciation within the Labridae family (Hanel *et al.* 2002). The studies showed that the biogeographic patterns of the Symphodus species group are dominated by speciation events driven by the closing and opening of the Mediterranean Sea and periodic glaciation events during the past 1 million years. The fourth species selected for this study is the Blackfaced blenny (*Tripterygion delaisi*) (Risso 1826). This species is distributed in shallow waters of the Mediterranean Sea, attaining a maximum length of 90mm (Zander 1986). Adults are strictly benthic and have 3 dorsal fins clearly separated (Fig. 4). It is commonly found in

shallow coastal waters living in rocky areas with reduced light such as under hanging rocks or cave entrances (Macpherson 1994; Writz 1978). Studies of this species have focused mainly on its reproductive system, morphometrics and speciation within the family (Carreras-Carbonell *et al.* 2005; Geertjes *et al.* 2001; Jonge *et al.* 1989). The comber (*Serranus cabrilla*) is the fifth species chosen (Fig. 5). The comber is a common demersal species inhabiting the eastern Atlantic Ocean and Mediterranean Sea. It is found in sea grass beds, rocky, sandy and muddy bottoms, with a wide bathymetric range (Tortonese 1986b). In the Canary Islands, it is considered as an important species of the artisanal small-scale fisheries (Brito *et al.* 2003). The Cardinal fish (*Apogon imberbis*) is the sixth species under study (Fig. 6). This species is endemic to the Mediterranean Sea usually inhabiting shallow rocky caves. This small fish (maximum 15 cm) is an oviparous species with internal fertilization and male mouthbrooding guarding. Larvae are pelagic once they hatch lasting in the plankton for up to 24 days (Tortonese 1986a). The Two-banded bream (*Diplodus vulgaris*) is the seventh species considered (Fig. 7). This bream species is one of the most frequent and abundant sparid fish in the coastal waters of southern Europe. This demersal species is distributed in the Mediterranean Sea and along the eastern Atlantic coast from France to Senegal (Bauchot & Hureau 1986) and it is often found in rocky bottoms with juveniles distributed in coastal lagoons and estuaries (Macpherson 1998). Finally, another sparid, the Saddled bream (*Oblada melanura*) is the eight species included in this study (Fig. 8). This diurnal schooling species is very common and abundant throughout the Mediterranean Sea and the Atlantic Ocean (Fischer *et al.* 1987). It can be found mainly over rocky bottoms and eel grass beads (Bauchot & Hureau 1986). Little information is available concerning its biology and population dynamics (Dufour *et al.* 1995; Lenfant & Olive 1998). Genetic analyses are scarce and have solely focused on resolving unclear phylogenetic relationships among sea bream species (Hanel & Sturmbauer 2000).

Fig. 4. Blackfaced Blenny (*Tripterygion delaisi*)

Fig. 5. Comber (*Serranus cabrilla*)

Fig. 6. Cardinal fish (*Apogon imberbis*)

Fig. 7. Two-banded bream (*Diplodus vulgaris*)

Fig. 8. Saddled bream (*Oblada melanura*)

1.5 CRITERIA FOR SPECIES SELECTION

In order to gain insight about how environmental features may affect gene flow patterns in species with different dispersal capabilities and how this knowledge may be applied into conservation-management strategies, I selected co-occurring commercial and non-commercial species that differ in their dispersal capabilities. The dispersal capabilities of each species are considered in terms of their egg type, (benthic-pelagic), the spatial distribution of larvae (inshore-offshore) and the pelagic duration (PD). The Peacock wrasse (*S. tinca*) has the shortest pelagic phase (7-13 d), and spawns in inshore waters. Adults are thought to be benthic and remain within spawning grounds (Costello 1991). Both mullets, in turn, spawn offshore and larvae travel through the ocean for long periods (>30 d) before recruiting to the juvenile-adult habitat. However, both the eggs and larvae of the Striped red mullet (*M.surmuletus*) are pelagic, whereas only postlarvae of Red mullet (*M. barbatus*) display a pelagic phase (Mamuris *et al.* 1998a). Both sparids, the Two-banded seabream (*D.vulgaris*) and the Saddled bream (*O.melanura*), posses pelagic eggs and offshore distributed larvae. However, the Two-banded seabream (*D.vulgaris*) exhibits a pelagic phase twice as long as the Saddled bream (*O.melanura*). Adults of these two species are mainly found in littoral waters on rocky or sandy bottoms. On the contrary, adults of both the Blackfaced blenny (*T.delaisi*) and the Comber (*S.cabrilla*) dwell in the benthos and are considered highly territorial with no apparent migratory movements (Heymer 1977). However, the Blackfaced blenny lays benthic eggs and larvae are distributed inshore at no more than a 100 m from the coastline, whereas the eggs of Comber are pelagic and larvae have been found at considerable distances from the coastline (Sabatés *et al.* 2004). The pelagic phase ranges from 16-21 days for the Blackfaced blenny and from 21-28 days for the Comber (Raventos & Macpherson 2001). Finally, Cardinal fish (*A. imberbis*) adults mainly inhabit caves and brood the eggs in the male's mouth until hatching (Tortonese 1986a). A summary of the species main life history traits is presented in table 1.

Various types of genetic markers are currently available for population studies and can be broadly subdivided into selected and neutral markers. Variation found at selected markers (i.e. genome fragments that are subject to selection) can provide valuable information for the identification of local adaptations, environmental change tolerance and are increasingly being used in aquaculture and sea-ranching programs (Bartley 1999; Kouttouki *et al.* 2006). Neutral markers, in turn, are assumed to follow a mutation-drift equilibrium neutral model (Charlesworth *et al.* 1995). Thus, genetic variation from neutral markers can be used to infer genetic relationships among populations under such a model in the absence of selection. Therefore, microsatellite DNA markers were chosen as the molecular tools for this study due to their high polymorphism, selective neutrality and fast evolving nature. The objective of this chapter is to describe the isolation and characterisation of polymorphic species-specific microsatellite loci from the subject species of this study.

1.6 PREDICTED DISPERSAL PATTERNS

According to the life history traits described above and in table 1, *D. vulgaris*, *M. surmuletus,* and *S. cabrilla*, species with extended PD, pelagic eggs and offshore distributed larvae should hold the greatest potential for dispersal, followed by *O. melanura* with pelagic eggs and offshore larvae but with a shorter pelagic duration. *M. barbatus*, is expected to be more restricted as it spawns offshore larvae but lays benthic eggs. *T. delaisi* and *S. tinca* on the other hand, should be fairly limited in their dispersal by producing benthic eggs, inshore distributed larvae and having shorter PD. Finally, *A. imberbis* being a mouth-brooder is the species with the least potential for dispersal in spite of its relatively long PD. A schematic representation of the species predicted dispersal is shown in Figure 9.

Table 1. Main life history traits of *Diplodus vulgaris*, *Mullus surmuletus*, *Serranus cabrilla, Mullus barbatus, Tripterygion delaisi, Oblada melanura, Symphodus tinca*, and *Apogon imberbis*. B: benthic, P: Pelagic, O: offshore > 2 km, I: Inshore < 2 km, n.d.a: no data available.

Specie	Family	Depth Range	Adult Habits	Egg type	Distribution of larvae	Pelagic duration	Refs.
Diplodus vulgaris	Sparidae	0-90 m	Littoral waters on rocky or sandy bottoms	P	O	29-58 days	Bauchot & Hureau 1986
Mullus surmuletus	Mullidae	10-100 m.	Demersal. Found in rocky bottoms.	P	O	28-35 days	Golani & Galil 1991
Serranus cabrilla	Serranidae	1-500 m	Benthic on the shelf and upper slope, on rocks, *Posidonia* beds, sandy and muddy bottoms	P	O	21-28 days	Brito *et al.* 2003
Mullus barbatus	Mullidae	100-300 m.	Demersal. Found in sandy-muddy bottoms	B	O	28-35 days	Mamuris *et al.* 1997
Tripterygion delaisi	Tripterygiidae	2-40 m	Bottom-living on rocky shores in biotopes with reduced light	B	I	16-21 days	Macpherson 1994; Writz 1978
Oblada melanura	Sparidae	0-30 m	Littoral waters above rocky bottoms and *Posidonia* beds	P	O	14-18 days	(Bauchot & Hureau 1986).
Symphodus tinca	Labridae	1-50 m.	Found near rocks and eel-grass beds	B	I	9-13 days	Gordoa *et al.* 2000
Apogon imberbis	Apogonidae	10-200 m.	On muddy or rocky bottoms and caves	Mouth-brooder	n.d.a	18-24 days	Tortonese 1986

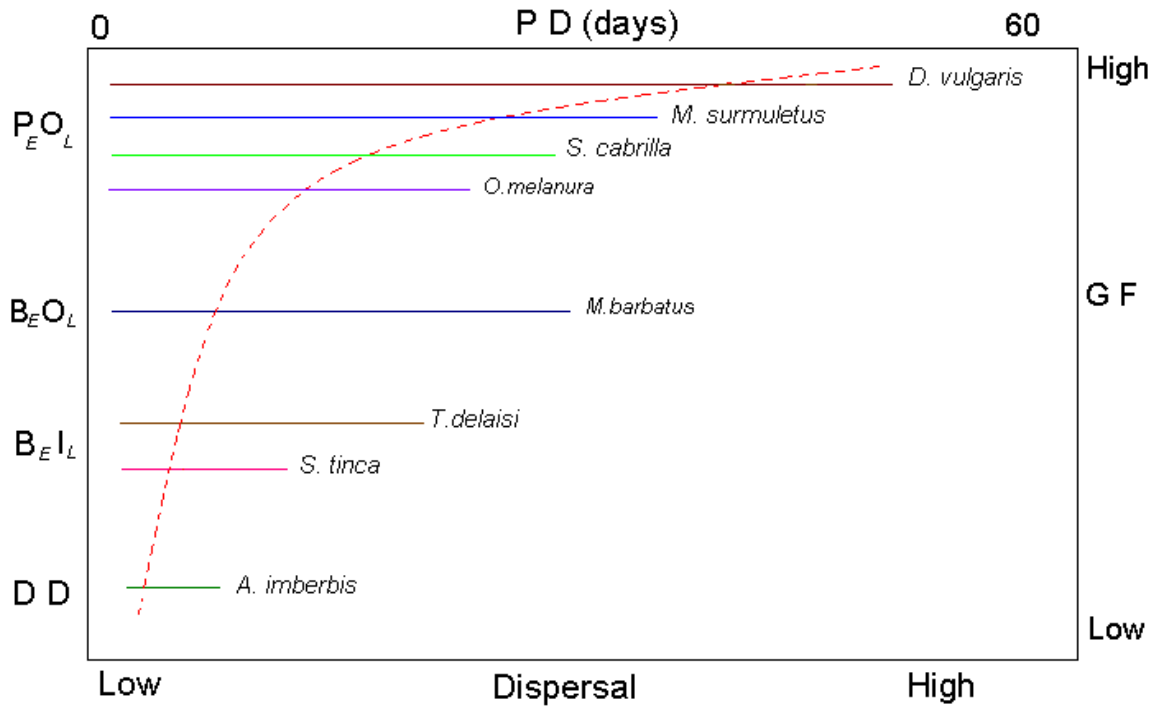


Figure 9. Schematic representation of predicted dispersal potential and expected gene flow of *D. vulgaris*, *M. surmuletus*, *S. cabrilla*, *M. barbatus*, *O.melanura*, *T. delaisi*, *S. tinca* and *A. imberbis*. X axis (top) = Pelagic duration in days. X axis (bottom) = Predicted dispersal potential (solid lines). Y axis (left) = Life history traits. P_E= Pelagic Eggs. B_E= Benthic Eggs. O_L= Offshore Larvae. I_L = Inshore Larvae. DD= Direct Development. Y axis (right) = Expected gene flow (dashed line). GF = Gene flow level.

1.7 STUDY SITE

With over 26,000 km of coastline, the Mediterranean Sea is a semi-enclosed sea with several distinct geographical sub-units with a turnover period of approximately 80 years for water entering through the Strait of Gibraltar. The Mediterranean Sea contains a narrow shelf, with wider shelves and areas of higher productivity in the Adriatic and in the Gulfs of Lions and Gabes. The Black Sea is linked to the Mediterranean through the Turkish straits, however, it is considered as a separate large marine ecosystem. The major inflow into the Mediterranean is oxygenated Atlantic surface water entering through the Strait of Gibraltar (Caddy & Griffiths 1990). Fresh water inflows are relatively low as the Mediterranean Sea receives from the rivers only about one-third of the amount of water that it loses by evaporation. Overall, the Mediterranean Sea is considered a low productivity ecosystem based on NASA's earth-orbiting ocean colour sensors from which primary productivity is inferred (SeaWiFS). Ecosystem parameters such as nutrient levels and chlorophyll concentrations are relatively low and decrease from west to east and from north to south (Azov 1990).

A vertical thermohaline density front separates the Mediterranean's coastal areas from the open sea and restricts the flow of terrigenous materials (nutrients and other river-borne substances) out of the coastal areas, which results in favourable conditions for spring blooms. In the summer, stratification occurs by vertically separating the warmer, less saline surface water from the deeper, colder and more saline water, resulting in autumnal algal blooms and extended hypoxia. This stratification occurs more frequently during extended periods of calm seas, high temperatures, and inflows of fresh water (UNEP/FAO 1990).

On the horizontal plane, lower salinity levels are found in the western Mediterranean and increase towards the northeast. Water entering from the Atlantic through the strait of Gibraltar displays a significant increase in salinity during the winter months at the northern

Mediterranean. Maximum salinity levels are found in the northeast during the summer due mainly to high evaporation and low continental water inflow (Fig.10).

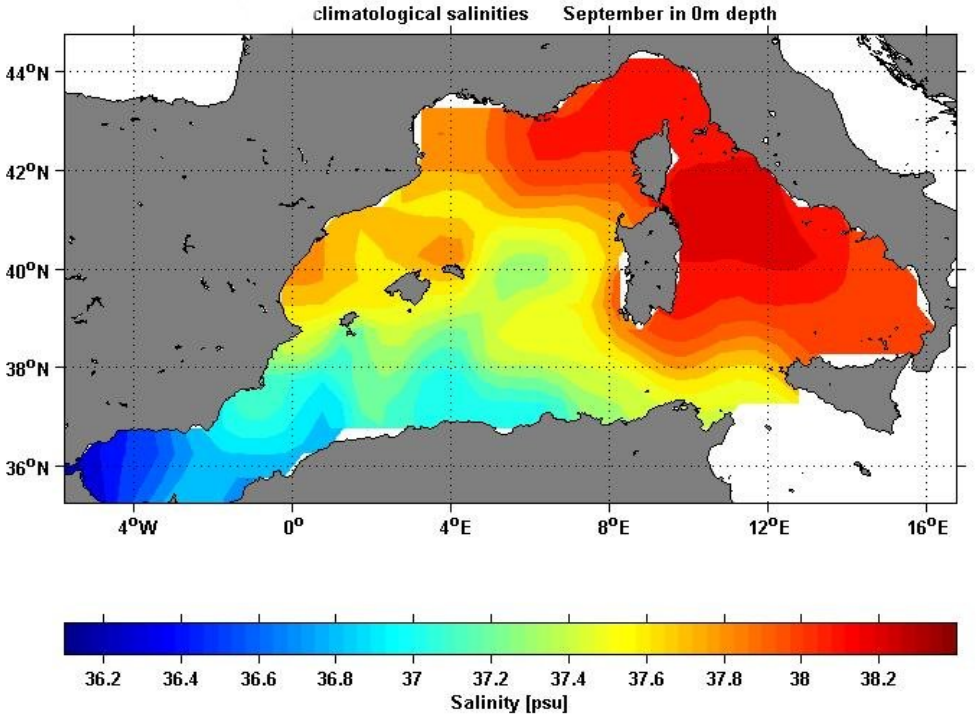


Fig. 10. Mediterranean's mean surface salinity contours (September, 2003). Data from NSF/NASA 2003

Surface temperatures, however, are more homogenous throughout the Mediterranean and remain constant during winter showing a slight increase at mid spring. Maximum temperatures are found in the southeast Mediterranean and northern coast of Africa in summer time (NSF/NASA 2003) (Fig.11).

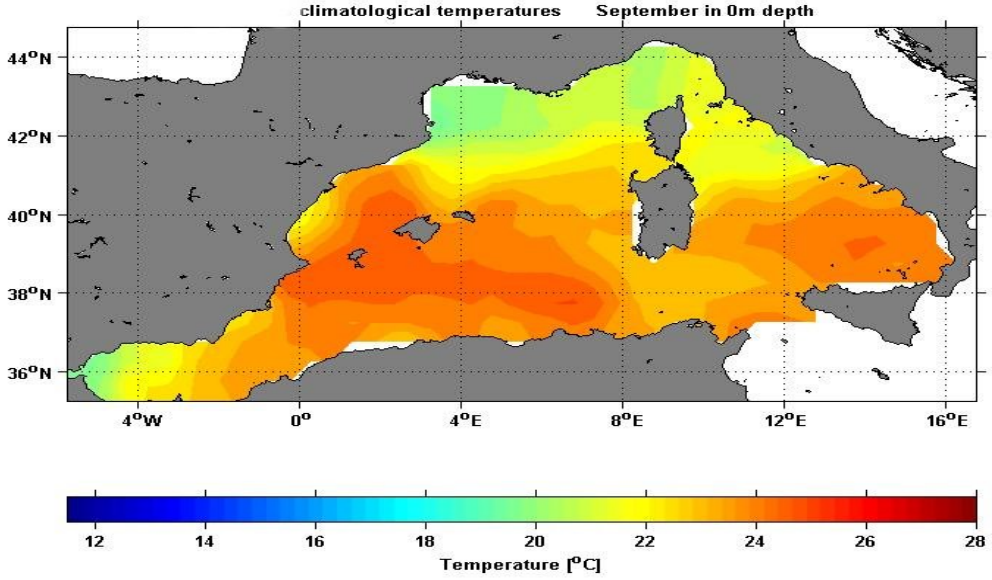


Fig. 11. Mediterranean's mean surface temperature contours (September, 2003). Data from NSF/NASA 2003

Predominant winds in the Midwest Mediterranean blow from the southeast from January until June, when a marked deviation towards the southwest occurs. During this change of direction there is also a significant decrease in wind intensity. The winds in the eastern Mediterranean remain constant throughout the year displaying southeast direction with steady velocity vectors (NSF/NASA 2003). The combined properties of water density (resulting from temperature-salinity gradients) and wind-forcing processes, confer the Mediterranean its peculiar surface circulation pattern (Fig. 12)

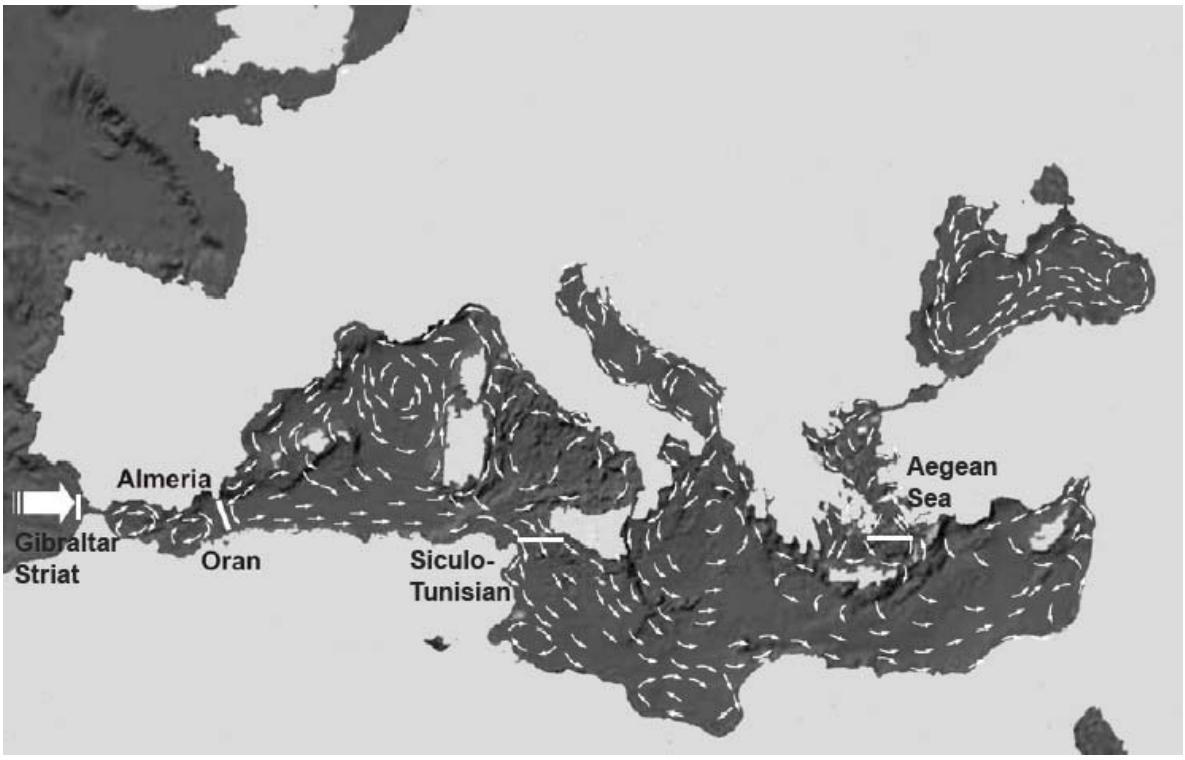


Fig. 12. Mediterranean surface circulation patterns and main oceanographic boundaries. Redrawn from Fernandez *et al*. (2005)

This pattern consists basically of a counter-clockwise movement of water. There is a continuous inflow of surface water from the Atlantic Ocean. After passing through the strait of Gibraltar, the main body of the incoming surface water flows eastward along the northern coast of Africa. This current is the most constant component of the circulation of the Mediterranean. It is most powerful in summer, when evaporation in the Mediterranean

is at its maximum. This inflow of Atlantic water loses its strength as it proceeds eastward, but it is still recognisable as a surface movement in the Sicilian channel and even off the Levantine coast. At the northern Mediterranean coastline, major currents are persistent throughout the year, flowing towards southwest reaching the southern coast of Spain and the strait of Gibraltar (Fig. 12). In summer, surface water becomes more saline through the intense evaporation, and correspondingly, its density increases. It therefore sinks, and the excess of this denser bottom water emerges into the Atlantic Ocean over the still forming strait of Gibraltar as a westward subsurface current below the inward current. (UNEP 1999).

1.8 GENETIC SUBDIVISION IN THE MEDITERRANEAN SEA

Within the Mediterranean genetic differentiation at the geographical level has been reported for several taxa at particular areas. The strait of Gibraltar is often reported as a biogeographic boundary for many species with Atlanto-Mediterranrean distributions. Numerous studies based on genetic markers have provided evidence for a genetic break between the Mediterranean and the Atlantic Ocean for species such as swordfish (*Xiphias gladius*), European hake (*Merluccius merluccius*), marine sponges (*Crambe crambe*) and sea stars (*Asterina gibbosa*) (Baus *et al.* 2005; Brutto *et al.* 2004; Duran *et al.* 2004; Kotoulas *et al.* 1995; Zane *et al.* 2000). However, recent studies have also provided evidence of extensive gene flow occurring between Mediterranean and Atlantic basins for chub mackerel (*Socomber japonicius*) and Norway lobster (*Nephrops norvegicus*) populations (Stamatis *et al.* 2004; Zardoya *et al.* 2004). In addition, contradictory results have also been reported for closely related species within this area. (Bargelloni *et al.* 2003) analysed genetic variation at nuclear and mitochondrial loci in five species belonging to the Sparidae family. The results provided evidence for a genetic break between the Mediterranean and the Atlantic Ocean for only two species of the five investigated.

Another frequently declared biogeographic boundary is the sea fraction between Almeria (Spain) and Oran (Morocco), known as the Almeria-Oran front. A recent review of genetic studies performed on this area has identify a sharp phylogeographical break between the Mediterranean and the Atlantic Ocean associated with this strait rather than with the strait of Gibraltar mentioned above (Patarnello *et al.* 2007). Genetic studies in have found significant genetic differentiation for a variety of marine taxa such as mussels (*Mytilus galloprovincialis*), scallops (*Pecten spp*.), European hake (*Merluccius merluccius*) and European sea bass (*Dicentrarchus labrax*) (Cimmaruta *et al.* 2005; Naciri *et al.* 1999; Quesada *et al.* 1995; Ríos *et al.* 2002). Regions such as the Sardinian channel and the

Aegean Sea are also suggested as biogeographic boundaries for anchovy (*Engraulis encrasicolus*) and gilthead sea bream (*Sparus auratus*) populations (Borsa 2002; De Innocentis *et al.* 2004). The Siculo-Tunisian strait has been reported to be the cause of genetic subdivision for Poor Cod (*Trisopterus minutus capelanus*) and Mackerel (*Scomber scombrus*) populations form eastern and western Mediterranean basins (Mattiangeli *et al.* 2004). Furthermore, intense debate still exists about genetic differences in populations of blue fin tuna (*Thunnus thynnus thynnus*) between these two basins (Alvarado Bremer *et al.* 1999; Broughton & Gold 1997; Pujolar *et al.* 2003; Viñas *et al.* 2003).

The above interpretations of observed patterns of genetic homogeneity/heterogeneity found within the Mediterranean include diverse hypothesis such as isolation by distance, secondary contact, hydrographical regimes, contact zones, climatic fluctuations etc. The lack of consensus regarding population structure of many ecologically similar species underscores the need for detailed genetic studies with comparable results. This is particularly true when dealing with heavily exploited species such as demersal fish, for which, significant reductions in their populations have been observed over the past decade (Myers & Worm 2003). The Mediterranean's demersal environment is occupied by more than 100 fish species that represent around 40 percent of the total reported catches. The overall classification status of demersal resources in the Mediterranean ranges from overexploited to fully exploited (FAO 2005). Highly appreciated gregarious species such as European hake (*Merluccius merluccius*), blue whiting (*Micromesistius poutassou*) and Red mullets (*Mullus spp*.) are the main demersal fish resources within the Mediterranean (FAO 2005). However, knowledge of genetic population structure is lacking for most of these species, including the extensively studied European hake (*Merluccius merluccius*), for which, some uncertainty remains concerning the genetic population subdivision between the Mediterranean and Atlantic populations (Cimmaruta *et al.* 2005).

CHAPTER II
Molecular tools

2.1 METHODOLOGY

In order to assess genetic variability in the target species, I isolated and characterised microsatellite loci through the development of enriched genomic libraries for *D.vulgaris, M. barbatus, S.tinca, O.melanura* and *A.imberbis* species. Further, microsatellite loci developed for *M.barbatus* were cross-amplified in its congener *M.surmuletus*. For *S.cabrilla* and *T.delaisi*, allelic variation was analysed in previously described microsatellite loci of these species (Carreras-Carbonell *et al.* 2004; Carreras-Carbonell *et al.* 2006). In addition, genetic variability of the loci isolated for *D.vulgaris* is reported in two related species *D.sargus* and *O.melanura.* Likewise, isolated primers for *O.melanura* were tested for variability in *D.sargus* and *D.vulgaris.*

Species-specific genomic libraries were developed as described by Glenn *et al.* (2000). DNA extractions were performed from lateral fin tissue out of 10 individuals of each species to assure high quality DNA availability. Approximately 10 µg of high molecular weight DNA was isolated by phenol-chloroform extraction (Sambrook *et al.* 1989). Simultaneous restriction-ligation of genomic DNA was carried out using the *RsaI* restriction enzyme and double stranded linker-adapted primers according to Hamilton *et al.* (1999). Ligated DNA was enriched with a biotin-labelled probe mixture consisting of $(GT)_{10}$, $(CT)_{10}$ and $(GATA)_{10}$ at 10µM each. The tetranucleotide repeat probe $(GATA)_{10}$ was tested only in *S.tinca* species with no significantly improved performance over dinucleotide repeat probes. Thus, dinucleotide repeat probes were used for the rest of the species. DNA fragments with repetitive sequences were then selectively captured by streptavidin-coated Dynabeads (Oxoid) and separated by a magnetic field. Enriched DNA was eluted in 200µL dH_2O from the bead probes and concentrated by vacuum centrifugation to a final concentration of ~100 ng/µL. Recovered DNA was then re-amplified by polymerase chain reaction (PCR), purified and ligated into a cloning vector

using pGEM-T Easy Vector II (Promega). A mean of 76 positive clones/species were screened and checked for inserts using ABI PRISM BigDye Terminator Cycle kit (Applied Biosystems) and resolved on an ABI 3100 Genetic Analyser (Applied Biosystems). Primer pairs for isolated microsatellite loci (mean 9.1/species) were designed using the software package OLIGO 6.4. Polymorphism was tested by multiplex PCR reactions performed in 20µl total volume, which include 50ng of DNA, 2mM of $MgCl_2$, 0.25µM of each primer, 200µM dNTP's, 1X reaction buffer [75 mM Tris-Hcl, 20 mM $(NH_4)_2SO_4$] and 0.5 units *Taq* polymerase (BIOTAQ). Different reaction conditions were used. For *M.barbatus* reactions conditions were as follow: an initial denaturation step of 5 min at 95 °C, 30 cycles consisting of 30 s at 92 °C, 30 s at 56.5 °C annealing temperature, and 30 s at 72 °C. For *S.tinca* reaction conditions were as follows: an initial denaturation step of 5 min at 95 °C, 30 cycles consisting of 30 s at 92°C, 30 s at 55 °C annealing temperature and 30 s at 72 °C. Whereas for *D.vulgaris* and *O.melanura*, reaction conditions consisted of: an initial denaturation step of 5 min at 95 C°, eight cycles consisting of 45 sec at 92 C°, 45 sec at 53 C° annealing temperature, 45 sec at 72 C° followed by an additional 24 cycles consisting of 30 sec at 92 C°, 30 sec at 56 C° annealing temperature, 30 sec at 72 C°. Finally for *A.imberbis,* reaction conditions consisted of: an initial denaturation step of 5 min at 95 C°, eight cycles consisting of 30 s at 92 C°, 30 s at 53.5 C° annealing temperature, 30 s at 72 C° followed by an additional twenty-eight cycles at 55.5 C° annealing temperature, 30 sec at 72 C°. Microsatellite variability was assessed in 30 individuals of each species. Individuals were genotyped by assessing allele size on an ABI 3100 Genetic Analyser (Applied Biosystems) using forward primers labelled with FAM (Sigma) and NED, PET and VIC (Applied Biosystems). Allele scoring was carried out using GENEMAPPER software version 3.5 (Applied Biosystems). Expected and observed values for heterozygosity were determined using ARLEQUIN version 2.0 (Schneider *et al.* 2000). The number of alleles

per locus, allele size range as well as deviations from Hardy-Weinberg expectations (HWE) and linkage disequilibrium between pairs of loci were estimated using FSTAT version 2.9 (Goudet 1995). All loci were polymorphic; the total number of alleles per locus, heterozygosities estimates as well as deviations from HWE for each species are listed in tables 2-8. No evidence of linkage disequilibrium was found between any locus pair.

Table 2. Characterization of eight Two banded bream (*Diplodus vulgaris*) microsatellite loci (N=30). Locus name, repeat motif, fluorescent dye-primer sequence, number of alleles, allele size range, H_O, observed heterozygosity; H_E, expected heterozygosity under Hardy-Weinberg equilibrium; F_{IS}, inbreeding coefficient, * P < 0.05.

