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A MULTIDISCIPLINARY APPROACH FOR PUZZLING OVER FISH CONNECTIVITY IN THE MEDITERRANEAN SEA: THE ROLE OF EARLY LIFE HISTORY STAGES OF RED MULLET (*MULLUS BARBATUS*)

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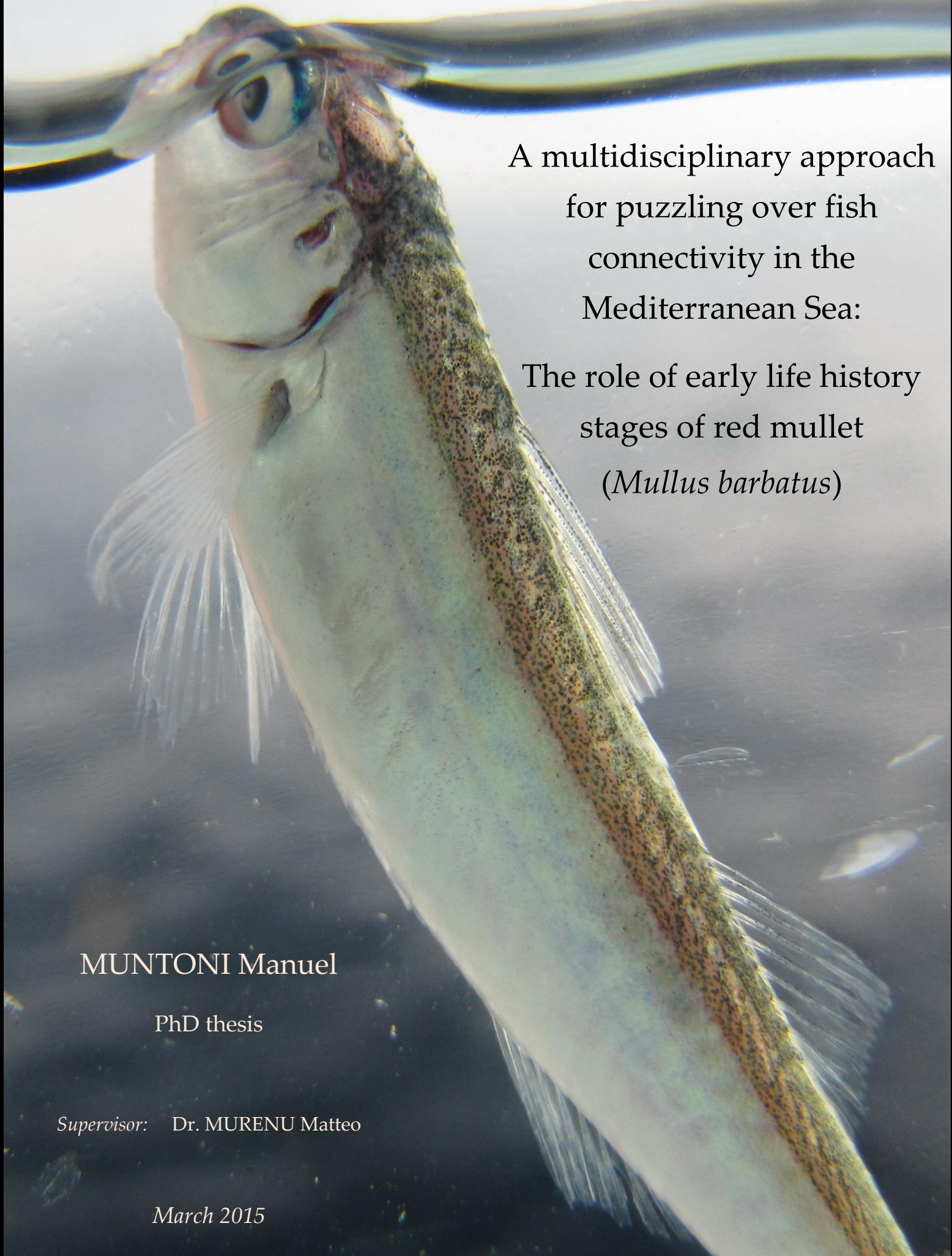

A multidisciplinary approach for puzzling over fish connectivity in the Mediterranean Sea:

The role of early life history stages of red mullet (*Mullus barbatus*)

MUNTONI Manuel

PhD thesis

Supervisor: Dr. MURENU Matteo

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A MULTIDISCIPLINARY APPROACH FOR PUZZLING OVER FISH CONNECTIVITY IN THE MEDITERRANEAN SEA: THE ROLE OF EARLY LIFE HISTORY STAGES OF RED MULLET (*MULLUS BARBATUS*)

ABSTRACT

Integrating connectivity patterns into marine ecosystem management is a fundamental step, specially for stock subjected to the combined impacts of human activities (overfishing, habitat degradation, etc.) and climate changes. Thus, management of marine resources must incorporates the spatial scales over which the populations are connected. Notwithstanding, studying these dynamics remains a crucial and hard task and the predictions of the temporal and spatial patterns of these mechanisms are still particularly challenging. This thesis aims to puzzle over the red mullet *Mullus barbatus* population connectivity in the Western Mediterranean Sea, by implementing a multidisciplinary approach. Otolith sclerochronology, larval dispersal modelling and genetic techniques were gathered in this study. More particularly, this research project focused on early life history stages of red mullet and their role in the characterization of connectivity dynamics. The results show that *M. barbatus* larval dispersal distances can reach a range of 200 km. The differences in early life traits (i.e. PLD, spawning and settlement dates) observed between various areas of the Western Mediterranean Sea suggest a certain level of larval patchiness, likely due to the occurrence of different spawning pulses during the reproductive period. The dispersal of individuals across distant areas, even not significant in demographic

terms, is accountable for the maintenance of the genetic flow among different demes. Fluctuations in the level of exchange among different areas, due to the variability of the source-sink dynamics, could have major implications in the population connectivity patterns. These findings highlight the reliability of combining several approaches and represent a benchmark for the definition of a proper resource management, with considerable engagements in effectively assuring the beneficial effects of the existent and future conservation strategies.

KEYWORDS

Connectivity, settlement, early life history stages, early life history traits, post-larvae, otolith sclerochronology, larval dispersal, modelling, genetics, Western Mediterranean Sea, *Mullus barbatus*.

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INTRODUCTION

WHAT IS CONNECTIVITY?

Marine species and discrete populations

Many marine fish species are distributed, within their spatial range, in geographically discrete populations (Hanski, 1998; Thomas and Kunin, 1999; Kritzer and Sale, 2004, 2006). Such structure in discrete populations, results from the interactions between the species' bio-ecological characteristics and the spatial heterogeneity of the marine habitats (Fahrig, 2003). The relationships among these discrete units are maintained through the movements of individuals at each of their life stages: eggs, larvae, juveniles or adults (Cowen and Sponaugle, 2009; Sale, 2004), determining how populations are naturally regulated and influencing their responses to natural or human disturbances (Mora and Sale, 2002a; Kritzer and Sale, 2006; Jones *et al.*, 2007; McCook *et al.*, 2009).

Connectivity definition

Commonly, connectivity is defined as the flux of items between separated locations, connecting spatially separated populations of a species within a unique metapopulation; this definition is valuable for nutrients, sediments, pollutants, and also individual dispersing organisms (Sale *et al.*, 2010). Population connectivity explicitly refers to the degree to which these spatially separated populations are linked by the dispersal of individuals (Palumbi, 2003; Cowen *et al.*, 2007).

Evolutionary and demographic connectivity

Connectivity plays a central role in the metapopulation dynamic and structure. The rate of individuals exchanged across different spatial scales can give indications on the independence of the local populations, i.e. if they can be considered as closed or as part of a unique metapopulation. Connectivity assumes different interpretations depending on the level to which exchanges influence the population dynamics. Evolutionary (i.e. genetic) connectivity is defined as the degree to which gene flow affects evolutionary processes within

metapopulation (Waples and Gaggiotti, 2006; Lowe and Allendorf, 2010) while, from an ecological point of view, demographic connectivity is defined as the degree to which population growth and vital rates are affected by dispersal (Lowe and Allendorf, 2010; Leis *et al.*, 2011).

Dispersal

For instance, the successful dispersal of individuals sets up the spatio-temporal scales of connectivity (Cowen *et al.*, 2007; Gaines *et al.*, 2007) and involves strong effects on local population dynamics (Fogarty and Botsford, 2007). Dispersal influences directly the geographic distribution of the species (Treml *et al.*, 2012) and regulates the entire demographic exchanges of individuals, shaping, at the same time, the genetic profile of the populations (Palumbi, 2003; Waples and Gaggiotti, 2006; Jones *et al.*, 2009; White *et al.*, 2010a).

Metapopulation structure and discrete populations

In many metapopulation organized in discrete local populations, the interactions between these different groups are mainly due to early life stages (Holbrook *et al.*, 1994; Hanski, 1998). This is the case of many coastal benthic fish species, generally sedentary: they usually produce benthic or pelagic eggs, followed, after spawning, by a pelagic larval stage (Leis, 2006). For species characterized by such bi-partite life cycles, population connectivity is mainly realized by the dispersion of the pelagic stages until the achievement of the settlement process (Pineda *et al.*, 2007; Sale, 2004). Thus, fish early life history traits are important parameters in the coastal benthic populations' dynamics, and in such cases, the level of population connectivity can be estimated as the flux, both genetic and demographic, of larvae among local populations (Sale, 2004).

Open vs closed populations

Various spatial structures can be observed, depending on the magnitude of the connectivity processes, ranging from very low levels of connectivity among populations, where the population structure and size are maintained only by self-recruitment (i.e. closed populations, Figure 1a) to high connectivity, occurring through large exchanges of larvae among populations (i.e. open populations, Figure 1b).

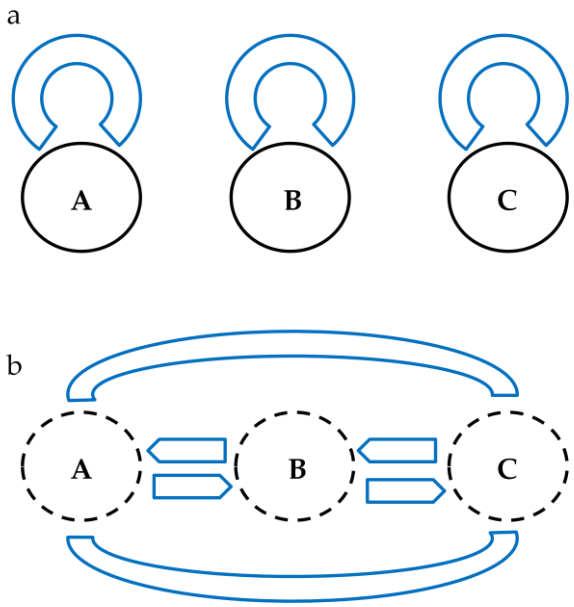


Figure 1. Schematic representation of closed (a) and open metapopulations (b). Blue lines represent the larvae movements before settlement, in the A, B and C populations.

MPAs and connectivity

A detailed knowledge of the magnitude and the spatio-temporal scale over which connectivity operates is crucial to comprehend the structure of marine populations and the relationship among them (Siegel *et al.*, 2008). Thus, understanding the connectivity patterns helps to provide the necessary knowledge on the scale at which marine resources should be managed and on the adapted management strategies to consider (Mora and Sale, 2002a; McCook *et al.*, 2009; Leis *et al.*, 2011; Magris *et al.*, 2014). Indeed, connections among different sites or populations are particularly relevant in the design of Marine Protected Areas (MPAs). Several studies have yet demonstrated the effectiveness of such management measures for the ecosystem conservation and the surrounding fisheries catches (Halpern and Warner, 2002, 2003; Gaines *et al.*, 2010a, 2010b; Sale *et al.*, 2010). Connectivity benefits from the expected higher biomass of target species (at larvae, juvenile or adults phases), exported from the reserve to the surrounding areas. Among the main effects of MPAs, recruitment subsidy is accountable for connecting sites at hundreds of kilometres away from MPAs where propagules originated, while spill-over may affect nearby MPA's areas at different spatial scales (from hundreds meter to few kilometres) (Halpern *et al.*,

2009).

Considering these parameters, the most important issues regarding the MPAs design, are the definition of their optimal size and the structure, which may affect their effectiveness. When designed for ecosystem conservation, MPAs should be large enough to retain a substantial portion of the local larval production to ensure adequate self-recruitment; when aiming to enhance fisheries, they should be sized so that a significant proportion of larvae can disperse to surrounding fished areas. If MPAs are designed as a network, they must be spaced at an appropriate distance to ensure connectivity via larval dispersal (Sale *et al.*, 2010). In the Figure 2 is reported the representation of the spatial structure of and ideal MPA (Sale *et al.*, 2010). The white circles represent reserve boundaries while the dome shapes represent the pattern and importance of larval dispersal, with higher numbers of larvae occurring at the birthplace (i.e. within the reserve), that gradually decrease in number with distance.

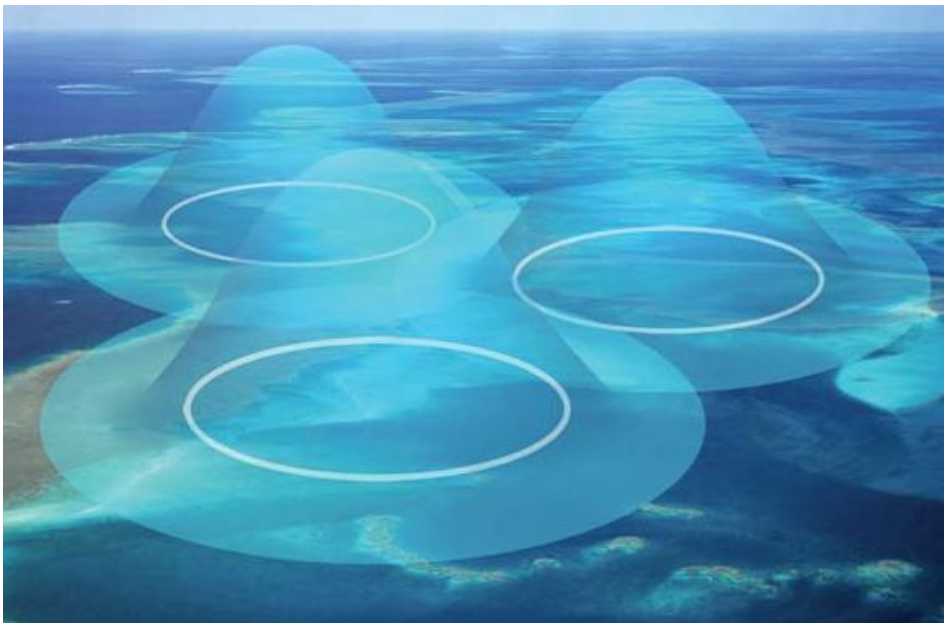


Figure 2. Ideal representation of the spatial structure of MPAs. Reproduced from Sale *et al.* (2010)

Thus, considering patterns of larval dispersal and the consequent connectivity trough different life-history stages of the targeted organisms is

fundamental for improving MPA's performance (Sale *et al.*, 2005).

Self-recruitment and local retention

Referring to connectivity, it is also important to introduce the concept of self-recruitment and local retention (Figure 3). Self-recruitment is defined as the proportion of the recruits returning to their natal origin location compared to the total number of larvae recruited in this same location (Jones *et al.*, 1999; Swearer *et al.*, 1999; Jones *et al.*, 2005; Almany *et al.*, 2007; Christie *et al.*, 2010). Local retention, refers to the proportion of larvae that settle in their natal origin in comparison with to the total number of larvae produced from that same location (Botsford *et al.*, 2009).