Locus	Repeat motif	Primer Sequence (5´-3´)	No. of alleles	Allele size (bp)	H_O	H_E	F_{IS}
Dvul1	$(CA)_{13}$	F:VIC- GGCCCGCTTTATTCTCAGTCTCAA R: TGCAGGGAACAGAGGGATGACAG	5	135-147	0.428	0.641	0.309
Dvul2	$(GT)_{28}$	F:VIC- CTGCGTTGATGGTTTTTCAGAATG R: CTGCGAAACATCTGGAGTTGTATT	11	180-206	0.571	0.843	0.319*
Dvul33	$(CA)_{11}$	F:FAM-GCCGGGCTCGACATTGACACTGAA R: GCAGCCAGCAGAGCTTAAAGAACT	17	260-300	0.885	0.918	0.037
Dvul4	$(CA)_{13}$	F:NED- GCGGTTATGTATACGTTGCGTTTA R: TTGGCGTTGAACAGAAGTCAGACA	16	244-276	0.914	0.923	0.01
Dvul61	$(GT)_{18}GA(GT)_8$	F:VIC- TGGGGACTCTCAGAATCATCACAA R: TGGAAAAAGCCCTCTGGACAAAAG	7	412-426	0.838	0.689	-0.221
Dvul63	$(CA)_{14}$	F:PET-GAGGAATGAGTAGAGAAAAGATGG R: ACCCCAACAACCAGAATACCTATA	13	190-234	0.575	0.840	0.315*
Dvul84	$(GT)_{15}$	F:PET- GCTCGACGTGCACTCTGCCCTTGA R: ATTCCCCAAATCCAGCACTCACAT	14	254-296	0.647	0.816	0.209
Dvul38	$(CA)_{16}$	F:FAM- TCGGGCACAGATAGAAAGAAACAC R: GAAGGAAGACGGATCTCAGGATGA	21	170-210	0.914	0.932	0.016

Table 3. Cross species amplification of 8 microsatellite loci from the Two banded bream (*Diplodus vulgaris*) in the white sea bream (*Diplodus sargus*) and the saddled bream (*Oblada melanura*). Locus name, number of alleles (N^a) allele size range. na indicates no amplification

Locus	*D. sargus* (n=7)		*O. melanura* (*n*=8)	
	N^a	Range	N^a	Range
Dvul11	2	144-146	3	144-150
Dvul2	3	182-186	4	182-192
Dvul33	8	282-302	10	274-304
Dvul4	8	248-280	7	248-268
Dvul61	6	390-440	6	404-450
Dvul63	na	na	na	na
Dvul84	7	254-274	7	256-284
Dvul38	2	170-174	4	170-176

Table 4. Characterization of ten Red mullet *Mullus barbatus* (N = 30 individuals) microsatellite loci and their variability on Striped red mullet *Mullus surmuletus* (N = 30 individuals). H_O ,Observed heterozygosity; H_E, Expected heterozygosity under Hardy–Weinberg equilibrium; F_{IS} Inbreeding coefficient, *P < 0.05

Locus/GenBank Accession No.	Locus	Repeat motif	Primer sequences (5´ → 3´)	Species	Number of alleles	Allele size (bp)	H_O	H_E	F_{IS}
DQ473548	Mbar3	$(CA)_{24}$	F:*PET*-GCTCCCCCGACACACTGTCT	*M.barbartus*	16	111-149	0.8793	0.9352	0.059
			R:ACCTTGGCCCTTCTTACGTC	*M.surmuletus*	8	115-145	0.5838	0.6480	0.179
DQ473549	Mbar11	$(GT)_{10}$ GC $(GT)_{10}$	F:*VIC*-TGACTGTCAGCACTTGCATT	*M.barbartus*	9	156-176	0.5691	0.5847	0.204
			R:CTGAGGAGAGTCATGAGT	*M.surmuletus*	16	162-208	0.8571	0.9227	0.060
DQ473551	Mbar14	$(AC)_{48}$ AT $(AC)_4$ AT $(AC)_4$	F:*FAM*-GATAGCGAGCCTGAAACCAC	*M.barbartus*	26	195-265	0.9285	0.9603	0.034
			R:CCCTCTGCTTGATATTCCT	*M.surmuletus*	16	193-235	0.6000	0.9163	0.347*
DQ473555	Mbar28	$(GT)_{12}$	F:*FAM*-AAAGGGAGAATGAGGTGAAA	*M.barbartus*	2	156-164	0.1034	0.1324	-0.037
			R:AAGCGCTCGCAACAAAGTC	*M.surmuletus*	2	164-166	0.0333	0.0661	0.000
DQ473552	Mbar46	$(GT)_{12}$	F:*NED*-CCCGAGCAGCAGAAAAA	*M.barbartus*	7	250-262	0.7666	0.8310	0.079
			R:CTTGCCCTCTGCCTCTG	*M.surmuletus*	7	236-244	0.4285	0.5539	0.229*
DQ473547	Mbar55	$(CA)_7CG(CA)_3$ TA$(CA)_6$	F:*NED*-TACACACAAACACTCACCCA	*M.barbartus*	12	146-176	0.8000	0.8717	0.074
			R:CGCAACCAATAGCACACTAC	*M.surmuletus*	7	142-166	0.5517	0.7489	0.267*
DQ473553	Mbar63	$(AC)_{10}$ AT $(AC)_8$	F:*VIC*-AACCAGCAGGTCTCACA	*M.barbartus*	11	301-337	0.7900	0.8621	0.186
			R:TTCATGCTCCTTTTGTTCC	*M.surmuletus*	14	269-327	0.8200	0.8887	0.091
DQ473550	Mbar130	$(AC)_{10}$	F:*NED*-GAGGGTAGATTTGGTTGCAG	*M.barbartus*	8	185-209	0.7583	0.7787	0.071
			R:AGAGTATTGCATTTTTCGCC	*M.surmuletus*	11	185-217	0.7955	0.8473	0.079
DQ473556	Mbar132	$(GT)_{10}$	F:*FAM*-GGAGCAAGGAAGAGGAGA	*M.barbartus*	10	112-132	0.7896	0.8324	0.162
			R: CTCTGCAGACCTGCTCAA	*M.surmuletus*	9	118-136	0.7641	0.8451	0.145
DQ473554	Mbar133	$(CA)_{14}$ CG $(CA)_5$	F:*PET*-CTCGGCACATCACAGAAAC	*M.barbartus*	11	226-266	0.7333	0.7847	0.065
			R:CCTCCCAAATTACACACATC	*M.surmuletus*	16	230-268	0.8247	0.8620	0.044

Table 5. Characterization of 8 Peacock wrasse (*Symphodus tinca*) microsatellite loci. GenBank Accession No., Locus name, repeat motif, fluorescent dye-primer sequence, number of alleles, Allele size range, H_O: observed heterozygosity, H_E: expected heterozygosity under Hardy-Weinberg equilibrium, F_{IS}: inbreeding coefficient, *P<0.05.

Locus/GenBank Accession No.	Locus	Repeat motif	Primer sequences (5´ → 3´)	Number of alleles	Allele size (bp)	H_O	H_E	F_{IS}
DQ324538	Stin155	$(GA)_{11}$	F:Fam-GAGGCAAACAAGTATTTCA R:AGGGACTGTGTTCTTGATG	11	138-168	0.8611	0.8798	0.054
DQ324537	Stin287	$(AC)_6GC(AC)_9$	F:Fam-AGCAGGTTCAATAAACACA R:GGGTGCTCAAGGTAATTGT	9	275-297	0.8055	0.8047	0.001
DQ324543	Stin368	$(AC)_{22}$	F:Fam-TCTGGCAGCCTTAGTCCTC R:GAGCGTCTTCAGAAGGACA	17	336-368	0.7500	0.8873	0.247*
DQ324540	Stin143	$(AC)_4G(AC)_{14}$	F:Hex-TGCAAGCAGAGTCTCAAACCC R:ATTTAACAGCTTGCTTGG	11	120-141	0.9166	0.8924	-0.027
DQ324542	Stin222	$(TG)_9C(TG)_{17}$	F:Hex-CCCGTGATAGATGAGGAG R:GGTGCTCCTGTGATATCT	11	206-231	0.7777	0.8321	0.159
DQ324539	Stin336	$(CA)_{36}$	F:Hex-GCTGGAAGAATAGAACATTC R:TTTTTTGCAAGCTTTTAGT	13	321-365	0.7714	0.8567	0.191
DQ324541	Stin245	$(GATA)_{30}$	F:Ned-TAGCCCCGCCCCCTAAAAA R:TGCTACTGGATGGCGCTGAA	12	218-314	0.8463	0.8961	0.084
DQ324536	Stin138	$(C)_{16}(CA)_{11}$	F:Ned-GCGTTTTTACGTCATGTTTT R:AGGTATGTGTCCGCTTTT	15	127-165	0.6571	0.9155	0.424*

Table 6. Characterization of 8 Cardinal fish (Apogon imberbis) microsatellite loci (N=30). GenBank Accession No., Locus name, repeat motif, fluorescent dye-primer sequence, number of alleles, Allele size range, H_O: observed heterozygosity, H_E: expected heterozygosity under Hardy-Weinberg equilibrium, F_{IS}: inbreeding coefficient, *: P < 0.05.

Locus/GenBank Accession No.	Locus	Repeat motif	Primer sequences (5´ → 3´)	Number of alleles	Allele size (bp)	H_O	H_E	F_{IS}
DQ822534	Aimb2	$(CA)_{12}$	F: *FAM*-AGCCGGTTCCTTTAGAGCATTCAA R: -GAGGCGTTTAGAGTGTGAGAAGGA	6	341-355	0.550	0.751	0.273
DQ822535	Aimb14	$(GT)_{21}$	F: *NED*-CACCCCACACTACATGCCCTTGAA R: -GCTGGCTGGCCTAGTTTGGGTCTC	11	316-358	0.800	0.876	0.084
DQ822536	Aimb17	$(CTAT)_{23}$	F: *NED*-TCGCTGGTGTTGTCTAATGCATTC R: -TGGGGAAGGAGAGCGATGCAGAAC	19	120-200	0.800	0.961	0.166
DQ822537	Aimb22	$(CA)_{14}$	F: *PET*-ACCGCTGCTGTCAGTCCTGTCACA R :- AACCGAGGCTGTTCCCATCAAATG	6	447-457	0.300	0.746	0.599*
DQ822538	Aimb28	$(CA)_3CT(CA)_9$	F: *PET*-CCGTTCTGCTCTGATTGGTCAACT R :- TCCTTTTGGCGCTGATTAGTTCAC	8	254-272	0.850	0.811	-0.052
DQ822539	Aimb29	$(CA)_{15}$	F: *FAM*-CTTGCCGTTTTTGCTACTATGTTCC R: -GCTGATTTTAAGCTACATTCTACCT	13	198-232	0.650	0.866	0.253*
DQ822540	Aimb41	$(GT)_{16}$	F: *VIC*-ACGGCTCAGAAGATGGTCCACACA R: -GTGCCATCCAATCTGTCCATCATA	13	335-377	0.850	0.850	-0.002
DQ822541	Aimb74	$(CA)_{11}TA(CA)_3$	F: *VIC*-CACCACAATAGTTAAATGCTCCCT R: -CTTCGCATCAGGGGTTAATCTCAA	6	210-240	0.650	0.680	0.035

Table 7. Characterization of eight saddled bream (*Oblada melanura*) microsatellite loci (N=48). Locus name, repeat motif, accession number, fluorescent dye-primer sequence, number of alleles, allele size range, H_O , observed heterozygosity; H_E , expected heterozygosity under Hardy-Weinberg equilibrium; F_{IS} , inbreeding coefficient, * $P < 0.05$.

Locus	Repeat motif	Access No.	Primer Sequence (5´-3´)	No. of alleles	Allele size (bp)	H_O	H_E	F_{IS}
Omel58	$(CA)_{13}$	EF064298	F:FAM-GGCATTATTGTTCCATCATTACTCC R: ATGGCATACAACCTGCATCAGAAG	10	284-304	0.723	0.768	0.069
Omel3	$(GT)_{14}$	EF064299	F:FAM-CCTCCGACATCATCAGTGTGTAAT TGGCATGCGAGGTTCAGTCTGTGC	9	393-409	0.806	0.818	0.029
Omel38	$(GT)_{17}$	EF064300	F:FAM-AGCCGGCTGAGCTCCATAATAACC R:TGCCCTCTTGTCACACCAGGTCAC	16	197-233	0.913	0.839	-0.077
Omel20	$(CA)_{12}$	EF064301	F:VIC- CAGGGTAGCAACAGGGTAACAATG R:GGCGGTTGAGGACACTGCAAAAAA	3	353-357	0.212	0.233	-0.086
Omel2	$(CA)_{10}$	EF064302	F:VIC- TGCCCCTGTCTGTTGGAGTATGAA R:AACCCCACTGACGTCTTTCTGAAC	18	226-264	0.766	0.900	0.159
Omel61	$(GT)_{21}$	EF064303	F:VIC- CAGCGGGGGATTAATCTGCATTTG R:GCCCGATTTATCTTCATCACCCAT	22	127-173	0.771	0.918	0.170*
Omel54	$(GT)_{14}$	EF064304	F:NED-TGGGGCACCAAAAGAGCGCGCGTG R:ACCCCCTGTCGCCTCCTCTCTTCC	20	197-241	0.783	0.918	0.158*
Omel27	$(CA)_{17}$	EF064305	F:NED-TTGGCTCATTAGACAAAGGCACAC R:GGGCGCTGAAACAATAGCCGTGTT	20	293-359	0.844	0.918	0.091

Table 8. Cross species amplification of 8 microsatellite loci from the saddled bream (*Oblada melanura*) in the white sea bream (*Diplodus sargus*) and the the two-banded sea bream (*Diplodus vulgaris*) Locus name, number of alleles (Na), allele size range. na indicates no amplification.

Locus	*D.sargus* (n = 7)		*D.vulgaris* (n = 8)	
	Na	Range	Na	Range
Omel58	4	288–296	4	290–310
Omel3	na		na	
Omel38	9	193–235	6	183–199
Omel20	10	349–385	na	na
Omel2	4	222–230	5	228–242
Omel61	8	139–161	6	137–179
Omel54	5	193–207	8	221–261
Omel27	na		10	291–319

CHAPTER III

Discordant patterns of population structure between two congeneric demersal species; the Red mullet (*Mullus barbatus*) and the Striped red mullet (*Mullus surmuletus*) from the Atlantic Ocean and the Mediterranean Sea.

Abstract

The accurate identification of gene flow patterns is of primarily importance when devising conservation-management effort for today's marine resources. The great variety of population structure patterns displayed in the demersal environment underscores the need for the identification of common gene flow patterns that can be found in most demersal species for conservation-management purposes. To this end, allele-frequency variation at 10 microsatellite loci of two congener demersal fish, the Red mullet (*Mullus barbatus*) and the Striped red mullet (*Mullus surmuletus*) from the Atlantic Ocean and the Mediterranean Sea was analysed. The results indicate that two different gene flow patterns exist between these species. *M.barbatus* genetic distribution was found highly structured resembling that of a metapopulation composed by largely independent self-recruiting subpopulations with some connections between them. Whereas *M.surmuletus* displayed less genetic heterogeneity within the Mediterranean Sea and a significant reduction of gene flow between the Atlantic Ocean and Mediterranean Sea. The results indicate that the patterns of gene flow in the demersal environment can be substantially different between closely related species, suggesting that biogeographic boundaries can affect demersal species in a different way despite common ecological features and spatial overlap. It can be suggested that the delimitation of such boundaries could be largely determined by the fine differences in life history traits between species. These results are discussed in relation to environmental features of the Mediterranean Sea and in terms of conservation and management of the current stocks of these species.

3.1 INTRODUCTION

The accurate identification of population genetic subdivision is of primarily importance when devising conservation-management effort for today's marine resources. Population genetic studies are increasingly being used for stock assessment providing valuable information for management and conservation purposes (Cimmaruta *et al.* 2005). For example, genetic methods can be useful in estimating a number of population parameters relevant to fishery management such as effective population size, gene flow and natural levels of genetic diversity. Loss of genetic diversity due to fishing pressure can lower the ability of a species to withstand changing environmental conditions (Conover & Munch 2002). Genetic diversity, in turn, is dependant on the number of adults spawning in the population or effective population size (N_e). This is an essential parameter since it reflects important biological aspects of the population such as the proportion of reproducing males and spawning females (Wright 1951). Therefore, N_e holds a direct influence on the variances in the number of offspring produced, and consequently, on the recruitment and spawning potential of the species been harvested (see box 1for methods to estimate N_e) .

Box 1. Current methods in estimating effective population size and reported values in major commercial marine fish species

The effective population (N_e) size is an important parameter towards conservation and management purposes. However, for wild marine populations (N_e) is notoriously difficult to estimate because of the highly stochastic nature of genetic and demographic processes affecting the N_e of a population (i.e. inbreeding, genetic drift, sex ratio, variance in reproductive success and mating system) (Engen *et al.* 2007; Wang & Whitlock 2003). Various methods to estimate N_e are currently available and can be broadly subdivided into temporal and non-temporal methods. Overall, temporal methods (Jorde & Ryman 1995; Nei & Tajima 1981; Waples 1989) measure the standardized variance in the temporal changes of allele frequencies from samples taken at different time periods. In the absence of mutation, selection and migration processes, the observed change in allele frequencies comes solely from genetic drift and can thus be used in estimating N_e (Wang 2005). Temporal methods also assume single discrete populations, no gene flow into the population and that samples were taken randomly from a single generation. However, these assumptions are unlikely to be valid for most wild populations. In species with overlapping generations and samples from different age classes, temporal changes in allele frequencies are not only affected by genetic drift but by the genetic differences between age classes (Jorde & Ryman 1995). Furthermore, temporal methods based on moment estimators tend to overestimate N_e when genetic drift is strong and when markers with high allelic diversity are used, resulting in bias and imprecision, especially when the sampling interval is short (Wang 2005). Recent probability methods have been developed to improve N_e estimates from temporal methods (Anderson *et al.* 2000; Beaumont 2003; Berthier *et al.* 2002; Wang 2001). Such methods rely on maximum-likelihood or Markov Chain Monte Carlo re-sampling techniques for probability calculation. The main advantages of probability methods over moment-based methods reside in that information from different temporal samples can be weighed for different sample sizes, the use of the whole data set instead of the standardized variance of allele frequencies and a flexible demographic model that can be easily modified to allow joint estimation of other parameters of interest such as population growth and migration rates (Beaumont 2003; Berthier *et al.* 2002; Wang & Whitlock 2003).

Non-temporal methods, on the other hand may be derived from heterozygosity excess and from linkage disequilibrium (Hill 1981; Luikart & Cornuet 1999). Both methods assume that neither mutation or selection processes are acting on a single isolated population in equilibrium (i.e. random mating) from which the samples originated. Thus, for neutral unlinked loci, heterozygosity excess and linkage disequilibrium come exclusively from genetic drift and can be used to infer *Ne*. These methods are only applicable to a single cohort and thus the assumption of no mutation or selection can be relaxed since only one generation is considered and markers are neutral (Waples 1991). However, when the assumption of a single isolated population is violated *Ne* can be underestimated because in addition to drift, migration-immigration processes can produce heterozygosity excess and linkage disequilibrium in finite populations. Other non-temporal methods estimate *Ne* from current genetic variation information. The underlying principle is that at drift-mutation equilibrium the amount of genetic variation is constant and can be determined by a single quantity $\theta = 4N_e\mu$ for diploid populations where μ is the mutation rate per generation per locus (Wang 2005). Bayesian and Maximum-likelihood are the most commonly used approaches to estimate θ (Beaumont & Rannala 2004; Beerli 2006).

Box 1 Continued

Species	Sampling Period	Method	Maker	***Ne (confidence interval)***	Reference
Cod (*G.morhua*)	1965,2002	Waples (1989) Wang (2001) Berthier *et al.* (2002)	Microsat	1068 (423-infinity**) 2067 (651–10 000**) 739 (412–973*)	Poulsen *et al.* (2006)
Cod (*G.morhua*)	1928,1997	Waples (1989) Wang (2001) Berthier *et al.* (2002)	Microsat	844 (428–2353**) 1193 (605–4680**) 766 (511–978*)	Poulsen *et al.* (2006)
Cod (*G.morhua*)	1954-1960 1960-1970	Waples (1989) Waples (1989)	Microsat Microsat	69 (30–222**) 121 (51–426**)	Hutchinson *et al.* (2003)
Flatfish plaice (*P.platessa*)	1950,1970 2002 1924,1948 1972,2002	Wang (2001) Wang (2001)	Microsat Microsat	19 535 (3435-70 000**) 1733 (1063-3598**)	Hoarau *et al.* (2005)
Mackerel (Scomber scombrus) Chub mackerel (*Scomber japonicus*)	2000,2001 1997 2000 2001	Beerli & Felsenstein (2001) Beerli & Felsenstein (2001)	MtDNA MtDNA	44 000 - 580 000** 180 000 – 610 000** 140 000 – 6 200 000** 290 000**	Zardoya *et al.* (2004)
Red Drum (*Sciaenops ocellatus*)	1986-1989	Waples (1989) Wang (2001) Beerli & Felsenstein (2001)	Microsat	2365 (833-infinity**) 3516 (1785-18148**) 1853**	Turner *et al.* (2002)
Chinook salmon (*Oncorhynchus tshawytscha*)	1978-1998	Waples (1989) Beerli & Felsenstein (2001)	Microsat	256.55§ 845.76§	Shrimpton & Heath (2003)
Sea trout (*Salmo trutta* L.)	1997,1998	Jorde & Ryman (1995)	MtDNA	< 30§	Laikre *et al.* (2002)
New Zealand snapper (*Pagrus auratus*)	1950-1986	Waples (1989) Nei (1975)	Microsat	176 (80-720**) 46	Hauser *et al.* (2002)

* 90% confidence interval. ** 95% confidence interval. § mean values within 95% confidence interval

Gene flow is another key parameter to marine fisheries since it provides the basis to identify the existence of sub-populations or groups of populations within the fishing area. However, a precise identification of population structure is more complex that previously recognized (Waples & Gaggiotti 2006). In the marine environment, the absence of obvious barriers to adult dispersal commonly allows individuals to intermix throughout the species range. In addition, physical processes such as winds and currents may favour passive transport of early-life stages such as eggs and larvae, away from their spawning location (Bostford *et al.* 2001; Richards *et al.* 2007; Underwood *et al.* 2007). Similarly, it has been demonstrated that even highly mobile marine organisms are capable of forming discrete stocks or populations within small areas despite the apparent connectivity of the oceans (Jorgensen *et al.* 2005; Knutsen *et al.* 2003). Consequently, these populations possess independent demographic parameters such as recruitment, mortalities and abundances. Hence, they ought to be regarded as unrelated breeding units or stocks for conservation and management strategies. Genetically distinct populations may also intermix during feeding or migration periods. However, these populations may be affiliated to different spawning locations, and thus, not conforming a single panmitic assemble within the same area (Lundy *et al.* 1999). Furthermore, populations may be geographically confined to certain areas allowing the accumulation of locally adapted traits and genetic diversity. Such populations could be at risk of loosing genetic diversity if adverse human-induced activities (i.e. overfishing) take place in that particular area (Grant *et al.* 1999). Nonetheless, local adaptation (i.e. change in a gene pool that occurs as a response to selective forces set up by local environmental factors) is difficult to detect in wild marine fish populations given the gene flow levels encountered in marine fish species (Conover *et al.* 2006). Most evidence of local adaptations in marine fish steams from common-garden experiments, in which the environmental factor that might have contributed to adaptation is controlled for. In such approaches, specimens from different locations for which phenotypic differences have been detected in the wild are reared simultaneously. In this way, any measurable quantitative

variance (Q_{ST}) between populations will reflect genetic adaptive variance (McKay & Latta 2002; Saether *et al.* 2007). Furthermore, Q_{ST} values can be compared with the variance measured at neutral genetic markers (F_{ST}) in order to disentangle the phenotypic variation induced by the environment from the variation at phenotypic traits that has a direct genetic basis (ICES 2006). Similarly, gene flow estimation can aid in monitoring re-stocking programs as well as in the tracking of invasive species (McQuaid & Phillips 2000). For all these reasons, it becomes apparent that genetic subdivision patterns in the marine environment must be carefully evaluated in order to make informed decisions towards resource management.

Genetic subdivision of a particular species within an area results from a significant reduction of gene flow occurring in that area. The Mediterranean is a semi-enclosed Sea where genetic differentiation at the geographical level has been reported for several taxa at particular areas. Numerous genetic studies have provided evidence that different biogeographic boundaries exist within the Mediterranean Sea as well as between the Mediterranean and the adjacent Atlantic Ocean (Alvarado Bremer *et al.* 2005; Bargelloni *et al.* 2005; Baus *et al.* 2005; Charrier *et al.* 2006; Cimmaruta *et al.* 2005; Grant 2005; Mattiangeli *et al.* 2004; Pujolar *et al.* 2003; Reuschel & Schubart 2006; Zardoya *et al.* 2004). The majority of studies have pointed at environmental conditions as the main cause of biogeographic boundaries occurrence. Therefore, knowledge of the genetic partitioning of species whose distribution ranges fall across environmentally heterogeneous areas is of major significance when devising conservation-management approaches. This is particularly true for demersal fish species where the absence or imprecise information could lead to overexploitation and subsequent resource collapse (Myers & Worm 2003). Furthermore, the great variety of population structure patterns displayed by demersal fish underscores the need for the identification of common gene flow patterns that can be found at the largest possible geographical extent. To this end, allele-frequency variation at 10

microsatellite loci of two co-occurring demersal fish, the Red mullet (*Mullus barbatus*) and its congener the Striped red mullet (*Mullus surmuletus*) was analysed.

M.barbatus and *M.surmuletus* are two of the main targets of the bottom trawl multi-species fishery in the Mediterranean Sea (Stergiou *et al.* 1997). Both species are frequently fished simultaneously with European hake (*M. merluccius*) by trawlers and a range of other small scale fishing vessels using a variety of gears (Martin *et al.* 1999). *M.barbatus* is now considered as overexploited or fully exploited throughout the Mediterranean, but no reliable assessments are currently available for *M.surmuletus* (FAO 2005). Within the Mediterranean no general quota systems are currently implemented for these species. Each country establishes its own regulations (i.e. fishing effort, age at first capture) according to the general recommendations proposed by the Food and Agriculture Organization of the United Nations (FAO). Despite their high economic value, most genetic studies have focused in taxonomical and intra-specific relationships (Mamuris *et al.* 1998a; Mamuris *et al.* 1999a; Mamuris *et al.* 1999b), whereas only a few population genetic studies have been performed within a small portion of their distribution ranges suggesting the existence of isolated populations of both species (Garoia *et al.* 2004; Mamuris *et al.* 1998b). Both Mullets co-occur over muddy and rocky substrates along the littoral zone (Hureau 1986) but differ in a number of life history traits. Both species have long larval phases (>30 d). However, both the eggs and larvae of *M.surmuletus* are pelagic, whereas only the postlarvae of *M.barbatus* displays a pelagic stage (Mamuris *et al.* 1998a). Both species are found at depths of less than 100 m, but the range of *M.barbatus* extends down to around of 300 m. (Hureau 1986; Özbilgin *et al.* 2004). Furthermore, there is no evidence of adult migratory behaviour in any of the species and it has been demonstrated that both species segregate their feeding niche by consuming different prey taxa (Labropoulou & Eleftheriou 1997). Based on the general biology of the two species, it could be expected that *M.surmuletus* would show lower levels of genetic differentiation than *M.barbatus* over comparable distances due mainly to the pelagic nature of its eggs and larvae. Thus, these

species represent a good opportunity to study gene flow patterns of closely related demersal species displaying slight differences in life history traits across a range of environmental conditions. The specific aims are to test (i) whether the patterns of population structure are the same for the two species, (ii) if any differences in population structure can be explained by their different life histories, (iii) if the observed patterns of genetic subdivision differ from those of other Mediterranean demersal species. These hypotheses are discussed in relation to environmental features of the Mediterranean Sea and in terms of conservation and management of the current stocks of these species.

3.2 MATERIALS AND METHODS

3.2.1 Sample collection and genetic analysis

During the period of 2003-2005, a total of 240 *M.surmuletus* and 222 *M.barbatus* adults were sampled in nine different locations from the eastern Atlantic Ocean and Mediterranean Sea (Fig. 13). Samples were obtained from commercial trawls as well as through local fisherman and by Scuba diving. Numbers of individuals per sample and species are listed in table 9. Geographic distances between sampling sites were estimated as the shortest coastline path between any two sites using the Mapping toolbox included in MATLAB v.7. (Mathworks). DNA extractions were performed from pectoral fin and barbel tissue following HotSHOT protocol (Truett *et al.* 2000). Ten microsatellite loci were successfully amplified by polymerase chain reaction as described in (Galarza *et al.* 2006). Amplified products were resolved on an ABI3100 Genetic Analyzer (Applied Biosystems). Allele scoring was carried out using GeneMapper v. 3.5 software (Applied Biosystems).

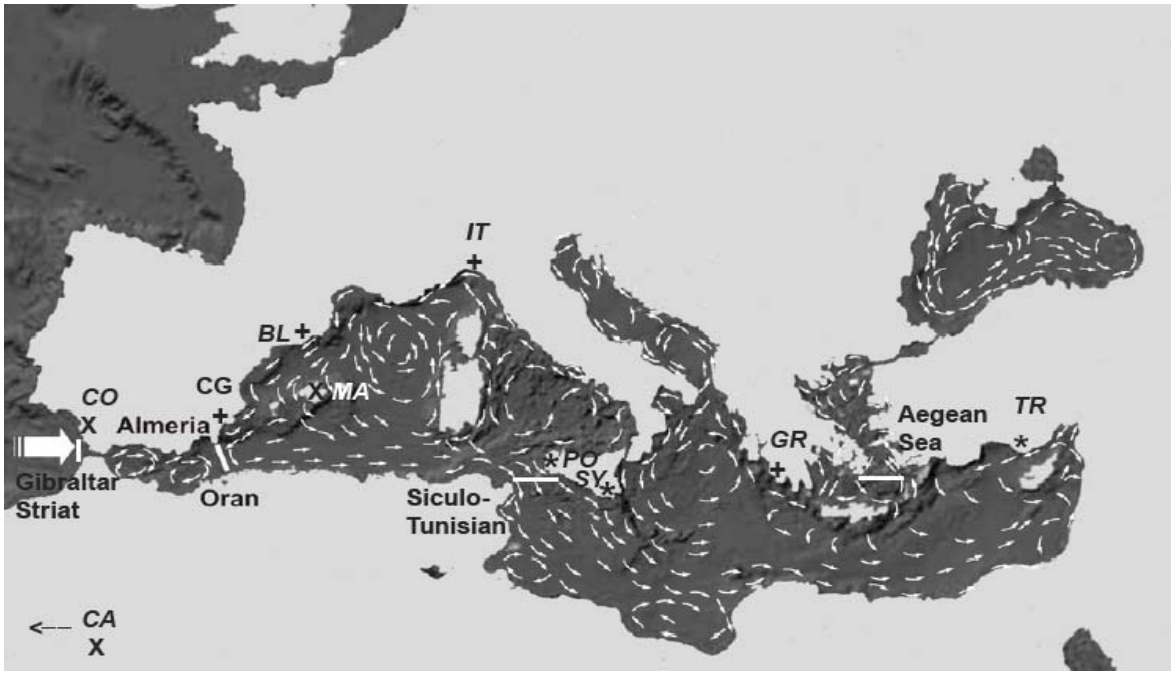


Fig.13 Sampling locations of *Mullus ssp*. from the Atlantic Ocean and the Mediterranean Sea. ***CA***=Canary Islands, ***CO***=Conil, ***CG***=Cabo de Gata, ***MA***=Mallorca, ***BL***=Blanes, ***IT***=Italy, ***PO***=Porticello, ***SI***=Syracuse, ***GR***=Greece, ***TR***=Turkey. X indicates *Mullus surmuletus* samples, * indicates *Mullus barbatus* samples, + indicates both species. Mediterranean major surface current patterns and major oceanographic boundaries are represented by arrows and solid lines respectively. Redrawn from Alhammoud *et a.l* (2005) and (Millot 1999).

3.2.2 Microsatellite Data Analysis

The total number of alleles per locus and sample was obtained using GENETIX v.4.01 (Belkhir *et al.* 1997). The program FSTAT v. 2.9.3 (Goudet 1995) was used to determine allelic richness, which quantifies the number of alleles independently of sample size. Observed and expected heterozygosity were calculated using the software package Arlequin v. 2.0 (Schneider *et al.* 2000). Deviations from Hardy-Weinberg expectations (HWE) were estimated according to the level of significance determined by means of 10,000 MCMC iterations using GENEPOP v.3.4. (Raymond & Rousset 1995). Bonferroni corrections were applied for multiple comparisons setting a significance level of 0.05 (Rice 1989).

3.2.3 Population Structure

The null hypothesis of non-differentiation of allelic and genotypic distributions across all sample pairs within the sampling area was tested. Significance of both tests was obtained by 10,000 iterations executed in GENEPOP v.3.4. (Raymond & Rousset 1995). To estimate the overall effects of gene flow among sampling locations, pairwise F_{ST}s and their estimated probabilities were calculated by 10,000 permutations using GENETIX v.4.02 according to (Weir & Cockerham 1984). The alternative hypothesis of significant genetic differentiation was accepted if the probability was equal or less than 0.05. Sequential Bonferroni corrections were applied for multiple tests (Rice 1989).

3.2.4 Spatial Structure

The R package GENELAND (Guillot *et al.* 2005) was used to test for the existence of genetically differentiated gene pools or populations across the sampling area based on geographical information and without predefined sampling pairs. The program makes use of a geographically constrained Bayesian model that explicitly takes into account the spatial position of sampled multilocus genotypes without any prior information on the number of

populations and degree of differentiation between them. Geographical coordinates were assigned to each individual by randomly choosing *n* spatial locations encompassed within a 10 km. radius from each of the sampling locations using the Mapping toolbox in MATLAB v.7. (Mathworks), where *n* equals the number of individuals/species sampled at a given location. The inference algorithm was launched the using the Dirichlet distribution as prior for allele frequencies with 20,000 MCMC iterations using spatial information.

Association between spatial locations and genetic similarities was tested through different approaches. The software package MSAnalyzer v.4.05 (Dieringer & Schlötterer 2003) was used to calculate inter-population genetic distance based on the proportion of shared alleles (D_{ps}) for all loci between all sample pairs (Bowcock *et al.* 1994). Distance matrices were then used to construct a neighbour-joining tree (Saitou & Nei 1987) with the program Neighbour in Phylip v.3.65 (Felsenstein 2004). Bootstrap values were calculated over 1000 replicates (SEQBOOT procedure of Phylip). Assuming that shared alleles result from common ancestry, the topology of the tree should reflect the connections of populations through recent dispersal events. The software MSAnalyzer v.2.39 (Dieringer & Schlötterer 2003) was also used to compute Cavalli-Sforza and Edwards' genetic distance (*Dc*) among population samples. This measure differs from D_{ps}, in the way that populations are represented as points in a multidimensional plane and the genetic distance between two points is measured by the geometric distance between the corresponding points in the space (Cavalli-Sforza & Edwards 1967). Simulation studies have shown that *Dc* has a linear relationship with time when the time since divergence between populations is long as well as a low coefficient of variation when highly polymorphic markers are used (Kalinowski 2002). Therefore, it may be more appropriate for inferring genetic divergence over a longer time-scale. A multidimensional scaling (MDS) analysis of *Dc* was performed to examine the population's genetic relationships on a two dimensional plane using MATLAB v.7.0 (Mathworks). Multidimensional scaling is a non-metric ordination technique that depicts genetic relationships among populations in two or three dimensions and can reduce the

distortion that may exist in phenograms because populations are represented in fewer dimensions. When genetic relationships among populations reflect non-hierarchical or semi-hierarchical pattern (i.e. weak population structure) multidimensional scaling diagrams are often a more effective means of showing these relationships than agglomerative clustering techniques (Lessa 1990).