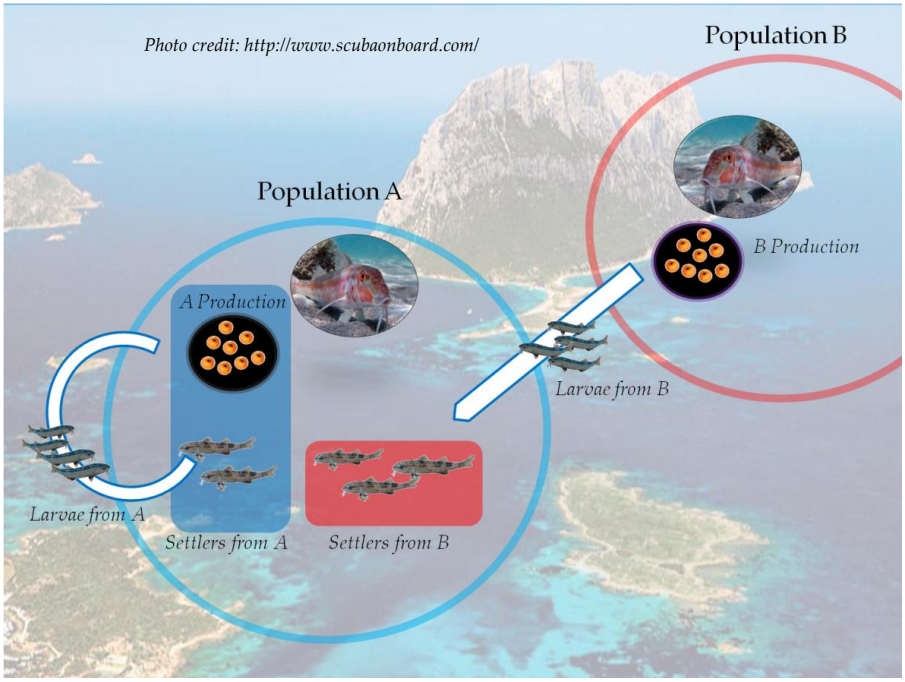


Figure 3. Representation of local retention (*Settlers from A* / *Production A*) and self-recruitment (*Settlers from PopA* / *(Settlers from A* + *Settlers from B)*), ideally assuming two populations

Larval dispersal and connectivity

From an ecological perspective, if the exchange is measured at the time of settlement, connectivity can be essentially considered as the spread of larvae from a spawning source to a settlement site (Pineda *et al.*, 2007). By this definition, larval dispersal determines i) the spatial scales over which different population units are connected, ii) the distribution and iii) the abundance of the considered species, all of these elements contributing to the spatial structuring of

the metapopulation (Leis *et al.*, 2011). Such larval connectivity can help to moderate the drift effect, i.e. the change in the frequency of a gene variant (allele) in a population due to random sampling in small populations, reducing the risk of extinction (Tallmon *et al.*, 2004).

Larval transport

The success of larval dispersal is conditioned by the larval transport, defined as the horizontal translocation of a larva between two points. Thus, physical processes such as oceanic currents and winds, as well as the larvae behaviour play an important role in the definition of the connectivity patterns. For facilitating dispersal over large distances, a significant larval transport is required, while conversely, it has been demonstrated that restricted dispersal does not necessarily imply little larval transport (Pineda *et al.*, 2007).

LARVAL CONNECTIVITY

Description of the Early Life History Stages (ELHS)

Fish life cycle and early life history stages

Marine benthic fish are characterized by complex life cycles. After the reproduction, eggs are liberated in the pelagic environment or kept attached to the substrate. After hatching, larvae generally go through several stages (Leis and Carson-Ewart, 2000; Leis and McCormick, 2002) corresponding to five ontogenetic development phases (Figure 4):

1) Yolk sac larva: Development stage beginning with hatching and ending with the ending of the yolk reserves; this whole stage is characterized by the presence of a yolk sac.

2) Preflexion larva: Development stage beginning at the ending of the yolk reserves and finishing with the beginning of the upward flexion of the notochord.

3) Flexion larva: Development stage beginning with the flexion of the notochord and ending when the hypural bones assume a vertical position.

4) Postflexion larva: Development stage beginning with the formation of the caudal fin (hypural element vertical) and ending when reaching the full

development of the external meristic complements (fin rays).

5) Post larva: also called competent larva, pre-settler or transitional larval stage, this is the stage characterized by the settling of the larvae in benthic habitats. At this stage, fins are completely developed and the scales begin to cover the body. The transition from the planktonic life to the benthic phase is normally reached through the definitive metamorphosis occurring at this stage.

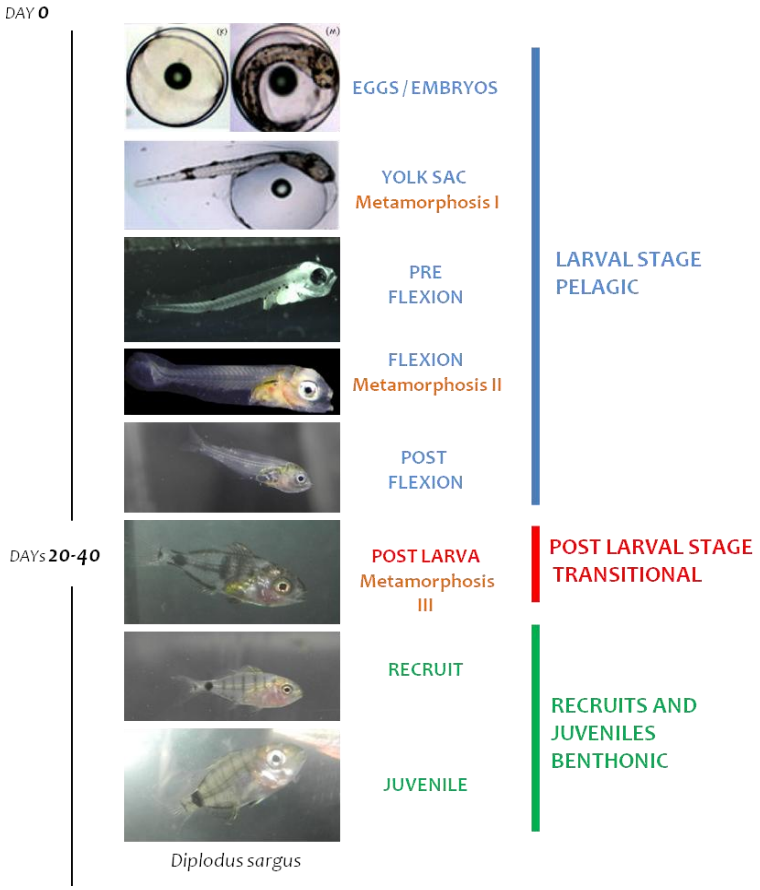


Figure 4. Phases of the ontogenetical development larval stages: example for *Diplodus sp.* (Sparidae). (Photos credits: Eggs and yolk sac larvae : Klimogianni *et al.* (2011). Flexion larvae: http://fisheggs-and-larvae.saiab.ac.za. Others: Manuel Muntoni)

All these phases are regulated by endogenous factors (species biology, ecology and behaviour) and exogenous factors (e.g. temperature, salinity, etc), which can lead to various spatio-temporal dispersal patterns (Cowen *et al.*, 2000; James *et al.*, 2002; Mora and Sale, 2002a).

During the fish early-life stages, it is possible to identify two crucial processes that can have strong implications on the connectivity patterns among population (Pineda *et al.*, 2009): (i) the larval pelagic period and (ii) the settlement and post-settlement processes.

Larval dispersal

Larval dispersal and Pelagic Larval Duration (PLD)

Larval dispersal has been recognized as one of the main driver helping to maintain the gene flow within local subpopulations (Pineda *et al.*, 2007). This dispersal pelagic phase is characterized by the transport of eggs and / or larvae from the spawning sites to the settlement sites and can begin with the passive dispersal of eggs when they are pelagic and not attached on the substrate. After few days, the new-hatched larvae spend a species-specific period in the pelagic environment, drifted until reaching the settlement area. The length of this period is known as the Pelagic Larval Duration (PLD).

Factors affecting larval dispersal

During this pelagic phase, larvae are strongly impacted by the hydrodynamic conditions of the water column and endure the ontogenetic, physiological and behavioural changes previously described, until the last metamorphosis that gives to the larvae (i.e. pre-settlers) the competences and capabilities to settle. Environmental factor (temperature, salinity, food availability, etc) and physical processes (advection, diffusion, coastal and ocean circulations, eddies and current, gyres, fronts, winds, *etc*) have been demonstrated to strongly affect the larval dispersal patterns (O'Connor *et al.*, 2007; White *et al.*, 2010b; Schunter *et al.*, 2011).

Coastal fish species present extremely variable spatial and temporal scales of larval dispersal (Sale, 2004) which can range from less than one meter and some minutes of dispersal until settlement to thousands of kilometers and various months, depending on the specific life-traits, behaviour and several exogenous factors (Fiksen *et al.*, 2007; Kinlan and Gaines, 2003).

Settlement and post-settlement processes

Settlement phase

For the coastal and demersal species, competent larvae (late larval stage or post-larval stage) are accountable for searching a suitable area to settle, moving from the pelagic environment to the benthos (Sale *et al.*, 1984). The settlement phase is often characterized by morphological and behavioural adaptations to the new benthic habitat (Leis and McCormick, 2002), where post-larvae will be exposed to complex interactions with different competitors and predators (Öhman *et al.*, 1998).

The importance of the settlement phase has been widely demonstrated (Doherty *et al.*, 2004; Hixon, 1998) and since benthic fishes are generally sedentary, a significant part of the recruitment to local reef fish populations is achieved through the settlement of new pelagic larvae.

Competency period

Particularly, the competency period, where competent larvae can delay metamorphosis (Chambers and Trippel, 1997), is an important trait regulating the arrival and the settlement of the larvae (Pineda, 2000). For example, a longer and more variable PLD may provide the flexibility to respond actively to favourable environmental cues which are also variable (Sponaugle and Cowen, 1994).

Factors affecting larval dispersal

The events occurring during this phase may impact the number of individuals reaching the adult phase (Fenberg and Roy, 2008). A wide spectrum of endogenous and exogenous factors impact the pre-settlement and post-settlement stages, inducing massive mortality and affecting the recruitment rates, distribution, growth and survival of the individuals (Pineda, 2000; Pineda *et al.*, 2007). In the first days after settlement, mortality rate is known to be high (Caley, 1998; Doherty *et al.*, 2004; Planes *et al.*, 2009) and is mainly attributable to strong predation (Almany and Webster, 2006; Doherty *et al.*, 2004; Holbrook and Schmitt, 2003; Planes *et al.*, 2000, 2009; Taylor, 1990). Moreover, other factors than predation can affect survival rates of the newly settled larvae, such as the own fish condition (Macpherson and Raventos, 2005; Raventos and Planes, 2008), its

parasites (Planes *et al.*, 2009), the settlement habitat type and structure (Almany *et al.*, 2007; Johnson, 2007; Juanes, 2007; Félix-Hackradt *et al.*, 2014) and the density-dependent phenomena (Nagelkerken, 2009; White *et al.*, 2010b).

Indeed, pre-settlement and post-settlement processes assume a fundamental role in the fish population dynamics of (Shima, 2001) and the issue of the recruitment depends on the success through these stages (Pineda *et al.*, 2009). However, estimating the effect on population dynamics of the pre and post-settlement processes it's still object of debate and the problematic of how many settlers will effectively join the adult population is a key component of the recruitment problem, but it is still hard to evaluate.

BACKGROUND AND DIFFICULTIES IN STUDYING CONNECTIVITY

Challenges in studying connectivity

Considering all these previous elements, it is clear that a central topic in ecology is to reveal the relationship between source populations of settling larvae and the settlement sites of the dispersing larvae. The main challenge is to provide an adequate understanding of the processes and scales controlling larval dispersal and connectivity (Magris *et al.*, 2014). Resolving the mechanisms commanding these processes would allow a more comprehensive understanding of the relevant ecological and physical actions and how they result in shaping the fish metapopulation.

Classical approach to study dispersal

Dispersal has been conventionally considered as the result of the simple combination of ocean dynamics and PLD, and larvae were assumed for a long time to be passive particles with no directional control of their own trajectories (Leis, 2006). In such context, reef fish populations have been viewed as unconditionally open and connected over large spatial scales, assuming the passive transport of pre-settlement stages (Caley *et al.*, 1996; Doherty and Williams, 1988; Sale, 1980, 1991; Williams *et al.*, 1984) and the distance between spawning and settlement sites considered to be linearly correlated to the PLD. Indeed, the major part of the literature focused on the definition of basic history traits such as pelagic larval duration (PLD) to evaluate realized dispersal

PLD and realized dispersal

distances (Kinlan and Gaines, 2003; Lester and Ruttenberg, 2005; Shanks, 2009; Shanks *et al.*, 2003; Weersing and Toonen, 2009).

In some cases, PLD has been shown to be directly related to dispersal and genetic distances, and also to the species' distribution area (Bohonak, 1999; Shanks *et al.*, 2003; Palumbi, 2004; Lester and Ruttenberg, 2005; Mora *et al.*, 2012), while other studies demonstrated the scarce consistency of these assumptions, reporting species presenting a relatively long PLD but high levels of self-recruitment and/or local retention (Swearer *et al.*, 1999; Bradbury and Snelgrove, 2001; Thorrold *et al.*, 2001; Almany *et al.*, 2007; Bradbury *et al.*, 2008; Christie *et al.*, 2010). These studies highlighted not only that fish populations could be relatively closed but also that levels of self-recruitment can be higher than previously thought (Jones *et al.*, 1999, 2005, 2009; Knutsen *et al.*, 2007).

Larval bio-ecology and behaviour

These findings are supported by an improved understanding of larval behavioural and ecological characteristics, and of their interactions with environmental cues (Leis and McCormick, 2002; Paris and Cowen, 2004; Leis *et al.*, 2006a, 2006b; Jones *et al.*, 2009). Indeed, it has been demonstrated that the larvae sensory systems and their swimming capabilities are determinant factors used to reach settlement areas and to survive in this new environment (Montgomery *et al.*, 2001; Atema *et al.*, 2002; Leis and McCormick, 2002; Montgomery *et al.*, 2006; Simpson *et al.*, 2010; Vermeij *et al.*, 2010; Mouritsen *et al.*, 2013; Paris *et al.*, 2013; Staaterman *et al.*, 2014). Active response of the larvae to environmental cues can significantly affect their final position and location (Paris and Cowen, 2004; Leis, 2007; Vikebø *et al.*, 2007), and influence the success of the dispersal phase. Active larvae movements can be vertical (e.g. diel migrations) or horizontal and they depend on the ontogenetic development (Irisson *et al.*, 2010; Leis, 2006). However, the extent to which the larval behaviour affects settlement and recruitment rates is not clear and is still under debate.

Difficulties in studying connectivity

Estimating the distances, the larval abundances, the distributions, and the relations between spawning and settlement areas are complicated by the intricacy of interrelated behavioural, biological, ecological and physical factors

(Reisser *et al.*, 2014). Most of the limitations in the study of such processes are due to the difficulties in obtaining direct observations of larvae (Sale and Kritzer, 2003), tracking their movements and defining the survival rates in this phase.

Main limitations

The main limitations and difficulties are:

- the lack of detailed information about the larval taxonomy, biology and ecology
- the lack of high resolution methods incorporating geography and oceanic currents
- the numerous interactions with a complex environment
- the high variability of spatial and temporal scales of larval dispersal (Werner *et al.*, 2007; Botsford *et al.*, 2009; Jones *et al.*, 2009)
- the evaluation of mortality rates during the early life stages

However, whereas measuring and predicting connectivity of marine populations is extremely difficult, it is also crucial (Sponaugle *et al.*, 2012), and various methodologies can be used.

METHODS TO STUDY CONNECTIVITY

Since the knowledge on connections over different spatial and temporal scales is essential for a comprehensive understanding of the population dynamics and for effective management of fish populations, several approaches have been developed and applied to obtain information on population connectivity: in situ or ex situ larval behavioural studies (Irisson and Lecchini, 2008; Igulu *et al.*, 2013), individual tagging (Jones *et al.*, 2005; Thorrold *et al.*, 2006; Cuif *et al.*, 2014) otoliths analyses (Swearer *et al.*, 2003), use of devices to track in situ larval trajectories (Irisson *et al.*, 2009), biophysical modelling (Cowen *et al.*, 2000; Mitarai *et al.*, 2009; Garavelli *et al.*, 2012a; Sponaugle *et al.*, 2012; Andrello *et al.*, 2013), population genetic approaches (Hedgecock *et al.*, 2007; Félix-Hackradt *et al.*, 2013a) and analytical approaches (Largier, 2003; Botsford *et al.*, 2009).

Some of these techniques of interest for this dissertation are more

precisely described thereafter and for an exhaustive review see (Leis *et al.*, 2011; Calò *et al.*, 2013).

Otolith studies

Description

Otoliths are hard calcium carbonate structures with balance, orientation, and sound detection function in fishes and are vastly used in ecological studies as biological natural markers (Campana and Thorrold, 2001). Fish otoliths present some characteristics particularly useful for furnishing reliable information on population dynamics (Campana, 2005):

- *Daily growth*: daily growth increment are generally deposed on a daily basis offering precise information on the fish age (sclerochronology) (Panfili *et al.*, 2002)
- *Chemical fingerprinting and metabolic inertia*: trace elements are indissolubly incorporated in the otolith matrix during its growth, reflecting the chemical composition of the surrounding environment; such information can be used as a proxy in the determination of different larval sources (Swearer *et al.*, 1999)
- *Easiness in conservation:* calcified structures are resistant to degradation
- *High specificity*: hard structures as otoliths can be used for stocks discrimination (e.g. morphological analyses on their shape)

ELHT, otoliths and connectivity

Some essential early life history traits such as PLD and spawning, hatching and settlement dates can be assessed by larval and post-settlers' otoliths sclerochronology (see Chapter II and III) (Panfili *et al.*, 2002; Raventos and Macpherson, 2001), providing critical information on the potential dispersal range and the distances covered by the larvae during its pelagic phase (Shanks, 2009). Due to their metabolic inertia, chemical elements included in otoliths can be used to obtain information on the larval history, dispersal and spawning patterns (Di Franco *et al.*, 2012b; Guidetti *et al.*, 2013) and to discriminate the individuals natal origin (Swearer *et al.*, 1999; Swan *et al.*, 2003; Barbee and

Swearer, 2007). Another technique that furnished significant advancements in the study of larval dispersal and population connectivity in marine fishes, is represented by the transgenerational marking, with ^{137}Ba isotopes, of embryonic otoliths (Thorrold *et al.*, 2006). This approach is based on maternal transmission of these marker from spawning females to egg material, that is ultimately incorporated into the otoliths of embryos produced by an individual after exposure to the isotope.

Larval dispersal modelling

Usefulness of dispersal modelling

Biophysical dispersal models are recently more and more used in connectivity studies and help to understand the past patterns and to simulate scenarios on eggs and larvae dispersal dynamics (Lett *et al.*, 2010). The possibility to couple biological, ecological and behavioural parameters with hydrographical models in the dispersal simulations has improved the quality of the estimates (Cowen *et al.*, 2006; Mitarai *et al.*, 2009; White *et al.*, 2011; Di Franco *et al.*, 2012a; Treml *et al.*, 2012).

Basic information and limitation

This approach can provide fine estimates about the spatial and temporal scales of relevant ecological processes such as connectivity and support the planning of appropriate sampling designs (Albert *et al.*, 2010). However, the lack of resolution of the basic knowledge information used as input parameters can strongly limit the use of such tools (Leis, 2007). Models are used worldwide, and whereas it is still rare, few studies have been done in the Mediterranean Sea (Andrello *et al.*, 2013; Di Franco *et al.*, 2012b).

Genetics

In the context of genetic and demographic connectivity, genetic studies are widely used for evaluating the structure of marine fish populations (Weersing and Toonen, 2009). Genetic markers can furnish an effective quantification of the gene flow (Waples and Gaggiotti, 2006) and the spatial genetic differentiation for several fish populations over different spatial scales (Hellberg *et al.*, 2002; Salas *et al.*, 2010); moreover they have been used to identify

the presence of bio-geographical barriers (Gaither *et al.*, 2009; Planes, 2002), cryptic species and areas of endemism (Rocha *et al.*, 2007).

Polymorphic markers such as microsatellites, displaying a high mutation rate, permit to detect the presence of fine population structures (Hauser and Carvalho, 2008), thus furnishing reliable information on the contemporary connectivity (van der Meer *et al.*, 2012). Other techniques, as allozymes or mtDNA, little sensitive to drift, particularly in large populations over short time periods, are used for investigating processes operating at evolutionary time scales (Mora and Sale, 2002; Leis *et al.*, 2011).

CASE OF STUDY: MULLUS BARBATUS IN THE WESTERN MEDITERRANEAN SEA

Spatial distribution and life cycle of *Mullus barbatus*

This dissertation is focused on the red mullet (*Mullus barbatus* Linnaeus, 1758), a common and targeted fish distributed all around the Mediterranean basin, the Black Sea and the eastern Atlantic from Scandinavia to Senegal (Froese and Pauly, 2014). The red mullet is mostly found in depths ranging from 10 to 300m, in sandy and muddy bottoms. In the Mediterranean Sea, the red mullet spawning events occur during spring, when it produces pelagic eggs. After hatching, pelagic larvae and post-larvae (i.e. pre-settlers) live close to the surface for a relatively variable pelagic larval duration, ranging from 25 to 42 days, meaning an extended competency period (17 days) (see Chapters II and III). The pelagic dispersal phase ends when pre-settlers, at a size of about 5 to 6 cm, dive from the surface to the settlement benthic areas.

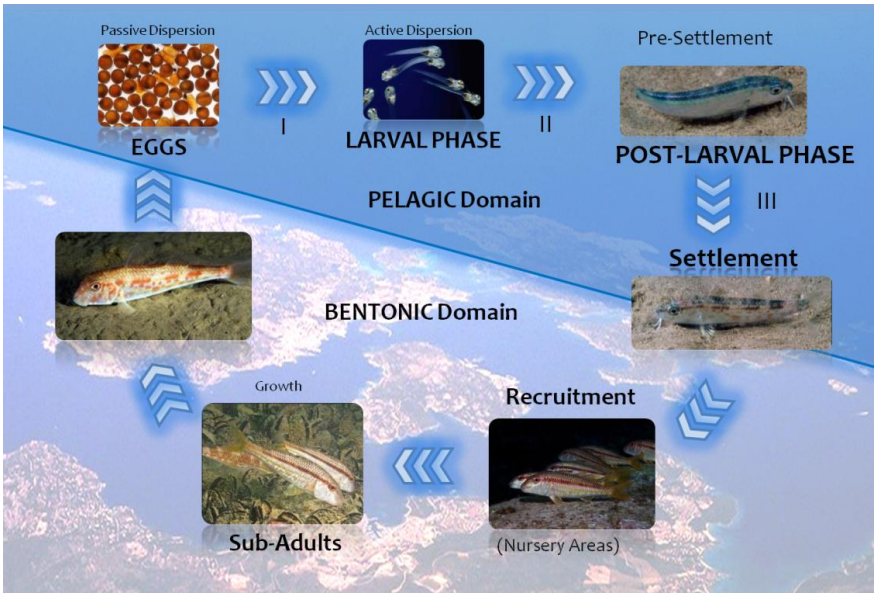


Figure 5. *Mullus barbatus* life cycle

Economical importance

This species has a high commercial value and is one of the main targeted species caught by demersal fisheries operating in the Mediterranean Sea (Hadjistephanou, 1992; Vassilopoulou and Papaconstaninou, 1992; Voliani, 1999; Tserpes *et al.*, 2002; Sieli *et al.*, 2011).

Literature background

Due to its relevance in the fisheries, *M. barbatus* has been highly studied in the Mediterranean context, and several aspects of its biology and ecology were evaluated. Different studies have been conducted on the red mullet adult growth (Bianchini and Ragonese, 2011; Fiorentino *et al.*, 2013; Kalagia *et al.*, 2004), reproduction and reproductive stocks (Fiorentino *et al.*, 2008), diet (Bautista-Vega *et al.*, 2008; Esposito *et al.*, 2013), stock-recruitment dynamic (Levi *et al.*, 2003), distribution patterns of adults (Vassilopoulou and Papaconstaninou, 1992; Lombarte *et al.*, 2000; Tserpes *et al.*, 2002; Maravelias *et al.*, 2007), nursery areas distribution (Carlucci *et al.*, 2009), larval distribution (Sabatés and Palomera, 1987) and early life history traits (such as PLD) (Chapter II, III).

Studies on connectivity

Additionally, various research focused on the definition of genetic population structure and connectivity across several Mediterranean localities, mainly through the use of microsatellites (Garoia *et al.*, 2004; Galarza *et al.*, 2007, 2009b; Maggio *et al.*, 2009; Félix-Hackradt *et al.*, 2013a) and allozymes (Arculeo *et*

al., 1999; Mamuris *et al.*, 1998). These studies provided some evidences about the complex genetic structure characterizing the red mullet metapopulation along the Mediterranean coasts. Separate stock units were detected in the Atlantic and in the Mediterranean Sea (Galarza *et al.*, 2009b). Isolated populations were found in the Adriatic Sea (Garoia *et al.*, 2004; Maggio *et al.*, 2009), while Félix-Hackradt *et al.* (2013) highlighted a micro-scale genetic homogeneity in the southern Spanish coasts analysing post-larval individuals.

Why this species?

Because of its ecological and economic importance, *Mullus barbatus* has been selected as a particularly interesting case study for investigating connectivity trough early life-history stages. Indeed, the red mullet presents a bi-partite life style, with relatively sedentary adults (this species performs small scale migration toward deeper spawning areas - Machias and Labropoulou, 2002), pelagic eggs, large-size pre-settlement stages (up to 6cm of total length) and a relative extended PLD and competency settlement period, which confer to early life history stages of this species, high potential dispersal capabilities (see Chapter III).

Moreover, this research focused on the Western Mediterranean Sea, which characteristics are particularly interesting for connectivity studies: it is an enclose basin of large surface, where various islands could be used as intermediary areas between very distant ones, facilitating connectivity patterns. In the Mediterranean Sea, studies on fish early-life stages have been conducted for various coastal rocky fish species (García-Rubies and Macpherson, 1995; Harmelin-Vivien *et al.*, 1995; Macpherson and Raventos, 2005, 2006; Félix-Hackradt *et al.*, 2013b, 2014), and more recent ones on connectivity (Di Franco *et al.*, 2012a, 2014; Félix-Hackradt *et al.*, 2013a). However, they rarely gather different approaches and methodologies to understand and explain the observed patterns.

OUTLINE OF THE THESIS

Challenges of the thesis

Considering all these elements, the challenge of my PhD research was to

provide a picture of the realized and potential red mullet population connectivity both at a sub-regional (from 10s to 100s of km; i.e. Sardinia) and regional scale (from 100s to 1000s km; i.e.Western Mediterranean Sea).

The general objectives of this research were to acquire the basic information on *M. barbatus* life history traits (PLD, competency phase estimation) and the effects of these parameters' variability on the dispersal processes and connectivity patterns. Dealing with the mechanisms involved in larval dispersal and population connectivity, a multi-disciplinary approach was implemented, by integrating information on sclerochronology, fish biology, physical modelling and genetics.

Specific questions

To achieve these general objectives, my work was structured in four main questions, corresponding to the four chapters of this manuscript, designed as an information-cascade where each answer composes the basic knowledge necessary to solve the following question:

(1) Which is the best otolith type for ageing post-larval individuals?

This question will be answered in: Chapter I - Sagittae *vs* Lapilli: Comparing red mullet (*Mullus barbatus*) settlers otoliths types

(2) Do spatial variations exist in Pelagic Larval Duration?

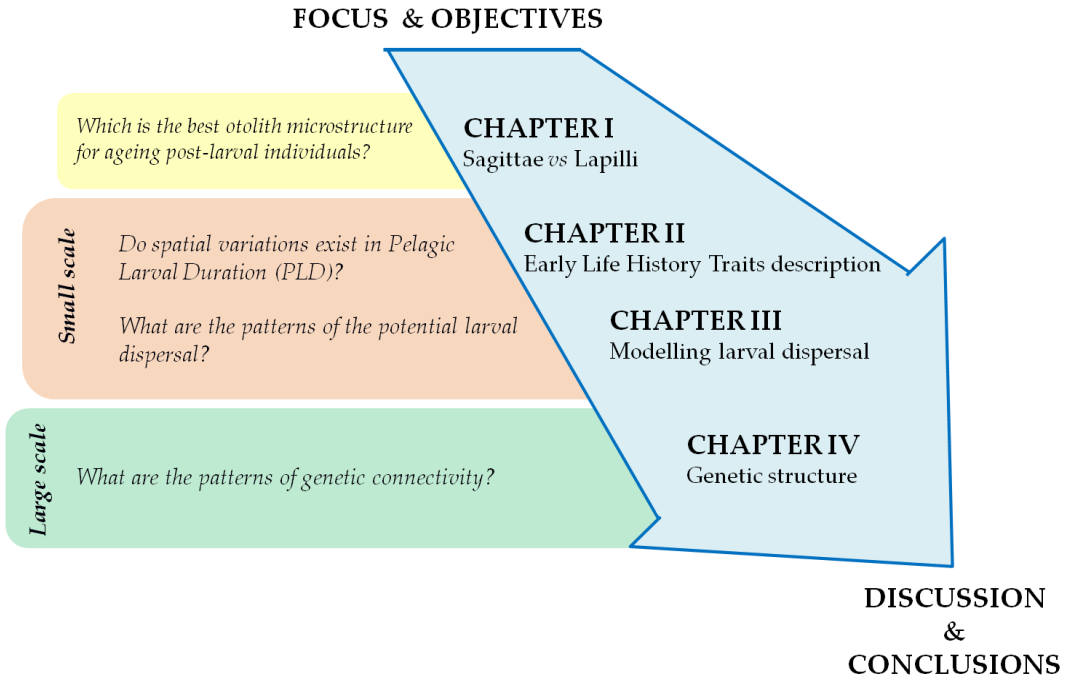
This question will be answered in: Chapter II - Settlement dynamics of *Mullus barbatus*: evidences from otolith sclerochronology

(3) What are the patterns of the potential larval dispersal?

This question will be answered in: Chapter III - Describing fish connectivity patterns: integration of otolith sclerochronology information in larval dispersal models

(4) What are the patterns of genetic connectivity?

This question will be answered in: Chapter IV - Genetic connectivity patterns at early life history stage: A broad scale case study.



Chapter I. Sagittae vs Lapilli: Comparing red mullet (Mullus barbatus) settlers otoliths microstructures

Among the variety of hard structures, otoliths are vastly used since their various microstructures can furnish daily-based estimates of the fish age. However, processing otoliths to obtain reliable information requires time, important economical and human resources and thus, designing a proper technique is a crucial issue for such study. Sagittae and lapilli of the red mullet (*Mullus barbatus*) were used to age settlers' individuals from the Western Mediterranean Sea coastal waters. In this chapter, to evaluate the quality of the different sclerochronogical information, we compare the reliability, the precision and the ability to describe larval growth of the different otoliths microstructures as well as the time investment needed for preparing the otoliths.