Finally, a genetic spatial autocorrelation analysis was performed in GenAlEx v.6 (Peakall & Smouse 2006). This multivariate procedure calculates the autocorrelation coefficient (*r*) between the population's genetic distances (F_{ST}) as a function of geographic distance classes. Statistical significance for the null hypothesis of no spatial structure was determined by creating 95 percent confidence intervals around *r*-values through 999 random permutations. If the calculated *r*-value falls outside these confidence intervals, then populations in that class are more similar than would be expected by chance and thus, significant spatial genetic structure is inferred. Further, the first intercept of the *r*-value with the *x*-axis represents the largest geographic distance at which populations become genetically independent. This distance has been interpreted as the average distance of gene flow per generation (Sokal & Wartenberg 1983), often referred to as neighborhood or genetic patch size (Gold & Turner 2002; Laikre *et al.* 2005; Palumbi 2004; Peakall *et al.* 2003).

3.2.5 Genetic demography

Effective population size (*Ne*) and gene flow rates between populations were estimated through a Markov Chain Monte Carlo (MCMC) maximum-likelihood approach based on coalescent theory as implemented in MIGRATE v.2. (Beerli & Felsenstein 1999). This method, unlike other methods that measure the standardized variance in the temporal changes of allele frequencies (i.e. (Jorde & Ryman 1995; Nei & Tajima 1981; Waples 1989), performs parameter inference from the current genetic variation information. The underlying principle is that at drift-mutation equilibrium, the amount of genetic variation is

constant and can be determined by a single quantity $\theta = 4N_e\mu$ for diploid populations where μ is the mutation rate per generation per locus (Beerli & Felsenstein 2001). When independent information about μ is available, N_e can be obtained from this estimate. Similarly, when μ is unknown, we can still get relative N_e estimates from different populations when the same markers are used to estimate θ (Wang 2005).

The program was run with the microsatellite model set to a threshold value of 30 for *M.barbatus* and 50 for *M. surmuletus*. This value refers to the maximum difference (in base pairs) between the smallest and the largest alleles. Probabilities of mutations are thus considered only within this range. Fifteen short chains (20,000 genealogies each) and five long chains (250,000 genealogies each) were recorded with a burn-in period of 35,000 genealogies. Further, we used the program STRUCTURE v.2 (Pritchard *et al.* 2000) to identify individuals of immigrant ancestry in the preceding generation. The program allows geographic information to be incorporated into the model and uses a Bayesian framework to compute the probability that an individual has at least one parent in another population (*Q*). Inference was performed with the USEPOPINFO option turned on and *K* set equal to the number of sampling locations (5 for *M.barbatus* and 7 for *M. surmuletus*). GENSBACK was set to 1 and MIGPRIOR was assigned a value of 0.1. Run length consisted of a burn-in period of 330,000 MCMC chains and 1,000,000 MCMC repeats after burn-in.

3.3 RESULTS

3.3.1 Microsatellite variability

The total number of alleles per locus ranged from 2 to 27 in *M.barbatus* and from 2 to 21 in *M. surmuletus* (Table 9). Expected heterozygosity and overall genetic variability as judged by allelic richness were slightly higher in *M.barbatus* than in *M. surmuletus* (Table 9). No of linkage disequilibrium was found between locus pairs. Significant deviations from Hardy-Weinberg expectations within sampling locations were observed in both species. Out of 140 tests performed across both species 47 remained significant after Bonferroni correction. Departures from Hardy-Weinberg expectations may be caused by several factors such as inbreeding, population sub-structuring (i.e Wahlund effect) and the presence of null alleles caused by technical issues. Inbreeding or population sub-structuring should be reflected in consistent deviations across most or all loci. Whereas null alleles caused by technical causes such as misscoring or poor amplification should result in variable deviations across loci and populations (Purcell *et al.* 2006). The program MICRO-CHECKER (van Oosterhout *et al.* 2004) was used to infer the most probable cause of departures from Hardy-Weinberg expectations. No evidence of large allele dropout at any locus could be observed. However, for locus Mb14, the results indicated possible scoring errors due to stuttering as suggested by an excess of large homozygote classes. This was confirmed by an in-depth examination of genotype plots of this locus. Therefore, locus Mb14 was removed from all subsequent analysis. The rest of loci showed random patterns of deviation across populations of both species. Moreover, MICRO-CHECKER indicated a general excess of homozygotes in most allele size classes. This suggests the presence of null alleles for those loci that showed significant departures from Hardy-Weinberg expectations. Multiplex PCR techniques could have contributed to the presence of null alleles by forcing simultaneous amplification of different loci at common annealing temperatures. The effect that such null alleles could have in the estimates of population

differentiation was assessed by two different approaches. First, a re-run of the F_{ST} permutation test was performed excluding those loci that presented evidence for null alleles. Second we estimated null allele frequencies for each locus and population using the program FreeNA (Chapuis & Estoup 2007). Frequencies corrected for null alleles were then used to compare F_{ST} estimates from corrected vs. non-corrected data sets as implemented in FreeNA (Chapuis & Estoup 2007). Both approaches showed similar results with changes in the magnitude of F_{ST} estimates but the pattern of population differentiation did not show significant differences by either approach. Finally, due to the relatively low number of individuals, and in order to avoid statistical sample-size artefacts, the two *M.barbatus* samples from Porticello and Syracuse (MbPO and MbSY, n=15 for each) were not included in further analyses. However, F_{ST} value for this population pair was highly significant (Data not shown).

Table 9. Summary statistics of ten microsatellite loci among sampling collections of *Mullus barbatus* and *Mullus surmuletus*. Species and sampling location; *n*, number of individuals, *a*, number of alleles; *Rs*, allelic richness; H_E, expected heterozygosity; Ho, Observed heterozygosity; F_{IS}, inbreeding coefficient (Weir & Cockerham, 1984). Asterisk represent significant *Fis* at α = 0.05. Significant values after Bonferroni correction are presented in bold italics

	Locus										Overall
Specie/sample	Mb3	Mb11	Mb14	Mb28	Mb46	Mb55	Mb63	Mb130	Mb132	Mb133	Loci
M.barbatus Cabo de Gata (MbCG)											
n	48	46	44	48	45	48	48	48	48	48	
a	17	9	31	2	7	12	11	11	10	12	12.2
Rs	12.823	6.254	19.014	1.699	6.41	9.715	8.919	7.259	8.223	8.557	8.887
He	0.909	0.600	0.953	0.063	0.815	0.867	0.852	0.714	0.808	0.796	0.738
Ho	0.383	0.520	0.911	0.065	0.733	0.833	0.729	0.307	0.717	0.729	0.593
Fis	***0.586****	0.113*	***0.563****	-0.023	0.111	0.033	0.135*	***0.567****	0.124	0.095	0.230
M.barbatus Blanes (MbBL)											
n	37	35	33	37	34	37	37	37	37	37	
a	15	11	16	3	6	15	11	7	11	10	10.5
Rs	13.457	9.463	14.996	2.845	5.776	13.063	9.916	5.987	10.472	8.755	9.473
He	0.916	0.826	0.915	0.332	0.734	0.893	0.845	0.723	0.856	0.8138	0.785
Ho	0.565	0.810	0.157	0.400	0.421	0.730	0.750	0.680	0.526	0.5652	0.542
Fis	***0.402****	0.004	***0.336****	-0.192	***0.448****	0.201*	0.196*	0.197*	***0.408****	***0.325****	0.233
M.barbatus Italy (MbIT)											
n	46	47	44	48	42	48	48	48	48	48	
a	19	8	27	4	7	13	14	14	8	16	13
Rs	13.792	5.902	16.499	2.401	6.661	11.383	9.554	10.204	6.988	9.798	9.318
He	0.914	0.585	0.934	0.109	0.743	0.894	0.849	0.845	0.722	0.817	0.741
	0.954	0.570	0.795	0.113	0.671	0.704	0.604	0.642	0.750	0.781	0.645
Fis	-0.033	0.003	***0.460****	-0.031	0.206*	0.224*	***0.299****	***0.251****	-0.027	0.178*	0.153
M.barbatus Porticello (MbPO)											
n	15	15	13	15	13	15	15	15	15	15	
a	10	5	8	3	6	5	7	3	6	8	6
Rs	12.194	5.925	13.362	1.748	4.295	10.467	7.891	7.465	4.297	8.437	7.608
He	0.875	0.656	0.836	0.225	0.723	0.653	0.704	0.375	0.777	0.820	0.664
Ho	0.833	0.566	0.428	0.183	0.647	0.485	0.714	0.291	0.888	0.700	0.511
Fis	0.091	0.165*	***0.544****	0.256*	0.242*	***0.313****	-0.063	0.07	-0.085	0.197*	0.173

M.barbatus Syracuse (MbSY)											
n	15	15	14	15	12	15	15	15	15	15	
a	8	3	5	2	4	3	2	4	2	3	3.3
Rs	12.253	4.734	13.785	1.948	4.729	10.634	6.925	7.552	4.893	8.681	7.613
He	0.846	0.611	0.777	0.218	0.533	0.682	0.632	0.726	0.593	0.625	0.624
Ho	0.8571	0.533	0.665	0.173	0.382	0.674	0.593	0.705	0.439	0.573	0.546
Fis	-0.065	0.210*	0.333*	***0.297****	***0.232****	0.048	0.02	0.092	0.198*	0.2	0.157
M.barbatus Greece (MbGR)											
n	48	48	45	47	39	48	48	48	45	48	
a	20	12	27	3	7	10	9	9	7	12	11.6
Rs	13.2	4.307	17.909	2.756	6.826	8.061	9	6.707	4.127	11.017	8.391
He	0.926	0.782	0.942	0.043	0.682	0.877	0.822	0.832	0.727	0.850	0.748
Ho	0.914	0.750	0.761	0.044	0.679	0.777	0.500	0.434	0.742	0.736	0.646
Fis	0.028	0.055	***0.360****	-0.006	0.0082	0.127	***0.319****	***0.447****	-0.007	0.146	0.148
M.barbatus Turkey (MbTR)											
n	48	43	48	48	41	48	48	48	48	48	
a	16	5	22	3	8	9	9	9	5	13	9.9
Rs	15.039	7.548	19.263	2.619	7.486	11.225	9.801	8.389	8.77	10.095	10.023
He	0.904	0.531	0.939	0.178	0.825	0.829	0.837	0.687	0.477	0.788	0.699
Ho	0.817	0.500	0.770	0.192	0.800	0.652	0.710	0.461	0.615	0.666	0.609
Fis	0.121*	0.077	0.222*	-0.059	0.051	***0.235****	0.115	***0.367****	-0.272	0.178*	0.104
M.surmuletus Canary Is. (MsCA)											
n	48	48	48	48	45	47	48	48	48	48	
a	5	12	20	3	6	8	14	16	10	10	10.4
Rs	2.078	4.619	5.865	2.26	2.579	3.777	5.793	4.874	5.041	4.78	4.166
He	0.287	0.761	0.881	0.390	0.526	0.737	0.891	0.777	0.839	0.805	0.689
Ho	0.243	0.657	0.833	0.206	0.521	0.688	0.674	0.200	0.818	0.310	0.497
Fis	0.166*	0.112	0.067	***0.213****	0.02	0.077	***0.254****	***0.748****	0.017	***0.625****	0.230
M.surmuletus Conil (MsCO)											
n	30	30	30	30	27	30	30	29	30	30	
a	7	11	16	3	6	6	13	10	11	14	9.7
Rs	2.79	4.862	5.729	2.107	3.284	3.606	4.527	4.707	5.181	5.511	4.230
He	0.478	0.880	0.877	0.509	0.873	0.718	0.734	0.78	0.846	0.867	0.726

	0.342	0.862	0.676	0.414	0.819	0.583	0.625	0.206	0.735	0.342	0.489
Fis	0.197*	0.078	***0.243****	0.195*	0.133	0.202*	0.164	***0.743****	0.146	***0.614****	0.262
M.surmuletus Cabo de Gata (MsCG)											
n	46	45	48	48	44	48	48	48	48	48	
a	7	18	17	2	5	7	16	15	10	17	11.4
Rs	2.963	6.029	5.923	1.251	2.424	3.434	5.481	5.219	4.712	5.749	4.318
He	0.560	0.901	0.892	0.065	0.524	0.703	0.868	0.834	0.816	0.881	0.704
Ho	0.500	0.439	0.681	0.068	0.452	0.558	0.750	0.314	0.690	0.428	0.488
Fis	0.119	***0.224****	***0.246****	-0.024	0.15	0.218*	0.147*	***0.632****	0.166	***0.522****	0.240
M.surmuletus Blanes (MsBL)											
n	48	48	48	48	44	48	48	48	47	48	
a	8	21	17	3	4	9	17	13	9	11	11.2
Rs	2.711	6.371	6.087	1.57	2.611	3.903	5.583	4.928	4.179	5.338	4.328
He	0.4933	0.919	0.905	0.160	0.579	0.748	0.8684	0.7992	0.7401	0.8612	0.707
Ho	0.4667	0.658	0.711	0.173	0.533	0.760	0.7111	0.3684	0.8261	0.2889	0.549
Fis	0.065	0.195*	0.126*	-0.073	0.091	-0.006	0.192*	***0.548****	-0.105	***0.671****	0.180
M.surmuletus Mallorca (MsMA)											
n	48	48	48	48	42	48	48	48	48	41	
a	8	11	17	3	5	10	18	14	12	21	11.9
Rs	2.733	4.517	6.114	1.756	2.373	3.731	6.423	4.727	5.416	6.502	4.429
He	0.458	0.744	0.908	0.219	0.501	0.706	0.924	0.783	0.868	0.925	0.704
	0.326	0.483	0.466	0.177	0.278	0.500	0.670	0.488	0.566	0.785	0.550
Fis	***0.299****	***0.364****	***0.495****	0.199*	***0.358****	***0.203****	***0.211****	***0.387****	***0.248****	0.163*	0.293
M.surmuletus Italy (MsIT)											
n	48	48	45	48	46	48	48	48	48	48	
a	8	17	19	4	4	6	15	8	9	13	10.3
Rs	2.415	5.935	6.207	1.899	2.418	3.601	5.657	4.522	4.585	5.444	4.268
He	0.3833	0.887	0.9102	0.266	0.481	0.725	0.877	0.79	0.797	0.865	0.698
Ho	0.428	0.678	0.810	0.277	0.533	0.688	0.864	0.361	0.694	0.181	0.515
Fis	-0.104	***0.252****	0.123*	-0.029	-0.09	0.103	0.028	***0.579****	0.143	***0.716****	0.172
M.surmuletus Greece (MsGR)											
n	48	41	48	48	47	48	48	48	48	48	

a	5	21	3	2	4	6	17	19	9	13	9.9
Rs	2.156	6.385	2.079	1.59	2.454	3.679	5.483	5.874	4.073	5.974	3.974
He	0.3435	0.917	0.510	0.18	0.507	0.729	0.864	0.883	0.727	0.900	0.656
Ho	0.3590	0.862	0.978	0.200	0.434	0.714	0.682	0.689	0.714	0.580	0.586
Fis	-0.032	0.171	-0.916	-0.097	0.165*	0.033	***0.222****	***0.236****	0.032	***0.456****	0.027

3.3.2 Population structure

The exact tests for homogeneity of allelic and genotypic frequency distributions indicated that single discrete populations exist within the sampling area for both species. Both exact tests showed significant differences between all sample pairs (significant at $P < 0.0001$ after sequential Bonferroni correction) except for the Italian and Greek *M.barbatus* samples, and for the Italian and the Spanish samples of Blanes and Cabo de Gata of *M.surmuletus*. Population pairwise F_{ST} values were congruent with both exact tests. For *M.barbatus*, differentiation was observed between all population pairwise comparisons performed except for the Italian and Greek samples (Table 10). The majority of comparisons among *M.surmuletus* populations were also significantly differentiated, the exceptions being those between the Spanish samples from Cabo de Gata and Blanes, but more surprisingly between those two samples and the sample from NW Italy (Table 10). Furthermore, no significant differentiation was observed between the Atlantic samples form the Canary Islands and Conil (Table 10).

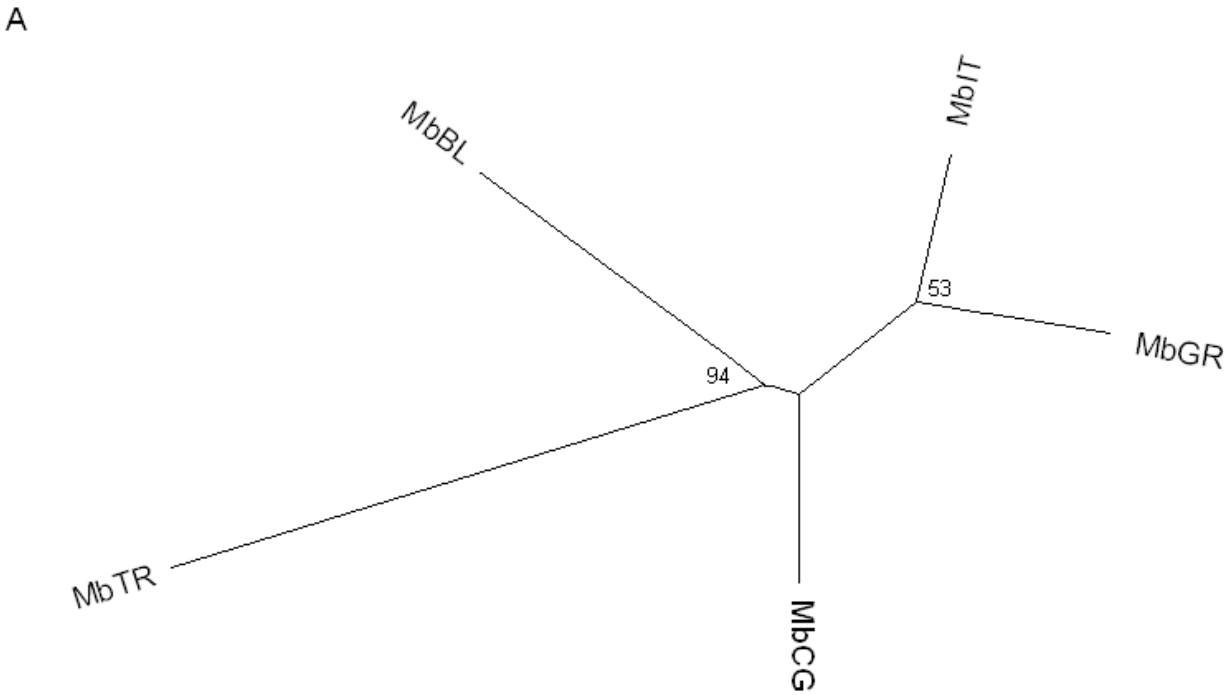
Table 10. Pairwise F_{ST} values (above diagonal) and geographic distances given in kilometres (below diagonal) among *M. barbatus* samples (a) and *M. surmuletus* samples (b). * Significant at $\alpha = 0.05$ after sequential Bonferroni correction.

a	MbCG	MbBL	MbIT	MbGR	MbTR
MbCG		0.022*	0.015*	0.021*	0.068*
MbBL	1086		0.031*	0.034*	0.079*
MbIT	1776	689		-0.006	0.080*
MbGR	3965	2879	2189		0.082*
MbTR	4998	3911	3222	1032	

b	MsCA	MsCO	MsCG	MsBL	MsMA	MsIT	MsGR
MsCA		0.0161	0.150*	0.153*	0.180*	0.152*	0.216*
MsCO	1175		0.086*	0.090*	0.140*	0.088*	0.156*
MsCG	1544	368		0.007	0.101*	0.017	0.066*
MsBL	2630	1455	1086		0.104*	0.019	0.067*
MsMA	2059	1069	700	385		0.105*	0.146*
MsIT	3320	2145	1776	689	1075		0.070*
MsGR	5510	4334	3965	2879	3264	2189	

3.3.3 Spatial structure

The Neighbour-joining tree based on the proportions of shared alleles showed different clustering patterns for both species. For *M.barbatus* the Turkish population (MbTR) was the most differentiated of this species, while the Italian (MbIT) and Greek (MbGR) populations showed moderate clustering (Fig. 14A). The two Atlantic populations of the *M.surmuletus* (MsCA and MsCO) clustered together showing the highest bootstrap support (Fig. 14B). The topology of the tree also indicated a clustering of the Italian (MsIT) *M.surmuletus* population with the Spanish populations from Cabo de Gata (MsCG) and Blanes (MsBL), as could be expected from their non-significant F_{ST} values.



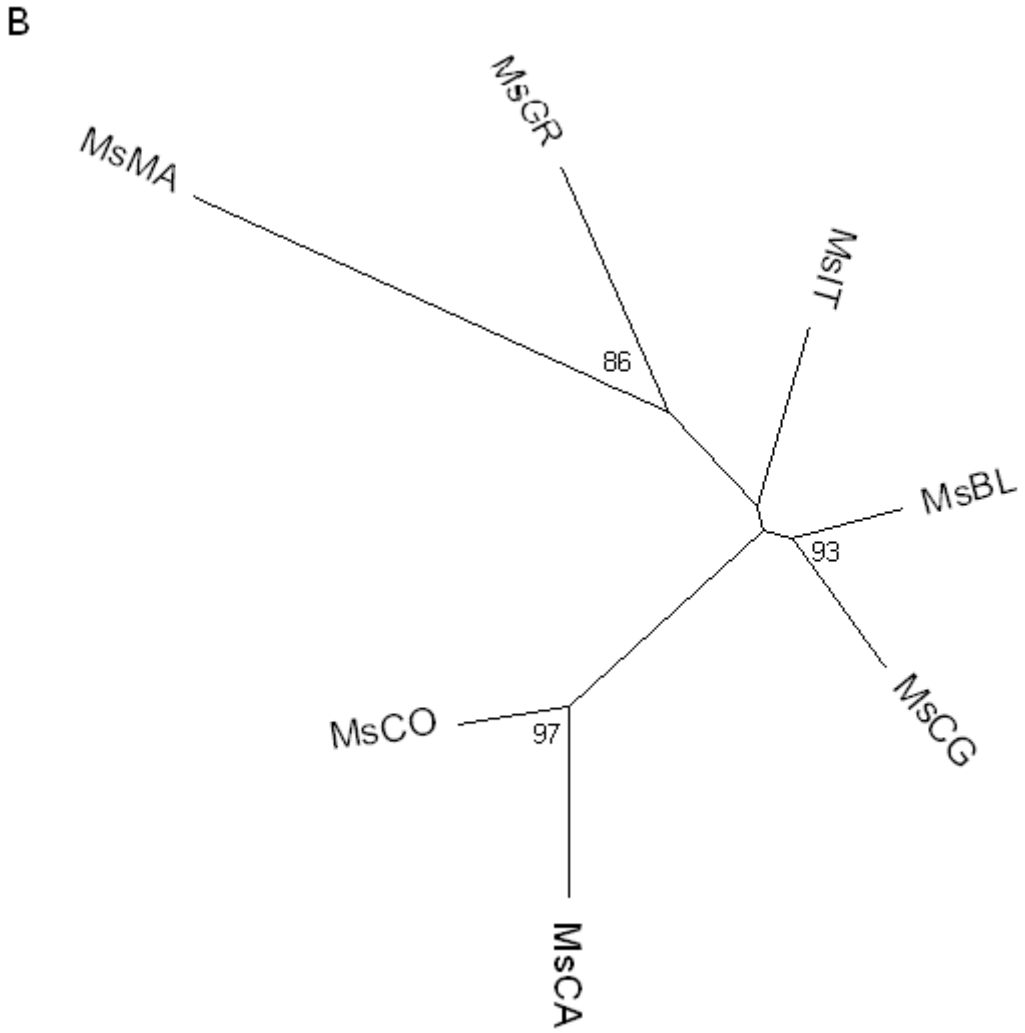


Fig. 14. Unrooted Neighbour-joining tree based on the proportion of shared alleles (Bowcock, 1994) between populations of *M.barbatus* (A) and *M.surmuletus* (B). Bootstrap values over 1000 replicates are shown when ≥ 50%. Population acronyms as in table 9.

MDS analysis showed a clear genetic grouping for *M.surmuletus* with the Atlantic populations of the Canary Islands and Conil (MsCA, MsCO) forming one group and the majority of the Mediterranean population samples Italy, Blanes and Cabo de Gata (MsIT, MsBL, MsCG) forming another. The Balearic population from Mallorca (MsMA) was the only exception to this pattern, whereas the Greek population (MsGR) clustered separately from the main Mediterranean grouping of this species (Fig. 15B). A more complex pattern was observed for *M.barbatus* with high genetic similarity displayed between the geographically distant Italian and Greek populations (MbIT, MbGR). Unsurprisingly, the

Turkish population (MbTR) was the most distinct, while Spanish population of Blanes (MbBL) clustered separately followed by Cabo de Gata (MbCG), which clustered relatively close to the Greek and Italian populations (Fig 15A).

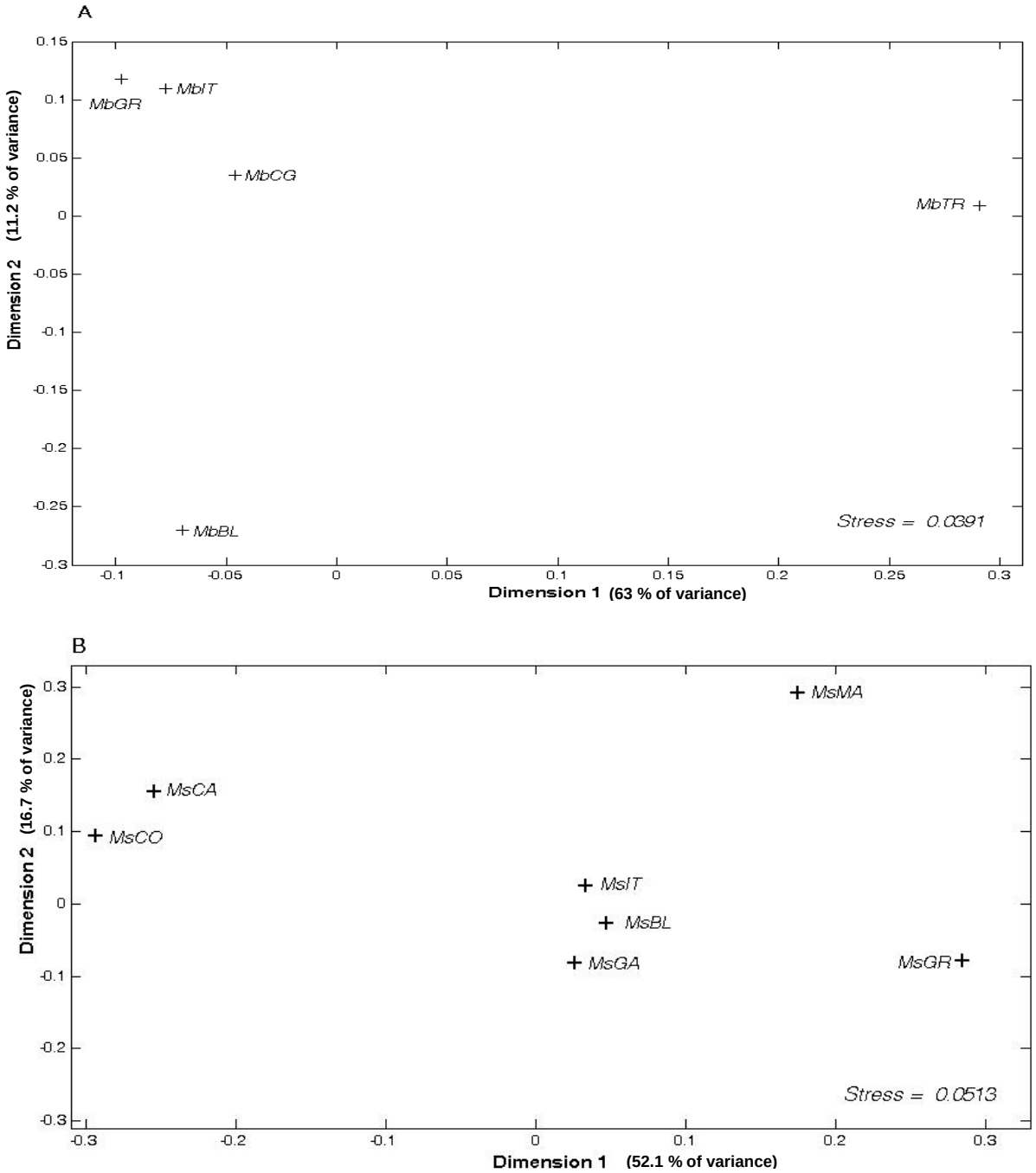


Fig. 15. Multidimensional scaling plot of population's genetic distance (*Dc*) for *M.barbatus* (A) and *M.surmuletus* (B).

Spatial autocorrelation analysis between populations revealed a significant positive genetic autocorrelation ($P < 0.05$) at the 1000 km distance classes for both species (Fig 16). No other significant autocorrelation was found for the rest of spatial distance classes in either species. The level of genetic autocorrelation decreased sharply for *M.barbatus* populations and more continuously for *M.surmuletus* with increased geographical distance. The correlogram curve first intercepted the *x*-axis at 1218 km for *M.barbatus* whereas for *M.surmuletus* it intercepted at 2357 km (Fig. 16A,B). This suggests a genetic patch size between 1000-1500 km for *M.barbatus* and 2000-2500 km for *M.surmuletus* (but see below).

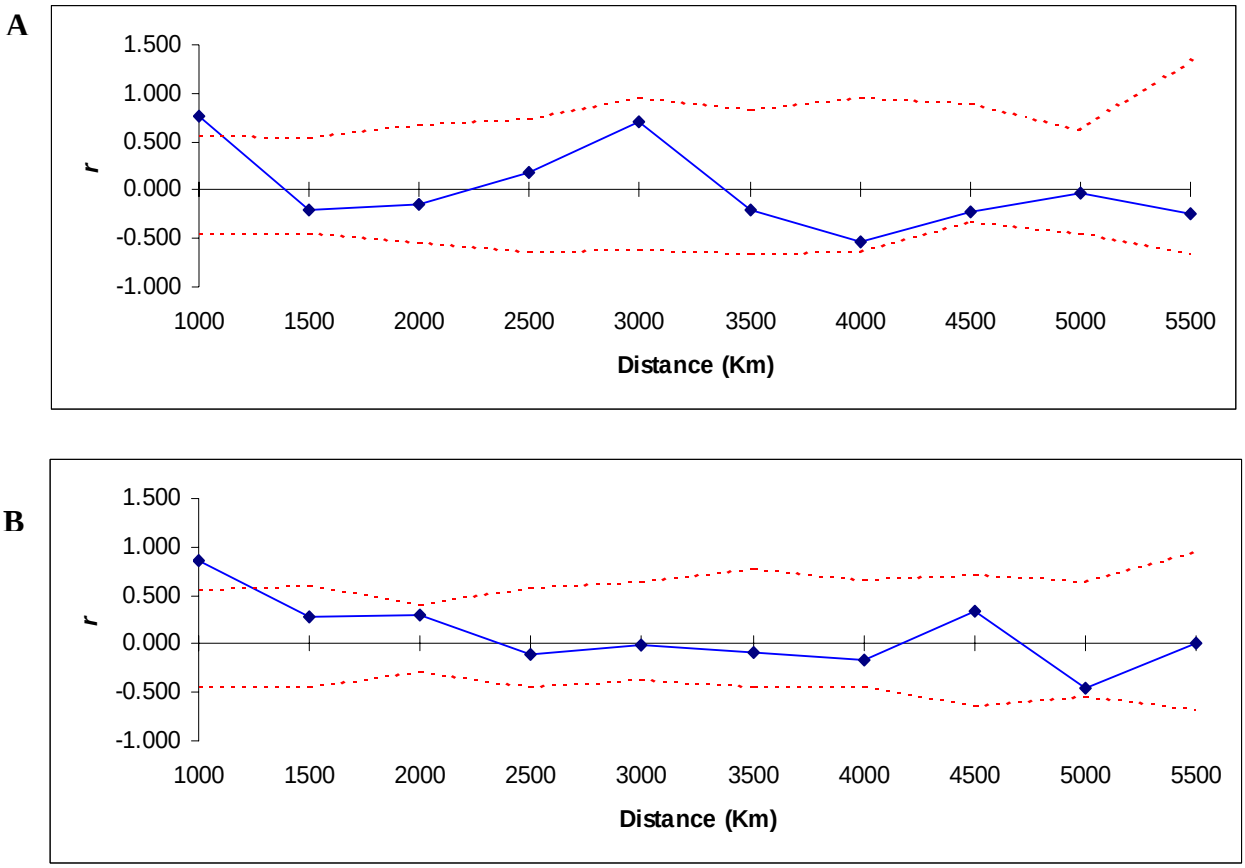


Fig. 16 Genetic autocorrelation (*r*) for *M.barbatus* (A) and *M.surmuletus* (B) populations as a function distinct geographic distance classes. Dotted grey lines indicate upper and lower 95% confidence intervals for the null hypothesis of no genetic structure as determined by bootstrapping.

3.3.4 Genetic demography

Absolute estimates of $\boldsymbol{\theta}$ were greater for *M.surmuletus* than for *M.barbatus*, while overall migration rates ($\mathbf{4}\boldsymbol{N_m}$) between populations were higher in *M.barbatus* (Table 11). None of the migration rates showed a clear symmetry in migrant exchange between populations of both species and no clear relationship between migration rates and potential transport current directionality could be observed except between populations of Cabo de Gata and Blanes for both species in which gene flow directionality occurred from Blanes to Cabo de Gata (Table 11, Fig 13). Within *M.barbatus,* the highest migration rates within species occurred between the nearest-neighbour populations with the exceptions of Italy (MsIT) that received most of its immigrants from Greece (MsGR) and vice versa. In *M.surmuletus,* in turn, the two Atlantic populations from the Canary Island (MsCA) and Conil (MsCO) showed the greatest migrant exchange between them, whereas the Mallorca (MsMA) population received most of its immigration was from Cabo de Gata (MsCG) rather than from the closer Blanes (MsBL) population (Table 11).

Table 11. Effective population (***Ne***) size and migration rates (4***Nm***) for *M.barbatus* (a) and *M.surmuletus* (b) populations. ‡Rows indicate receiving populations. All values within 95% confidence intervals.

a			4***Nm***‡				
Population	$\boldsymbol{\theta}[4N_e\mu]$	***Ne****	MbCG	MbBL	MbIT	MbGR	MbTR
MbCG	0.415	1.03875×10^3		30.632	18.452	17.045	13.212
MbBL	0.347	$.8696 \times 10^3$	25.597		19.765	15.701	12.684
MbIT	0.402	1.00737×10^3	20.893	28.667		51.170	22.069
MbGR	0.287	$.718675 \times 10^3$	26.212	15.352	53.100		26.101
MbTR	0.419	1.0485×10^3	13.786	15.013	19.881	22.367	

b			4***Nm***‡						
Population	$\boldsymbol{\theta}[4N_e\mu]$	***Ne****	MsCA	MsCO	MsCG	MsBL	MsMA	MsIT	MsGR
MsCA	0.571	1.429125×10^3		7.855	5.686	6.001	4.156	5.994	3.568
MsCO	0.705	1.763575×10^3	7.120		5.870	2.677	5.502	3.269	2.255
MsCG	0.713	1.7849×10^3	5.284	6.350		8.311	3.464	3.851	5.098
MsBL	0.809	2.0238×10^3	3.111	3.093	7.507		2.827	2.254	3.071
MsMA	1.075	2.689075×10^3	2.030	2.082	3.003	1.282		1.775	2.416
MsIT	0.574	1.4374×10^3	6.127	3.256	7.454	4.552	3.938		5.065
MsGR	0.759	1.89835×10^3	2.413	2.870	5.428	3.692	4.221	7.676	

*Assuming a mutation rate of 10^{-4} per generation/locus (Hendry *et al.* 2002).

Similarly, the test performed to estimate the probability that individuals from a given population have recent ancestry in another population showed an identical pattern to that observed in migration rates. Average posterior probabilities among population pairs (*Q*) indicate that recent migration events (preceding generation) are more likely to have occurred between those population pairs showing the highest migration rates (Table 12).

Table 12. Average posterior probability of having recent ancestry in another population (*Q*) among samples of *M.barbatus* (a) and *M.surmuletus* (b). Rows indicate receiving populations

a					
Population	MbCG	MbBL	MbIT	MbGR	MbTR
MbCG		0.0090	0.0051	0.0073	0.0072
MbBL	0.0079		0.0064	0.0066	0.0046
MbIT	0.0074	0.0046		0.0101	0.0021
MbGR	0.0072	0.0044	0.0085		0.0049
MbTR	0.0084	0.0057	0.0082	0.0086	

b							
Population	MsCA	MsCO	MsCG	MsBL	MsMA	MsIT	MsGR
MsCA		0.006	0.0035	0.00271	0.00166	0.00262	0.00117
MsCO	0.01136		0.004	0.00330	0.00086	0.00525	0.00283
MsCG	0.00486	0.00570		0.00729	0.00068	0.00513	0.00361
MsBL	0.00741	0.00471	0.00785		0.00108	0.00721	0.00206
MsMA	0.00114	0.00261	0.00385	0.00191		0.00280	0.003
MsIT	0.00105	0.00452	0.00631	0.00621	0.00223		0.00389
MsGR	0.00336	0.00270	0.00390	0.00331	0.00354	0.005	

3.4 DISCUSSION

In this study, a comprehensive genetic evaluation of the Mediterranean populations of *M.barbatus* and *M.surmuletus* was carried out covering a large fraction of the distribution ranges of these species. The results showed high genetic variability in both species as well as effective population sizes (N_e) comparable to other highly exploited marine fish species such as cod (Box 1). These results are in line with previous genetic studies where large numbers of alleles per locus and high heterozygosity values have been found in both species using allozymes (Mamuris *et al.* 1998b; Mamuris *et al.* 1999b) as well as microsatellites in *M.barbatus* (Garoia *et al.* 2004). No previous microsatellite studies are available for *M.surmuletus*. Nonetheless, overall genetic variability values were also high relative to those of *M.barbatus* (Table 9). Furthermore, effective population size (N_e) estimates were noticeably higher in *M.surmuletus* than in *M.barbatus.*

Marine fish species with large population sizes are expected to exhibit little intra-specific genetic structuring even over large geographic distances (Ward *et al.* 1994). However, recent studies have demonstrated that fine scale genetic structuring may occur even for highly dispersive marine fish species (Jorgensen *et al.* 2005; Knutsen *et al.* 2003). The results are in congruence with this latter statement. Different patterns of population structure were observed for the two species investigated. *M.barbatus* showed a clear pattern of genetic partitioning along its Mediterranean distribution. *M.surmuletus*, in turn, showed a less heterogeneous pattern within the Mediterranean and a clear subdivision between Atlantic and Mediterranean populations. Overall, the red mullet displayed a more structured pattern relative to *M.surmuletus*, as it would be expected according to their respective pelagic dispersal strategies. This is supported by pairwise comparisons as well as by the spatial autocorrelation analysis, which indicates a larger genetic patch size for *M.surmuletus*. It is important to notice, however, that caution should be taken when interpreting this result. A single correlogram may not reflect accurately the true non-

random spatial genetic pattern. The geographic distance at which the *r*-value intercepts with the *x*-axis largely depends on the extent of genetic structure, the size of the distance class chosen and the associated number of samples per distance class (Peakall & Smouse 2006). Consequently, sampling at distance intervals larger or smaller than the actual scale of genetic structure as well as choosing different distance class sizes may lead to different values for the *x*-axis intercept (Peakall *et al.* 2003). However, in this case, it may be appropriate to interpret these results as evidence that gene flow primarily occurs over shorter geographical distances for *M.barbatus* relative to *M.surmuletus*.

From a single species perspective, the overall results of *M.barbatus*, are in good agreement with those previously reported for this species where discrete populations have been found in the Aegean and Ionian Seas as well as within the Adriatic Sea as inferred from genetic data (Garoia *et al.* 2004; Mamuris *et al.* 1998a). However, all analysis performed for *M.barbatus* showed significant differences in all but one sample pair. Interestingly, the single comparison that showed non-significant differences occurred between samples from the eastern (Greece) and western (Italy) Mediterranean (Table 10, Figs. 14-15). This finding is puzzling assuming a lower dispersal potential of *M.barbatus* relative to *M.surmuletus* and considering that these sampling locations are separated by more than 2100 kilometres with no apparent surface circulation pattern interconnecting them (Fig. 13, Table 10). Likewise, it seems unlikely that allelic diversity have converged into similar states (homoplasy) for this population pair in particular. The Mediterranean's eastern and western basins are separated by the strait of Sicily, also referred to as the Siculo-Tunisian strait. Hydrographic measurements show that surface Atlantic water follows the African coast eastwards entering the western basin through this strait to balance the water-mass deficit due to evaporation (Alhammoud *et al.* 2005). This phenomenon is commonly identified as subsurface minimum of salinity between 30 and 200 m. depth. Its salinity increases and its depth range decreases as it flows eastwards due to the high evaporation (Ozsoy *et al.* 1989). Various studies have pointed at these peculiar oceanographic

conditions taking place at the Siculo-Tunisian strait as the main cause of limited gene flow between eastern and western Mediterranean basins. Populations of species such as Sea bass (*Dicentrarchus labrax*), Poor Cod (*Trisopterus minutus capelanus*) and Mackerel (*Scomber scombrus*) have all showed a clear genetic discontinuity between eastern and western basins (Bahri-Sfar L *et al.* 2000; Mattiangeli *et al.* 2003; Zardoya *et al.* 2004). Direct comparisons, however, cannot be precisely established with the above studies because of the use of different molecular markers and/or sampling schemes. Furthermore, there are differences in life history traits between *M.barbatus* and those species, which include pelagic forms with schooling and homing behaviour. Nevertheless, Mamuris *et al*. (1998b; 1999) also found a clear genetic subdivision pattern for *M.barbatus* populations between the eastern and western Mediterranean basins, using enzyme-coding loci and random amplified polymorphic DNA (RAPD).

Two main hypotheses may be established to explain the results obtained in the present study. I) Our results indicate that the possibility of gene exchange through non-sampled populations cannot be excluded. Gene flow estimates, as measured by the $4N_m$ parameter, showed that the highest migrant exchange is found between samples from Greece and Italy (Table 11). Thus, it can be suggested that intermediate populations occurring along the Mediterranean coast of Italy may assist in the exchange of migrants between these populations, and thereby, maintaining similar frequencies of neutral alleles. II) Even though only a few breeding individuals per generation are sufficient to prevent genetic divergence among populations (Wright, 1951), migration at the rate observed here is very unlikely to be achieved in one generation by individuals moving between Greece and Italy. Particularly when considering the geographic distance and ocean current regimes prevailing between these sampling locations (Fig. 13). This notion is further supported by the fact that neither the test for recent ancestry in another population or the neighbour-joining tree yielded conclusive evidence that there has been recent dispersal events between these sampling locations (Table 12, Fig. 14). On the other hand, the close clustering of *M.barbatus*

populations from Greece and Italy on the MDS analysis indicates that genetic similarities have existed over time scales longer than a few generations (Fig. 15). Thus, it can be hypothesized that these populations were historically linked but currently are in the process of splitting. In any event, the fact that the transition zone reported at the Siculo-Tunisian strait does not seems to be effective for *M.barbatus* whereas apparently it holds true for *M. surmuletus* warrants further investigation by means of temporal sampling replicates as well as by the analysis of intermediate populations along potential transport currents.

Within *M.surmuletus* samples, a clear genetic assembly was formed by samples from NW Italy, Blanes and Cabo de Gata in Spain (Figs. 14-15). This grouping was further supported by the F_{ST} pairwise permutation test, showing no significant differences between pairwise comparisons (Table 10). These samples are located along the Liguro-Provençal-Catalan (LPC) current which follows a quasi-permanent SW trajectory alongside the coasts of Italy, France and Spain at a mean surface velocity of 0.35 m^{-1} (Mounier *et al.* 2005) (Fig.13). The LPC is one of the major current systems in the Mediterranean Sea. The core of the current extends from 25 to 45 km distance from the coast to a depth range between 400 and 650 m. (Conan & Millot 1995; Mounier *et al.* 2005). Its average transport ranges from 1 to 2.5 Sv which is of the same order of magnitude as the incoming Atlantic water transport at Gibraltar (Lacombe *et al.* 1981; Taupier-Letage & Millot 1986). Therefore, considering the long pelagic stage of *M.surmuletus*, it can be suggested that drifting eggs and larvae could potentially be transported along this current system homogenizing neutral allele frequencies into a single gene pool within this region. In contrast, significant genetic differences were observed with the adjacent sample from the Balearic Islands (Figs. 14, 15). One explanation could be the presence of several submarine canyons of more than 1000 m. depth that separate the Balearic islands from the coasts of France and Spain (Palanques *et al.* 2005). Several studies have demonstrated that deep water can act as strong barrier to genetic exchange across a variety of marine organisms (Doherty *et al.* 1995; Shaw *et al.* 2004; Stepien & Rosenblatt 1991). The maximum depth distribution of *M.surmuletus* is recorded

at 150 m, with the majority of the adult specimens usually living in water shallower than 100 m (Hureau 1986). Therefore, this could represent a natural barrier to adult movement to and from the islands. Furthermore, the Balearic Islands display a narrow shelf bound and there is evidence that they are, to some extent, hydrodynamically isolated from the continent (Jordi *et al.* 2005). This could also pose a barrier to passive dispersal of eggs and larvae. Estimates of gene flow (**$4N_m$**), indicate that there is some exchange with neighbouring populations (Table 11). However, levels of genetic exchange may be too low to prevent drift, perhaps allowing local adaptation and enhancing recruitment of locally produced larvae as suggested by its relatively large F_{IS} values (Table 9).

A sharp genetic divide was detected when comparing samples originating from the Atlantic Ocean and samples originating from the Mediterranean Sea. This same pattern has been previously observed for other demersal species with Atlantic and Mediterranean distributions. Species such as European Hake (*Merluccius merluccius*), Sea breams (*Diplodus puntazzo, Sparus auratus*) and various other Sparid species (*Lithognathus mormyrus, Spondyliosoma cantharus, Dentex dentex*) all have shown genetic differentiation between Atlantic and Mediterranean populations (Bargelloni *et al.* 2005; Bargelloni *et al.* 2003; Brutto *et al.* 2004; Castillo *et al.* 2004; Cimmaruta *et al.* 2005; De Innocentis *et al.* 2004). However, discrepancies still exist about the precise location of the boundary separating Atlantic and Mediterranean populations. The Mediterranean Sea is connected to the Atlantic Ocean through the strait of Gibraltar. This strait has been hypothesized to represent the division between two biogeographic regions, the north-east Atlantic and the Mediterranean (Quignard 1978). For other demersal species such as European Hake (*Merluccius merluccius)*, protein coding loci and mtDNA analysis suggest that the strait of Gibraltar may be considered a break point area to gene flow (Brutto *et al.* 2004). However, a recent allozyme-based study identified the boundary between Atlantic and Mediterranean Hake populations more precisely in the Almeria-Oran front (AOF), inside the Mediterranean instead of at the Strait of Gibraltar (Cimmaruta *et al.* 2005). In this area, a

strong inflow transports less saline surface Atlantic waters into the Alboran Sea, creating an Atlantic domain within the Mediterranean and partially isolating the Alboran Sea from the other Mediterranean basins (Tintoré *et al.* 1988). The sampling scheme for *M.surmuletus* included one location (Cabo de Gata) at the Mediterranean extreme of the Alboran Sea (Fig.13). The results show that this sample showed more genetic similarities with Mediterranean samples than with the Atlantic samples (Figs. 14, 15). Therefore, it can be suggested that the subdivision between Atlantic and Mediterranean populations of *M.surmuletus* occurs between the Strait of Gibraltar and the Alboran Sea. However, intermediate samples would be needed to identify precisely the location of the boundary. Occasional adult dispersal and larval transport may occur from adjacent Atlantic samples as indicated by the $\mathbf{4}N_m$ and Q parameters, although this may not be enough to prevent short-term genetic drift among Atlantic and Mediterranean populations (Tables 11,12). Furthermore, the present pattern of marine currents in the area seems to assist in maintaining this differentiation (Fig.13).

3.5 CONCLUSIONS

The results show that the patterns of genetic distribution in the marine environment may be substantially different between closely related species. This suggests that marine biogeographical boundaries can affect in a different way species sharing many ecological features and spatial overlap. Furthermore, the delimitation of such boundaries could be largely influenced by the fine differences in life history traits between species.

These results can have relevant implications from a conservation-management perspective of both species. The patters of population structure observed here are not in complete agreement with those previously reported for other commercially important demersal species within the Mediterranean. The overall results coincide with the bulk of studies that have found a reduction of gene flow between the Atlantic Ocean and Mediterranean Sea for demersal species. This is consistent with the current policy for these species, in which Atlantic and Mediterranean stocks are considered separate stocks. Nonetheless, within the Mediterranean Sea, the species showed different structuring patterns, neither of which was similar to those of other commercially exploited demersal fish species in the area. The genetically homogeneous *M.surmuletus* populations from the Northwest Mediterranean ought to be considered as a shared stock between countries or regions for management purposes, whereas populations originating from the Balearic Islands and Greece should be managed as regionally segregated populations that have little or no intermixing with their respective surrounding populations. The genetic distribution of *M.barbatus* resembles that of a metapopulation structure composed by largely independent self-recruiting subpopulations, with some connections between them. Therefore, each of these subpopulations could have a different response to environmental variables and human induced activities (i.e. fishing pressure), and consequently, all must be monitored as separate units in order to obtain more information towards management strategies. Overall, genetic population structure patterns in the marine realm are best explained when

considered in combination with hydrogeographic and hydrodynamic factors that may be contributing to gene flow dynamics, and thus, influencing the microevolutionary processes of the species.

CHAPTER IV

A multi-species approach to determine the role of early-life history traits and oceanographic boundaries on population genetic structure and its implications in the design of marine protected areas.

Abstract

Managed resource protected areas (MRPA) have gained popularity in the last decades as management and conservation tools of marine resources. An accurate understanding of gene flow patterns is crucial to make informed decisions towards their connectivity with other protected areas as well as with non-protected areas. Such patterns are determined by the interplay between biological processes inherent to marine species (i.e. life-history traits) and environmental discontinuities (i.e. oceanographic barriers) found across the species' distribution range. However, determining the relative influence that these two factors have in outlining genetic connectivity and its demographic consequences remains a major challenge for marine ecologist. To this end, a comparison of gene flow patterns of six littoral fish species with distinct early-life history traits sampled at the same locations across common environmental discontinuities in the form of oceanographic fronts in the western Mediterranean is presented. The results show that different species may respond in a similar way to common environmental features regardless of their distinct early-life history traits. No evident relationship between dispersal potential and realized gene flow could be observed across the species. This suggests that genetic connectivity patterns cannot be predicted confidently by the species' dispersal potential as determined by their early-life characteristics. Furthermore, our results provide evidence that small-scale hydrographic variability and local processes can have ecologically meaningful effects on population connectivity in marine species. These results highlight the importance of employing multi-species approaches towards the devise and subsequent implementation of MRPA where multiple species co-occur.

4.1 INTRODUCTION

Marine reserves, no-take zones, marine refuges and other kinds of marine protected areas, have become fashionable in the past two decades as conservation and management tools of marine resources. Their increasing popularity as an alternative to traditional management-conservation methods such as closures and quota systems has lead to an establishment of a categorization according to their function and objectives. Marine protected areas (MPA) may be established for the maintenance of habitats or specific species, to preserve representative ecosystems and areas of cultural significance, and to protect areas managed for the sustainable use of natural resources (Kelleher & Kenchington 1999). This latter category is termed Managed Resource Protected Area (MRPA) and its ultimate goal is to protect biodiversity while ensuring long-term fishery sustainability (IUCN 1994). However, its devise and subsequent implementation are particularly challenging since discrepancies often exists between conservation and exploitation interests. Hence, the present arrangement of MRPAs frequently results from a consensus agreement between a variety of standpoints, mainly from the political, the socio-economic and the scientific sectors. In this regard, the main contribution of the scientific sector to MRPAs design has relied mainly on the development of theoretical models to estimate parameters such as the amount of habitat needed to be protected (Mangel 1998; Mangel 2000), reserves configuration (Bostford *et al.* 2001), optimal sizes of protected areas (Watson *et al.* 2000) and habitat selection (Lundberg & Jonzén 1999). Moreover, numerous studies have evaluated the performance of already established MRPAs through a great variety of approaches including, abundance and diversities estimates, (Claudet *et al.* 2006; Narvarte *et al.* 2006; Shears *et al.* 2006), effects of fishing protection on genetic structure (Perez-Ruzafa *et al.* 2006), socio-economic success (McClanahan *et al.* 2006), habitat and assemblages distributions (Fraschetti *et al.* 2005) as well as anthropogenic impacts (Casu *et al.* 2006). Overall,

these studies show that MRPAs can be of grate value for conservation-management purposes, particularly when analysed over a long-term period. However, *a priori* empirical information on the potential effectiveness of MRPAs to be established within a particular ecosystem has received little attention.

Ultimately, the effectiveness of MRPAs may be measured in terms of the impact that organisms protected within the reserve have outside of it, whether reserves will be self-seeding, whether they will accumulate recruits from surrounding exploited areas, and whether reserve networks can exchange recruits (Palumbi 2003). Therefore, an understanding of the connectivity of a MRPA with its surrounding environment is important to maximize its efficacy within and/or beyond its boundaries. At this point, two types of connectivity may be distinguish; demographic connectivity and genetic or evolutionary connectivity. The former implies the movement of individuals (i.e. migration events, spawning aggregations) that spend at least part of their life cycle within the MRPA. Whereas the latter deals with the exchange of genes between populations within the MRPA and adjacent populations. Demographic connectivity may be practical to evaluate the performance of the MRPA in terms of exports of individuals to non-protected areas. This export may be either by adults or juveniles dispersing actively or by passive transport of eggs and larvae (Palumbi 2004). However, individual movements to neighbouring populations cannot be taken as an indicator that effective biological links exists, nor that they are maintained over time through this demographic exchange. Therefore, to ensure long-term sustainability and biodiversity protection, demographic processes affecting MRPAs must be evaluated through a genetic perspective.

The emergence of molecular techniques such as polymerase chain reaction (PCR) and DNA sequencing have become a complementary, but essential tool for an efficient

management of marine resources in the last decades. Through this approach, an estimate on the degree of connectivity between populations via gene flow can be obtained. Furthermore, it allows for the assessment on the spatial distribution of genetic diversity, or genetic seascape, within and between geographical areas where MRPAs can potentially be established. This information enables managers to distinguish single panmitic assembles from fragmented breeding populations whose identification is fundamental for conservation and management (Grant *et al.* 1999). For many years, it was assumed that marine populations operate as an open system where pelagic stages represent the main cause of gene flow due to their high potential for dispersal (Roberts 1997; Scheltema 1965; Shulman & Bermingham 1995). However, mounting evidence shows that pelagic stages often fail to fully achieve their dispersal potential (Barber *et al.* 2002; Froukh & Kochzius 2007; Jin-Xian *et al.* 2007; Jones *et al.* 2005; Swearer *et al.* 2002), suggesting that the relationship between dispersal potential and realized gene flow among marine populations is more complex than previously assumed.

Gene flow among marine populations may be influenced by different factors, being water flow dynamics and pelagic stage characteristics determinants of population genetic connectivity (Palumbi 2004). However, decoupling the influence that each of these factors has in determining gene flow remains a major challenge for marine ecologists. Although significant progress has recently been made in our ability to accurately track pelagic stages (Becker *et al.* 2007; Patterson & Swearer 2007) as well as in determining genetic connectivity patterns by combining ocean and genetic data (Galindo *et al.* 2006; Hohenlohe 2004), we still have a limited understanding about how oceanographic conditions may influence gene flow among marine populations by posing barriers to the dispersal of pelagic stages, and even less about how species with different pelagic characteristics may respond to such barriers. Therefore, the identification of gene flow trends across species with varying dispersal potential under the same oceanographic

conditions is fundamental for implementing appropriate conservation-management strategies within an ecosystem where multiple species co-occur. Here, a comparison of genetic connectivity patterns of six littoral fish species with different dispersal potential sampled at the same geographical locations in the Western Mediterranean is performed. Further, I consider how such patterns can be explained by reference to pre-existing knowledge of their early-life history characteristics and in the presence of oceanographic barriers in the form of two density fronts of different magnitude found within the region.

4.1.1 The species

It is well known that pelagic stage characteristics vary greatly among marine species. While some species may spawn benthic eggs from which pelagic larvae hatch, other species may display life cycles where both the eggs and the larvae are pelagic, whereas other species may have evolved peculiar forms of parental care such as mouth-guard or brood sacks and bear live young (Bonhomme & Planes 2000; Riginos & Victor 2001). In addition, the pelagic duration (PD) and the spatial distribution of pelagic stages (i.e. inshore-offshore) also show great variability among marine species (Johannes 1978; Kingsford & Choat 1989; Knutsen *et al.* 2004; Sabatés 1990; Sabatés *et al.* 2004). Under this scenario, it is expected that species with extended pelagic phases and offshore distributed larvae would be more apt to disperse over greater distances that species with shorter or absent pelagic phases and inshore distribution. However, contradictory results regarding expected vs. effective dispersal are often found when multiple species with varying dispersal potential are compared simultaneously (Bargelloni *et al.* 2005; Bargelloni *et al.* 2003; Charrier *et al.* 2006b; Doherty *et al.* 1995; Hickford & Schiel 2003; Kinlan & Gaines 2003; Waples 1987). In this study, six co-occurring species from five different families representing a wide spectrum of the main early life-history characteristics found in littoral fish are considered. The species considered are the

Common two-banded bream (*Diplodus vulgaris*), the Comber (*Serranus cabrilla*), the Blackfaced blenny (*Tripterygion delaisi*), the Saddled bream (*Oblada melanura*), the Peacock wrasse (*Symphodus tinca*) and the Cardinal fish (*Apogon imberbis*). *D.vulgaris* and *S.cabrilla*, species with extended pelagic duration, pelagic eggs and offshore distributed larvae are considered to have a greatest dispersal potential followed by *O.melanura* which spawns pelagic eggs and offshore larvae but with a shorter pelagic duration. *T.delaisi* and *S.tinca* are more restricted in their dispersal by producing benthic eggs, inshore larvae and having shorter PD. *A.imberbis*, in turn, being a direct development mouth-brooder species presents the least potential for dispersal. A summary of the species main life history traits is presented in table 13.

4.1.2. The Fronts

The western Mediterranean, is a well studied geographical area considered as a "hot-spot" in marine biodiversity (Macpherson 2002) with a high level of endemism and where conservation strategies require the inclusion of ocean circulation and population genetic data. The western Mediterranean is characterized by a particular geomorphology and associated oceanography, mostly conditioned by the inflow of Atlantic water through the Gibraltar Strait (Millot 2005). The interaction between lighter Atlantic water with higher density Mediterranean water generates two oceanographic fronts, separated by ca. 600 km and potentially affecting dispersal capabilities (Fig. 17A). The first front (Almeria-Oran Front, AOF), situated ca. 400 km east from the Gibraltar Strait, is very strong, nearly permanent throughout the year with density gradients reaching to 1.5 σT units and present in the upper 300 m (Tintoré *et al.* 1988) (Fig. 17C). The AOF has been reported to act as a barrier to gene flow in numerous species (Charrier *et al.* 2006a; Cimmaruta *et al.* 2005; Naciri *et al.* 1999; Quesada *et al.* 1995; Ríos *et al.* 2002). East to the AOF, the Atlantic water flows north-eastward reaching the Balearic Islands and

forming a second density front. This second front (Balearic Front, BF) is weaker, with density differences of 0.5 σT units between water masses, and characterized by intense mesoscale variability where dynamic processes are not constant through the year (Pinot *et al.* 1994) (Fig 17B). Traditionally, BF has not been considered as a factor for population differentiation, although it plays an important role in delimiting the distribution boundaries for Atlantic-Mediterranean fauna (Abelló *et al.* 2002). The particular characteristics of each front provide a good opportunity to test their potential effects on gene flow among marine populations. Specifically, the following questions are asked: Do fronts affect all species equally irrespective of their dispersal potential? Are species with higher dispersal potential less affected by weaker fronts than species with restricted dispersal? Do populations separated by fronts show less degree of connectivity than populations not divided by fronts?

Table 13. Main life history traits of *Diplodus vulgaris*, *Serranus cabrilla, Tripterygion delaisi, Oblada melanura, Symphodus tinca*, and *Apogon imberbis*. B: benthic, P: Pelagic, O: offshore > 2 km, I: Inshore < 2 km, n.d.a: no data available.

Species	Family	Depth Range	Adult Habitat	Egg type	Distribution of larvae	Pelagic duration	Refs.
Diplodus vulgaris	Sparidae	0-90 m	Littoral waters on rocky or sandy bottoms	P	O	29-58 days	Bauchot & Hureau 1986
Serranus cabrilla	Serranidae	1-500 m	Benthic on the shelf and upper slope, on rocks, *Posidonia* beds, sandy and muddy bottoms	P	O	21-28 days	Brito *et al.* 2003
Tripterygion delaisi	Tripterygiidae	2-40 m	Bottom-living on rocky shores in biotopes with reduced light	B	I	16-21 days	Macpherson 1994; Writz 1978
Oblada melanura	Sparidae	0-30 m	Littoral waters above rocky bottoms and *Posidonia* beds	P	O	14-18 days	(Bauchot & Hureau 1986)
Symphodus tinca	Labridae	1-50 m.	Found near rocks and eel-grass beds	B	I	9-13 days	Gordoa *et al.* 2000
Apogon imberbis	Apogonidae	10-200 m.	On muddy or rocky bottoms and caves	Mouth-brooder	n.d.a	18-24 days	Tortonese 1986

4.2 MATERIAL AND METHODS

4.2.1 Sample collection and genetic analysis

During 2003-2007, a total of 1055 adults from six different marine fish species (mean 44inds/site/species) were sampled at the same four locations along the Spanish Mediterranean coast and the Balearic Islands (Fig. 17A). All samples were obtained by scuba diving. Precise number of individuals per site and species are listed in Appendix 3.

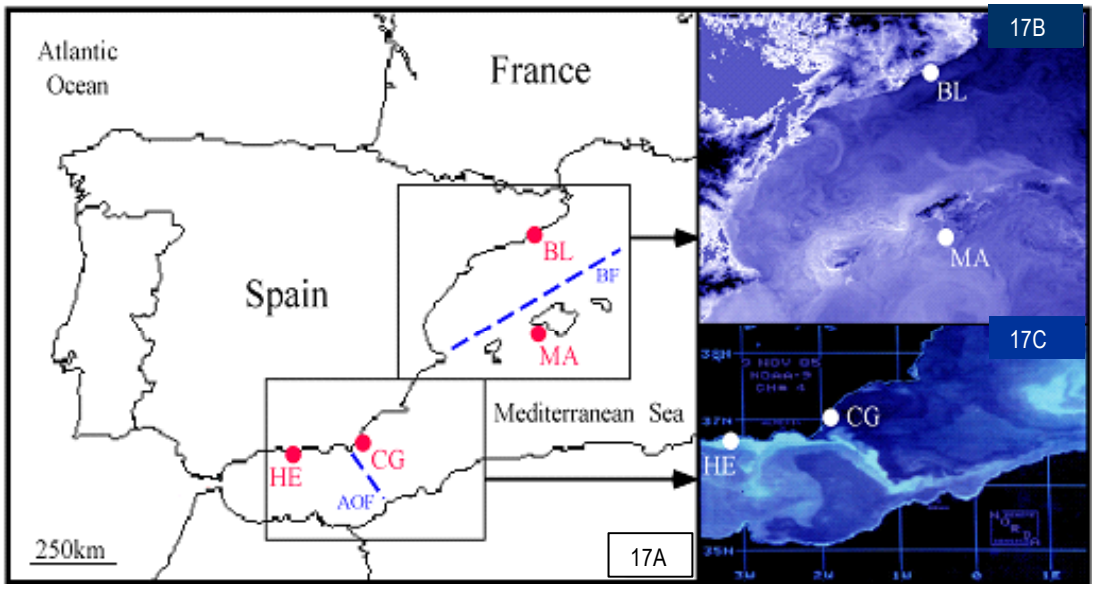


Figure 17 A . Sampling locations for the six species. HE: Herradura, CG: Cabo de Gata, MA: Mallorca, BL: Blanes and the locations of oceanographic fronts. Figure 17 B showing Balearic front (BF). Figure 17 C showing Almeria-Oran front (AOF)

4.2.2 Microsatellite Data Analysis

DNA extractions were performed from pectoral fin tissue following HotSHOT protocol (Truett *et al.* 2000). A total of 53 microsatellite loci (mean 9 loci/species) were successfully amplified by polymerase chain reaction according to Carreras-Carbonell *et al.* (2004); Carreras-Carbonell *et al.* (2006a); Galarza *et al.* (2006); Galarza *et al.* (2007); Roques *et al.* (2007a); Roques *et al.* (2007b). Amplified products were resolved on an ABI3100 Genetic Analyser (Applied Biosystems). Allele scoring was carried out using GeneMapper v 3.5 software (Applied Biosystems).

The total number of alleles per locus and sample was obtained using GENETIX v.4.02 (Belkhir *et al.* 1997). The program FSTAT v. 2.9.3 (Goudet 1995) was used to determine allelic richness, which estimates the number of alleles independently of sample size. Observed and expected heterozygosities were determined using the software package Arlequin v. 2.0 (Schneider *et al.* 2000). Deviations from Hardy-Weinberg expectations (HWE) were estimated according to the level of significance determined by a Markov chain method using GENEPOP v. 3.4. (Raymond & Rousset 1995). Bonferroni corrections were made for multiple tests.

4.2.3 Spatial Genetic Structure

The effect of the oceanographic fronts in gene flow levels within and among species was assessed through four different approaches, at both, the population and individual levels. First, at the population level, pairwise F_{ST}s and their estimated probabilities were calculated within species by 10,000 permutations using GENETIX v. 4.02 (Belkhir *et al.* 1997). Permuted F_{ST} values were compared with the observed values, to estimate the probability of randomly achieving a value greater than or equal to the observed. The alternative hypothesis of significant genetic differentiation was accepted if this probability was equal to or less than 0.05. Sequential Bonferroni corrections were made for multiple tests setting an initial α value of 0.05 (Rice 1989).