The specific objectives are:

(i) to test the usefulness of different otoliths' microstructures (i.e., sagittae and lapilli) for describing the growth and estimating the age of the red mullet early life stages

(ii) to compare the age estimates obtained from both structures

(iii) to estimate the reading precision for each type of otolith

(iv) to evaluate the time-investment for the preparation of each otolith type

Chapter II. Early life history traits of Mullus barbatus: evidences from otolith sclerochronology

In this chapter, I acquired the information on the settlement phase by means of otoliths analysis. Using post-larval otolith sclerochronology, this part of the thesis aimed to fill the existing gaps on the bio-ecological basic knowledge of *Mullus barbatus* early life-history traits (timing of settlement, competency period, pelagic larval duration and spawning date) at sub-regional (Sardinia) and regional scale (Western Mediterranean Sea). The empirical information obtained in this section will be then implemented in Chapter III, aiming to model larval dispersal at sub-regional scale. The specific objectives are to:

(i) estimate the PLD and the competence extension of the species

(ii) determine the spawning and settlement dates at local level (Sardinia, Italy)

(iii) check for differences in PLD among the sampled sites

(iv) highlight large scale differences in the considered early life history stages traits for 3 sites of Western Mediterranean

Chapter III. Describing fish connectivity patterns: integration of otolith sclerochronology information in larval dispersal models

Starting from the information obtained in the previous chapters, this part of the manuscript deals with modelling the larval dispersal patterns and studying the source-sink dynamics. In this chapter are reported the results of biophysical models based on empirical information on PLD for representing *M. barbatus* larval dispersal patterns at a sub-regional scale.

Indeed, for improving the reliability of the models' predictions, they were calibrated by implementing the information previously obtained. More particularly, the purpose of this study was to investigate, at relatively small scale (about 200 km), the dispersal patterns and the connectivity rates of *Mullus barbatus* between various areas during the early life history stages, and how they can be affected by the hydrographical circulation patterns.

The specific objectives are:

(i) to propose various scenarios of small-scale dispersal patterns,

(ii) to define the patterns of larval exchange and local retention, by describing the source–sink dynamics

(iii) to identify the potential spawning areas

Chapter IV. Genetic connectivity and early life history stages: the case of red mullet (Mullus barbatus) in the Western Mediterranean Sea

This last chapter provides an overview of the genetic connectivity of *M. barbatus* settlers caught in 4 distant sites of the Western Mediterranean Sea. This part of the work tries to define the role of early life history stages in shaping population structure over large scale. The population structure was investigated using 10 microsatellite markers specifically developed for this species.

The specific objectives are:

(i) to characterize the genetic pool of the settlers' population of *M. barbatus*

(ii) to detect significant genetic variability among different sites

(iii) to investigate the most probable populations sources

(iv) to assess the genetic differences related to the spatial distance between sites

CHAPTER I

SAGITTAE *VS* LAPILLI:

COMPARING RED MULLET SETTLERS OTOLITHS' TYPES

SAGITTAE *VS* LAPILLI: COMPARING RED MULLET (*MULLUS BARBATUS*) SETTLERS OTOLITHS' TYPES

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ABSTRACT

Reliable age estimates are of primary importance in the understanding of ecological and oceanographic processes influencing the early life of marine fishes and their recruitment. Among the variety of hard structures, otoliths are vastly used since their microstructure can furnish daily-based estimates of the fish age. Processing otoliths to obtain reliable information requires time and funding resources; however, for some species, it still lacks of accuracy and precision for obtaining relevant early life history traits estimations such as pelagic larval duration (PLD), spawning and settlement dates, etc.. Most research carried out so far used sagittae or lapilli data, but the potential differences in reliability and time costs when using these different otoliths have been rarely tested. In our study we compared and discussed the usefulness of sagittae and lapilli microstructure for ageing red mullet (*Mullus barbatus*) early life stages. Settlers of red mullet from coastal waters of the Western Mediterranean Sea were used to assess for differences in estimation of growth and age

as well as preparation time associated with different otolith type. Our results showed that lapilli and sagittae present the same level of precision (i.e. repeatability of the reading) while estimating the fish age, but the preparation time is significantly lower for the lapilli. Individuals smaller than 50 mm showed the highest precision and preparation time-saving for both sagittae and lapilli. Lapilli were also better growth descriptors, being their length significantly correlated with the fish total length. Overall, lapilli can be considered as more suitable for estimating early life history traits of *Mullus barbatus*. However, in the face of unclear readings situations, a comparative approach through the use of both sagittae and lapilli is recommended for a finest interpretation of daily increments.

KEYWORDS

Daily ring increment, ageing, comparison, sagittae, lapilli, variation coefficient, *Mullus barbatus*, Mediterranean Sea

INTRODUCTION

Accurate and precise ageing of fish is a critical step in the study of life histories to understand population dynamics and, consequently, to ameliorate the management of the marine resources (Campana and Thorrold, 2001; Green, 2009). Since four decades, with the publication of a reference study (Pannella, 1971), otoliths sclerochronology on early life history stages of teleostean fishes has been widely applied; indeed, it was found to offer reliable information on age, size, growth and development as well as on dispersal during their pelagic phase and on the population connectivity at a larger scale (Campana and Thorrold, 2001; Campana, 2005; Begg *et al.*, 2005; Sponaugle, 2010; Calò *et al.*, 2013).

Otoliths provide some of the best examples of bio-chronology, since they form daily rings generally difficult to identify in other hard structures (fish scales and bones) which do not produce such daily increments (Campana and Thorrold, 2001). A proper reading of otoliths daily increments gives detailed and crucial information on larval and juvenile fishes early-life history traits, such as the precise age and the time spent in the pelagic environment (e.g. Wellington and Victor, 1989; Wellington and Victor, 1992; Raventos and Macpherson, 2001). Otolith's ageing requires some basic assumptions: a) specific competences and skills of the operator (Campana, 2001); b) the selection of an adequate protocol for preparing the otoliths (Narimatsu *et al.*, 2007), depending on their intrinsic structural characteristics. As a consequence, the results of the ageing process could be affected by the otolith's type that is chosen (asterisci, sagittae, or lapilli) (Fey *et al.*, 2005; Joh *et al.*, 2005) and the research objectives and the investigated fish species (Morioka and Machinandiarena, 2001).

As a consequence, the selection of an ageing technique should be based on various criteria:

(1) the easiness of processing and reading, (2) the time-investment and (3) the robustness of the ageing process (Campana, 2001). An evaluation of the precision – through various independent increment counting per otolith – and accuracy – estimation deviation from real age– of the different otolith type reading should be also performed to consider the bias of the estimates (Campana, 2001; Panfili *et al.*, 2002; Fey *et al.*, 2005).

Most of the age and growth literature of larval and juvenile fish are done using the sagittae (Campana and Neilson, 1985). However, it has also been reported that the age estimation of various fish species can be accurately achieved by using the lapilli microstructures,e.g. Hoff *et al.*, 1997; Bestgen and Bundy, 1998; Morioka and Machinandiarena, 2001. In those studies, lapilli were considered as good estimators for fish early life history (ELH) parameters, providing the same accuracy and precision than sagittae, with an easier age-estimation reading process (e.g. Ichimaru and Katsunori, 1995; Morioka and Machinandiarena, 2001). In the Mediterranean context, the daily ageing using otoliths microstructures has been conducted for different species (Morales-Nin, 1992; Raventos and Macpherson, 2001; Di Franco and Guidetti, 2011) but few of these studies considered a comparison of the results obtained using different structures and/or reading techniques (Morales-Nin *et al.*, 1999).

Our study has been conducted on the red mullet (*Mullus barbatus*, Linnaeus, 1758), a demersal fish species inhabiting the south east and north east Atlantic and the whole Mediterranean Sea. This species is mainly targeted by coastal fisheries for its high commercial value (Tserpes *et al.*, 2002). Early-life stages (i.e. eggs, larvae and post-larvae) of *M. barbatus* are pelagic and are found in the upper layer of the water column during the summer months (Sabatés and Palomera, 1987). Post-larvae up to 50 mm of total length (TL) are still pelagic and they will be

able to settle at a size of 50 to 60 mm (Vassilopoulou and Papaconstaninou, 1992; Voliani, 1999; Kalagia *et al.*, 2004).

The literature on stock dynamics of red mullet is large and ranges from the description of the juvenile spatial distribution to the description of their recruitment and connectivity patterns (Fiorentino *et al.*, 2008; Carlucci *et al.*, 2009; Félix-Hackradt *et al.*, 2013). However, otoliths studies on red mullets in the Mediterranean Sea have been only performed on adult stages (Polat *et al.*, 2005; Aguirre and Lombarte, 1999; Mahé *et al.*, 2012). At nowadays, studies on early stages ageing and assessment of the information reliability provided by different otoliths were not documented in literature.

The present study was performed for addressing the following specific objectives: (i) to test the usefulness of different otolith types (i.e., sagittae and lapilli) for describing the growth of the red mullet early life stages, (ii) to compare the precision of the age estimates obtained from each type of otolith, and (iii) to evaluate the time-investment for the preparation of each otolith type.

MATERIAL AND METHODS

Ethic statement

The sampling activity did not involve neither protected nor endangered species in the sampled areas. As a standard protocol, in accordance with the ethical justifications (Metcalfe and Craig, 2011), fishes were immediately anaesthetised and then euthanized by reaching a ratio of 1/3 of ethanol and 2/3 of sea water. After the sacrifice, they were stored in ethanol (96%) for conservation before otolith extraction.

Sampling, data collection and otolith processing

Settlers of *M. barbatus* have been caught during the summers of 2013 and 2014 in the Gulf of Cagliari located in the south of Sardinia (Italy). The specimens were collected using light traps (CARE^{RM}, Ecocean, Montpellier, France) and hand nets. The total length (TL) of each fish was measured to the nearest millimetre and their weight (W) to the nearest 0.001 g. Otoliths were extracted from the cranium by a longitudinal cut from the snout to a position posterior to the occipital (operculum), following (Secor *et al.*, 1992) method. The extracted otoliths were measured to the nearest 0.001 mm along the longest axis.

The morphological differences lead to the different approaches in the preparation for the age estimation for both types of otoliths. Following Morales-Nin *et al.*, (1999), the otoliths were placed in slides with heated thermoplastic glue (CrystalBond™). The sagittae transversal section of *M. barbatus* was obtained by removing rostrum and post-rostrum sections by methodical polishing (Secor *et al*., 1992). First, the sagittae were placed on the edge of a glass slide with the post rostrum free and the nucleus just in the slide side of the edge. Then, the post rostrum part was grinded and polished. Next step implies reheat the slide and move the otolith from the edge to the centre of the slide and placed ground side down and grinded the protruded section until a thin section including all rings and nucleus is obtained. A finest smoothing was necessary when approaching closest to the core, in order to obtain the primordium and the first increment clearly visible (mark which defines the outer edge of the nucleus), with the best reading-plane, focused on the edge. Lapilli were gradually polished on both sides, until the achievement of the thinnest sagittal section corresponding to the optimal reading plane for the incremental daily rings identification.

The grinding was performed by using wet sandpaper (grit sizes ranging from 1, 3 to 30

μm). The preparation time of each otolith, from the inclusion of the otolith in the slides to the mounted finished section, was recorded in minutes (min) for the subsequent analysis.

After the preparation, four independent daily increments readings were performed for each otolith. Counts were made by one expert reader using a transmitted-light microscope (Leitz Dialux 20EB, 250× magnification) equipped with an high-resolution video camera (Zeiss Axiocam ERC5s, 5 Megapixel) and a live monitor system. In order to obtain a clear image, the settings of the microscope (exposure, contrast, white balance, etc.) were tailored to each sample. Because of the elliptic shape of sagittae, that implies a widest and non-compressed deposition, daily growth otolith increments were counted along the longest axis of sagitta transversal section, starting from the nucleus and ending to the edge. Daily increments were counted without prior knowledge by the reader of the size or collection date of the individual.

Statistical analysis

The age of fishes was estimated for each otolith as the mean value of the number of increments evaluated by the whole set of the four readings. Standard deviation (SD) associated with the age was also calculated for each individual. Pearson's product-moment correlation and linear regression were used to assess the relationships between TL and otolith length (OL) and to test the age-TL relationship.

The agreement among otolith readings was estimated as the percentage of the number of observations showing similar evaluations (differences less than 1 day) between different otoliths types. In order to evaluate the correspondence of the age information obtained from different otolith types, we tested the relationship among the estimations obtained for each otolith type by

Pearson's product-moment correlation. Linear regression was also used to describe the relation among otolith readings.

As a measure of the repeatability of the readings, precision of increment counts from different otoliths was evaluated by contrasting the mean Coefficient of Variation (CV) (Chang, 1982). The CV was calculated by using the four increment counts made for each individual and type of otolith as a mean value in the formula SD/mean age.

Student's test was preliminary conducted, both for CV and preparation time, to assess the differences in the mean values between sagittae and lapilli.

Then, the differences for each response variable, respectively CV and preparation time, were examined by a two-way ANOVA test. Two fixed and orthogonal factors were used in the analysis, the otolith type (i.e. with two levels, sagittae *vs*. lapilli) and the fish size categories, which included two categories (≤ 50 mm; *and* ≥50 mm), for which the threshold size of 50 mm corresponds to the total length at settlement reported for this species in the Mediterranean (Vassilopoulou and Papaconstaninou, 1992). Homogeneity of variances was checked with a Cochran's C test (Underwood, 1997). When significant differences were detected, pairwise comparisons were performed by means of Student Newman–Keuls (SNK) test. All the statistical analyses were performed using the R statistical package (R Development Core Team, 2011).

RESULTS

A total of 46 settlers of *Mullus barbatus* were analysed. The TL of the specimens ranged from 27 to 62 mm with a mean value of 47.15 mm (SD = 8.81 mm). For 7 specimens (15.21% of the total samples) the measurements and the mounting times of the otoliths were not available and they were not included in the growth analysis.

The sagittae (Figure 1a) are elliptic-shaped, strongly compressed laterally, with a pronounced rostral and anti-rostral complex, which becomes more evident as fish size increases. Lapilli (Figure 1b) were spherical or heart-shaped forms, presenting a simpler global morphology than sagittae.

The lengths of the sagittae ranged from 0.798 to 1.656 mm, while the lapilli length ranged from 0.262 to 0.534 mm. The mean length of the sagittal otoliths was 1.131 mm (SD = 0.228 mm) while that of the lapilli was 0.400 mm (SD = 0.062 mm).

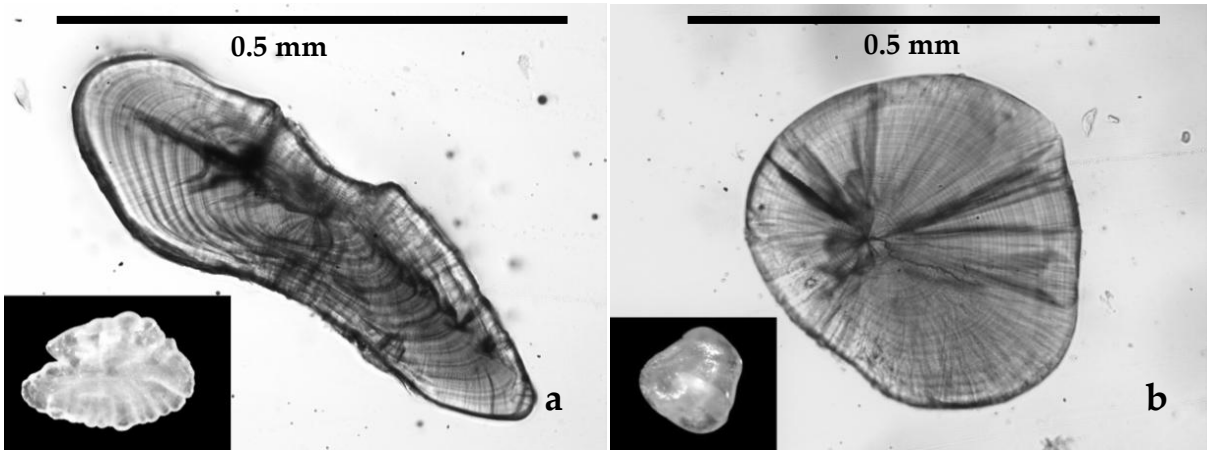

Figure 1. (a) Sagitta's transversal section (250×). Total sagitta length = 0.83 mm and (b) lapillus's sagittal section (250×). Total lapillus length = 0.44 mm. In the frame the view of the whole otolith.