Second, the effect that early-life history traits have on genetic differentiation (F_{ST}) across fronts was estimated through a Spearman's rank correlation test. While it is virtually impractical to perform standard statistical analysis on data that is far from normal (i.e. meristic vs. descriptive, in this case F_{ST} vs. life history traits) one can assesses for an association of these variables using a Spearman's rank correlation test (r). This non-parametric procedure does not require any assumptions about the frequency distribution of the variables nor that the relationship between the variables is linear (Zar 1999). Thus,

it can be applied to nominal data such as the ranked measurements of the variables. Data on table 13 was used to rank the species in order of increasing dispersal potential as judged by, i) inshore-offshore distributed larvae, ii) egg type benthic-pelagic, iii) pelagic larval duration PLD. Then, their respective F_{ST} value from table 15 was ranked in increasing order and a Spearman's rank correlation test was performed on these rankings. In theory, a negative correlation is expected between the species' dispersal capability and genetic differentiation (i.e. the higher the dispersal capability, the lower the genetic differentiation).

Third, the program MSAnalyzer v.2.39 (Dieringer & Schlötterer 2003) was used to compute inter-population genetic distance matrices based on the proportion of shared alleles (D_{ps}) for all loci between all population pairs within species. Assuming that shared alleles result from common ancestry, the topology of the tree should reflect a connection between those populations not separated by the fronts as a result of recent dispersal events. Whereas a separate clustering is expected for pairs of populations divided by the fronts. Distance matrices were permutated 5000 times and dendrograms were constructed by the neighbour-joining method (Saitou & Nei 1987) for each species with the program Neighbour in Phylip v.3.65 (Felsenstein 2004). A consensus dendrogram was obtained using the program Consense in Phylip v.3.65 (Felsenstein 2004) and graphically displayed using MEGA v. 3.1 (Kumar *et al.* 2004).

Fourth, at the individual level, spatial locations of genetic discontinuities (i.e. genetic boundaries) along the sampling area were determined using the R package GENELAND (Guillot *et al.* 2005). This program makes use of a geographically constrained Bayesian model that explicitly takes into account the spatial position of sampled multilocus genotypes without any prior information on the number of populations and degree of differentiation between them. Geographical coordinates were assigned to each individual

by randomly choosing *n* spatial locations encompassed within a 10 km. radius from each of the four sampling locations using the Mapping toolbox in MATLAB v.7. (Mathworks), where *n* equals the number of individuals/species sampled at a given location. Thus, the same geographic coordinates were used for individuals of all species sampled at the same site. The inference algorithm was launched using the Dirichlet distribution as prior for allele frequencies with 20,000 MCMC iterations using spatial information. Then, the algorithm was re-run with an additional 20,000 MCMC iterations, fixing the value of *K* (i.e. number of populations) to that determined by the mode of the posterior distribution of the MCMC chain and setting the poisson processes equal to the number of sampled individuals per species.

4.3 RESULTS

4.3.1 Microsatellite variability

High levels of polymorphism were registered at the majority of the assayed microsatellite loci. The total number of alleles per locus, observed and expected heterozygosity as well as Hardy-Weinberg expectations are listed in Appendix 3. No evidence of linkage disequilibrium was found between locus pairs. Significant deviations from Hardy-Weinberg expectations within sampling locations were observed in all species. Out of 188 tests across species, 48 remained significant after Bonferroni correction. Within species more than 70% of the deviations were observed in one or two locus, which showed consistent departures in most or all sampling sites and thus, were removed from further analysis (Table 14). The loci excluded were Aimb17, Aimb22, Dvul2, Dvul63, Sc05, Td09 and St138.

Table 14. Loci presenting significant deviations from Hardy-Weinberg expectations after Bonferroni correction for multiple tests

D.vulgaris			*S.cabrilla*			*T.delaisi*			*O.melanura*			*S.tinca*			*A.imberbis*		
Locus	F_{IS}	Site	Locus	F_{IS}	Site	Locus	F_{IS}	Site	Locus	F_{IS}	Site	Locus	F_{IS}	Site	Locus	F_{IS}	Site
Dvul1	0.192	HE	Sc05	0.274	HE	td01	0.022	MA	Omel2	0.24	HE	St138	0.378	HE	Aimb14	0.48	MA
Dvul2	0.341	HE	Sc05	0.253	CG	td06	0.081	CG	Omel2	0.157	BL	St138	0.179	CG	Aimb17	0.138	HE
Dvul2	0.372	CG	Sc05	0.198	MA	td07	0.155	CG	Omel27	0.251	HE	St138	0.332	MA	Aimb17	0.137	CG
Dvul2	0.462	MA	Sc06	0.251	BL	td08	0.194	MA	Omel27	0.193	BL	St138	0.414	BL	Aimb17	0.437	MA
Dvul2	0.413	BL				td08	0.214	BL	Omel38	0.367	BL	St368	0.133	MA	Aimb17	0.089	BL
Dvul61	0.239	HE				td09	0.131	HE	Omel54	0.22	CG	St368	0.266	BL	A.imb2	0.462	MA
Dvul61	0.151	BL				td09	0.071	CG	Omel58	0.313	BL				Aimb22	0.505	HE
Dvul63	0.285	HE				td09	0.375	MA							Aimb22	0.064	CG
Dvul63	0.193	CG				td09	0.513	BL							Aimb22	0.35	MA
Dvul63	0.355	BL													Aimb22	0.583	BL
															Aimb28	0.327	HE
															Aimb41	0.284	CG

Departures from Hardy-Weinberg expectations may be caused by several factors such as inbreeding, population sub-structuring (i.e. Wahlund effect) and by the presence of null alleles caused by technical issues. The program MICRO-CHECKER (van Oosterhout *et al.* 2004) was used to infer the most probable cause of departures from Hardy-Weinberg expectations. No evidence of large allele dropout could be observed at any locus. However, a general excess of homozygotes was found in most allele size classes, suggesting the presence of null alleles for the remaining loci that showed significant departures (Aimb2, Aimb28, Aimb41, Dvul1, Dvul61, Sc06, Td01, Td06, Td07, Td08, St368, Omel2, Omel28, Omel38, Omel54 and Omel58). Multiplex PCR techniques employed could have contributed to the presence of null alleles by forcing simultaneous amplification of different loci at common annealing temperatures. The effect that such null alleles could have in the estimates of population differentiation was assessed by three different approaches. First, the F_{ST} permutation test was re-run excluding those loci that presented evidence for null alleles. Second the program FreeNA (Chapuis & Estoup 2007) was used to obtain genotype frequencies corrected for null alleles and then compare F_{ST} estimates from corrected vs. non corrected data sets. Both approaches showed similar results with changes in the magnitude of F_{ST}. Non-corrected data set tended to slightly overestimate F_{ST} values. However, the pattern of significant population differentiation did not show differences by either approach. Third, a likelihood approach was used to assess for the probability that the number of significant test obtained exceed the 0.05 significance level by chance. This probability was calculated through the binomial likelihood function

$$L = \sum_{i=r}^{n} C(1-\alpha)^{n-r}(\alpha)^{r}$$

where *n* is the number of total tests, *r* is the fraction of significant tests at $\alpha \leq 0.05$ and C is the factorial constant (n!/(r!(n-r)!) (Chapman *et al.* 1999; Kinnison *et al.* 2002). This approach allowed us to examine the likelihood (*L*) that *r* of *n* loci (i.e. 3 out 10) would exhibit significant differences by chance alone. This likelihood was calculated for all pairwise population comparisons within species across all loci. The numerical solution to the likelihood function shows that when 1/3 of the loci involved in independent tests exceed the $\alpha \leq 0.05$, we should conclude that the populations are genetically distinct (Chapman *et al.* 1999). Counts of significant loci involved in pairwise tests for all species showed that samples differ by more loci than expected by chance (mean 3/4 per species). These results suggests that the pattern of genetic differentiation observed here is not affected significantly by the presence of null alleles and also, that it is very unlikely that chance alone could have generated such a pattern ($P < 0.001$ by the Likelihood method).

4.3.2 Spatial genetic structure

Starting from an among-species perspective, population pairwise F_{ST} values showed significant differences for all species across the AOF with the exception of *O.melanura* populations (Table 15). Comparisons across the BF exhibit more variable results with Cabo de Gata-Blanes showing more significant differences in allelic frequencies in the majority of the species, whereas between Mallorca-Blanes, locations also affected by the BF, half of the species analysed presented significant genetic differences. In contrast, results from Cabo de Gata-Mallorca, locations not separated by any of the fronts (NF), indicated that significant genetic differentiation exist for the majority of the species (Table 15). Within species, *T.delaisi* was the species that showed significant genetic differences between all of its populations, followed by *A.imberbis* for which, only Cabo de Gata-Blanes showed no significant differences (Table 15). On the other hand, *S.tinca*

was the species less structured with only populations across the AOF being significantly differentiated (Table 15).

Table 15. Pairwise F_{ST} values for the different species across the Almeria-Oran Front (AOF), the Balearic Front (BF) and across no fonts (NF). Significant values at α = 0.05 after sequential Bonferroni correction are shown in bold italics.

Species	AOF	BF		NF
	Herradura - Cabo de Gata	Mallorca - Blanes	Cabo de Gata - Blanes	Cabo de Gata - Mallorca
D. vulgaris	***0.060***	0.004	***0.008***	0.001
S. cabrilla	***0.014***	0.009	***0.059***	***0.036***
T.delaisi	***0.018***	***0.027***	***0.038***	***0.034***
O.melanura	0.001	***0.17***	***0.052***	***0.155***
S. tinca	***0.033***	0.002	0.001	0.001
A. imberbis	***0.011***	***0.060***	0.005	***0.052***

Results from the Spearman Rank correlation test, show that early-life history traits, do not have an effect on genetic differentiation across the AOF as shown by the positive correlation values (Table 16). Similarly, no single early-life history trait could be identified to have an effect on genetic divergence across the BF. Congruently, the same result was obtained when traits were analysed across no fronts (NF) (Table 16). This suggests that genetic differentiation cannot be directly related to an early-life history trait (i.e. larvae distribution, egg type or pelagic duration) when analyzed across strong, weak or absent oceanographic barriers.

Table 16. Spearman's Ranked correlation tests for the different life history traits across the Alemria-Oran front (AOF), Balearic front (BF) and no fronts (NF). Correlation values (*r*) between life history traits and genetic differentiation (F_{ST}) are shown in bold. I-O: Inshore-Offshore, B-P: Benthic-Pelagic, PD: Pelagic duration

AOF (F_{ST} from HE-CG)						
Species	Larvae distribution (I-O)		Egg type (B-P)		PD	
	Rank	Ranked F_{ST}	Rank	Ranked F_{ST}	Rank	Ranked F_{ST}
A.imberbis	2	2	1	2	4	2
S.tinca	2	5	2.5	5	1	5
O.melanura	5	1	5	1	2	1
T.delaisi	2	4	2.5	4	3	4
S.cabrilla	5	3	5	3	5	3
D.vulgaris	5	6	5	6	6	6
r values		**0.028**		**0.128**		**0.257**
BF (F_{ST} from MA-BL)						
Species	Larvae distribution (I-O)		Egg type (B-P)		PD	
	Rank	Ranked F_{ST}	Rank	Ranked F_{ST}	Rank	Ranked F_{ST}
A.imberbis	2	5	1	5	4	5
S.tinca	2	1	2.5	1	1	1
O.mel	5	6	5	6	2	6
T.delaisi	2	4	2.5	4	3	4
S.cabrilla	5	3	5	3	5	3
D.vulgaris	5	2	5	2	6	2
r values		**0.2**		**0.014**		**0.08**
BF (F_{ST} from CG-BL)						
Species	Larvae distribution (I-O)		Egg type (B-P)		PD	
	Rank	Ranked F_{ST}	Rank	Ranked F_{ST}	Rank	Ranked F_{ST}
A.imberbis	2	2	1	2	4	2
S.tinca	2	1	2.5	1	1	1
O.mel	5	5	5	5	2	5
T.delaisi	2	4	2.5	4	3	4
S.cabrilla	5	6	5	6	5	6
D.vulgaris	5	3	5	3	6	3
r values		**0.714**		**0.7**		**0.314**
No-front (F_{ST} from CG-MA)						
Species	Larvae distribution (I-O)		Egg type (B-P)		PD	
	Rank	Ranked F_{ST}	Rank	Ranked F_{ST}	Rank	Ranked F_{ST}
A.imberbis	2	4	1	4	4	4
S.tinca	2	1.5	2.5	1.5	1	1.5
O.mel	5	5	5	5	2	5
T.delaisi	2	3	2.5	3	3	3
S.cabrilla	5	2	5	2	5	2
D.vulgaris	5	1.5	5	1.5	6	1.5
r values		**0.242**		**0.01**		**0.1**

Neighbour-Joining dendrograms based on proportions of shared alleles between populations showed a marked effect of the AOF on *D.vulgaris,* and *S.tinca*. Populations of these species displayed identical clustering patterns with sampling locations Cabo de Gata and Mallorca clustering together and Herradura being clearly differentiated (Fig.18). The BF influence was reflected in *O.melanura*, *A.imberbis* and to a lesser degree in *T.delaisi* populations. These species showed similar clustering patterns with Blanes and Cabo de Gata clustering together and Mallorca and Herradura on separate branches. The clustering patterns of *S.cabrilla*, on the other hand, did not show any evident relation to any of the fronts. However, a clear pattern was observed between localities mostly influenced by Atlantic waters and those found within Mediterranean waters (Fig. 18). Populations not separated by any of the fronts (Cabo de Gata-Mallorca) clustered together only for *D.vulgaris* and *S.tinca* species.

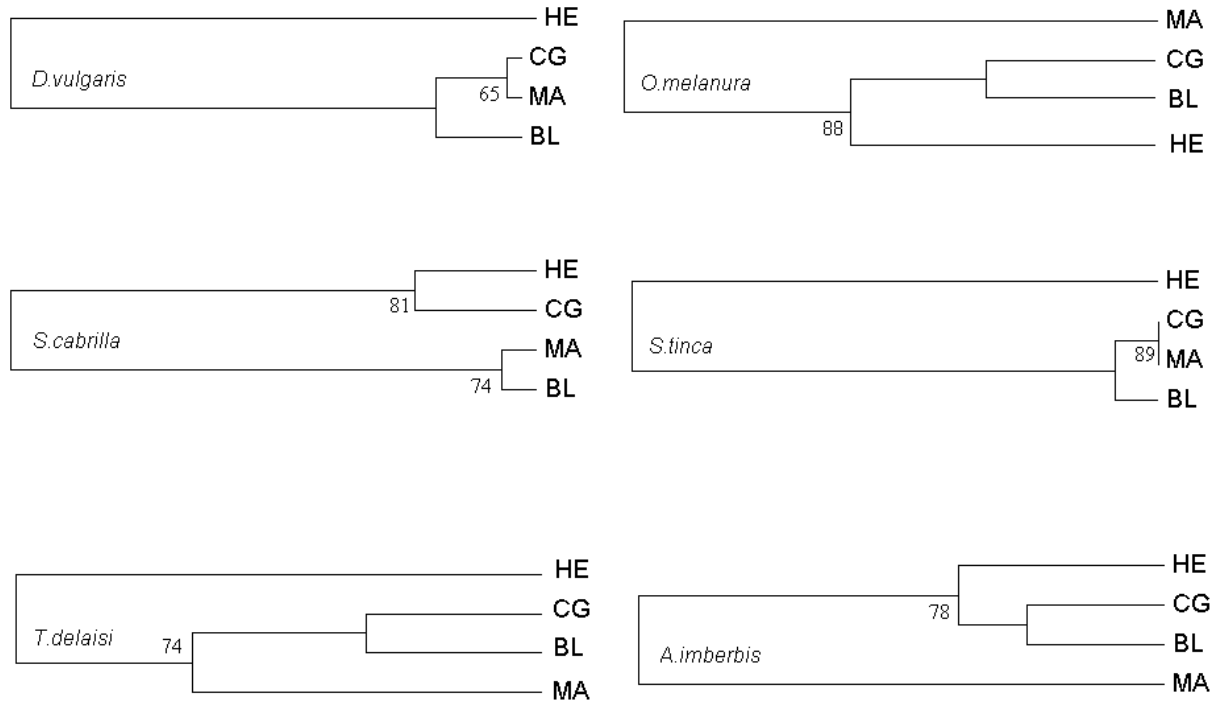


Fig. 18. Neighbour-joining dendrograms based on the proportion of shared alleles (Bowcock, 1994) between populations of *D.vulgaris, S.cabrilla, T.delaisi, O.melanura, S.tinca and A.imberbis* species. Bootstrap values over 5000 replicates are shown when ≥ 50%.

Results from the geographically constrained Bayesian model suggested the existence of two genetically differentiated subunits (i.e. two populations) within all species across the sampling area. The posterior probabilities of individual membership to each subunit and the spatial location of the genetic discontinuities between subunits are presented in Figure 19. Such genetic discontinuities varied spatially among species. A strong genetic discontinuity congruent with the location of the AOF was observed between Herradura and Cabo de Gata for *D.vulgaris* and *S.tinca* species (Fig. 19 A,D). The model also showed the presence of a genetic discontinuity between Mallorca and Cabo de Gata, locations divided by the BF, for *A.imberbis* and *O.melanura* species (Fig. 19 E, F). Similarly, the model suggested that genetic boundaries exist between locations not separated by any of the fronts (Cabo de Gata-Mallorca) for all species with the exception of except for *D.vulgaris* and *S.tinca* species (Fig. 19 A,D). Interestingly, these genetic boundaries coincide with the highest genetic differentiation values obtained by F_{ST} procedures (Table 15).

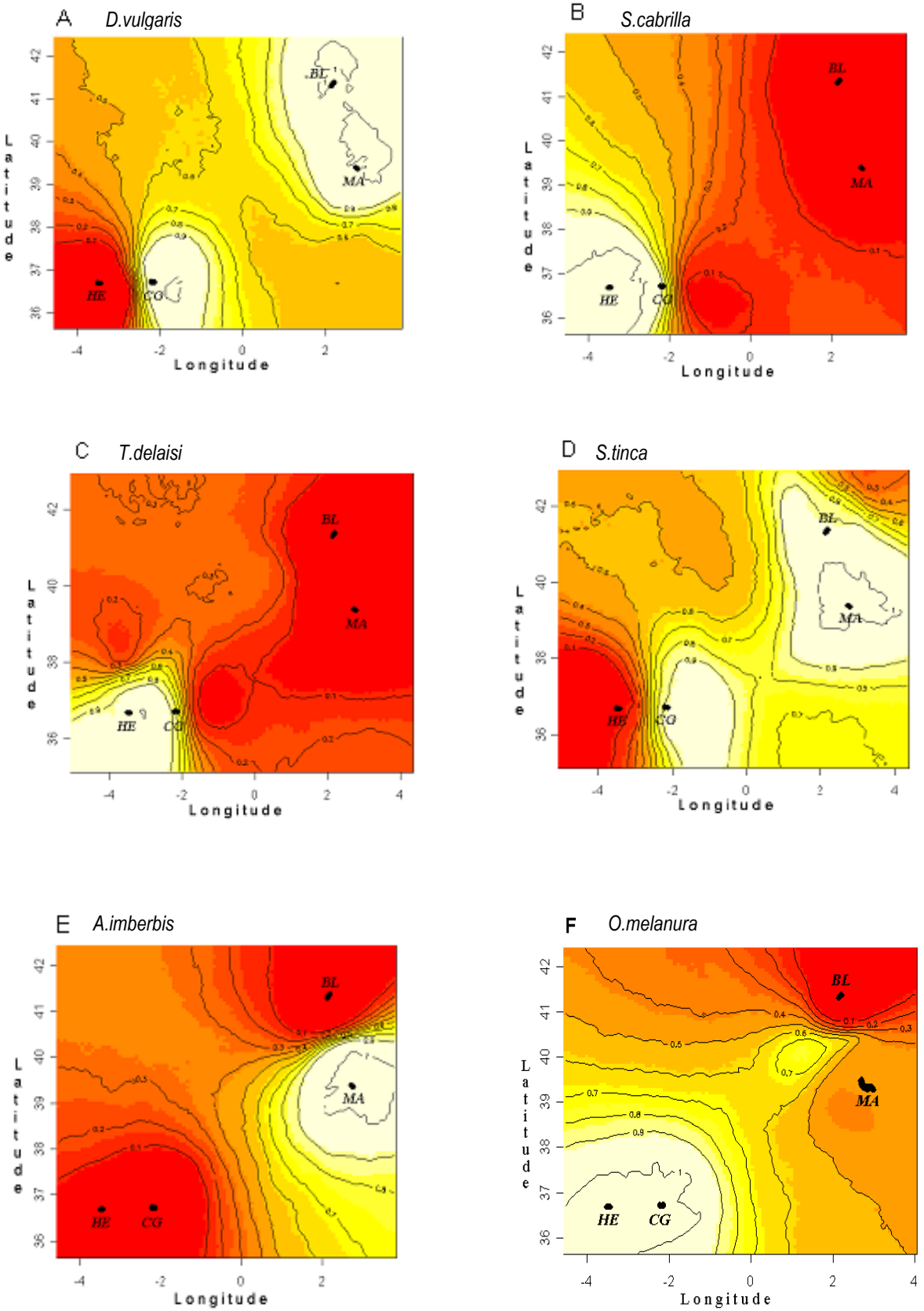


Fig. 19. Map of posterior probabilities of population membership and spatial location of genetic discontinuities for *D.vulgaris* (A), *S.cabrilla* (B), *T.delaisi* (C), *S.tinca* (D), *A.imberbis* (E) and (F) *O.melanura* species. Contour lines indicate spatial position of genetic discontinuities. Lighter colours indicate higher probabilities of population membership.

4. 4 DISCUSSION

Both fronts appeared to have a distinct influence on population genetic structure of the different species. This influence was more noticeable in the strongest and less variable front (AOF), decreasing clearly the genetic flux in all species with the exemption of *O.melanura*, whereas the weaker front (BF), only diminishes the flux in some species. The AOF seems to have a greater effect on the populations of *D.vulgaris* and *S.tinca,* whereas the BF affected mainly *A.imberbis O.melanura* and *T.delaisi* populations. Populations of *S.cabrilla*, in turn, showed inconsistent patterns across both fronts. As suggested by the results, the AOF can potentially affect species with pelagic stages irrespective of their durations and spatial distribution. This is easily illustrated by populations of *D.vulgaris* and *S.tinca*, which showed identical patterns of genetic structuring in spite of having substantial differences between their pelagic stages (Table 13). However, an exemption was found in *O.melanura,* an intermediate restricted species for which, its populations appear to experience extensive gene flow across this barrier. In the last decades, numerous studies based on different genetic markers have been performed in various taxa across the AOF. Appendix 2 presets a list of the species analysed in such studies including their main life history traits, the markers utilized as well as the study's main findings. The results are in congruence with the majority of these studies, which have shown evidence that significantly differentiated populations can be found across this front even for species that include very long pelagic stages (≈ 4 months) in their early-life histories (Appendix 2). Results of no genetic differentiation, however, have also been reported for species with relatively smaller dispersal capabilities having shorter pelagic durations (≈ 30 days), and in some cases, laying benthic eggs (Appendix 2). Interestingly, when closely related species have been analysed simultaneously, incongruent results are commonly observed between species. By using mitochondrial genetic markers Charrier *et al.* (2006) found that significant

differences exists across the AOF for populations of one anglerfish species (*Lophius budegassa)* whereas the opposite was true for populations of its congener *(Lophius piscatorius)* which possess a qualitatively similar life history. Bargelloni *et al.* (2003) analysed 5 sparid species using mitochondrial genetic markers. The results show evidence that a gene flow break exists for three out of the five species investigated. In a similar study, Bargelloni *et al.* (2005) analysed another two sparid species with highly comparable biology by means of mitochondrial and nuclear (allozymes) genetic markers. Both markers showed significant genetic differences across the AOF for the populations of only one of the species. In this study, hyper-variable genetic markers were used and the results concerning the two sparid species (*D.vulgaris* and *O.melanura*) also show striking inter-specific differences. Contrary to expectations, *O.melanura* showed nearly no genetic differentiation across the AOF in spite of its presumed limited dispersal capabilities relative to those of *D.vulgaris* (Table 15). These results complements previous findings and highlights the fact that although the AOF can be an effective barrier to gene flow for most marine species, exemptions to this trend exists and cannot be attributable solely to the dispersal capabilities expected from early-life history traits or the genetic markers used for inference.

Other environmental and biological factors, in addition to the AOF, may have also contributed to the genetic structure observed in these species. The AOF front sets the limits of the Alboran Sea where the main circulation is characterized by two semi permanent anticyclonic vortices that give rise to the western and eastern Alboran gyres (Viúdez *et al.* 1998). Here nutrient enriched water produced by upwelling and Atlantic inflow is recirculated before it propagates eastwards along the African coast (Béranger *et al.* 2005). This flow pattern coupled with the frontal density formation may induce retention/concentration mechanisms of pelagic larvae within this enriched water. Hence, favorable conditions for eggs and larval development arise, promoting nearby

recruitment by potentially retaining early life stages long enough to develop. Likewise, juvenile and adult individuals may also benefit from these retention/concentration mechanisms by encountering favorable foraging conditions in these highly productive waters, and thus, remaining within the area.

The BF on the other hand, appears to affect mostly intermediate and strongly restricted species with short or nil pelagic stages. Although there are a few documented cases where high gene flow levels have been observed in restricted species such as brooders and invertebrates (Ayre & Hughes 2000; Richards *et al.* 2007; Sponer & Roy 2002), the results suggest that species with constrained pelagic stages are on average more affected by the BF than broadcast spawning species. The effect of the BF on restricted species may be intensified by the existence of two potential habitat discontinuities between insular and continental populations. One is represented by open water as such, whose main oceanographic features can vary substantially from that of coastal systems (Millot 2005). Thus, the likelihood of inshore-spawned larvae to survive in oceanic conditions may be reduced compared to offshore-spawned larvae. The other is the presence of a deep-water trench of more than 1000 m. depth that separates the Balearic Islands from the continent (Palanques *et al.* 2005) and prevents adult movement. In view of the relative shallow depth range of *A.imberbis*, *O.melanura* and *T.delaisi*, deep water could pose a natural barrier to adult movement to and from the island restricting genetic flow as it has been shown for other marine species (Doherty *et al.* 1995; Shaw *et al.* 2004; Stepien & Rosenblatt 1991). On the other hand, *D.vulgaris* and *S.cabrilla* also possess a substantially shallower depth range than the aforementioned deep-water trench and no genetic discontinuity seems evident for populations of these species. The Balearic Islands display a narrow shelf bound and there is evidence of partial hydrodynamic interactions occurring with the continent (Jordi *et al.* 2005). Hence, this intermittent connection may allow for passive transport of early life stages as the most likely

mechanism of gene flow between the Balearic Islands and the continent for the populations of these species.

The finding of high gene flow levels between continental and insular populations of *S.tinca* is puzzling since it is one of the species least likely to be affected by passive transport (the shortest PD and inshore larval distribution). One possible explanation could be given by the occurrence of favorable transporting circulation patterns during the onset of larvae and throughout its pelagic stage. However, *S.tinca* as well as *S.cabrilla* spawn from spring to early summer, whereas spawning season of *D.vulgaris* lasts from autumn until winter (Bauchot & Hureau 1986). Thus, no evident association can be established between seasonal circulation patterns and spawning time of the species. Another explanation could be given by the mating and settlement behaviour of the species. As in other species of the genus *Symphodus*, spawning takes place in nests built with branching algae that are often destroyed and transported by tides (Bauchot & Hureau 1986). These branching algae are also the habitat for settlers and thus passive transportation through drifting algae can occur in these two stages. However, this hypothesis merits further research.

Overall, two common patterns of genetic partitioning could be observed in this study. Interestingly, shared patterns were found between species considered to be highly dispersive (*D.vulgaris*) and fairly restricted (*S.tinca*), as well as between intermediate dispersive (*T.delaisi*) and highly restricted species (*A.imberbis*). Similarly, no conclusive evidence was found that a single life history trait could be directly associated to population genetic differentiation when analysed across strong (AOF) weak (BF) and non-existent (NF) oceanographic barriers. The failure to see the predicted relationship between dispersal capability and realized gene flow provides evidence that different species may respond in a similar way to common environmental features regardless of

their dispersal potential. Furthermore, the results highlight the importance of understanding small-scale hydrographic variability and local processes since these can have ecologically meaningful effects on population connectivity in marine species and therefore, should be considered in the devise and implementation of MRPA's. As suggested by the results, circulation processes may sweep away inshore and/or short-lived larvae allowing it to settle in distant habitats contributing to genetic homogenisation of distant populations in spite of non-continuous habitat and the species' limited dispersal. Equally, the existence of oceanographic barriers may account for population self-recruitment of species with extended pelagic stages resulting in limited connectivity with adjacent populations. Therefore, detailed regional ocean circulation patterns should be taken into account when devising MRPA's, even within small geographical regions. Finally, the results highlight the importance of employing multi-species approaches in the understanding of gene flow patterns occurring within an ecosystem where multiple species co-occur. The results demonstrate that these patterns may not be predicted confidently by the species' dispersal potential as determined by their early-life history characteristics. Therefore, spatially oriented management of marine resources should be devised according to the scale of genetic connectivity and detailed information of environmental features ranging within the area.

CHAPTER V
General Discussion

5.1 COMPLEXITY AMONG DISPERSAL AND GENE FLOW PATTERNS IN THE MARINE REALM.

Population structure is dependent on the interaction of the biology of a species and the environment in which resides (Palumbi 2003). In terrestrial environments, groups of individuals of the same species are often isolated from other groups either by physical barriers or by sufficiently large distances that, in many cases, mixing and interbreeding among groups occurs rarely. These groups are therefore well defined and outline populations that can be recognized as distinct from each other showing a relatively clear structuring. In the marine environment, however, population structure is not as apparent as in its mainland counterpart. Factors affecting gene flow patterns in the marine realm can be broadly subdivided into biotic and abiotic categories. Apart from human activities (overfishing, translocation, ecosystem degradation) (Beddington *et al.* 2007), abiotic factors influencing population genetic structure in wild marine populations include water flow dynamics (ocean currents, gyres, eddies, upwelling processes)(Barber *et al.* 2000; Carlsson *et al.* 2004; Grant 2005; Zardoya *et al.* 2004), habitat discontinuities (bottom topography, substrate distribution) (Beldade *et al.* 2006; Dawson 2001; Hemmer-Hansen *et al.* 2007; Medina *et al.* 2007; Riginos & Nachman 2001; Shaw *et al.* 2004), geographical distance (Hellberg 1996; Lavery *et al.* 1995; Medina *et al.* 2007), and environmental stressors (temperature and salinity gradients) (Cimmaruta *et al.* 2005; Florin & Hoglund 2007; Foll & Gaggiotti 2006). Biotic factors include those referring to the particular biology of the species such as early-life history traits, which act as the main means of dispersal for most marine species (Strathmann 1980). The interaction between these two factors is the driving force that outlines gene flow patterns in the marine realm. Typically, these two factors separately or in combination, are often invoked to interpret the observed gene flow patterns. However, these interpretations

should be taken with caution due to our limited understanding of biotic factors and their response to highly variable abiotic factors, particularly at short temporal-spatial scales. For example, tide regimes are largely dependant on the interaction between gravitational forces and can be confidentially predicted for periods of up to 90 years (Stewart 2000). However, the effect that incoming tides could have on local hydrography of small estuaries or bays on a daily bases can be hardly predicted due to local particularities of the area such as coastline shape, substrate, depth, and winds shifts (Fernández *et al.* 2005). Thus, larvae spawned at a particular point could have a different fate from larvae spawned at the same point at a different time (i.e. on the next high tide). For marine organisms that rely on planktonic stages as their main mechanism of dispersal, it is at this scale where most ecological connections between populations are most likely to be established (Sale *et al.* 2005). Hence, the highly stochastic nature of abiotic processes ranging at this scale, coupled with the fact that obtaining reliable estimates of effective dispersal rates by means of pelagic stages is notoriously difficult (see section 1.2 for a review), results in a complex relationship which must be carefully evaluated when planning management-conservation actions.

5.2 THE MULTI-SPECIES APPROACH, EARLY-LIFE HISTORIES AND GENE FLOW

To understand how gene flow patterns relate to the variation observed in pelagic stage characteristics and how this relationship can be translated into conservation-management strategies, a few studies have adopted a multi-species approach. The rationale of this approach is to analyse multiple species displaying a range of pelagic stage characteristics exposed to common abiotic factors to examine if relationships can be identified between observed gene flow patterns and the different pelagic stage characteristics. The

theoretical expectation is that species with long and/or offshore-spawned pelagic stages would display higher gene flow levels, and thus, higher connectivity than species with more restricted pelagic stages under the same environmental conditions. However, empirical multi-species studies have often found contrasting or inconclusive evidence to support this expectation. The first relevant study was conducted by (Waples 1989) in which, allozyme variation at 10 shore fish species was examined along the California current system. The results showed an overall agreement between dispersal capabilities and gene flow patterns despite the fact that most species were estimated to have high gene flow levels among localities. In a similar study, (Doherty *et al.* 1995) analysed allozyme variation of 7 reef fish species with distinct dispersal strategies across the Great Barrier Reef and observed a chaotic pattern between gene flow levels and dispersal strategies, although, the authors observed a positive relationship between genetic differentiation and the total pelagic larval duration among the species. (Shulman & Bermingham 1995) examined mitochondrial DNA variation to estimate the degree of genetic differentiation among 8 tropical reef fish species that differed in their egg type and planktonic duration. The authors concluded that neither egg type nor the planktonic duration could be related to genetic structure. In contrast, (Riginos & Victor 2001) analysed control region sequences of 3 blennioid fish species with different dispersal potential measured as duration of larval phase and spatial distribution in the Gulf of California and observed a good agreement between levels of genetic differentiation and dispersal potential. In two separate studies, (Bargelloni *et al.* 2005; Bargelloni *et al.* 2003), analysed mitochondrial and allozyme data in 7 Sparid species from the Atlantic Ocean and the Mediterranean Sea. The results showed incongruence between the observed gene flow patterns and those expected by the species' early-life history traits. Recently, (Charrier *et al.* 2006) examined mitochondrial sequences of 2 anglerfish species with comparable pelagic characteristics in the western Mediterranean observing

significant discrepancies among the gene flow patterns between the two species. In the present study, microsatellite loci also revealed contrasting results to those expected by analysing a total of 8 littoral fish species within the Mediterranean Sea. Overall, the cumulative evidence obtained from these studies shows that no obvious relationships can be inferred between early-life history traits and observed gene flow patterns (Fig. 20). Even within those studies that have shown some degree of agreement between these variables (i.e. Chapter III; Riginos & Victor 2001, Waples 1987), numerous exceptions are reported that show substantial departures from this expected relationship. In addition, the overall conclusion of positive association between gene flow and life histories reached by Waples (1987) and Riginos & Victor (2001), could be largely influenced by the inclusion of viviparous species as well as species with facultatively pelagic juveniles within their species choice. Congruently, after subsequent analysis, (Waples & Rosenblatt 1987) concluded that no major spatial pattern could be attributed directly to dispersal strategy.