The correlation between otolith length and fish size was significant both for lapilli (r = 0.88; t = 11.21; R^2 = 0.78; *p-value* < 0.001) and for sagittae (r = 0.80; t = 8.81; R^2 = 0.65;*p-value* < 0.001) (Figure 2 and Tab. 1). Wider data dispersion was observed for the sagittae length of the individuals larger than 50 mm of TL.

Table 1. Linear relationships and comparison of the slope between the total fish length and otolith length

	Otolith	*r*	t	*p-value*	Equation	R^2
OL – TL	Sagittae	0.80	8.12	1.43E-06	y = 0.191 + 0.020 x	0.65
	Lapilli	0.88	11.21	3.93E-10	y = 0.117 + 0.006 x	0.78
AGE - TL	Sagittae	0.74	7.35	3.43E-09	y = 13.03 + 0.47 x	0.55
	Lapilli	0.73	7.10	7.89E-06	y = 11.57 + 0.50 x	0.53

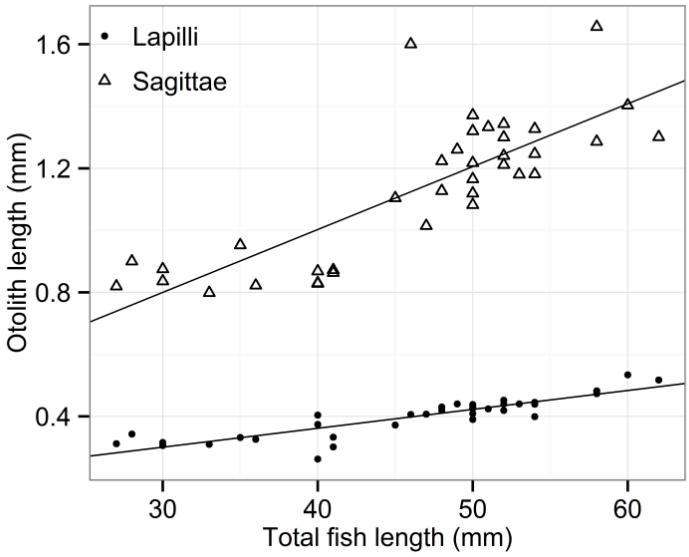

Figure 2. Relationship between fish total length and otolith length for both structures assessed.

The age of the fish individuals sampled ranged from 26 to 49 days (Figure 3). Wider data dispersion was observable for the individuals larger than 50 mm of TL. The mean ages revealed by the sagittae and lapilli readings were respectively 35.42 days (SD = 5.63) and 35.37 days (SD = 6.08). A relative low goodness of fitting was found between the estimated ages and the total fish size, both from sagittae (R^2 = 0.55, *p-value* < 0.001) and lapilli (R^2 = 0.53, *p-value* < 0.001) (Figure 3 and Tab. 1).

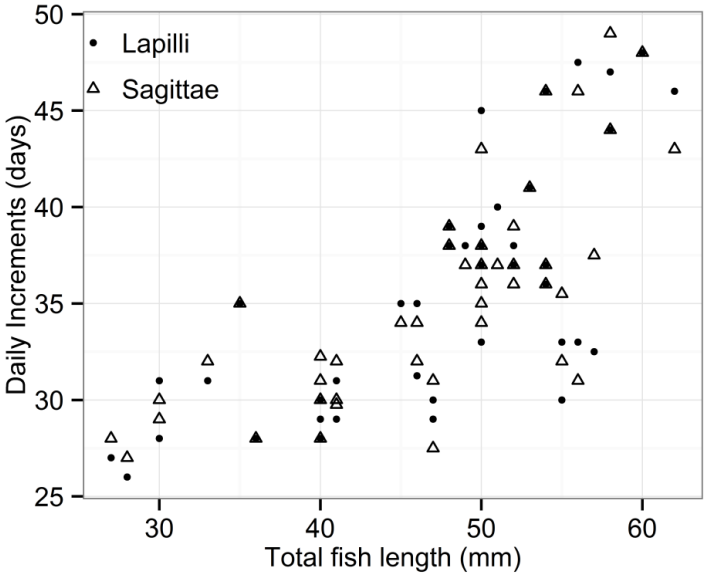

Figure 3. Plot of the number of the increment rings in the otoliths against total length (mm) of the fish.

The 67.3% of the samples showed no differences between the age estimations obtained from different otoliths (Figure 4). Increment counts from sagittae and lapilli showed high degree of correlation ($r = 0.964$, *p-value* < 0.001) (Figure 5).

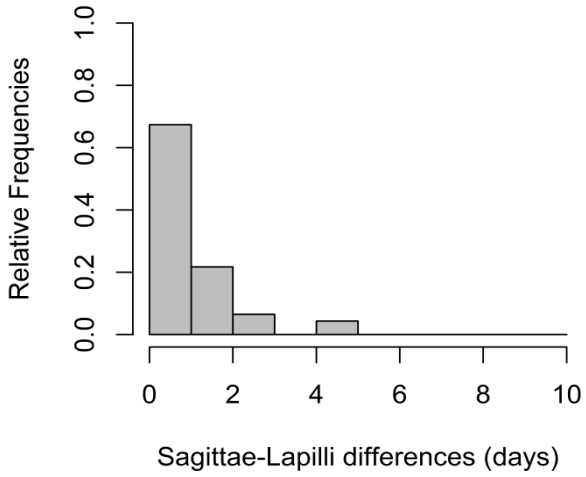

Figure 4. Frequencies of the differences in the age estimated from sagittae and lapilli (days)

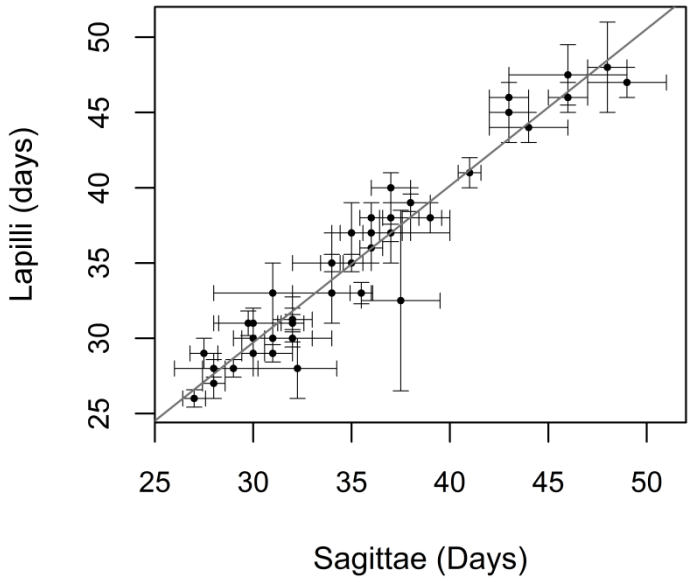

Figure 5. Agreement plot. Estimation of the mean age and standard deviation of *M. barbatus* settlers (in days) evaluated by comparing increment counts in sagittae and lapilli.

The CV calculated for the readings conducted on individuals larger than 50 mm presented higher values, while a better reading precision was found for smaller individuals. With the exception of few difficult readings, the precision of estimates was high and the observed CV was 3.55% for lapilli and 3.43% for sagittae (Figure 6). The 89.13% of the lapilli showed a CV lower than 5%, while sagittae revealed a lower value (78.26%).

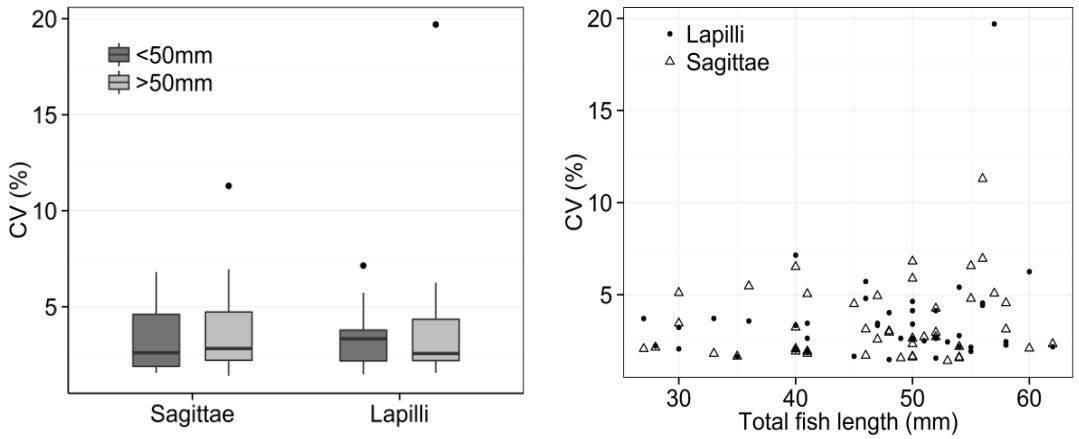

Figure 6. Boxplot of the coefficient of variation (CV) for otolith type (left side). The different box-colours represent the different size categories. It is also reported the distribution of the total length (TL) of fishes (right side).

The Student test performed on the mean CV values of both sagittae and lapilli did not show any statistical significant differences (t = -0.3095, df = 108.854, *p-value* = 0.7576). The ANOVA did not detect any significant differences of the CV for the factors otolith type and total length of the fish (Tab. 2). However, the CV calculated on the readings of individuals ≥50 mm showed, generally, higher values (CV = 3.91) than those of the individuals ≤50 mm (CV = 3.22).

The mean preparation time was 36.30 min (SD = 14.27) and 27.35 min (SD = 10.51) for sagittae and lapilli, respectively. The sagittae-preparation time ranged from 14 to 72 minutes and from 11 to 48 min for the lapilli (Figure 7).

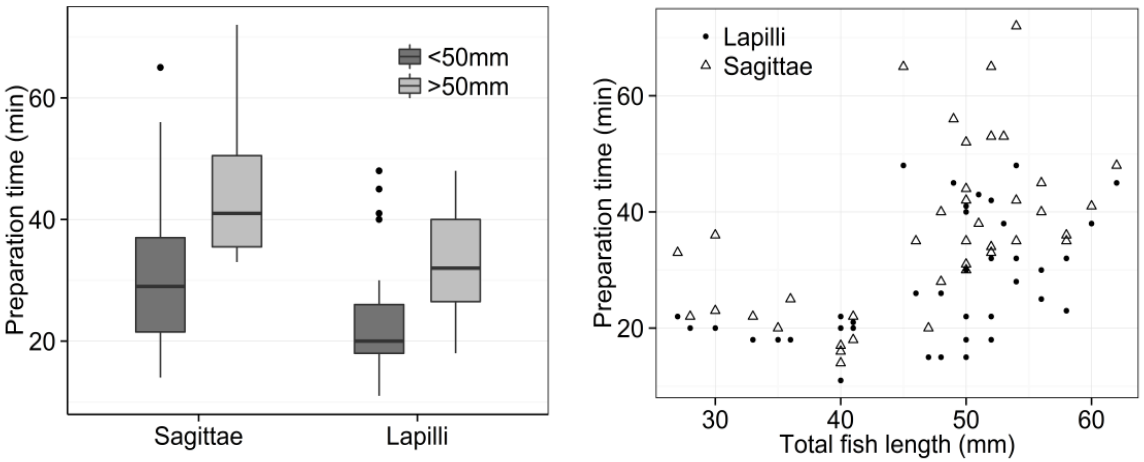

Figure 7. Boxplot of the preparation time for otolith type (left side). The different box-colours represent the different size categories. It is also reported the distribution of the total length (TL) of fishes (right side).

The ANOVA detected significant differences in preparation-time for both otolith type and the fish size (Tab 2).

Table 2. Results of ANOVA performed on the values of CV and preparation time.

Variation Coefficient (CV)	**Degrees of freedom**	**F *Value***	***p-value***
Otolith Type	1	0.049	0.826
Size Category	1	1.783	0.185
Otolith Type ⊚ Size Category	1	0.014	0.907
Residuals	88		

Preparation Time	**Degrees of freedom**	**F *Value***	***p-value***
Otolith Type	1	12.233	0.00079
Size Category	1	18.892	4.34E-05
Otolith Type ⊚ Size Category	1	0.6071	0.41528
Residuals	74		

No significant values emerged from the Cochran C test applied to test the homogeneity of the variances. The result of the SNK pairwise comparison between the levels of each factor, highlighted values of preparation time significantly longer for sagittae and for larger individuals (*p-value* < 0.001). No correlation was found between the preparation time of the otoliths and their CV.

DISCUSSION

Sagittae and lapilli length showed significant correlation with the fish size and, as reported for other species (Morales-Nin and Moranta, 2004, Narimatsu *et al.*, 2007, Gallardo-Cabello *et al.*, 2014), otolith size can be used as estimator of the growth rates during the settlement phase. Contrarily, the moderate relationship of the age and the body size, showed by both otoliths, highlighted the variability in the somatic growth rate at the settlement stage of this species.

Compared with the age, the OL was more reliable in the estimation of the TL of the fish. These findings are related with the allometric growth characterizing early fish stages that, due to endogenous or environmental determinants (American Fisheries Society, 2004), can act differentially on somatic and otolith growth (Hare and Cowen, 1995).

Our study showed the consistency of estimates from both sagittae and lapilli and the absence of differences in the precision of ageing between both structures. The observed correlation between sagittae and lapilli readings can be considered as a direct mutual verification of the pattern in the deposition of the rings between the two otoliths considered. Allochronic deposition patterns have been reported for several species and may cause over- or under-estimations in the ageing process (Campana and Neilson, 1985; Rae *et al.*, 1999; Morales-Nin *et al.*, 1999). The agreement in the readings excluded differences due to asynchronies in the deposition of the rings between the two otoliths during the ontogenetic development (e.g. pre- or post-hatching or first feeding increment formation). Nevertheless, to confirm the daily deposition rate assumed in this study, the increment formation periodicity still needs to be corroborated by validation. In spite of the daily nature of the otolith deposition increments (Campana, 2001), a correction factor may be required in order to reduce the uncertainty and to increase the accuracy of the age estimations.

Some sagittae and lapilli showed high differences in the independent repeated readings (CV>5%), evidencing some difficulties in the reading process. In fact, these otoliths were characterized by areas with no clearly visible increment rings, resulting in a lower level of precision of the readings. More particularly, the part of the sagittae located around the core presented different degrees of densities of the organic matrix. Such misleading structures may reflect biological and/or ecological processes, such as habitat shifts, environmental or physiological

changes occurring during the first stages of the fish life history (Geffen, 1992, Morales-Nin, 2000, Green *et al.*, 2009). These events, occurring during the ontogenetic development, induce some variations in the otolith matrix composition and in the daily increments deposition rate (Morales-Nin, 2000) that may increase uncertainty in reading processes.

Processing otoliths and counting their daily increments require specific skills and can be extremely difficult and time consuming (Cadrin *et al.*, 2013). The preparation time for the *Mullus barbatus* otoliths varied depending on the otolith type and on the fish size. The more complex shape and microstructure characterizing the sagittae resulted in a longer preparation time, while the section of the lapilli was generally easier and faster to prepare. Preparation time for larger fish was found to be longer than for smaller ones. Therefore, the lapilli of smaller individuals (< 50 mm) required less preparation time, and were the otolith usually easier to read.