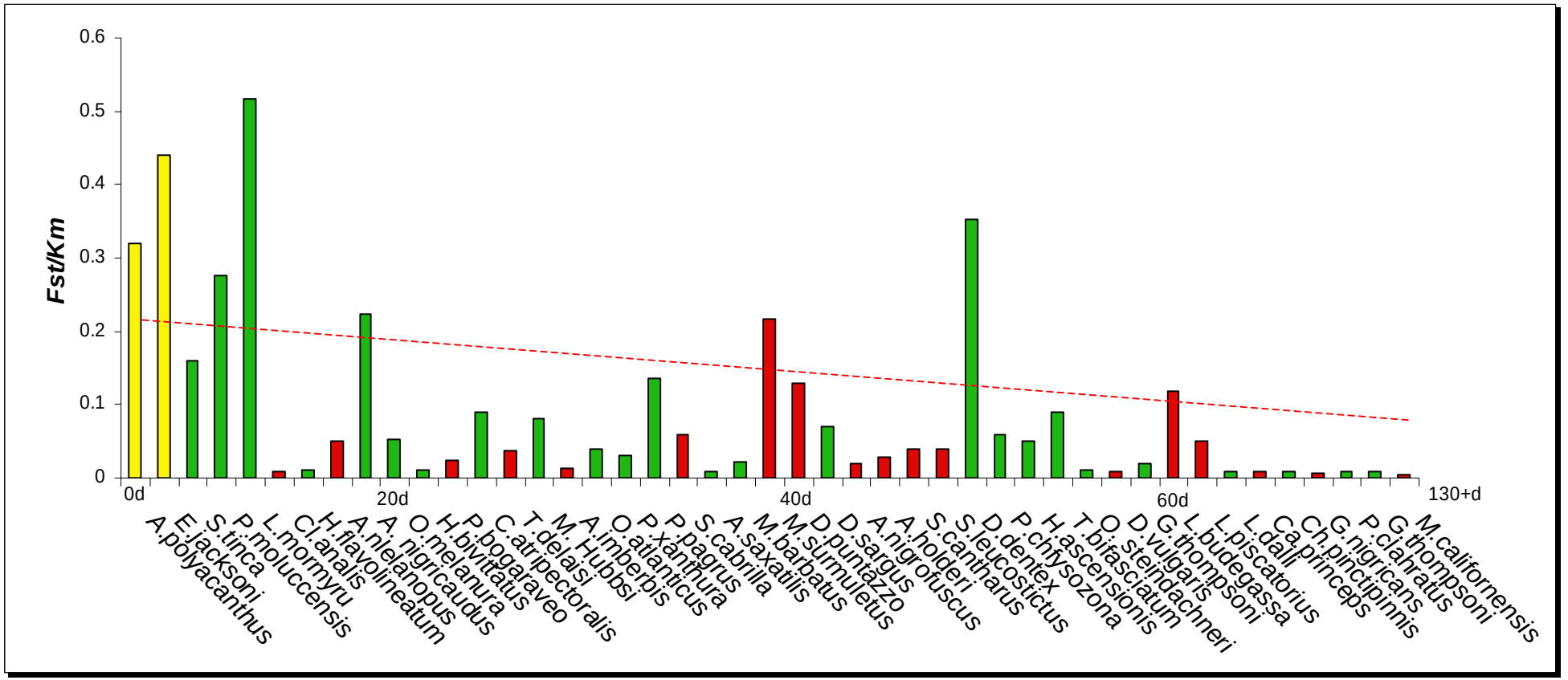
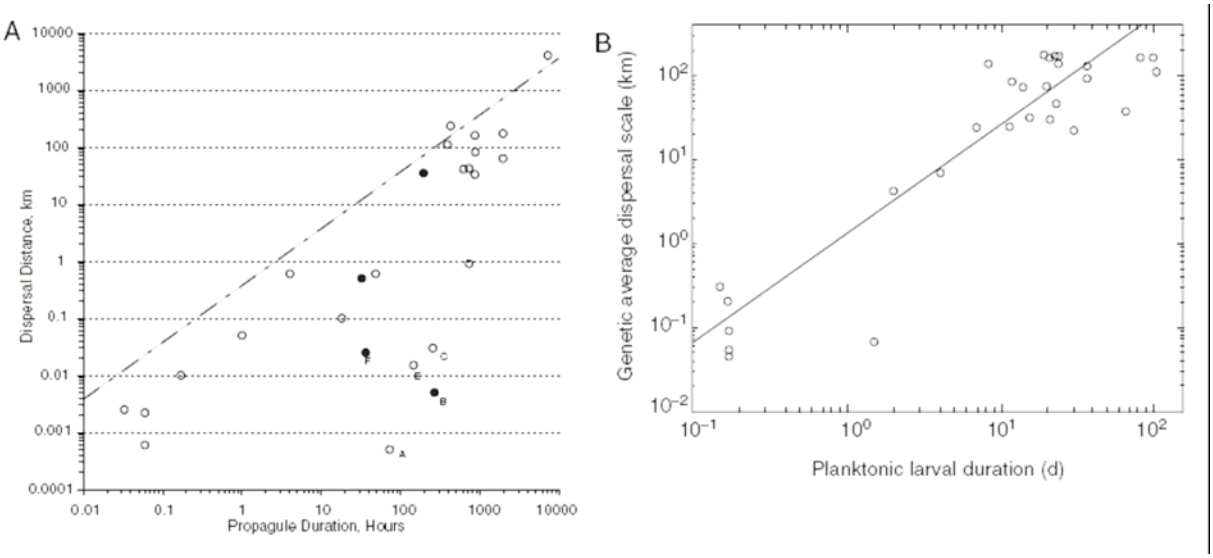


Figure 20. Expected (dotted line) and observed (column bars) gene flow patterns in relation to the pelagic duration (in days) among the species surveyed at multi-species studies (x axis). For each data set, the reported parameter of genetic differentiation (F_{ST} or analogous) was divided by the mean geographic distance surveyed in order to achieve standardization across all data sets. Geographic distances were obtained directly from references, measured from published site maps or calculated using Matlab v.7 (Mathworks) based on reported locations of study sites. Yellow column bars indicate viviparous species, red column bars indicate benthic-spawned species, whereas green column bars indicate pelagic-spawned species. Data from Waples 1987; Doherty 1995; Riginos & Victor 2001; Bargelloni *et al.* 2001; Bargelloni *et al*. 2005; Charrier et al 2006; Chapter III and Chapter IV.

By analysing previous multi-species results in combination with the present study, two main conclusions can be proposed. First, it seems evident that comparisons between species with extreme variations in their pelagic stages often conform to expected gene flow patterns, whereas for species with intermediate pelagic stages such expectation does not hold true (Fig. 20). This observation is congruent with three recent studies that have reviewed the relationship between pelagic durations and dispersal estimates (Lester *et al.* 2007; Shanks *et al.* 2003; Siegel *et al.* 2003). The three studies agreed demonstrating an overall positive relationship between these two variables (Fig. 21). However, it must be recognized that in these studies, data was merged from a wide variety of marine taxa including highly restricted organisms such as ephemeral algae and echinoderms. Thus, a wide spectrum of planktonic stage characteristics -including extreme ends- was compared. Likewise, data was obtained from a number of different sources other than genetic data such as direct observations, studies of distributions in nature and experimental studies. Nonetheless, in addition to the positive relationship inferred between pelagic duration and dispersal estimates, these studies also identified a number of species whose observed dispersal distances fell well outside their theoretical expected range of dispersal. (Fig. 21).



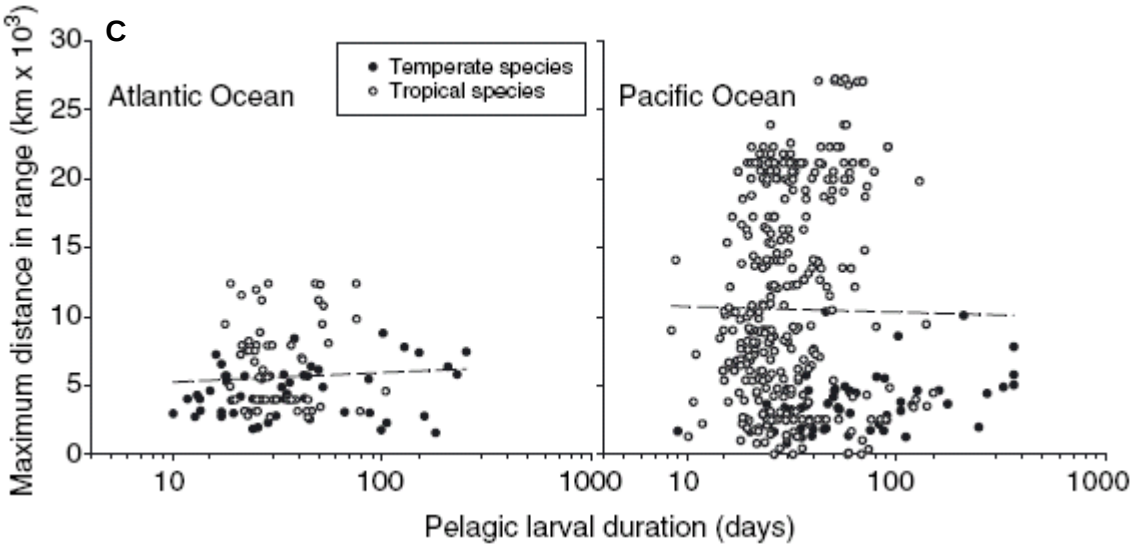


Figure 21. Relationship between pelagic larval duration and dispersal distance. **A:** from Shanks *et al.* (2003), **B:** from Siegel *et al.* (2003), **C:** from Lester *et al.* (2007).

Second, no direct relationship can be expected between the spatial distribution of the pelagic stages and the patterns of genetic structure (Fig. 20). The majority of multi-species studies that have surveyed species with pelagic stages report a substantial number of disparities amongst their comparisons. Nearly identical gene flow patterns have been observed between some or most inshore vs. offshore and benthic vs. pelagic spawned species. In the present study, unexpected results were also observed when comparing gene flow patterns between species with slight differences in their early-life history traits (i.e. Chapter III) as well as between species with highly contrasting pelagic stages (i.e. Chapter IV). Thus, an examination of the possible causes for these many atypical results is instructive. Firstly, multi-species approaches explore the correlation between summary statistics measuring levels of genetic differentiation and independent estimates of dispersal capability. Such a correlation is expected if genetic drift and gene flow are the primary agents affecting allele frequencies (Waples 1987). Therefore, it is possible to envision some scheme involving natural selection affecting such a correlation, particularly in those studies that have relied on gene expression methods for

inference (i.e. Waples 1987; Doherty 1995). However, the patterns described in these studies were largely unaffected by variation in the number and type of gene loci included in the analysis (i.e. enzymes or general proteins). Furthermore, results using different subsets of loci indicated internal consistency in the data sets, as it would be expected under a drift model but not under a model involving selection at some loci. Equally, the rest of multi-species studies showed a qualitatively similar pattern of discrepancies using gene loci widely accepted as neutral. Thus, the congruency in the results across the different studies suggest that no obvious relationship can be observed between gene flow and early-life history traits irrespective of the genetic method used and the species analysed.

A more likely explanation can be found by examining the wide range of upper and lower values reported for the pelagic stage duration of the species investigated (Fig. 22). In all studies (except see Doherty, 1995) no precise estimation was made on the pelagic stage duration of the various species surveyed. Consequently, available mean reported durations were used in the analysis. Figure 22 shows that most species surveyed have a mean pelagic duration between 10-60 days whereas a few species posses mean pelagic durations >70 days. It is worth noting that for those species with mean pelagic durations between 10-60 days, the upper and lower reported values largely overlap (Fig. 22). Similarly, the same pattern is repeated for the group of species with longer mean durations. This is illustrative because it highlights the great uncertainty in defining pelagic durations and at the same time, it discloses a wide range of possible results if different values are chosen within those ranges. Assumption

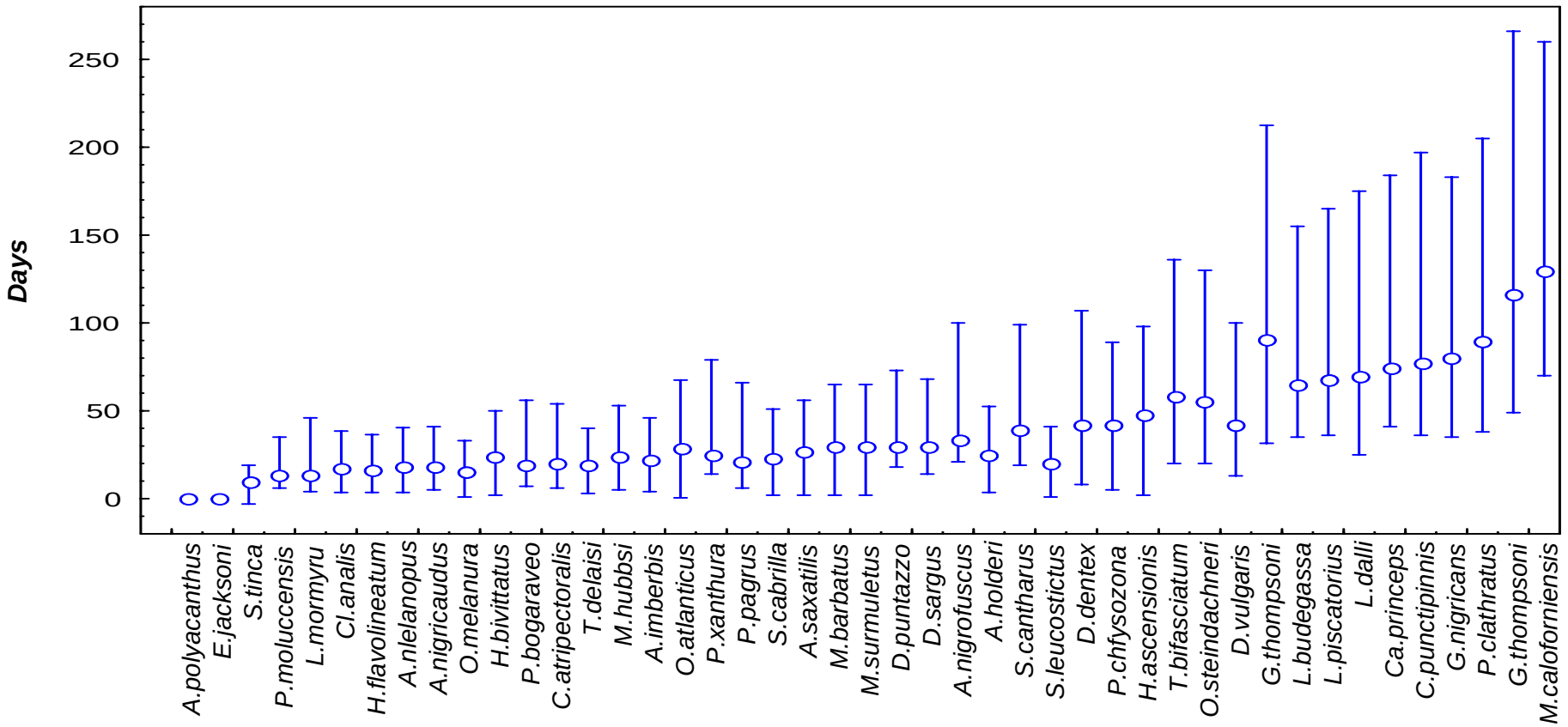


Figure 22. Mean pelagic durations (open circles) among the species surveyed at multi-species studies. Error bars indicate upper and lower reported values of pelagic duration. Data from Waples 1987; Doherty 1995; Riginos & Victor 2001; Bargelloni *et al*. 2001; Bargelloni *et al*. 2005; Charrier et al 2006; Chapter III, Chapter IV and references therein.

Behavior and ontogeny of early-life stages may also be invoked to account for the observed discrepancy between presumed dispersal capabilities and realized gene flow. It has been demonstrated that fish larvae are capable of executing vertical migrations as a response to a variety of abiotic and biotic factors such as currents, stratification, light, oxygen, temperature and predator-prey distributions (Bradbury *et al.* 2006b; Norcross & Shaw 1984). However, information on the vertical distribution of larvae in nature and how it varies with ontogeny and across species is limited. Furthermore, ontogeny changes with size, which in turn, is largely dependant on age (Carr *et al.* 2007). Thus, for species with wide reported ranges of larval durations, it is difficult to estimate with precision when passive dispersal ends and active habitat selection begins. Newly hatched larvae are not capable of complicated swimming performances. However, owing to rapid morphological development, it is often a matter of days before swimming execution has much improved (Urho 1999). In laboratory swimming chambers, for example, it has been shown that larvae of some marine fish taxa (Sciaenidae, Sparidae) are capable of swimming more than 10 km at speeds which exceed the average currents in their coastal environments (Clark *et al.* 2005). In addition, not only larvae are able to execute active migrations but also the egg stages have been shown to vary their position in the water column through buoyancy (Alvarez *et al.* 2001; Norcross & Shaw 1984). Buoyancy also changes with ontogeny, thus as egg stages develop, their spatial distribution varies and this can be used to exploit counter-currents or layering of the water column so that some degree of controlled dispersal is achieved (Goodwin *et al.* 2005). Alternatively, habitat complexity (i.e. gyres, shifting currents, suitable habitat fragmentation) may overcome the larvae's dispersal potential if such complexity is of sufficient magnitude and sustained for time periods longer that the larval stages.

In sum, the failure to observed the expected gene flow patterns between marine fish species with varying dispersal capability as presumed by their early-life history traits stems mainly from the absence of precise information on the general biology of the species (i.e. pelagic durations, spatial distributions, ontogeny) as well as from the highly variable physical processes in the environment in which the early-life stages develop.

5.2.1 The Multi-species approach and resource management

The role that the application of genetic markers can play in the sustainable use and conservation of marine resources is being increasingly appreciated by resource managers, policy makers and by the international community in general. Gene and genotype frequency data can provide valuable information on species identification (Rehbein *et al.* 2002), population stock structure (Ayllon *et al.* 2006; Fritsch *et al.* 2007), hybridisation (van Herwerden *et al.* 2006), inbreeding (Hoarau *et al.* 2005), genetic impacts of fishing and aquaculture (Ayllon *et al.* 2006; Hauser *et al.* 2002; Li *et al.* 2006) and population connectivity via gene flow (Roberts *et al.* 2004; Vollmer & Palumbi 2007). In this regard, the main advantage of multi-species approaches over single-species studies resides in that the former allows direct comparisons with other species relative to the same area. In this way, a simultaneous estimate can be obtained about the relative importance that geographical distance, habitat discontinuities and life history traits have in the genetic connectivity of marine populations found within a particular area. This parallels current ecosystem approaches in which integrative management strategies are considered between areas (Laffoley *et al.* 2004) and it is in line with the notion that the multi-species nature of today's fisheries demands that managed areas accommodate different life histories (Palumbi 2004).

Two main differences can be highlighted between the Multi-species approach employed in chapter IV and those previously reported. First, none of the previous studies had all the samples collected at exactly the same locations. The sampling scheme presented here is tailored to facilitate comparisons among species and reduce error when implementing statistical analyses that make use of spatial information (see below). Second, contrary to previous studies in which gene flow patterns were surveyed across relatively large geographical areas (i.e. meso-scale current systems) without *a priori* identified barriers to gene flow, this study was performed within a small hydrodynamic system in which the effect of two well-defined potential barriers could be compared against barrier-free results. Thus, comparisons are not only possible among species but among different oceanographic conditions and different areas. This is particularly useful towards management strategies since it provides an overall appreciation on the degree of genetic connectivity within and between areas, which can then be identified as potentially effective for conservation and management purposes.

Marine protected areas may be established for a number different goals and objectives (see section 4.1). Hence, different criteria exist towards evaluating their performance and effectiveness (Kelleher & Kenchington 1999). Overall, protected areas meant for the conservation and management of economically important fish resources have been positively evaluated through the analysis of demographic parameters (Claudet *et al.* 2006; Fraschetti *et al.* 2005; Shears *et al.* 2006). (Narvarte *et al.* 2006), for example, found a higher recruit/spawner ratio inside MPA compare to an unprotected area. Evaluations through genetic methods, however, are scarce despite the fact that protected areas are thought to be an efficient means to maintain genetic diversity (Palumbi 2003). Inappropriate fisheries management can have important adverse genetic consequences on the harvested species (see section 3.1). (Perez-Ruzafa *et al.* 2006) evaluated the effects

of fishing protection on the genetic structure of fish populations in protected and non-protected areas of the western Mediterranean. The results showed that protected areas had significantly higher allelic richness compared to non-protected areas. In contrast, (Lenfant 2003) found no significant genetic differences between protected and non-protected areas in the French Mediterranean coast. Interestingly, both studies analysed the same species *(Diplodus sargus*) using enzyme coding loci. The discrepancy among studies is probably influenced by geographic variability since at large distances the existence of exclusive alleles determined most of the genetic differentiation among populations in both studies. This highlights the importance of understanding the scale of genetic connectivity between protected areas and their surrounding environment.

5.3 SEASCAPE GENETICS

As stated above, understanding the relationship between gene flow patterns and dispersal capabilities is not a trivial task. In marine species with planktonic larvae, genetic studies have often found minimal levels of geographic differentiation in species with presumed low dispersal potential and vice-versa. To understand how larval dispersal relates to geographical patterns of genetic structure, (Palumbi 2003) developed a method based on an isolation-by-distance (IBD) framework. In this method, genetic differentiation is calculated among a set of simulated populations along a stepping stone lattice with equal spacing between. Populations are assumed to exchange larvae as a function of the distance between them. Then, through a series of simulations, spacing between populations is varied and the relationship between this spacing and genetic differentiation is calculated. The rationale is that over time, the balance between gene flow (dispersal) and random genetic drift results in a spatial pattern of isolation by distance within the genetic structure of neutral alleles which can be directly related to

dispersal distance (Epperson 2003; Wright 1943). Specifically, the slope of the IBD relationship can be related to the mean effective dispersal distance averaged over many generations by means of numerical simulations of various population genetic structure scenarios (Palumbi 2003). A fundamental limitation of this approach, however, is that the geographical structure of natural populations may not fit the stepping-stone model accurately. Marine populations are hardly evenly distributed along a continuous environment. Coastline contour, bathymetric variations, substrate homogeneity and water properties all influence natural population's geographical structure. Therefore, marine populations are not likely to exchange migrants exclusively with their nearest neighbours a assumed in the model. Furthermore, although mean dispersal distances can be estimated, there is no way to accurately capture maximum dispersal distances (Lester *et al.* 2007).

Other statistical procedures are now being implemented to study the spatial variation of genetic diversity among wild marine populations. These new methods are the result of the combination between landscape ecology and population genetics (Manel *et al.* 2003). In the marine field they are collectively known as "Seascape Genetics", a term borrowed from their mainland counterpart "Landscape Genetics", field in which they have been widely applied (Latta 2006; Pannell & Dorken 2006; Sork & Smouse 2006). This approach aims to provide information about the interaction between seascape environmental features and microevolutionary processes, such as gene flow, genetic drift and selection. It also aids in identifying cryptic boundaries, which can be either breaks in the gene flow across populations without any obvious cause, or secondary contact among previously isolated populations (Manel *et al.* 2003). Hence, two data sets are needed, a genetic one and a seascape ecological one. The key aspect is to identify breaks, gradients or transitions that coincide in these two data sets (Holderegger & Wagner 2006). A

number of different methods are becoming available mainly due to the development of sophisticated statistical tools and increasing computational power. Below is a summary of the most commonly used methods as well as a brief description of their underlying principle.

- **Genetic Spatial Autocorrelation:** This multivariate approach estimates simultaneously the spatial signal generated by multiple genetic loci producing an overall autocorrelation coefficient. The autocorrelation coefficient provides a measure of the genetic similarity between pairs of populations whose geographic separation falls within a given distance class and the results are then summarized by a correlogram (Peakall *et al.* 2003; Smouse & Peakall 1999).

- **Monmonier algorithm**: In this approach, samples (i.e. individuals) are located on a map according to their relative geographical position. A Delaunay triangulation approach is used to connect the adjacent geographical positions of the samples on a map, resulting in a network that connects all the samples (Manni *et al.* 2004). Then, the Genetic distances between neighbouring samples are calculated and associated to each edge of the network. Finally, boundaries between groups of samples are identified through the Monmonier's maximum difference algorithm (Miller 2005).

- **Voronoi tessellation**: This method is design to seek population structure from individual multilocus genotypes sampled at different geographical locations. As opposite to the Monmonier method, this method is based on a hierarchical mixture model whose neighborhood system is obtained from the Voronoi

tessellation (Chen *et al.* 2007). Clustering of multilocus genotypes is then performed through a Hidden Markov Random Field (HMRF) on these tessellation network (Francois *et al.* 2006).

- **Partial Mantel tests:** In addition to ordinary Mantel tests which test for isolation-by-distance patterns by assessing the correlation between genetic and geographic distance matrices, this technique introduces a third indicator variable matrix with complementary population pairwise values such as habitat discontinuities or alternate measures of population connectivity (Raufaste & Rousset 2001). The effect that such indicator variable has on the genetic structure of the populations as a function of geographical distance is then calculated through the partial correlation coefficient of these three matrices. This procedure allows to assess the effect of landscape configuration disentangled from a mere effect of geographic distance (i.e. isolation by distance) (Bohonak 2002).

- **Canonical correspondence analysis:** This analysis can be performed as a complement to partial Mantel tests. In spite of their usefulness, partial correlation coefficients can only provide evidence that a particular factor has a significant effect on population genetic structure. Canonical correspondence analysis (CCA), in turn, can analyze simultaneously a number of environmental factors and quantify the amount of genetic variation explained by each factor (Angers *et al.* 1999). Thus, it enables genetic diversity to be related to environmental factors and provides an indication of those factors that contribute significantly to the explanation in the variation in genetic distribution (Annan *et al.* 2007).

- **Interpolation:** The use of interpolation techniques is increasingly being used in the population genetics field. Interpolation routines can be applied to locate boundaries across a surface for an interpolated variable (i.e. allele frequency surfaces) by searching for regions in which the absolute value of the surface slope is large (also known as Womble) (Crida & Manel 2007). Multivariate statistics can also benefit from interpolation techniques to visualize spatial patterns of genetic data. For example, principal component analysis (PCA) can be used to cluster allele frequencies of spatially referenced populations. Then, the first two principal components can be interpolated to visualize clines in allelic data across the sampled populations (Bucci *et al.* 2007).

- **Geographical Information Systems:** The increasing availability of geostatistical tools provides an efficient and visually appealing way to identify genetic boundaries throughout a seascape (Spear *et al.* 2005). By means of interpolation techniques, genetic data can be overlaid into geographical maps containing information about one or more environmental variables (Epps *et al.* 2007). The information can be used to create synthesis maps from which genetic barriers can be inferred and also to generate hypothesis about the cause of such genetic boundaries.

Other alternative and complementary methods based on assignment tests and numerical recognition methods are now being developed (Legendre P & Legendre 2006; MANEL *et al.* 2007). Overall, seascape genetic techniques make use of environmental, physical, ecological or spatial information which is directly integrated with genetic data. Numerous genetic studies performed on marine fish species such as Herring (*Clupea harengus*) Northern pike (*Esox lucius*), Yellow perch (*Perca flavensces*) and

anadromous smelt (*Osmerys mordax*) have found such techniques successful when investigating spatial genetic structure (Bradbury *et al.* 2006a; Gagnon & Angers 2006; Jorgensen *et al.* 2005; Laikre *et al.* 2005). In the present study, two seascape genetic methods were employed at both, the population and the individual level. The genetic spatial autocorrelation analysis performed in Chapter III calculates the autocorrelation coefficient between the population's genetic distances (F_{ST}) as a function of geographic distance classes. Furthermore, it provides an estimate of the geographic distance at which the genetic cohesion among the populations is no longer significant. Nonetheless, this distance should be treated with caution due to the arbitrariness in selecting the number of distance classes between the populations. Selecting distance classes greater than the scale of genetic connectivity will fail to detect genetic structure, while distance classes well below the scale of genetic connectivity may be associated with unnecessarily small sample sets, and therefore limited statistical power (Peakall & Smouse 2006). However, when two overlapping species are compared simultaneously over the same distance classes, their autocorrelation coefficient provides a good relative measure of the genetic size range of the species. Thus, differences between the species become apparent regarding their effective dispersal and population connectivity via gene flow in spite of the spatial overlap and ecological affinities. This information can be useful to fishery managers, especially those dealing with multi-species fisheries as in the case of the species considered in chapter III to delimit the geographical extent of fishing grounds where both species can be managed simultaneously.

At the individual level, the spatial statistical procedure implemented in chapter IV (Geneland) makes use of georeferenced multilucous genotypes (i.e. individuals) which are considered as a spatial mixture of panmictic populations. Then, the spatial organization of populations is modeled through the Voronoi tessellation method

described above. The main advantage of this approach is that inference is made without assuming predefined populations. Hence, the spatial location of genetic boundaries as well as the number of panmitic units or populations are inferred using the information on the spatial position of the individuals sampled (Guillot *et al.* 2005). The potential of the method to detect spatial genetic structure has successfully been tested in terrestrial organisms (Coulon *et al.* 2006; Faubet *et al.* 2007; Guillot *et al.* 2005; Pilot *et al.* 2006; Rowe & Beebee 2007; Zannese *et al.* 2006). Likewise, the efficacy of the method has been positively evaluated through simulation approaches (Chen *et al.* 2007). However, applications of the method in marine organisms are lacking. This could be due in part to the difficulty in assigning spatial coordinates to individual samples from wild marine populations. In this study, this issue was addressed with the help of a geostatsitical analysis software (Mapping toolbox in Matlab) to assign each individual a latitude and a longitude virtual coordinates. Such coordinates were allowed to vary within a 10 km radius from their original position to take into account the uncertainty in the exact positioning of the individual (i.e. no GPS available) as well as other sampling error artifacts such as the collection of individuals in different zones of a beach or different inlets. The results showed a good agreement between the spatial position of genetic discontinuities identified by the method and the actual location of the oceanographic density fronts. This provides evidence that individual-based methods that make use of spatial information can be successfully applied to marine species for which geo-referencing of individuals is logistically difficult. Furthermore, the information obtained can be directly translated into spatially oriented management. Results of chapter IV, for example, show that the AOF constitutes a natural break for continuous populations and thus, management should be devised considering two separate areas, one on each side of the front.

5.4 LIMITATIONS OF THE PRESENT STUDY

5.4.1 Historical events

Although ocean currents and habitat discontinuities may be effective in promoting and/or restricting gene flow, their occurrences may not entirely explain the patterns of genetic partitioning observed here. Genetic homogeneity between distant populations does not necessarily mean that separate breeding units do not exist. Populations which may have not been isolated long enough to establish differences in allele frequencies or that gene flow between them has been kept at low but constant levels could probably be regarded as genetically homogeneous populations by the genetic markers utilised here.

The observed patterns of population structure could be influenced by isolation during historical events. For example, past episodes of dispersal at times of lower sea levels which have not been erased by subsequent dispersal through present day circulation. Numerous changes have taken place throughout the recent geological history of the Mediterranean Sea and the adjacent Atlantic Ocean, resulting in a dynamic geographical configuration. During the last glaciation lowered sea levels modified substantially the western Mediterranean coastline causing the connection of southern Italy with Sicily and North Africa (Bonatti 1966; Thiede 1978). Similarly, the Gibraltar Strait has experienced repeated closures isolating the Mediterranean Sea from the Atlantic Ocean (Maldonado 1985). Significant geological changes have also occurred at the eastern Mediterranean basin due to the lowered sea level. At the height of the last glaciation, about 15 000 years BP, the sea level in the Aegean Sea dropped by about 120 m (Van Andel & Lianos 1984). As a result, the Euripos Strait and the Oreos Channel were closed and the north Euboikos Gulf and areas such as Platania became isolated brackish lakes (Stergiou *et al.*

1997). Under this scenario, populations from the eastern and western basins as well as from the Eastern Atlantic and Western Mediterranean potentially underwent various secondary contact events due to the repeated closures and fluctuating sea levels (as hypothesised in Chapter III). However a genetic pattern representative of such scenarios cannot be detected by the genetic markers utilized here due mainly to the high mutation rate they experience and their fast-evolving nature. Such a pattern could be observed if assessed through an evolutionary approach using genetic markers capable of retaining the history of past isolation, even in the event of contemporary admixture of populations, such as mtDNA. Even though the implementation of such an approach is beyond the scope of this project, it merits further research since it can provide useful complementary information in understanding the past and current processes that generate the observed genetic patterns of Mediterranean populations.

5.4.2. Null alleles

In this study numerous cases of null alleles were observed across loci in all species. As it has been suggested in sections 3.3 and 4.3, multiplexing techniques could have greatly contributed to this overall excess of null alleles. Other probable causes such as miss-scoring, short allele dominance and inconsistent amplification were statistically evaluated and corrected where appropriate. Likewise, the extent to which null alleles could have influenced the observed genetic structure patterns was analyzed thoroughly for the F_{ST} permutation tests (see sections 3.3 and 4.3). Thus, for tests that rely on F_{ST} values for inference (i.e. Spatial Autocorrelation, Spearman's correlation) the potential error introduced by null alleles can be safely ignored since null alleles have a negligible effect on the statistical significance of F_{ST} values. Similary, for methods that infer genetic relations among populations based on genetic distance matrices (i.e. D_{ps} and Dc),

null alleles may have little or no effect since matrices and subsequent dendrograms are constructed solely on the basis of the shared allelic frequencies of visible allele size classes.

For the rest of tests, however, it remains unclear how the presence of null alleles could have influenced the results. Presumably, for tests that define populations by clustering multilocus genotypes at Hardy-Weinberg equilibrium such as GENELAND and STRUCTURE, the presence of null alleles could have affected the results by overestimating the number of clusters (i.e. populations) due to null alleles inducing Hardy-Weinberg deviations. Nonetheless, within population substructure (i.e. Wahlund effect), inbreeding, or selection at or near a microsatellite locus may cause HWE departures as well (Poulsen *et al.* 2006).

5.4.3. Management implications

It is important to realise, however, that the genetic data obtained in the present work cannot be applied in the same way towards the conservation and/or management of the various species investigated. This is mainly due to the different biological and ecological characteristics of each species as well as to their particular situation as an anthropogenic resource. For example, the two Mullet species are part of major commercial fisheries throughout the Mediterranean and the eastern Atlantic Ocean, whereas the Cardinal fish and the Blackfaced blenny are of little commercial value and currently are not subject to exploitation. The Peacock wrasse and the Comber, in turn, are part of artisanal and recreational spear angling fisheries respectively. Finally, the two sparid species belong to small-scale commercial fisheries and have recently gained considerable importance as aquaculture species (see section 1.4 for detailed information on each species). Hence, no

single general conservation-management strategy can be drawn from the present study. In turn, the genetic information presented here should be integrated into conservation and/or management strategies according to the goals and objectives of such strategies and considering the biological and ecological characteristics of each individual species (i.e. Chapter III). Nonetheless, genetic information from various species may also be used simultaneously when conservation-management goals include the identification of biogeographical areas or the estimation of connectivity levels between areas or regions (see chapter IV for an example).

5.4 CONCLUSIONS & OUTLOOK

Understanding population connectivity mechanisms has been of great interest for marine ecologists in the last decades. Planktonic stages have been the subject of choice since they are believed to be the primary agents of connectivity between marine populations. New findings in this field are motivating paradigm shifts concerning the openness of marine populations, the role of early-life histories in dispersal, and the significance of environmental variables in determining connectivity. This study shows that planktonic stages are not good predictors of population connectivity and that environmental variables play a major role in determining connectivity among marine populations. It also highlights the difficulty in interpreting genetic connectivity patterns based on presumed dispersal capabilities and in relation to environmental features. The absence of techniques to study larval behavior, ontogeny and spatial distribution in the field as well as the highly stochastic nature of physical processes at short spatio-temporal scales impede our ability to make informed predictions about connectivity patterns through planktonic stages. Future studies trying to elucidate the relationship between dispersal

capability of planktonic stages and realized gene flow must exercise special care in the following aspects:

Oceanographic variability: Physical processes should be extensively evaluated at time and space scales relevant to planktonic stages. Variance of environmental conditions at the onset of planktonic stages may have a direct influence in population connectivity and species range expansion.

Time series studies: The temporal stability of observed genetic connectivity on seasonal and interanual scales needs to be determined with time series studies. Seasonal shifts in current patterns and episodic events may have profound consequences for the transport and recruitment success of larvae.

Larval ontogeny: Special attention needs to be given to the precise estimation of planktonic stage duration. Counting of daily increments in the otoliths taken from newly settled individuals can provide precise information on the duration of the planktonic stage.

Non-genetic data: Parallel methods of assessing differences between populations should be carried out simultaneously with genetic methods. Genetic methods may fail to distinguish morphologically and behaviourally differentiated sub-groups of individuals. Therefore, geographic variability in behaviour, morphology and elemental composition should be analysed whenever possible, since it may reveal population structure that is not apparent with genetic methods alone.

Determining what constitutes a natural break in continuous marine populations and delineating evolutionary significant units that form population subdivision are major objectives of population genetics and evolutionary biology. Significant methodological advances have been recently made possible by the amalgamation of data from distinct disciplines such as physical oceanography, geostatistics, environmental data and population genetics. The challenge is now to synthesize these data into coherent management and conservation strategies. Decisions made under the assumption of continuously distributed or regionally independent populations may lead to erroneous actions if the assumption is wrong. An integration of approaches across space and timescales offers the greatest potential for making informed decisions towards conservation and management of marine resources. Such integration will undoubtedly provide unexpected results, raise new questions, and dispel incorrect beliefs.

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Appendix 1

Note: Both the first and second authors contributed equally to the development of the primer notes in which the first author is not the author of this thesis

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PRIMER NOTE

Isolation and characterization of nine polymorphic microsatellite markers in the two-banded sea bream (*Diplodus vulgaris*) and cross-species amplification in the white sea bream (*Diplodus sargus*) and the saddled bream (*Oblada melanura*)

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Abstract

We have developed nine new microsatellite markers for the two-banded sea bream (*Diplodus vulgaris*) from an enriched genome library protocol. All these loci are polymorphic, with mean allelic diversity of 13 (range 5–21), and expected and observed heterozygosities from 0.641 to 0.932 and 0.428 to 0.914, respectively. Cross-species tests in two close-related species of the genus *Diplodus* (*D. sargus* and *O. melanura*) revealed successful amplifications at 8 out of 9 loci, with mean allele number of 4.75 (range 2–8) and 5.50 (range 3–10), respectively. These results are consistent with the close phylogenetic relationships between the three species, indicating this set of primers might prove useful for studying the levels of genetic diversity and population differentiation in these three species and in other phylogenetically close species of the genus *Diplodus* and *Sparus*.

Keywords: *Diplodus*, microsatellite, *Oblada melanura*, sea bream, Sparidae

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The family Sparidae is represented in the Mediterranean Sea by 11 genera and 24 species that usually inhabit coastal areas and produce pelagic eggs and larvae (Bauchot *et al*. 1986). Sea breams are commercially important species and have also recently gained considerable importance for aquaculture (Fischer *et al*. 1987). The two-banded sea bream, *Diplodus vulgaris* is a demersal species distributed in the Mediterranean and along the eastern Atlantic coast from France to Senegal (Bauchot & Hureau 1986). It can be found close to rocky and sandy bottoms, and juveniles often live in coastal lagoons and estuaries (Macpherson 1998). Although it is one of the most frequent and abundant sparid fish in the coastal waters of southern Europe, the amount of information available on several important aspects of its biology and on their stock assessment is very scarce. Furthermore, while several species of the same genus have been genetically extensively studied by means of allozymes and mitochondrial DNA for species differentiation (Reina *et al*. 1994; Alarcon & Alvarez 1999), phylogeography (Bargelloni *et al*. 2005) and genetic structure (Lenfant & Planes 1996, 2002; Lenfant 1998, 2003; Planes & Lenfant 2002; Ben Slimen *et al*. 2004; Gonzalez-Wanguemert *et al*. 2004; Perez-Ruzafa *et al*. 2006), genetic information is scarce for *D. vulgaris*. A single genetic work using allozymes markers revealed that *D. vulgaris* is genetically structured within the east Mediterranean basin (Arculeo *et al*. 2003), but more results are needed throughout its entire range. Marine species exhibit a great variability in population structure and genetic diversity patterns, and it is important to address specific questions for management purposes in commercially important species. Moreover, genetic populations' analyses aimed at identifying regional patterns of genetic connectivity between marine populations are critical for setting the appropriate geographical scales in which marine reserve systems will be best effective. In

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Table 1 Characterization of eight two-banded sea bream (*Diplodus vulgaris*) microsatellite loci ($N = 30$). Locus name, repeat motif, fluorescent dye-'primer sequence, number of alleles, allele size range, H_O, observed heterozygosity; H_E, expected heterozygosity under Hardy–Weinberg equilibrium; F_{IS}, inbreeding coefficient, $^*P < 0.05$

Locus	Repeat motif	Accession no.	Primer sequence (5'–3')	No. of alleles	Allele size (bp)	H_O	H_E	F_{IS}	*P* values
Dvul11	$(CA)_{13}$	EF064289	F: VIC-GGCCCGCTTTATTCTCAGTCTCAA R: TGCAGGGAACAGAGGGATGACAG	5	135–147	0.428	0.641	0.309	0.075
Dvul2	$(GT)_{28}$	EF064290	F: VIC-CTGCGTTGATGGTTTTTCAGAATG R: CTGCGAAACATCTGGAGTTGTATT	11	180–206	0.571	0.843	0.319*	0.0062
Dvul33	$(CA)_{11}$	EF064291	F: FAM-GCCGGGCTCGACATTGACACTGAA R: GCAGCCAGCAGAGCTTAAAGAACT	17	260–300	0.885	0.918	0.037	0.2625
Dvul4	$(CA)_{13}$	EF064292	F: NED-GCGGTTATGTATACGTTGCGTTTA R: TTGGCGTTGAACAGAAGTCAGACA	16	244–276	0.914	0.923	0.01	0.3625
Dvul61	$(GT)_{18}GA(GT)_8$	EF064293	F: VIC-TGGGGACTCTCAGAATCATCACAA R: TGGAAAAAGCCCTCTGGACAAAAG	7	412–426	0.838	0.689	–0.221	1
Dvul63	$(CA)_{14}$	EF064294	F: PET-GAGGAATGAGTAGAGAAAAGATGG R: ACCCCAACAACCAGAATACCTATA	13	190–234	0.575	0.84	0.315*	0.0062
Dvul84	$(GT)_{15}$	EF064295	F: PET-GCTCGACGTGCACTCTGCCCTTGA R: ATTCCCCAAATCCAGCACTCACAT	14	254–296	0.647	0.816	0.209	0.0125
Dvul38	$(CA)_{16}$	EF064296	F: FAM-TCGGGCACAGATAGAAAGAAACAC R: GAAGGAAGACGGATCTCAGGATGA	21	170–210	0.914	0.932	0.016	0.4063
Dvul6	$(CA)_{14}$	EF064297	F: FAM-GGGCAAACAGGAGCAAAAAGCCAG R: AGCCGCAGTTGATTTACAGAGTGT	2§	419–425	NC	NC	NC	NC

§, only two individuals were genotyped at this locus.

order to assess the genetic variability of *D. vulgaris*, we developed a set of dinucleotide microsatellites markers and test for their variability in two closely relative species, the white sea bream (*Diplodus sargus*) and the saddled bream (*Oblada melanura*).

Microsatellite markers were identified through the development of an enriched genomic library as described by Glenn *et al*. (2000). DNA extractions were performed from lateral fin tissue out of 10 *D. vulgaris* individuals from the western Mediterranean, and approximately 10 µg of high molecular weight DNA was isolated by phenol-chloroform extraction (Sambrook *et al*. 1989). Simultaneous restriction–ligation of genomic DNA was carried out using the *Rsa*I restriction enzyme and double-stranded linker-adapted primers according to Hamilton *et al*. (1999). Ligated DNA was enriched by with a biotin-labelled probe mixture consisting of $(GT)_{10}$ and $(CT)_{10}$ at 10 µM each. DNA fragments with repetitive sequences were then selectively captured by streptavidin-coated Dynabeads (Oxoid) and separated by a magnetic field. Enriched DNA was eluted in 200 µL dH_2O from the bead probes and concentrated by vacuum centrifugation to a final concentration of ~100 ng/µL. Recovered DNA was then re-amplified by polymerase chain reaction (PCR), purified and ligated into a cloning vector using pGEM-T Easy Vector II (Promega). A total of 98 positive clones were screened and checked for inserts using ABI PRISM BigDye Terminator Cycle Kit (Applied Biosystems) and resolved on an ABI 3100 Genetic Analyser (Applied Biosystems). Primer pairs for nine potentially usable microsatellite loci were designed using the software package OLIGO 6.4. Polymorphism was tested by multiplex PCRs performed in 20 µL total volume, which include 50 ng of DNA, 2 mM of $MgCl_2$, 0.25 µM of each primer, 200 µM dNTP's, 1× reaction buffer [75 mM Tris-Hcl, 20 mM $(NH_4)_2SO_4$] and 0.5 U *Taq* polymerase (BIOTAQ). Reaction conditions were as follows: an initial denaturation step of 5 min at 95 °C, eight cycles consisting of 45 s at 92 °C, 45 s at 53 °C annealing temperature, 45 s at 72 °C followed by an additional 24 cycles consisting of 30 s at 92 °C, 30 s at 55 °C annealing temperature, and 30 s at 72 °C. Microsatellite variability was assessed in 30 individuals from the western Mediterranean (Blanes). Individuals were genotyped by assessing allele size on an ABI 3100 Genetic Analyser (Applied Biosystems) using forward primers labelled with FAM (Sigma) and NED, PET and VIC (Applied Biosystems). Allele scoring was carried out using GENEMAPPER version software version 3.5 (Applied Biosystems). Expected and observed values for heterozygosity were determined using ARLEQUIN 2.0 (Schneider *et al*. 2000). The number of alleles per locus, allele size range as well as deviations from Hardy–Weinberg expectations and linkage disequilibrium between pairs of loci were estimated using FSTAT version 2.9 (Goudet 1995). All loci were polymorphic; the total numbers of alleles per locus and heterozygosity estimates are listed in Table 1. We found no evidence of linkage disequilibrium between locus pairs. Two loci showed significant departures from Hardy–Weinberg equilibrium (Dvul2, Dvul63) likely due

Table 2 Cross-species amplification of nine microsatellite loci from the two banded bream (*Diplodus vulgaris*) in the white sea bream (*Diplodus sargus*) and the saddled bream (*Oblada melanura*). Presented are locus name, number of alleles (N_a), allele size range; NA indicates non-amplification

	D. sargus ($n = 7$)		*O. melanura* ($n = 8$)	
Locus	N_a	Range	N_a	Range
Dvul11	2	144–146	3	144–150
Dvul2	3	182–186	4	182–192
Dvul33	8	282–302	10	274–304
Dvul4	8	248–280	7	248–268
Dvul61	6	390–440	6	404–450
Dvul63	NA	NA	NA	NA
Dvul84	7	254–274	7	256–284
Dvul38	2	170–174	4	170–176
Dvul6	2	345–349	3	341–349

to the presence of null alleles. Cross-species amplification was examined in two closed relatives (*D. sargus* and *O. melanura*) using the same conditions detailed for *D. vulgaris*. All except one locus (Dvul63) amplify in both species. All loci were polymorphic in both species, with allele number ranging from two to 10, depending on species and locus (see Table 2). This is consistent with the close phylogenetic relationships between the three species (De la Herran *et al*. 2001; Summerer *et al*. 2001; Day 2002). They provide a useful set of markers for studying the genetic diversity, population differentiation and for the genetic monitoring of farm populations of these three species, and might even proved useful in other phylogenetically close species of the genus *Diplodus* and *Sparus*.

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PRIMER NOTE

Cross-amplification of 10 new isolated polymorphic microsatellite loci for red mullet (*Mullus barbatus*) in striped red mullet (*Mullus surmuletus*)

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Abstract

Ten polymorphic dinucleotide microsatellite loci were isolated and characterized for the red mullet (*Mullus barbatus*). Allele variability was tested on both the red mullet and its congener the striped red mullet (*Mullus surmuletus*). Characterization of 30 individuals of both species from the western Mediterranean showed moderate to high allelic diversity ranging from two to 26 alleles per locus (mean 10.9). Three loci showed departures from Hardy–Weinberg proportions. No evidence of significant association between genotypes at pairs of loci was observed. These polymorphic loci could be suitable for population genetic assessments of both species.

Keywords: microsatellite, Mullidae, *Mullus barbatus*, *Mullus surmuletus*

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Belonging to the family Mullidae, the red mullet (*Mullus barbatus*) and striped red mullet (*Mullus surmuletus*) are distributed across the eastern Atlantic Ocean and the Mediterranean and Black Seas (Hureau 1986). These demersal species are subject of commercial exploitation of bottom trawl multispecies fisheries throughout their geographical distribution range (Stergiou *et al*. 1997). Numerous studies have been carried out on various aspects of these specie's ecology, biology, taxonomic relationships and population structure (Labropoulou & Eleftheriou 1997; Mamuris *et al*. 1998, 1999; Özbilgin *et al*. 2004). However, despite their high economic value, no fishery genetic assessments exist for the striped red mullet and only one study has been performed on the red mullet within a small portion of its distribution range (Garoia *et al*. 2004). Here, we introduce a set of 10 polymorphic dinucleotide microsatellite markers developed for the red mullet and report their variability on both the red mullet and in the striped red mullet.

We developed an enriched genomic library as described in the protocol of Glenn *et al*. (2000), available at www.uga.edu/srel/DNA_Lab/protocols.htm. Particular modifications to the protocol were performed as follows. DNA extractions were carried out from lateral fin tissue out of 10 *M. barbatus* individuals from the western Mediterranean by phenol–chloroform method (Sambrook *et al*. 1989). Genomic DNA was simultaneously digested with *Rsa*I restriction enzyme and ligated to double-stranded linker-adapted primers (Hamilton *et al*. 1999). Restricted-ligated DNA fragments were then amplified with single-stranded linker-adapted primers and hybridized with a biotinylated enriched probe mixture consisting of $(GT)_{10}$ and $(CT)_{10}$ at 10 μM each. DNA fragments with repetitive sequences were selectively captured by streptavidin-coated Dynabeads (Oxoid) and separated by a magnetic field. Enriched DNA was eluted in 200 μL dH_2O from the bead probes and concentrated by vacuum centrifugation to a final concentration of ~100 ng/μL. Subsequent ligation of enriched DNA into a cloning vector was carried out using a TOPO TA cloning kit (Invitrogen) following the manufacturer's protocol. More than 110 positive clones were obtained. All clones were sequenced and checked for inserts using ABI PRISM BigDye Terminator Cycle kit (Applied Biosystems) and resolved on an ABI PRISM 3100 Genetic Analyser (Applied Biosystems).

Polymerase chain reaction (PCR) primers were designed using OLIGO 6.4 software. PCRs were performed in 25-μL

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Table 1 Characterization of 10 red mullet *Mullus barbatus* ($N = 30$ individuals) microsatellite loci and their variability on striped red mullet *Mullus surmuletus* ($N = 30$ individuals). H_O, observed heterozygosity; H_E, expected heterozygosity under Hardy–Weinberg equilibrium; F_{IS} inbreeding coefficient; *, $P < 0.05$

Locus/GenBank Accession no.	Locus	Repeat motif	Primer sequences (5′–3′)	Species	Number of alleles	Allele size (bp)	H_O	H_E	F_{IS}
DQ473548	Mbar3	$(CA)_{24}$	**F:** ***PET***-GCTCCCCCGACACACTGTCT	*M. barbartus*	16	111–149	0.8793	0.9352	0.059
			R: ACCTTGGCCCTTCTTACGTC	*M. surmuletus*	8	115–145	0.5838	0.6480	0.179
DQ473549	Mbar11	$(GT)_{10}GC(GT)_{10}$	**F:** ***VIC***-TGACTGTCAGCACTTGCATT	*M. barbartus*	9	156–176	0.5691	0.5847	0.204
			R: CTGAGGAGAGTCATGAGT	*M. surmuletus*	16	162–208	0.8571	0.9227	0.060
DQ473551	Mbar14	$(AC)_{48}AT(AC)_4AT(AC)_4$	**F:** ***FAM***-GATAGCGAGCCTGAAACCAC	*M. barbartus*	26	195–265	0.9285	0.9603	0.034
			R: CCCTCTGCTTGATATTCCT	*M. surmuletus*	16	193–235	0.6000	0.9163	0.347*
DQ473555	Mbar28	$(GT)_{12}$	**F:** ***FAM***-AAAGGGAGAATGAGGTGAAA	*M. barbartus*	2	156–164	0.1034	0.1324	−0.037
			R: AAGCGCTCGCAACAAAGTC	*M. surmuletus*	2	164–166	0.0333	0.0661	0.000
DQ473552	Mbar46	$(GT)_{12}$	**F:** ***NED***-CCCGAGCAGCAGAAAAA	*M. barbartus*	7	250–262	0.7666	0.8310	0.079
			R:CTTGCCCTCTGCCTCTG	*M. surmuletus*	7	236–244	0.4285	0.5539	0.229*
DQ473547	Mbar55	$(CA)_7CG(CA)_3TA(CA)_6$	**F:** ***NED***-TACACACAAACACTCACCCA	*M. barbartus*	12	146–176	0.8000	0.8717	0.074
			R: CGCAACCAATAGCACACTAC	*M. surmuletus*	7	142–166	0.5517	0.7489	0.267*
DQ473553	Mbar63	$(AC)_{10}AT(AC)_8$	**F:** ***VIC***-AACCAGCAGGTCTCACA	*M. barbartus*	11	301–337	0.7900	0.8621	0.186
			R: TTCATGCTCCTTTTGTTCC	*M. surmuletus*	14	269–327	0.8200	0.8887	0.091
DQ473550	Mbar130	$(AC)_{10}$	**F:** ***NED***-GAGGGTAGATTTGGTTGCAG	*M. barbartus*	8	185–209	0.7583	0.7787	0.071
			R: AGAGTATTGCATTTTTCGCC	*M. surmuletus*	11	185–217	0.7955	0.8473	0.079
DQ473556	Mbar132	$(GT)_{10}$	**F:** ***FAM***-GGAGCAAGGAAGAGGAGA	*M. barbartus*	10	112–132	0.7896	0.8324	0.162
			R: CTCTGCAGACCTGCTCAA	*M. surmuletus*	9	118–136	0.7641	0.8451	0.145
DQ473554	Mbar133	$(CA)_{14}CG(CA)_5$	**F:** ***PET***-CTCGGCACATCACAGAAAC	*M. barbartus*	11	226–266	0.7333	0.7847	0.065
			R: CCTCCCAAATTACACACATC	*M. surmuletus*	16	230–268	0.8247	0.8620	0.044

total volume, which included 50 ng of DNA, 2 mM of $MgCl_2$, 0.75 μM of each primer, 200 μM dNTP's, 1× reaction buffer [75 mM Tris-HCl, 20 mM $(NH_4)_2SO_4$] and 0.5 U *Taq* polymerase (BIOTAQ). Reaction conditions were as follows: an initial denaturation step of 5 min at 95 °C, 30 cycles consisting of 30 s at 92 °C, 30 s at 56.5 °C annealing temperature, and 30 s at 72 °C.

Microsatellite variability was assessed in 30 individuals of both species from the western Mediterranean. Observed and expected heterozygosities were calculated using ARLEQUIN version 2.0 (Schneider *et al*. 2000). The number of alleles per locus, allele size range as well as deviations from Hardy–Weinberg expectations and linkage disequilibrium between pairs of loci were estimated using FSTAT version 2.9 (Goudet 1995). Locus polymorphism ranged from moderate to high. Allele variability and heterozygosity estimates are listed in Table 1. We found no evidence of significant association between genotypes at pairs of loci within each sample. Three loci showed departure from Hardy–Weinberg proportions for the striped red mullet (Mb11, Mb46, Mb55). This could be due to the presence of null alleles segregating at high frequencies. Nonetheless, the results suggest that most of these loci are suitable for population genetic assessments of both species.

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TECHNICAL NOTE

Isolation of eight microsatellites loci from the saddled bream, *Oblada melanura* and cross-species amplification in two sea bream species of the genus *Diplodus*

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Abstract We have developed eight new microsatellite markers for the saddled bream (*Oblada melanura*) from an enriched genome library protocol. All these loci are polymorphic, with mean allelic diversity of 14.75 (range 3–22), and expected and observed heterozygosities from 0.233 to 0.918 and 0.212 to 0.913, respectively. Cross-species tests in two close relatives of the genus *Diplodus* (*D. sargus* and *D. vulgaris*) revealed successful amplifications at 6 out of 8 loci, with means allele number of 6.67 (range 4–10) and 6.50 (range 4–10), respectively. These results are consistent with the close phylogenetic relationships between the three species, indicating this set of primers might proved useful for studying the levels of genetic diversity and population differentiation in these three species and in other phylogenetically close species of the genus *Diplodus* and *Sparus*.

Keywords Microsatellite · *Oblada melanura* · *Diplodus* · Sea breams

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The saddled bream, *Oblada melanura*, belongs to the Sparidae family, which includes commercially important species and has also recently gained considerable importance for aquaculture throughout the Mediterranean (Fischer et al. 1987). *O. melanura* is a diurnal schooling species, very common and abundant throughout the Mediterranean Sea and the Atlantic Ocean (Bay of Biscay and from the Strait of Gibraltar to Angola, Madeira, Cape Verde and the Canary Islands). It is considered a gregarious species and can be found over rocky bottoms and seagrass beds (*Zostera* and seaweeds) (Bauchot and Hureau 1990). They feed almost exclusively on small crustaceans and other zooplanktonic animals, which they graze from the substrata when juveniles, but when adults they feed mainly on vegetable matter. Apart from the feeding habits and the species distribution in the Adriatic region (Pallaoro et al. 1998, 2003, 2004), little information is available concerning its biology and population dynamics (Dufour et al. 1995; Lenfant and Olive 1998). Genetic analyses are scarce and have solely focused on resolving unclear phylogenetic relationships among sea bream species (Hanel and Sturmbauer 2000; Summerer et al. 2001). Here, we report the development and characterisation of 8 microsatellite loci for *O. melanura* and present estimates of allelic variability of these loci and their cross-amplification in two close relatives, the white sea bream (*Diplodus sargus*) and the two-banded sea bream (*Diplodus vulgaris)*. While the limited knowledge on the species' ecology can make a priori predictions about the population structure problematic, the characterisation of microsatellites variation in *O. melanura* and related taxa may give insights into the level of genetic diversity, the amount of gene flow and genetic structuring of these exploited marine

species, that may be of great concern for their conservation.

Microsatellite markers were identified through the development of an enriched genomic library as described by Glenn et al. (2000). DNA extractions were performed from fin tissue and approximately 10 μg of high molecular weight DNA was isolated by phenol–chloroform extraction (Sambrook et al. 1989). Simultaneous restriction-ligation of genomic DNA was carried out using the *Rsa*I restriction enzyme and double stranded linker-adapted primers according to Hamilton et al. (1999). Ligated DNA was enriched with a biotin-labelled probe mixture consisting of $(GT)_{10}$ and $(CT)_{10}$ at 10 μM each. DNA fragments with repetitive sequences were then selectively captured by streptavidin-coated Dynabeads (Oxoid) and separated by a magnetic field. Enriched DNA was eluted in 200 μl dH_2O from the bead probes and concentrated by vacuum centrifugation to a final concentration of ~100 ng/μl. DNA was then reamplified by polymerase chain reaction (PCR), purified and ligated into a cloning vector using pGEM-T Easy Vector II (Promega). A total of 65 positive clones were screened and checked for inserts using ABI PRISM BigDye Terminator Cycle kit (Applied Biosystems) and resolved on an ABI 3100 Genetic Analyser (Applied Biosystems). Primer pairs for 8 potentially usable microsatellite loci were designed using the software package OLIGO 6.4. Polymorphism was tested by multiplex PCR reactions performed in 20 μl total volume, which include 50 ng of DNA, 2 mM of $MgCl_2$, 0.25 μM of each primer, 200 μM dNTP's, 1× reaction buffer [75 mM Tris-Hcl, 20 mM $(NH_4)_2SO_4$] and 0.5 units *Taq* polymerase (BIOTAQ). Reaction conditions were as follows: an initial denaturation step of 5 min at 95°C, eight cycles consisting of 45 s at 92°C, 45 s at 53°C annealing temperature, 45 s at 72°C followed by an additional 24 cycles consisting of 30 s at 92°C, 30 s at 55°C annealing temperature, 30 s at 72°C. Microsatellite variability was assessed in 48 individuals from the western Mediterranean coast (Tarifa). Individuals were genotyped by assessing allele size on an ABI 3100 Genetic Analyser (Applied Biosystems) using forward primers labelled with FAM (Sigma) and NED, PET and VIC (Applied Biosystems). Allele scoring was carried out using GENEMAPPER software version 3.5 (Applied Biosystems). Expected and observed values for heterozygosity, number of alleles per locus, allele size range as well as deviations from Hardy–Weinberg expectations (HWE) and linkage disequilibrium between pairs of loci were estimated using GENETIX version 4.05 (Belkhir et al. 2004). Significance was assessed using permutation procedures. All loci were

Table 1 Characterisation of eight saddled bream (*Oblada melanura*) microsatellite loci ($N = 48$)

Locus	Repeat motif	Accession No.	Primer sequence (5′–3′)	No. of alleles	Allele size (bp)	H_o	H_E	F_{IS}
Omel58	$(CA)_{13}$	EF064298	F:FAM-GGCATTATTGTTCCATCATTACTCC R: ATGGCATACAACCTGCATCAGAAG	8	292–306	0.714	0.733	-0.009
Omel3	$(GT)_{14}$	EF064299	F:FAM-CCTCCGACATCATCAGTGTGTAAT TGGCATGCGAGGTTCAGTCTGTGC	8	393–407	0.831	0.759	0.104
Omel38	$(GT)_{17}$	EF064300	F:FAM-AGCCGGCTGAGCTCCATAATAACC R:TGCCCTCTTGTCACACCAGGTCAC	10	197–219	0.846	0.861	0.071
Omel20	$(CA)_{12}$	EF064301	F:VIC- CAGGGTAGCAACAGGGTAACAATG R:GGCGGTTGAGGACACTGCAAAAAA	3	353–357	0.212	0.233	-0.086
Omel2	$(CA)_{10}$	EF064302	F:VIC- TGCCCCTGTCTGTTGGAGTATGAA R:AACCCCACTGACGTCTTTCTGAAC	15	226–286	0.862	0.667	0.242*
Omel61	$(GT)_{21}$	EF064303	F:VIC- CAGCGGGGGATTAATCTGCATTTG R:GCCCGATTTATCTTCATCACCCAT	16	129–159	0.909	0.8	0.137
Omel54	$(GT)_{14}$	EF064304	F:NED-TGGGGCACCAAAAGAGCGCGCGTG R:ACCCCCTGTCGCCTCCTCTCTTCC	16	197–229	0.904	0.6	0.351*
Omel27	$(CA)_{17}$	EF064305	F:NED-TTGGCTCATTAGACAAAGGCACAC R:GGGCGCTGAAACAATAGCCGTGTT	14	295–335	0.907	0.7	0.244*

Locus name, repeat motif, fluorescent dye-primer sequence, number of alleles, allele size range, H_O, observed heterozygosity; H_E, expected heterozygosity under Hardy–Weinberg equilibrium; F_{IS}, inbreeding coefficient; * $P < 0.05$

Table 2 Cross species amplification of 8 microsatellite loci from the saddled bream (*Oblada melanura*) in the white sea bream (*Diplodus sargus*) and the the two-banded sea bream (*Diplodus vulgaris*)

Locus	*D. sargus (n = 7)*		*D. vulgaris (n = 8)*	
	na	Range	na	Range
Omel58	4	288–296	4	290–310
Omel3	na		na	
Omel38	9	193–235	6	183–199
Omel20	10	349–385	na	
Omel2	4	222–230	5	228–242
Omel61	8	139–161	6	137–179
Omel54	5	193–207	8	221–261
Omel27	na		10	291–319

Locus name, number of alleles (N^a), allele size range. na indicates non amplification

polymorphic; the total numbers of alleles per locus and heterozygosity estimates are listed in Table 1. We found no evidence of linkage disequilibrium between locus pairs. Nonetheless, three loci (Omel2, Omel54 and Omel27) showed significant deviation from HWE, both showing heterozygote deficit.

Cross-species amplification was examined in two closed relatives (*D. sargus* and *D. vulgaris)* using the same conditions detailed for *O. melanura*. All except one locus (Omel3) amplify in both or one of the species. All loci are polymorphic in both species, with allele number ranging from four to ten, depending on species and locus (Table 2), consistent with the close phylogenetic relationships between the three species (Day 2002; De la Herran et al. 2001; Summerer et al. 2001). This set of markers can be useful for studying the genetic diversity, population differentiation and for the genetic monitoring of farm populations of these three species, and might even proved useful in other phylogenetically close species of the genus *Diplodus* and *Sparus*.