We concluded that sagittae and lapilli provided the same precision in the estimation of the age, indicating that the analyses of their microstructures can furnish meaningful estimates. However, as reported for other species (Ichimaru and Katsunori, 1995; Morioka and Machinandiarena, 2001; Fey *et al.*, 2005; Narimatsu *et al.*, 2007), lapilli provide an easier and faster processing for ageing *Mullus barbatus* settlers. Nonetheless, depending on the objectives of the study, the analysis of both otoliths can improve the reliability of the information, so that the mutual verification of different methods is recommendable in the otolith ageing of ELH stages. This comparative approach would result in a finest interpretation of daily increment, being helpful in the case of unclear-reading situations. This could be the case for the larger settlers, for which the daily increments in the peripheral region of the sagittae are not clear and could be disentangled by using the lapilli readings. Future studies will benefit from using the lapilli for ecological studies of

the early life history of red mullet, but we recommend combining it to the sagittae reading as a verification method for the larger individuals.

ACKNOWLEDGMENTS

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In the previous chapter, I compared the reliability, precision, the time investment of different otoliths' microstructures (i.e., sagittae and lapilli), for describing the growth and estimating the age of the red mullet early life stages.

Lapilli as the best solution for aging *M. barbatus* settlers

Sagittae and lapilli showed similar estimations of the age and the growth but overall, lapilli offer an easier and faster processing for ageing *Mullus barbatus* settlers. Thus, considering overall the results, lapilli were elected as the best solution for ageing red mullet settlers.

The next step

By these findings, these chapter represented the first step of the study, providing the methodological support for the next one, where a more specific evaluation of the settlement patterns across the western Mediterranean Sea will be conducted.

CHAPTER II

SETTLEMENT DYNAMICS OF *MULLUS BARBATUS*:

EVIDENCES FROM OTOLITH SCLEROCHRONOLOGY

EARLY LIFE HISTORY TRAITS OF *MULLUS BARBATUS*: EVIDENCES FROM OTOLITH SCLEROCHRONOLOGY

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ABSTRACT

Settlement is a critical phase in the life history of benthic fishes. Therefore, the settlement process is likely to control the dynamics of recruitment-limited populations to a large extent. This is specially the case in heavily exploited demersal populations of marine fishes. Despite the central importance of recruitment in population dynamics and fisheries science, the available information about settlement of commercially exploited species is scanty. Here, early life history traits of the red mullet (*Mullus barbatus*), one of the most heavily fished species in the Mediterranean Sea, was characterized through sclerochronology of otolith from post-larval specimens. Spawning date and pelagic larval duration (PLD) were inferred from otolith readings. Spatial variability among 3 studied areas in the Western Mediterranean was then determined. The present findings showed that the Mediterranean population of Mullus barbatus displays wide variability in the duration of the pelagic larval stage. Estimations of early life history traits confirmed such variability, supporting the hypothesis that red mullets start their larval phase in different pulses within a wide temporal window, which results larger than previously though.

KEYWORDS

Early life history traits, Pelagic Larval Duration, spawning date, settlement date, *Mullus barbatus,* Mediterranean Sea

INTRODUCTION

A complex life cycle with multiple ontogenetic shifts is a common characteristic among marine organisms (Sale, 2004). Generally life cycle of fish includes, a pelagic larvae stage which lasts until they settle onto suitable habitats where to spend the juvenile phase (Leis, 2006). In the case of demersal fishes, the settlement phase represent the transition phase from the plankton to the benthos domain (Leis and McCormick, 2002). During this transition, post-larvae (i.e pre-settlers) are characterized by rapid and deep morphological, ethological and physiological changes (Hunter, 1981; Sale, 1993) and they exert a critical influence on several ecological processes. For example, the settlement cause an ontogenetic niche shift that alter inter- and intra-specific trophic relationships (Nakazawa *et al.*, 2010).

The morphological changes related to ontogenetic process of these stages are reflected in the otoliths, where a rapid change in chemical composition and width of daily increments has been stated as a clear biological mark for settlement (Wilson and McCormick, 1997). Moreover, starting from the embryonic development, otoliths are reliable biological fingerprints of the processes occurring during early life stages (Wilson and McCormick, 1997; Morales-Nin, 2000).

Otoliths "keep memory" of the larval history, giving reliable information on main early life history traits (spawning date, pelagic larval duration, etc.).

Since settlement can occur in episodic pulses (reviewed in Doherty and Williams, 1988) or events synchronized with lunar or tidal cycles (e.g. McFarland *et al.*, 1985; Robertson *et al.*, 1988, 1999; Sponaugle and Pinkard, 2004) it may be characterized by high variable patterns. By the way, changes in settlement rates and spatiotemporal extent are key contributor factors to the variability of recruitment (Sale, 2004), the process by which juveniles are incorporated into adult cohorts (Limburg, 2009).

There are several evidences suggesting that a better understanding of how pre- and post-settlement processes affect populations (Roughgarden *et al.*, 1988; Menge, 2000) can advantage modelling of fish population dynamics itself (Leis *et al.*, 2011). In particular reliable information on early life traits such us the planktonic larval duration (PLD), the lengthiness of the competence period (CP), the size at settlement (SAS) and the spawning date are crucial for improve modelling of dispersal and connectivity among populations. Significant improvement to population

modelling, comes from the implementation of these information in individually-based models (IBM) (Letcher *et al.*, 1996; Chambers and Trippel, 1997; Letcher *et al.*, 1998; Rose *et al.*, 1999) and stage-based models (Butler and Nishimoto, 1997; Morris et al., 2010).

Despite numerous approaches have been developed and applied to obtain reliable information on these processes (see reviews Sale, 2004; Leis *et al.*, 2011) a lack of knowledge of basic information and a relatively poor understanding remains in many regions and many fish species (Sale *et al.*, 2005).

Whereas most of the studies on growth have been carried out on juveniles and adults (Aguirre and Lombarte, 1999; Polat *et al.*, 2005; Mahé *et al.*, 2012), studies on early stages ageing remains lacking for that species.

This part of the thesis aimed to fill the existing gaps on the bio-ecological basic knowledge of *Mullus barbatus* early life-history traits. We implemented a detailed sampling design in Sardinia, considering 3 sites, in order to obtain specific information about the local pelagic larval durations, settlement and spawning dates of the early life history stages of *M. barbatus*. These empirical information will be implemented in Chapter III, aiming to model larval dispersal at sub-regional scale. In particular, we i) preliminary validated the daily deposition of the otolith increments, ii) estimated the PLD and the competence extension of the species, iii) determined the spawning and settlement dates for each considered Sardinian site, and iv) checked for differences in PLD among the sampled sites. Moreover, we compared Sardinian samples with specimens collected in 2 other areas of the Western Mediterranean Sea for v) highlighting large scale differences in the considered early life history stages traits.

MATERIALS AND METHODS

Sample collection

The large sampling scheme includes 3 sampling areas in the Western Mediterranean (Figure 1), namely Murcia (MUR, Spain), Sicily (SIC, Italy) and Sardinia (SAR, Italy). In each area, a random site was chosen to sample fish individuals over the settlement phase (i.e. pre and post-settlers). In Sardinia, considering the objectives of the study, 3 sites were selected: Porto Pino (PP), Poetto (PO) and Capo Boi (CB).

Using the information on the arrival period of *M. barbatus* post-larvae in southern Mediterranean obtained by Murenu and Muntoni (2011), light traps (CARE^{RM}, Ecocean, Montpellier, France) and hand nets were deployed, during the summer months (from June to September 2013) and until December 2013, respectively, to collect both post-larval stages (hereafter pre-settlers, PRE) and post-settlers (hereafter SET). Catalan *et al.* (2014) stated that, to collect pre- and post-settlement stages of fish, a proper technique is to combine light traps and epi-benthic trawling (here equivalent to hand net collection). Because of the principles regulating the functioning of the CARE light traps (i.e. phototropism and tigmotropism), we assumed that, at the time of sampling, the individuals caught by this tool, were almost ready and/or competent to settle.

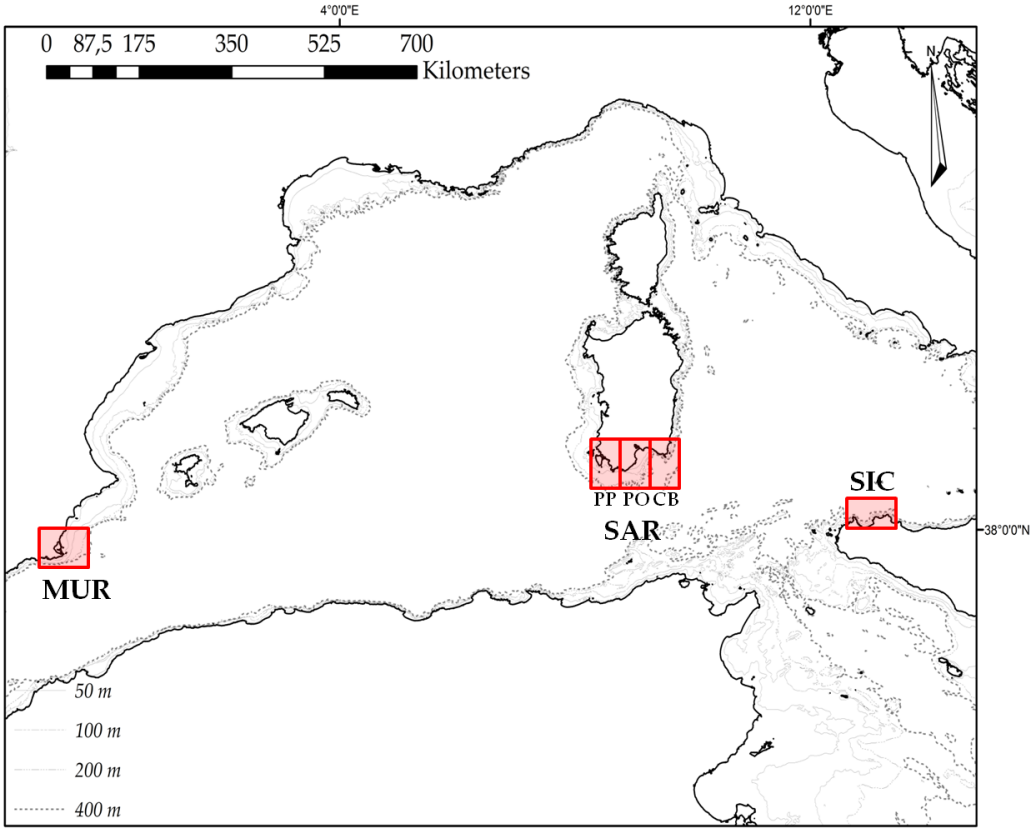

Figure 1. Sampling areas: Murcia (MUR), Sardinia (SAR), Sicily (SIC). Sardinian sites: Porto Pino (PP), Poetto (PO) and Capo Boi (CB)

Light traps were deployed at night during the new moon period. Floating at the sea surface, light traps were moored on the bottom at depths greater than 25 m to avoid confounding

effects of any vertical migration of fish that have already settle.

Hand nets were managed by scuba divers at night, before the sunrise, to easily catch *Mullus barbatus* settlers on shallower coastal bottoms (depth < 5m). All the specimens were collected and preserved in 95% ethanol. Then, taking advantage of the information acquired in the first part of the PhD project, lapilli were prepared as reported in the Chapter I.

Validation of the daily increments

After the capture, in order to preliminary validate the daily deposition of the otolith's increments (specifically lapilli), 2 fish were placed to an aquarium with pure seawater. Then, after the acclimation period, fish were transferred to an aquarium containing dissolved Alizarin complexone (ALC) (ICN Biomedicals, Inc., Costa Mesa, California) at a concentration of 100 mg/L for 24 h (Tsukamoto 1988; Thomas et al. 1995; Beckman and Schulz 1996; Sugeha et al. 2001, Durham and Wilde, 2008). This aquarium remained in the shade throughout all the experiment period, during which ambient temperatures ranged from 24 to 26°C. Then fish were replaced in the pure seawater and water exchanges were performed every 8 h for the next 24 h because of leaching of residual alizarin from the fish tissue. These operation were performed at 10-d interval and then fish were sacrificed and preserved in a 95% solution of ethanol for the successive readings as reported in Chapter I. The red fluorescent mark resulting from ALC treatment was located with the use of a light microscope equipped with an ultraviolet (UV) light source as described by Tsukamoto (1988). The validation has been carried out by verifying if the number of increments between the two ALC marks corresponded with the 10 day interval between the two ALC expositions.

Statistical analysis

Fish age was estimated as the mean value of the number of daily increments observed by each lapillus done by three independent readings. We considered for the rest of the study only the otoliths for which the coefficient of variation (CV) was less of 5% (see Chapter I). After the otolith reading, all the specimens without settlement mark were coded as pre-settlers (PRE), while

individuals displaying the settlement mark were coded as settlers (SET). For specimens captured before the settlement (i.e. PRE), thus which did not end their pelagic phase, we used the number of Daily increments After Hatching (DAH) as an indication of the time spent in the pelagic environment until being caught. For SET, the PLD coincides with the number of daily increment rings from the primordium until the settlement mark. (for details about otolith microstructure see Panfili *et al.*, 2002).

We calculated the competency period (Sale, 2002), i.e. the period during which the larvae are ready to perform the settlement and to join the benthic environment, as the range of PLD values estimated among the settlers.

The spawning date (SPD) was directly derived from the catch date by subtracting the total number of daily rings counted in the otolith. The settlement date (SED), evaluated when the settlement mark was present, was calculated from the catch date by subtracting the number of daily rings counted after the settlement mark.

One-way analysis of variance (ANOVA) was used to examine the partitioning of the variability in PLD among the 3 sampled areas and within Sardinia sites. A Shapiro-Wilk's test was preliminary conducted to test the normality of the data, while a Bartlett's test were used to test the homoscedasticity before to perform the ANOVA.

All the statistical analyses were performed using the R statistical package (R Development Core Team, 2014).

RESULTS

As the first step, we intended to validate the daily deposition rate of the increments in the otoliths. *M. barbatus* individuals were successfully marked with an ALC solution. At the end of the 10 d experiment, two clear marks were detected in the otolith. Ten increments were counted within the range between the two marks, coinciding with the days-interval between the two exposition to ALC.

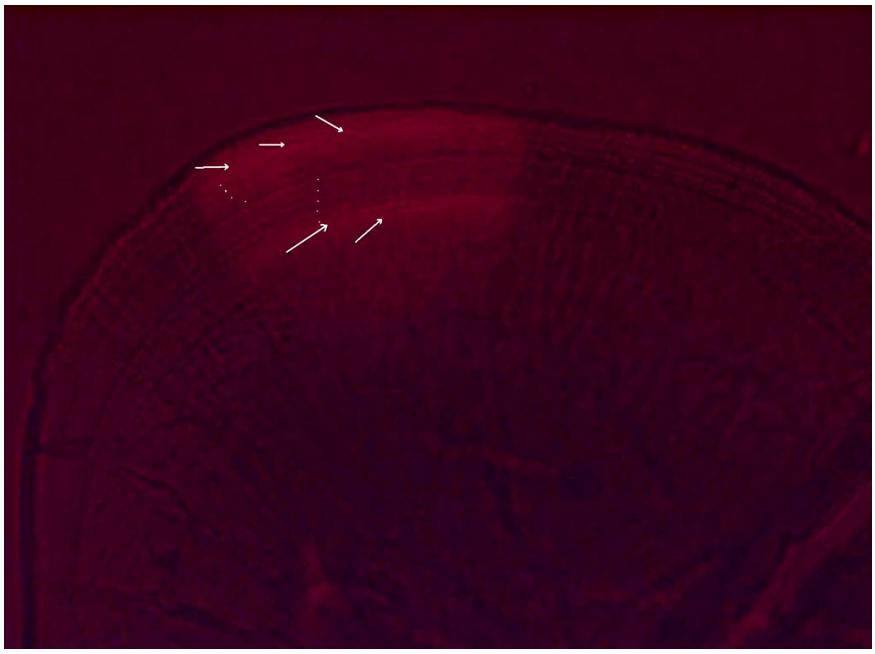

Figure 2. The red fluorescent mark resulting from ALC treatment located with the use of a light microscope equipped with an ultraviolet (UV) light source

A total of 129 specimens were collected in the 3 areas, respectively 16 for Murcia (MUR), 48 for Sardinia (SAR) and 65 for Sicily (SIC). After the readings, 84 individuals were coded as pre-settlers and 45 as settlers. Total lengths (TL) of individuals ranged from 23 to 104 mm, with a mean length of 58.8 ± 18.1 mm (Figure 3). Mean length of settlers and recruits were 48.4 ± 6.3 mm and 72.4 ± 19.4 mm, respectively.

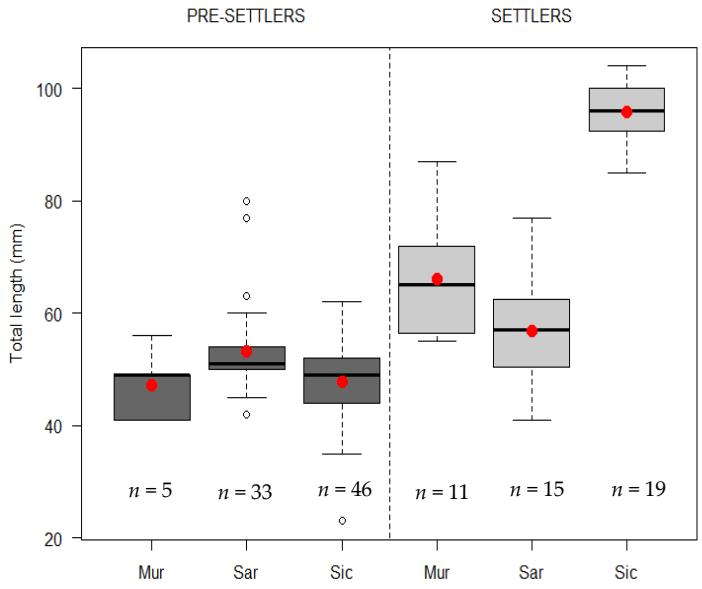

Figure 3. Sampling numbers and TL per area for each considered stage

The PLD estimated for settlers among the Mediterranean areas ranged from 25 to 45 days (mean = 33.98 ± 4.97 days). Overall, the red mullet competency period resulted to be 20 days long. This suggests that larvae are able to settle in a temporal window about 1/3 (± 10 days) around the mean larval duration detected by our study.

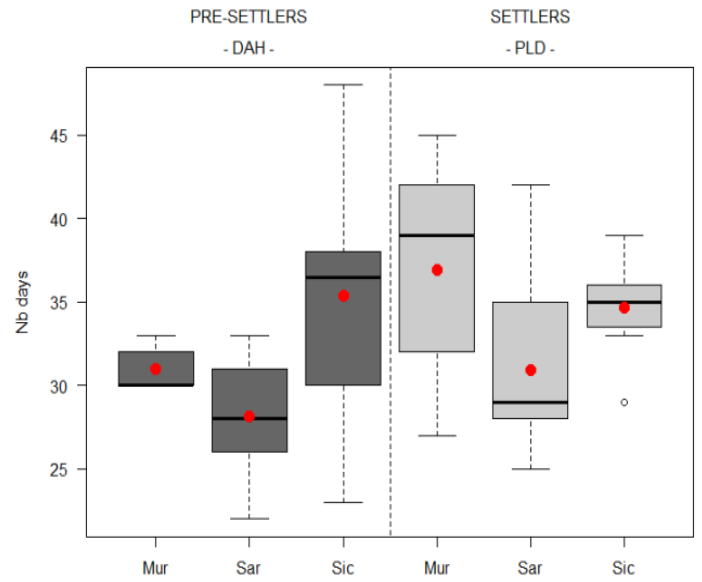

Figure 4. DAH and PLD range estimated per area. Red dots represent the mean value

Settlers from MUR and SAR showed a longer competency window (18 and 17 days, respectively) if compared with SIC specimens, for which the ability to settle was limited to 3 days. Inversely, considering the PLD, SAR show the lowest values (30.93 ± 4.89 days), while SIC value was attested to be around 37 days (Table 1).

Table 1. Competency periods based on SET for each Mediterranean sampled area. Values are expressed in days

Area	**Competency Period**	**PLD (mean ± sd)**
MUR	27 - 45	36.9 ± 6.18
SAR	25 - 42	30.93 ± 4.89
SIC	29 - 32	34.68 ± 2.64

In the figure 5 are reported the number of increments for each stage sampled across Sardinian sites. Also in this case, as previously observable for the others Mediterranean areas, the DAH showed a limited days range, likely due to the capacity of the sampling devices (particularly CARE) to catch individuals which were going to settle. The site CB showed the minimum range in the DAH for the pre-settlers, while conversely, PO showed a wider range (22 – 33 days).

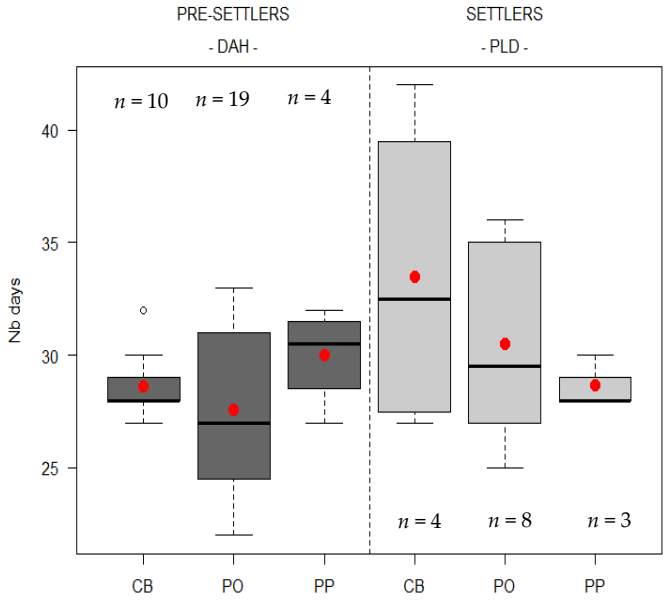

Figure 5. Sampling numbers and PLD range for each Sardinian sampled site. Red dots represent the mean value

When observing the competency period and the PLD for Sardinian settlers (Table 2), the site PP showed a very short pelagic phase (28.67 ± 1.15) and competency period, equal to 2 days. Contrarily, CB revealed an extended competency phase, equal to 15 days, likewise a prolonged PLD (28.67 ± 1.15 days).

Table 2. Competency periods based on SET for each Sardinian sampled site. Values are expressed in days

Area	Competency Period	PLD (mean ± sd)
CB	27 - 42	33.5 ± 7.23
PO	25 - 36	30.5 ± 4.34
PP	28 - 30	28.6 ± 1.15

In the table 3 are reported the PLD revealed for the Sardinian settlers, in order to summarize the information in respect of the settlement date.

Table 3. Settlement dates and PLD of *M. barbatus* estimated by post-settlers lapilli readings

Settlement Date	CB	PO	PP
15/07		36	
19/07		25	
22/07			28
25/07		30	
26/07			28
31/07		25	
04/08	42	26	
06/08		29	
07/08		29	
09/08		34	
10/08	37	28	
11/08			30
16/08	28		
18/08	29		
29/08		30	

By the estimation of the settlers' spawning and settlement dates, it has been possible to follow the source-sink temporal dynamics in the Sardinian area. The highest spawning frequencies were revealed during the end of June and the first half of July, while the settlement peak was observed during the first part of August, with a maximum during the week of the 3rd to 10th (Figure 6).

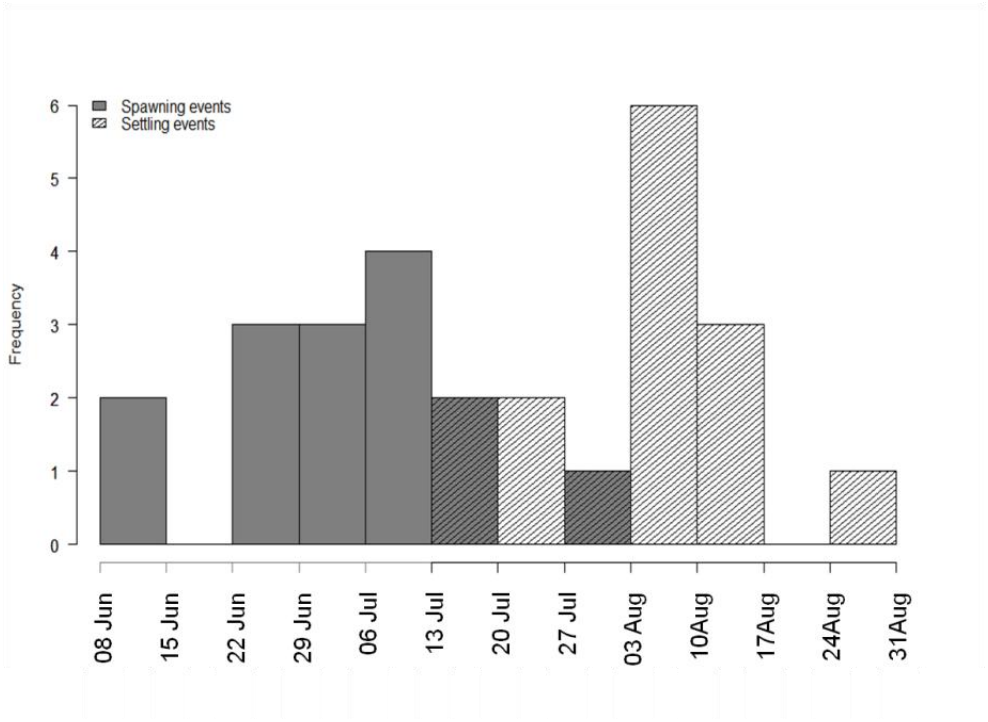

Figure 6. Spawning and settlement dates occurred in Sardinia

Examining in detail the spawning events, we can see that most of the individuals, both pre-settlers and settlers, were born in July 2013, between the 4th and the 13th (Figure 7a). Moreover, it is possible to observe that individuals coming from the same reproductive pulse showed a wide range of PLDs (22 – 37 days).

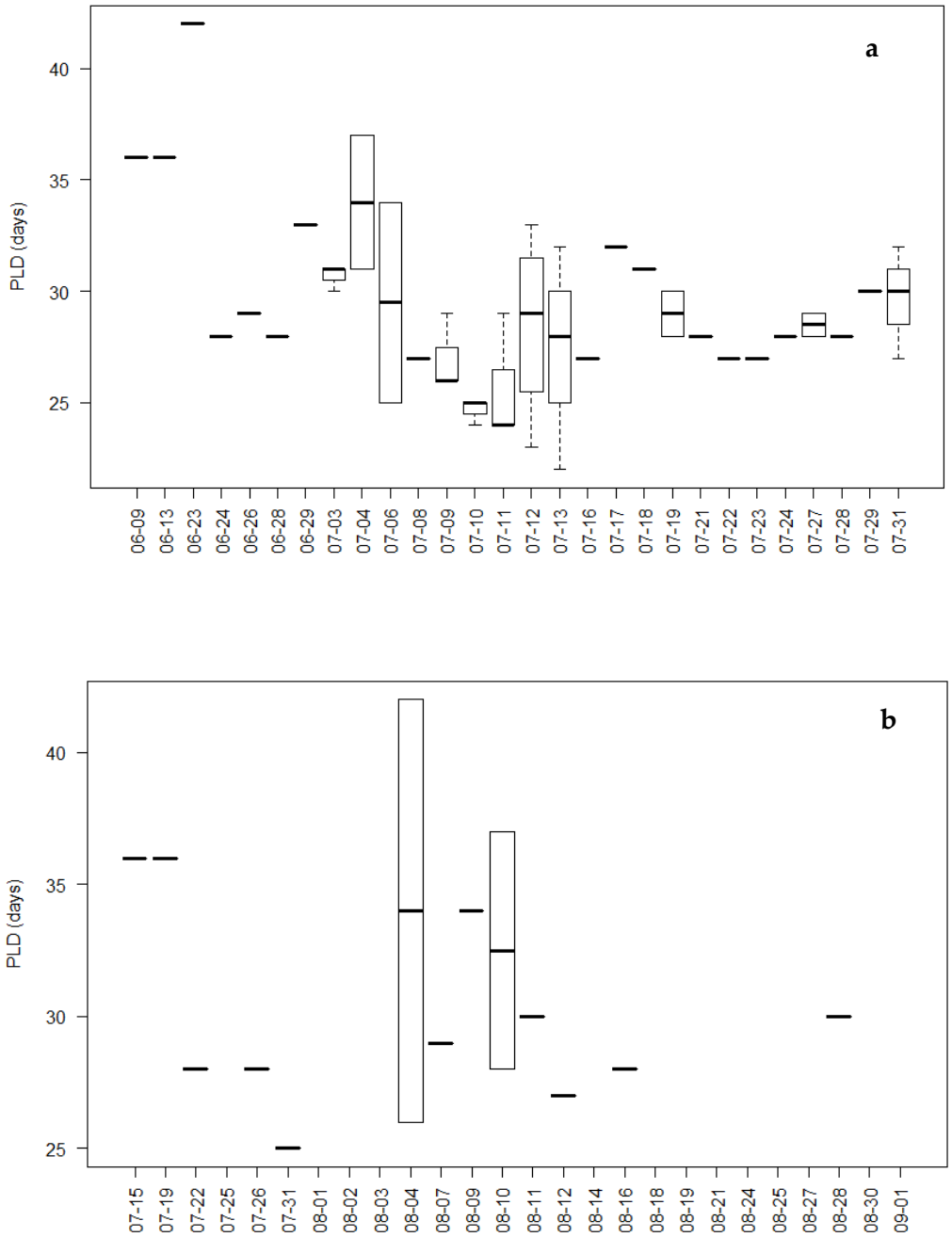

Figure 7. PLD per spawning (a) and settlement event (b) for the Sardinian samples. Dates are reported in the month-day format

When focusing on the settlers, we observed that individuals settling in the same day (4th and 10th of August) can present highly different PLD values. In the first case specimens displayed 26 and 42 days of PLD, while at the second date the PLDs were equal to 28 and 37 days.

The ANOVA performed to check for variation in life traits under the null hypothesis of no differences in the PLDs among the 3 investigated areas.

Table 4. ANOVA results among Mediterranean areas

	Degrees of freedom	Sum of Squares	Mean square	F value	*p-value*
Area	2	243.0	121.52	6.047	0.00492 **
Residuals	42	843.9	20.09		

Table 5. ANOVA results among Sardinian sites.

	Degrees of freedom	Sum of Squares	Mean square	F value	*p-value*
Area	2	43.27	21.63	0.59	0.436
Residuals	12	291.67	24.31		

As reported in Table 4, the ANOVA detected significant differences (*p-value* < 0.001) of the PLDs among the different areas across the Mediterranean Sea; however, no significant differences were found among the PLDs of the considered Sardinian sites (Table 5).

DISCUSSION

Firstly, the periodicity of the deposition of the increment were tested. The results, even if preliminarily and without a statistical robustness because of the few sampled available, suggest that during the early life history stages of *M. barbatus*, otolith's increments are deposited daily. On the other hand these preliminary results put the basis for more detailed and accurate studies on the deposition rate of this species, confirming the usefulness of ALC in validating growth increments for this species, as found in other studies for other species as cyprinids (e.g. Beckman and Schulz, 1996; Durham and Wild, 2008).