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PRIMER NOTE

Isolation and characterization of polymorphic microsatellite markers for peacock wrasse (*Symphodus tinca*)

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Abstract

Eight polymorphic microsatellite loci were isolated and characterized for the peacock wrasse (*Symphodus tinca*), a labrid fish inhabiting the Mediterranean and Black seas. Characterization of 35 individuals from the western Mediterranean indicated a relatively high allelic diversity (mean = 12.4, range 9–17), and observed heterozygosity ranging from 0.65 to 0.91. We found no evidence of linkage disequilibrium between pairs of loci. Two loci showed significant departure from Hardy–Weinberg equilibrium. These polymorphic markers can be useful in most basic population genetic applications.

Keywords: enrichment, microsatellites, *Symphodus tinca*, wrasse

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The peacock wrasse (*Symphodus tinca*) is a marine fish of the family Labridae attaining a maximum length up to 35 cm. This species occurs in the eastern Atlantic and the Mediterranean and Black seas (Quignard & Pras 1986). It is not subject to targeted food fisheries. However, it is not rare to find it as a by-catch of fisheries focused on other species and its catches contribute significantly to recreational angling and spearfishing activities (Gordoa *et al*. 2000).

The peacock wrasse is found mainly on reefs and shallow coastal areas. Males are territorial and conspicuous male nuptial colouration and courtship have been observed (Costello 1991). The spawning season begins in April and ends in late June (Warner & Lejeune 1985). Planktonic larval duration ranges from 9 to 13 days and settlement takes place from early May onwards (Raventos & Macpherson 2001). Genetic studies are scarce and have relied mainly on mitochondrial DNA (mtDNA) to infer phylogenetic relationships within Labrids (Hanel *et al*. 2002). With the aim of estimating genetic variability at the nuclear level, here we report eight new polymorphic microsatellite DNA loci for the peacock wrasse.

An enriched genomic library was constructed following the methods of Glenn (2000) available at www.uga.edu/srel/DNA_Lab/protocols.htm, with modifications to the protocol as detailed in the succeeding text. Approximately 10 μg of high molecular weight DNA of 10 individuals from Blanes (Western Mediterranean) was isolated by phenol–chloroform extraction followed by ethanol precipitation (Sambrook *et al*. 1989). DNA extractions were performed from pectoral fin tissue, RNase treatment was applied where required and recovered DNA was diluted to ~200 ng/μL final concentration. The genomic DNA was simultaneously digested with *Rsa*I restriction enzyme and ligated to double-stranded linker-adapted primers (Hamilton *et al*. 1999). Restricted-ligated DNA fragments were amplified with linker-adapted primers and hybridized with a biotinylated probe mixture consisting of $(GT)_{10}$ and $(GATA)_{10}$ $(CT)_{10}$ at 10 μM each. DNA fragments with repetitive sequences were selectively captured by streptavidin-coated Dynabeads (Oxoid) and separated by a magnetic field. DNA was eluted in dH_2O from the bead probes and concentrated by vacuum centrifugation to a final concentration of ~100 ng/μL. DNA was then reamplified by polymerase chain reaction (PCR) using linker-adapted primers. Subsequent ligation of enriched/recovered DNA into a cloning vector was carried out using a TOPO TA cloning kit (Invitrogen) following the manufacturer's protocol.

More than 180 positive clones were obtained. All clones were sequenced and checked for inserts using ABI PRISM BigDye Terminator Cycle kit (Applied Biosystems) and

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Table 1 Characterization of eight peacock wrasse (*Symphodus tinca*) microsatellite loci. GenBank Accession no., locus name, repeat motif, fluorescent dye-primer sequence, number of alleles, allele size range, H_O, observed heterozygosity; H_E, expected heterozygosity under Hardy–Weinberg equilibrium; F_{IS}, inbreeding coefficient, *$P < 0.05$

GenBank Accession no.	Locus	Repeat motif	Primer sequences (5′–3′)	No. of alleles	Allele size (bp)	H_O	H_E	F_{IS}
DQ324538	Stin155	$(GA)_{11}$	F: FAM-GAGGCAAACAAGTATTTCA R: AGGGACTGTGTTCTTGATG	11	138–168	0.8611	0.8798	0.054
DQ324537	Stin287	$(AC)_6GC(AC)_9$	F: FAM-AGCAGGTTCAATAAACACA R: GGGTGCTCAAGGTAATTGT	9	275–297	0.8055	0.8047	0.001
DQ324543	Stin368	$(AC)_{22}$	F: FAM-TCTGGCAGCCTTAGTCCTC R: GAGCGTCTTCAGAAGGACA	17	336–368	0.7500	0.8873	0.247*
DQ324540	Stin143	$(AC)_4G(AC)_{14}$	F: HEX-TGCAAGCAGAGTCTCAAACCC R: ATTTAACAGCTTGCTTGG	11	120–141	0.9166	0.8924	−0.027
DQ324542	Stin222	$(TG)_9C(TG)_{17}$	F: HEX-CCCGTGATAGATGAGGAG R: GGTGCTCCTGTGATATCT	11	206–231	0.7777	0.8321	0.159
DQ324539	Stin336	$(CA)_{36}$	F: HEX-GCTGGAAGAATAGAACATTC R: TTTTTTGCAAGCTTTTAGT	13	321–365	0.7714	0.8567	0.191
DQ324541	Stin245	$(GATA)_{30}$	F: NED-TAGCCCCGCCCCCTAAAAA R: TGCTACTGGATGGCGCTGAA	12	218–314	0.8463	0.8961	0.084
DQ324536	Stin138	$(C)_{16}(CA)_{11}$	F: NED-GCGTTTTTACGTCATGTTTT R: AGGTATGTGTCCGCTTTT	15	127–165	0.6571	0.9155	0.424*

resolved on an ABI 3100 Genetic Analyser (Applied Biosystems). PCR primers were designed using OLIGO 6.4 software. PCRs were performed in 25 μL total volume, which includes 50 ng of DNA, 1 mM of $MgCl_2$, 0.75 μM of forward and reverse universal M13 primers, 200 μM dNTP's, 1×reaction buffer [75 mM Tris-Hcl, 20 mM $(NH_4)_2SO_4$] and 0.5 U *Taq* polimerase (BIOTAQ). Reaction conditions were as follows: an initial denaturation step of 5 min at 95 °C, 30 cycles consisting of 30 s at 92 °C, 30 s at 55 °C annealing temperature and 30 s at 72 °C. Individuals were genotyped by assessing allele size on an ABI 3100 Genetic Analyser (Applied Biosystems) using forward primers labelled with FAM, HEX (Sigma) and NED (Applied Biosystems). Allele scoring was carried out using GENEMAPPER version 3.5 software (Applied Biosystems).

Microsatellite variability was tested in 35 individuals from Blanes. Observed and expected heterozygosities as well as deviations from Hardy–Weinberg expectations were calculated using ARLEQUIN (Schneider *et al*. 2000). The number of alleles per locus and allele size range were estimated using FSTAT version 2.9. (Goudet 1995). All loci were polymorphic, the number of alleles per locus, expected and observed heterozygosities are listed in Table 1. There was no evidence of linkage disequilibrium between locus pairs. Two loci showed significant departures from Hardy–Weinberg equilibrium. This could be due to segregation of null alleles or population subdivision. With the present data set, we cannot distinguish conclusively between these alternatives, but the likeliest explanation is presence of null alleles segregating at high frequencies. Nonetheless, these results suggest that most of the loci characterized could be useful for most population genetics applications.

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TECHNICAL NOTE

Polymorphic microsatellite loci for the cardinal fish (*Apogon imberbis*)

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Abstract Eight polymorphic microsatellite loci were isolated and characterized for the Cardinal fish (*Apogon imberbis*), a coastal-reef fish endemic to the Mediterranean Sea. Characterization of 30 Cardinal fish individuals form the western Mediterranean showed moderate to high allelic diversity ranging from 6 to 19 alleles per locus. Two loci showed significant departures from Hardy-Weinberg equilibrium presumably due to null alleles. No evidence of linkage disequilibrium was found for any locus pairwise comparasions. This microsatellite set could be useful for any basic population genetic studies of this species.

Keywords Microsatellite · *Apogon imberbis* · Cardinal fish · Apogonidae

Cardinal fishes are coastal-reef marine fish of the genus *Apogon*. This genus comprises a large number of species (174) distributed from the tropical Indo-West Pacific to the Atlantic Ocean and the Mediterranean Sea (Kuiter and Kozawa 1999). However, only one species, *Apogon imberbis*, exists within the Mediterranean Sea (Tortonese 1986). All species appear to have a peculiar reproductive strategy in which internal fertilization is achieved by transferring the sperm into the oviduct of the female through the male's ventral fins (Thresher 1984). The fertilized eggs are then released by the female and picked up by the male who broods them in his mouth until hatching. This form of male parental care has probably been responsible for the considerable interest in the genus, resulting in studies of mating behavior (Kuwamura 1983), filial cannibalism (Smith 1992), gamete biology (Lahnsteiner 2003), parental care effort (Okuda 2001) and molecular phylogeny (Mabuchi et al. 2006). Little attention, however, has been paid to the Mediterranean species for which only one study covering basic aspects of its reproductive behavior and growth rate pattern is available (Garnaud 1962). Furthermore, nuclear genetic information is scarce with just a single study reporting variability at nuclear loci for a single species of the genus (Miller-Sims et al. 2004). Here, we report the characterization of eight microsatellite loci developed in *A. imberbis* with the aim of evaluate dispersal strategies in this marine mouth-brooding fish.

Microsatellite markers were identify through the development of an enriched genomic library as described in Glenn et al (2000). DNA was extracted from 10 individuals from the Western Mediterranean by the phenol-chloroform method (Sambrook et al. 1989). Simultaneous restriction-ligation of genomic DNA was carried out using *RsaI* restriction enzyme and double stranded linker-adapted primers according to Hamilton et al (1999). Ligated DNA was size selected

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Table 1 Characterization of 8 Cardinal fish (*Apogon imberbis*) microsatellite loci (N = 30)

Locus/GenBank Accession No.	Locus	Repeat motif	Primer sequences ($5' \rightarrow 3'$)	Number of alleles	Allele size (bp)	H_O	H_E	Fis
DQ822534	Aimb2	$(CA)_{12}$	**F:** ***FAM***-AGCCGGTTCCTTTAGAGCATTCAA **R:** -GAGGCGTTTAGAGTGTGAGAAGGA	6	341–355	0.550	0.751	0.273
DQ822535	Aimb14	$(GT)_{21}$	**F:** ***NED***-CACCCCACACTACATGCCCTTGAA **R:** -GCTGGCTGGCCTAGTTTGGGTCTC	11	316–358	0.800	0.876	0.084
DQ822536	Aimb17	$(CTAT)_{23}$	**F:** ***NED***-TCGCTGGTGTTGTCTAATGCATTC **R:** -TGGGGAAGGAGAGCGATGCAGAAC	19	120–200	0.800	0.961	0.166
DQ822537	Aimb22	$(CA)_{14}$	**F:** ***PET***-ACCGCTGCTGTCAGTCCTGTCACA **R :-** AACCGAGGCTGTTCCCATCAAATG	6	447–457	0.300	0.746	0.599*
DQ822538	Aimb28	$(CA)_3CT(CA)_9$	F: ***PET***-CCGTTCTGCTCTGATTGGTCAACT **R :-** TCCTTTTGGCGCTGATTAGTTCAC	8	254–272	0.850	0.811	–0.052
DQ822539	Aimb29	$(CA)_{15}$	**F:** ***FAM***-CTTGCCGTTTTTGCTACTATGTTCC **R:** -GCTGATTTTAAGCTACATTCTACCT	13	198–232	0.650	0.866	0.253*
DQ822540	Aimb41	$(GT)_{16}$	**F:** ***VIC***-ACGGCTCAGAAGATGGTCCACACA **R**: -GTGCCATCCAATCTGTCCATCATA	13	335–377	0.850	0.850	–0.002
DQ822541	Aimb74	$(CA)_{11}TA(CA)_3$	**F:** ***VIC***-CACCACAATAGTTAAATGCTCCCT **R:** -CTTCGCATCAGGGGTTAATCTCAA	6	210–240	0.650	0.680	0.035

GenBank Accession No., Locus name, repeat motif, fluorescent dye-primer sequence, number of alleles, Allele size range, H_O: observed heterozygosity, H_E: expected heterozygosity under Hardy-Weinberg equilibrium, F_{IS}: inbreeding coefficient, $^*P < 0.05$

and enriched by magnetic bead selection with a biotin-labeled probe mixture consisting of $(GT)_{10}$ and $(CT)_{10}$ at 10 μM each. Enriched DNA was eluted in 200 μl dH_2O from the bead probes and concentrated by vacuum centrifugation to a final concentration of ~100 ng/μl. Recovered DNA was then purified and cloned using pGEM-T Easy Vector II (Promega). A total of 56 positive clones were screened and checked for inserts using ABI PRISM BigDye Terminator Cycle kit (Applied Biosystems) and resolved on an ABI 3100 Genetic Analyser (Applied Biosystems). Primer pairs for eight potential usable microsatellite loci were designed using OLIGO 6.4 software. Polymorphism was tested by multiplex PCR reactions performed in 25 μl total volume, which include 50 ng of DNA, 2 mM of $MgCl_2$, 0.75 μM of each primer, 200 μM dNTP's, 1X reaction buffer [75 mM Tris–Hcl, 20 mM $(NH_4)_2SO_4$] and 0.5 units *Taq* polimerase (BIOTAQ). Reaction conditions were as follows: an initial denaturation step of 5 min at 95°C, eight cycles consisting of 30 s at 92°C, 30 s at 53.5°C annealing temperature, 30 s at 72°C followed by an additional twenty eight cycles at 55.5°C annealing temperature. Microsatellite variability was assessed in 30 individuals from the western Mediterranean. Individuals were genotyped by assessing allele size on an ABI 3100 Genetic Analyser (Applied Biosystems) using forward primers labelled with FAM (Sigma) and NED, PET and VIC (Applied Biosystems). Allele scoring was carried out using GENEMAPPER version 3.5 software (Applied Biosystems). Expected and observed values for heterozygosity were determined using ARLEQUIN V.2.0 (Schneider et al. 2000). The number of alleles per locus, allele size range as well as deviations from Hardy-Weinberg expectations and linkage disequilibrium between pairs of loci were estimated using FSTAT V.2.9 (Goudet 1995). All loci were polymorphic, the total number of alleles per locus and heterozygosities estimates are listed in Table 1. We found no evidence of linkage disequilibrium between locus pairs. Two loci showed significant departures from Hardy–Weinberg equilibrium (Aimb22, Aimb29). This could be due to the presence of null alleles or the inclusion of individuals from different populations in the analysis.

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Appendix 2

Appendix 2. Review of marine species analysed across the Almeria-Oran oceanographic front AOF.

Species/Family	Dispersal Capability	Marker	Separated by AOF	F_{st}/Analogous (range)	Reference
European Hake *(Merluccius merluccius)*	Eggs & larvae planktonic	Allozymes	Yes	F_{st} (0.005-0.205)	(Cimmaruta *et al.* 2005)
Sardine *(Sardina pilchardus)*	Larval duration 41 days. Pelagic eggs	mtDNA	NO	Φ_{st} (0.003-0.013)	(Atarhouch *et al.* 2005)
Sharpsnout sebream *(Diplodus puntazzo)*	Pelagic larval phase ≈30 days	Allozymes mtDNA	Yes Yes	F_{st} (0.130) Φ_{st} (0.120)	(Bargelloni *et al.* 2005)
White seabream *(Diplodus sargus)*	Pelagic larval phase ≈30 days	Allozymes mtDNA	No No	F_{st} (0.07) Φ_{st} (0.0)	(Bargelloni *et al.* 2005)
Sea Bass *(Dicentrarchus labrax)*	Pelagic Eggs & larvae. Planktonic larval stage 1-2 months	Microsats	Yes	F_{st} (0.024-0.033)	(Naciri *et al.* 1999)
Sparid *(Lithognathus mormyrus)*	Planktonic larvae	mtDNA Allozymes	Yes Yes	Φ_{st} (0.741) F_{st} (0.817)	(Bargelloni *et al.* 2003)
Sparid *(Spondyliosoma cantharus)*	Planktonic larvae Benthic eggs	mtDNA Allozymes	Yes No	Φ_{st} (0.786) F_{st} (0.039)	(Bargelloni *et al.* 2003)
Sparid *(Dentex dentex)*	No info	mtDNA Allozymes	Yes Yes	Φ_{st} (0.820) F_{st} (0.952)	(Bargelloni *et al.* 2003)
Sparid *(Pagrus pagrus)*	Planktonic larvae	mtDNA Allozymes	No Suggestive	Φ_{st} (0.003) F_{st} (0.135)	(Bargelloni *et al.* 2003)
Sparid *(Pagellus bogaraveo)*	Planktonic larvae	mtDNA Allozymes	No No	Φ_{st} (0.022) F_{st} (-0.006)	(Bargelloni *et al.* 2003)
Crustacean (*Meganyctiphanes norvegica)*	Planktonic larvae	mtDNA	Yes	Φ_{st} (0.012-0.293)	(Zane *et al.* 2000)
Sea urchin *(Paracentrotus lividus)*	Planktonic larvae. Planktonic larval stage 20-30 days	mtDNA	Yes	Φ_{st} (0.011-0.034)	(Duran *et al.* 2004)
Mussel (*Mytilus galloprovincialis)*	Pelagic larvae	mtDNA	Yes	Only P-values are reported	(Quesada *et al.* 1995)
Anglerfish (*Lophius budegassa)*	Planktonic eggs. Larval pelagic stage 2-4 months	mtDNA	Yes	Φ_{st} (-0.009-0.119)	(Charrier *et al.* 2006)
Anglerfish *(Lophius piscatorius)*	Planktonic eggs. Larval pelagic stage 2-4 months	mtDNA	No	Φ_{st} (-0.001-0.05)	(Charrier *et al.* 2006)
Cuttlefish (*Sepia officinalis)*	Benthic eggs. No planktonic stage	Microsats mtDNA	Yes Yes	F_{st} (0.01) Φ_{st} Values in supplementary material. Don't	(Perez-Losada *et al.* 2002; Perez-losada *et al.* 2007)

				have access	
Scallops, *(Pecten jacobaeus)*	Pelagic larval stage 24 days	Allozymes	Yes	F_{st} (0.041)	(Ríos *et al.* 2002)
Srtriped red mullet *(Mullus surmuletus)*	Pelagic eggs and larvae. Pelagic stage duration 20-30 days	Microsats	Suggestive	F_{st} (0.086)	This study Chapter III
Blackfaced Blenny *(Tripterygion delaisi)*	Benthic eggs. Planktonic larval stage 16-21 days	Microsats	Suggestive	F_{st} (0.046)	(Carreras-carbonell *et al.* 2006b)
Sea Bass *(Dicentrarchus labrax)*	Pelagic Eggs & larvae. Planktonic larval stage 1-2 months	MtDNA Microsats	Yes Yes	Φ_{st} (0.061) F_{st} (0.008)	(Lemaire *et al.* 2005)

Appendix 3

Appendix 3. Summary statistics for fifty three microsatellite loci among sampling collections of *Diplodus vulgaris, Serranus cabrilla, Tripterygion delaisi, Oblada melanura, Symphodus tinca* and *Apogon Imberbis* species. n: number of individuals, a: number of alleles, Rs: allelic richness, H_O: Observed heterozygosity, H_E: expected heterozygosity F_{IS}: inbreeding coefficient (Weir & Cockerham, 1984). Asterisk represents significant F_{IS} at = 0.05. Significant values after Bonferroni corrections are shown in bold italics.

Species	**Locus**								
D.vul (HE)	**Dvul1**	**Dvul2**	**Dvul33**	**Dvul4**	**Dvul61**	**Dvul63**	**Dvul84**	**Dvul38**	**Overall**
n	48	48	47	48	43	44	46	47	
a	5	11	17	16	8	16	17	21	13.875
Rs	4.998	10.884	16.639	15.667	8	15.886	16.591	20.808	13.684
H_O	0.5	0.542	0.872	0.875	0.86	0.591	0.674	0.915	
H_E	0.635	0.824	0.9	0.898	0.696	0.827	0.824	0.932	
F_{IS}	***0.192****	***0.341****	0.032	0.027	***0.239****	***0.285****	0.183*	0.016	0.12
D.vul (CG)									
n	47	46	48	47	46	44	44	44	
a	2	10	15	16	26	15	15	13	14
Rs	2	9.927	14.769	15.731	25.652	15	14.909	12.789	13.847
H_O	0.298	0.5	0.875	0.894	0.913	0.75	0.682	0.897	
H_E	0.303	0.803	0.892	0.918	0.952	0.933	0.824	0.923	
F_{IS}	-0.044	***0.372****	0.015	0.024	0.042	***0.193****	0.163*	0.013	0.11
D.vul (MA)									
n	47	46	47	45	46	47	47	45	
a	4	12	16	14	26	17	19	17	15.625
Rs	3.993	11.92	15.488	13.908	25.576	16.81	18.299	18.197	15.523
H_O	0.277	0.435	0.851	0.911	0.826	0.809	0.809	0.927	
H_E	0.269	0.805	0.893	0.906	0.947	0.927	0.815	0.935	
F_{IS}	-0.101	***0.462****	0.036	-0.009	0.126	0.128*	0.008	0.134	0.111
D.vul (BL)									
n	48	43	47	48	48	44	48	48	
a	4	10	13	15	28	14	22	18	15.5
Rs	3.895	10	12.889	14.674	27.105	13.932	20.814	17.734	15.13
H_O	0.229	0.488	0.957	0.917	0.813	0.591	0.771	0.871	
H_E	0.246	0.841	0.875	0.882	0.957	0.919	0.756	0.917	
F_{IS}	0.066	***0.413****	-0.095	-0.041	0.151*	***0.355****	-0.019	0.121	0.127

Appendix 3 continued

S.cab (HE)	**Sc03**	**Sc04**	**Sc05**	***Sc06***	**Sc07**	**Sc08**	**Sc11**	**Sc12**	**Sc13**	**Sc14**	**Sc15**	
n	32	32	32	31	32	32	32	30	30	28	31	
a	8	7	17	10	6	7	4	4	12	16	8	9
Rs	7.5	6.861	16.579	9.613	5.986	6.875	3.986	4	11.73	16	7.797	8.811
H_O	0.688	0.75	0.656	0.871	0.719	0.688	0.719	0.6	0.9	0.821	0.613	
H_E	0.662	0.804	0.939	0.818	0.801	0.741	0.671	0.591	0.868	0.916	0.641	
F_{IS}	-0.039	0.068	***0.274****	-0.07	0.096	0.07	-0.086	-0.049	-0.037	0.1	0.045	0.046
S.cab (CG)												
n	30	30	30	29	30	30	30	29	29	29	29	
a	8	11	20	12	9	11	6	4	12	16	6	10.454
Rs	7.733	10.6	19.46	11.929	8.733	10.663	5.927	3.965	11.929	15.826	5.931	10.245
H_O	0.767	0.867	0.733	0.724	0.8	0.667	0.6	0.345	0.897	0.793	0.552	
H_E	0.7	0.832	0.946	0.883	0.814	0.797	0.728	0.559	0.872	0.908	0.691	
F_{IS}	-0.124	-0.041	***0.253****	0.169*	0.018	0.162	0.163	0.173*	-0.03	0.129	0.19	0.107
S.cab (MA)												
n	30	30	30	30	30	30	30	30	30	30	30	
a	7	9	21	14	9	9	5	6	11	19	5	10.454
Rs	6.86	8.863	20.526	13.796	8.8	8.796	4.867	5.863	10.733	18.196	4.93	10.202
H_O	0.7	0.833	0.767	0.933	0.8	0.7	0.333	0.6	0.867	0.867	0.433	
H_E	0.688	0.846	0.954	0.888	0.816	0.728	0.38	0.574	0.816	0.892	0.484	
F_{IS}	-0.018	0.008	***0.198****	-0.051	0.01	0.039	0.051	-0.085	-0.062	0.03	0.06	0.019
S.cab (BL)												
n	30	30	30	30	30	29	30	30	30	30	30	
a	7	8	20	12	7	8	3	5	9	12	4	8.636
Rs	6.8	7.863	19.719	11.73	6.867	7.861	2.997	4.93	8.863	11.723	3.933	8.48
H_O	0.533	0.7	0.833	0.633	0.767	0.552	0.2	0.5	0.767	0.733	0.333	
H_E	0.579	0.771	0.952	0.829	0.763	0.67	0.215	0.621	0.799	0.822	0.401	
F_{IS}	0.038	0.094	***0.251****	0.239	-0.004	0.162	0.072	0.198*	0.033	0.11	0.105	0.11

Appendix 3 continued

T.del (HE)	**Td01**	**Td02**	**Td04**	**Td05**	**Td06**	**Td07**	**Td08**	**Td09**	**Td10**	***Td11***	
n	32	32	34	33	32	34	33	34	34	35	
a	14	12	19	27	31	8	23	9	31	10	18.4
Rs	13.905	11.875	18.437	26.561	30.559	7.735	22.624	8.558	29.722	9.507	17.948
H_O	0.938	0.969	0.824	1	1	0.794	0.813	0.618	1	0.486	
H_E	0.906	0.856	0.92	0.971	0.969	0.76	0.933	0.701	0.966	0.531	
F_{IS}	-0.034	-0.133	0.099	-0.03	-0.032	-0.061	0.127*	***0.131****	-0.036	0.087	0.004
T.del (CG)											
n	33	32	33	32	32	32	31	32	32	32	
a	14	13	17	25	23	7	26	6	27	8	16.6
Rs	13.815	12.843	16.801	24.684	22.778	6.969	26	5.968	26.652	7.938	16.444
H_O	0.879	0.906	0.909	0.906	0.875	0.656	0.903	0.531	0.875	0.75	
H_E	0.904	0.83	0.873	0.927	0.953	0.774	0.952	0.727	0.956	0.694	
F_{IS}	0.028	-0.092	-0.041	0.023	***0.081****	***0.155****	0.052	***0.249****	0.083	-0.081	0.045
T.del (MA)											
n	42	41	42	42	42	42	42	42	42	42	
a	16	12	18	25	24	7	20	5	26	7	16
Rs	14.146	10.661	15.894	22.799	21.505	6.456	18.27	4.738	23.228	6.992	14.468
H_O	0.881	0.854	0.881	0.952	0.881	0.548	0.738	0.643	1	0.738	
H_E	0.9	0.734	0.891	0.95	0.938	0.615	0.922	0.564	0.946	0.71	
F_{IS}	***0.022****	-0.165	0.012	-0.002	0.06	0.09	***0.194****	***0.375****	-0.058	-0.039	0.002
T.del (BL)											
n	47	47	47	46	47	47	47	47	47	47	
a	12	16	20	25	31	8	29	9	27	10	18.7
Rs	11.28	15.416	17.867	22.505	26.374	6.865	24.589	8.181	23.897	9.383	16.635
H_O	0.872	0.787	0.915	0.978	0.915	0.702	0.745	0.362	0.894	0.681	
H_E	0.876	0.913	0.917	0.936	0.961	0.683	0.951	0.749	0.953	0.735	
F_{IS}	0.005	0.138	0.003	-0.045	0.046	-0.042	***0.214****	***0.513****	0.063*	0.062	0.092

Appendix 3 continued

O.mel (HE)	**Omel2**	**Omel3**	**Omel20**	**Omel27**	**Omel38**	**Omel54**	**Omel58**	**Omel61**	
n	43	40	41	43	40	43	43	41	
a	15	10	3	13	10	16	8	15	11.25
Rs	12.94	9.215	2.785	12.254	8.751	14.407	6.882	13.676	10.113
H_O	0.698	0.872	0.186	0.847	0.83	0.707	0.744	0.884	
H_E	0.925	0.885	0.23	0.925	0.86	0.906	0.785	0.904	
F_{IS}	***0.24****	0.086	0.088	***0.251****	0.106	0.155*	0.035	0.145	0.15
O.mel (CG)									
n	43	43	41	41	43	41	43	42	
a	17	8	3	15	10	16	9	15	11.62
Rs	14.939	8.351	2.511	14.632	8.646	14.021	7.786	13.164	10.50
H_O	0.905	0.868	0.22	0.893	0.744	0.805	0.721	0.891	
H_E	0.908	0.887	0.223	0.906	0.833	0.928	0.746	0.927	
F_{IS}	0.003	0.019	0.088	0.137	0.166	***0.22****	0.053	0.145	0.133
O.mel (MA)									
n	46	45	47	47	45	47	46	46	
a	17	8	3	14	13	16	12	15	12.25
Rs	15.856	7.836	2.816	13.742	13.426	14.955	8.481	14.453	11.446
H_O	0.934	0.812	0.085	0.896	0.733	0.765	0.673	0.717	
H_E	0.917	0.827	0.103	0.913	0.852	0.889	0.732	0.919	
F_{IS}	-0.022	0.095	0.025	0.01	0.136	0.138	0.08	0.22	0.085
O.mel (BL)									
n	34	34	34	33	34	31	34	34	
a	13	9	3	14	15	16	9	17	12
Rs	11.047	8.793	3	13.917	14.028	14.587	8.404	15.152	11.116
H_O	0.706	0.872	0.318	0.759	0.588	0.848	0.788	0.911	
H_E	0.837	0.905	0.385	0.892	0.927	0.926	0.858	0.916	
F_{IS}	0.157*	0.021	0.179	***0.193****	***0.367****	0.081	0.113*	0.004	0.164

Appendix 3 continued

S.tin (HE)	**St138**	**St143**	**St155**	**St222**	**St287**	**St368**	**St245**	**St336**	
n	37	37	39	39	40	39	37	39	
a	15	9	11	10	9	17	12	13	12
Rs	15	9	10.897	9.895	8.915	16.791	11.987	12.879	11.92
H_O	0.568	0.595	0.897	0.821	0.825	0.846	0.872	0.845	
H_E	0.911	0.788	0.883	0.83	0.796	0.914	0.8961	0.8567	
F_{IS}	***0.378****	0.249	-0.023	0.005	-0.036	0.075	0.84	0.191	0.111
S.tin (CG)									
n	54	51	58	57	57	55	54	57	
a	17	11	13	12	11	17	15	13	13.625
Rs	15.625	10.916	12.174	10.847	9.471	15.448	14.724	12.677	12.735
H_O	0.759	0.843	0.897	0.895	0.789	0.836	0.847	0.798	
H_E	0.926	0.86	0.891	0.825	0.786	0.89	0.86	0.824	
F_{IS}	***0.179****	0.018	-0.005	-0.085	-0.008	0.061	0.008	0.017	0.03
S.tin (MA)									
n	45	47	47	47	47	46	45	47	
a	15	12	11	12	9	17	12	11	12.375
Rs	14.575	11.778	10.903	10.934	8.566	16.701	11.878	10.902	12.029
H_O	0.6	0.915	0.83	0.745	0.723	0.783	0.864	0.757	
H_E	0.902	0.886	0.878	0.796	0.793	0.904	0.878	0.769	
F_{IS}	***0.332****	-0.032	0.05	0.066	0.076	***0.133****	0.06	0.078	0.106
S.tin (BL)									
n	51	49	51	51	45	50	49	51	
a	19	12	13	13	10	16	13	17	14.125
Rs	17.092	11.446	12.554	11.802	9.584	15.716	12.677	15.098	13.246
H_O	0.529	0.878	0.882	0.686	0.8	0.68	0.915	0.773	
H_E	0.907	0.874	0.894	0.8	0.792	0.926	0.927	0.784	
F_{IS}	***0.414****	-0.003	0.013	0.143	-0.012	***0.266****	0.112	0.015	0.141

Appendix 3 continued

A.imb (HE)	**Aimb14**	**Aimb17**	**Aimb2**	**Aimb22**	**Aimb28**	**Aimb29**	**Aimb41**	**Aimb74**	
n	32	31	32	32	32	32	32	31	
a	13	20	8	5	7	13	15	9	11.25
Rs	12.874	20	7.968	5	7	12.874	14.842	9	11.194
H_O	0.843	0.806	0.75	0.312	0.531	0.812	0.812	0.709	
H_E	0.876	0.933	0.771	0.646	0.79	0.879	0.866	0.727	
F_{IS}	0.032	***0.138****	0.028	***0.505****	***0.327****	0.064	0.055	-0.005	0.133
A.imb (CG)									
n	48	48	48	45	48	47	48	47	
a	13	20	8	5	6	18	14	8	11.5
Rs	11.729	18.599	7.926	5	5.863	15.91	11.955	7.609	10.573
H_O	0.854	0.812	0.75	0.6	0.645	0.914	0.604	0.744	
H_E	0.879	0.939	0.805	0.64	0.723	0.901	0.841	0.644	
F_{IS}	0.024	***0.137****	0.065	***0.064****	0.105	-0.016	***0.284****	-0.179	0.135
A.imb (MA)									
n	46	42	48	47	48	48	48	48	
a	10	21	7	6	8	18	7	8	10.625
Rs	9.473	19.498	6.712	5.621	7.518	15.533	6.518	7.263	9.767
H_O	0.434	0.523	0.637	0.404	0.77	0.895	0.729	0.541	
H_E	0.833	0.934	0.736	0.622	0.822	0.895	0.617	0.624	
F_{IS}	***0.48****	***0.437****	0.194*	***0.35****	0.057	-0.006	-0.209	0.133	0.216
A.imb (BL)									
n	47	47	46	42	47	45	46	47	
a	14	25	7	6	8	16	15	9	12.5
Rs	12.889	22.961	6.957	5.737	7.531	14.36	12.595	7.828	11.357
H_O	0.872	0.872	0.772	0.872	0.872	0.862	0.872	0.872	
H_E	0.836	0.958	0.843	0.74	0.749	0.873	0.79	0.677	
F_{IS}	0.084	***0.089****	0.142*	***0.583****	-0.052	0.103*	0.145	-0.011	0.159