The interval between hatching and settlement, namely the larval duration, is one of the

most extensively studied traits of the fish early life history stages. The existing spatial variability in such life trait may have important consequences for recruitment and population dynamics (Caley *et al.*, 1996; McCormick and Hoey, 2004; Shima and Findlay, 2002) and provide significant information about pre- and post-settlement processes (Chambers and Trippel, 1997).

This study evaluated the main early life history traits (deposition rate of otolith increments, PLD, competency period, spawning and settlement dates) of *Mullus barbatus*, for which no information were previously reported. Some information are available for the congeneric species *Mullus surmuletus*, for which PLD was estimated to be around 28 to 35 days (Macpherson and Raventos, 2006) in the northwest part of the Mediterranean Sea, but without taking into account a possible spatial variability of this trait. Overall, *M. barbatus* has an extended competency period (20 days), that let larvae the possibility to delay the settlement settle in a temporal window ranging from 25 to 45 days. Cowen (1991) suggested, that long competent period may be related to the need to find a suitable settlement site within the appropriate stage of their development and that aptitude to delay the pelagic-benthonic transition may ensuring the correct fulfilment of settlement phase, and it's strongly linked with the pre-competent phase.

By comparing the 3 studied areas, has been possible to identify a clear spatial variability of larval duration of *M. barbatus* in the Western Mediterranean Sea that was not observed, at a smaller spatial scale, among Sardinian sites. In 2013, the widest competency period was displayed by the samples from Murcia, also presenting the highest mean PLD, of nearly 37 days. In Sardinia, the mean PLD was found to be the lowest, of about 31 days, while presenting also a wide range of values and Sicily was characterized by a very narrow competency period. Such spatial variability has been yet observed for other species (Bay *et al.*, 2006; Di Franco and Guidetti, 2011), and may be induced by environmental conditions affecting the early life stages (Öhman *et al.*, 1998; Robertson *et al.*, 1999; Bergenius *et al.*, 2005; Raventos and Macpherson, 2005; Rankin and Sponaugle, 2014). Environmental factors as temperature food availability etc., may also affect growth rate, but their relationship with developmental rate has not been explicitly examined here.

Moreover, the information obtained on the settlement events, where larvae from different reproductive pulses settle in the same period, suggested the presence of patches of individuals of individuals with different ages. The formation and the maintenance of these groups may be due to behaviour-mediated aggregation or passive accumulation (Paris and Cowen, 2004; Di Franco *et al.*,

2013) or to environmental stimuli.

The results of the study are in agreement of what reported for red mullet on the reproductive period in the Mediterranean Sea (see Introduction) and provide reliable and precise empirical information on early life history stages of this species. Such information will be used in the next chapter (III) as input biological parameters for modelling red mullet larval dispersal.

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The initial point

The previous chapter describes the settlement phase by the use of post-larval otolith sclerochronology. This part of the dissertation aimed to fill the existing gaps on the bio-ecological basic knowledge of *Mullus barbatus* early life-history traits (timing of settlement, competency period, pelagic larval dispersal and spawning date) at sub-regional (Sardinia) and regional scale (Western Mediterranean Sea).

Early life history traits of *Mullus barbatus*

Red mullet showed an extended competency period (20 days), with a PLD range of 25-45 days, that furnish to the pre-settlers individuals the capability to delay metamorphosis until to find a suitable habitat to settle. The settlement phase seems to be, thus, mediated by environmental stimuli, that directly affect the behavior of competent larvae, that can actively decide when perform the transition.

Moreover, this part of the work highlighted that the red mullet early life history traits (as PLD) may significantly vary at regional scale (Western Mediterranean basin), but not at the local one.

The next step

The empirical information obtained in this section will be now implemented in Chapter III, aiming to model larval dispersal at sub-regional scale.

CHAPTER III

DESCRIBING FISH CONNECTIVITY PATTERNS: INTEGRATION OF OTOLITH SCLEROCHRONOLOGY INFORMATION IN LARVAL DISPERSAL MODELS

DESCRIBING FISH CONNECTIVITY PATTERNS: INTEGRATION OF OTOLITH SCLEROCHRONOLOGY INFORMATION IN LARVAL DISPERSAL MODELS

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ABSTRACT

Understanding connectivity patterns among coastal fish populations is a crucial step in the management of marine resources. However, quantifying and measuring the exchange among fish populations is a hard task because of the difficulties in tracking the trajectory and fate of the larval phase. Moreover, there is a lack of basic knowledge about the bio-ecological traits of many marine coastal exploited species, usually related to their bi-partite life cycles. To fill these gaps, a variety of approaches have been developed to assess the fish larval dispersal and to describe the patterns of fish population connectivity. The use of models as a tool to identify and measure these patterns in the marine environment is continuously increasing. However, the accuracy of the models is strongly dependent from the precision of the input parameters, that often are not available at local scale. One of the most important parameter for evaluating larval dispersal is the pelagic larval duration (PLD). Here, we used the post-larval otolith sclerochronology of the species *Mullus barbatus* to evaluate the spawning date and the PLD in our area of interest, for which these information were not available. Then, we calibrated the dispersal model using the information previously obtained for improving the reliability of the model's prediction. The combination of these approaches is useful to reconstruct the history of the larvae and to describe the patterns of connectivity.

KEYWORDS

Connectivity, dispersal modelling, settlers, spawning areas, *Mullus barbatus*, western Mediterranean Sea.

INTRODUCTION

Evaluating the connectivity scale among fish populations is known as one of the most challenging topic at this time in marine ecology (Sale, 2004). The connectivity processes determine the scales and structures at which populations of marine fishes operate (Leis *et al.*, 2011). The acquisition of reliable information on population connectivity is a mandatory step for understanding the community and population dynamics, genetic and demographic structures, and for evaluating the resilience of natural populations to environmental or anthropogenic cues (Cowen *et al.*, 2007). Because of the central role of these dynamics, one of the main challenge for scientist is to couple oceanographic data with the life-history and behavioural characteristics of the species (Cowen *et al.*, 2007; Fogarty and Botsford, 2007), for providing a more realistic representation of dispersal and connectivity of fish populations. The implementation of information about these processes into marine ecosystem management became crucial in the definition and the optimization of proper and sustainable politics, considering that biogeographical distributions rarely coincide with political boundaries (Kough *et al.*, 2013) and that furthermore, population boundaries are difficult to identify (Cowen and Sponaugle, 2009)

In many demersal teleost fishes displaying benthic or relatively sedentary adult phases, the connectivity processes are mainly mediated by dispersal of larval stages that take place in the pelagic domain (Leis and McCormick, 2002). The connectivity paths can be affected by fluctuations of the larval dispersal success, which can, consequently, have a strong repercussion on the dynamics of the fish population, which induce great implications in the decision-making processes of the fisheries management (Werner *et al.*, 2007). For populations maintained by a widespread dispersal, a high exchange rate can mask the presence of distinct subpopulations (Kritzer and Sale, 2004) that should be managed at a broader scale, while populations with an accentuated self-recruitment could be managed locally (Botsford *et al.*, 2003). The variability of the dispersal processes and settlement success is linked to the environmental conditions (White *et al.*, 2010; Schunter *et al.*, 2011), the larval biology, the behaviour and the interactions among all these factors. Larval responses to the variations of environmental cues may imply variations in some of the main larval history traits such as the Pelagic Larval Duration (PLD) (Bergenius *et al.*, 2005) or in the definition of dispersal trajectories (Irisson, 2008). Physical processes such as winds and currents

may favour the transport of early-life stages - eggs and larvae - away from their spawning location (Leis, 2006; Underwood *et al.*, 2007) and induce adaptations on reproductive strategies of the species in order to optimize the reproductive success (Catalán *et al.*, 2008).

In the last decades, the number of tools designed to study connectivity and the influence of dispersal patterns on such dynamics is rapidly grown (Lett *et al.*, 2010; Leis *et al.*, 2011; Calò *et al.*, 2013). Biophysical models are vastly used in demographic and genetic connectivity studies to explore, understand, forecast and simulate scenarios of the dispersal dynamics of eggs and larvae (Lett *et al.*, 2010; Leis *et al.*, 2011). Models, thus, help in evaluating and defining influences of biological and physical factors on larval dispersal and settlement (Lett *et al.*, 2009).

The possibility to couple biological, ecological and behavioural parameters with hydrographical physical models in simulating dispersal, has improved the quality of spatial and temporal scales' estimations of relevant ecological processes (such as connectivity) (Botsford *et al.*, 2009). On the other hand, the accuracy and precision of these estimations are strongly affected by the quality of the base-knowledge-information, both bio-ecological and environmental, used as input for the modelling processes (Werner *et al.*, 2007). Biological information mainly implied in modelling connectivity regards life history traits such as the PLD, growth, survival per stage, larvae swimming capabilities and behaviour (North *et al.*, 2009). Ecological information commonly refers to the spatio-temporal distribution of the spawning events, settlement and nursery areas and reproductive traits, as strategies, intensity and periodicity.

Despite the improvements of the techniques for studying connectivity (genetics, modelling, micro-structural and micro-chemical otoliths information, tagging, etc) (Leis *et al.*, 2011; Calò *et al.*, 2013), detailed information about larval history traits are still limited and at the present, there is a lack either for bio-ecological features (number of eggs and larvae, mortality, larval behaviour, exchange rate, etc) and for oceanographic high resolution data (Sale and Kritzer, 2003).

Most of the limitations in acquiring information about larval stages are due to the difficulty in obtaining direct *in situ* measurements of the larvae exchanged (Mora and Sale, 2002; Sale and Kritzer, 2003) by tracking their movements (Levin, 2006) and in defining the survival rates at this stages (Jones *et al.*, 2009; Harrison *et al.*, 2012). Moreover, estimating the distances covered by the pelagic larvae and the number of larvae dispersed away from their spawning areas is complicated

by the complexity of interrelated behavioural, biological, ecological and physical factors (Reisser *et al.*, 2014) that can imply substantial variations in the PLD (Macpherson and Raventos, 2005). Because of the constraints in terms of time, resources and specific competences (e.g. analyses on otolith microstructure) required to obtain reliable information (Di Franco *et al.*, 2012), most of the model applications use PLD values and spawning dates acquired over distinct spatio-temporal scales. This lack of fine information about the bio-ecology of the early life history traits represents a strong limitation for the use and application of these tools (Werner *et al.*, 2007) and few studies were conducted until now in the Mediterranean Sea (Calò *et al.*, 2013). For some mediterranean species, larval and post-settlers' otoliths sclerochronology has been used in order to estimate spawning, hatching and settlement dates by back-calculations made on PLDs (Chapter II; Raventos and Macpherson, 2001). PLD has been considered as a good estimator of the potential dispersal range and the distances covered by the larvae (Shanks, 2009). However, even if connectivity is related to PLD, the distribution area of the species is not only dependent on it (Macpherson and Raventos, 2006). For long distance dispersers, furthermore displaying an extended competency period, variation in the PLD can determine substantial changes in the dispersal range of the larvae (Corell *et al.*, 2012), with obvious implications on the recruitment dynamics. Since PLD may significantly vary in time and space, the models parameterization using local information is preferred to reach more realistic predictions (Fiksen *et al.*, 2007; Werner *et al.*, 2007).

Focusing on the species *Mullus barbatus* in the Sardinia Island area (Mediterranean Sea, Italy) as case study, we investigated larval dispersal gathering a biophysical model with a Lagrangian larval transport model, specifically parameterized using local biological empirical data. This paper aims to integrate empirical information to model the first phases of the red mullet larvae life history and to describe the sub-regional patterns of connectivity. Our specific objectives were: (i) to draw the potential larval dispersal of *M. barbatus* settling in the South of Sardinia, (ii) to define the patterns of larval exchange among areas and local retention for describing the source–sink dynamics (i.e. spawning-settlement dynamics) and (iii) to identify the potential larval sources (i.e. spawning areas).

MATERIALS AND METHODS

Study area

The study area is located in Sardinia, western Mediterranean Sea. This area is characterized by a multifaceted topography and a complex hydrodynamic regime (Malanotte-Rizzoli, 2001). The coastal area is dominated by sandy and rocky bottoms associated with *Posidonia oceanica* beds, which are the preferred settlement grounds of *M. barbatus* (Wheeler, 1969; Lombarte *et al.*, 2000). *M. barbatus* settlers were sampled at three sites located along the southern coast of the island: Porto Pino (PP), Poetto (PO) and Capo Boi (CB) (Figure 1 and Table 1). These sites were distant of at least 30 km between each other.

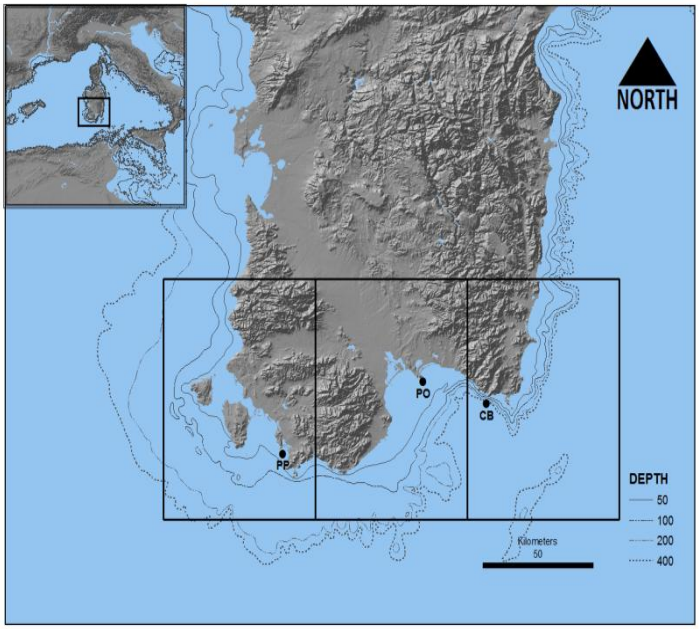

Figure 1. Study area and *M. barbatus* sampled settlement sites. PP: Porto Pino; PO; Poetto; CB: Capo Boi.

Table 1. *M. barbatus* sampled settlement sites localization

Site	Latitude	Longitude
Capo Boi (CB)	39.06	9.26
Porto Pino (PP)	38.55	8.34
Poetto (PO)	39.10	9.26

Biological and ecological characteristics of Mullus barbatus

We used the PLD range of the post-settlers of the species *Mullus barbatus* estimated in the previous study (Chapter II).

Considering the preliminary information obtained in the previous chapter about the daily deposition of the increments, settlement dates were calculated by subtracting the number of daily increments counted after the settlement mark, from the catch date. In a similar way, spawning dates were obtained by subtracting the total number of daily increments (i.e. total age) from the catch date. For each considered site, the PLDs and the settlement dates reported in Table 2 were used as initial parameters of the of the simulations.

Table 2. Settlement dates and PLD of *M. barbatus* estimated by reading the post-settlers otoliths increments

Settlement Date	CB	PO	PP
15/07		36	
19/07		25	
22/07			28
25/07		30	
26/07			28
31/07		25	
04/08	42	26	
06/08		29	
07/08		29	
09/08		34	
10/08	37	28	
11/08			30
16/08	28		
18/08	29		
29/08		30	

Hydrodynamic model

The hydrodynamic model used in the study is the Mediterranean Sea regional configuration (MED12) of the ocean general circulation model NEMO (Nucleus for European Modelling of the Ocean) (Arsouze *et al.*, 2012; Beuvier *et al.*, 2012). NEMO-MED12 (resolution of 1/12 degree) is an extraction from the global Ocean General Circulation Model (OGCM) in the configuration ORCA12 ORCA 1/12 grid. This model resolves the primitive equation under the assumption of spherical-earth approximation, the thin-shell approximation, the turbulent closure hypothesis, the Boussinesq hypothesis, the hydrostatic hypothesis and the incompressibility hypothesis (Madec, 2012). The resolution of 1/12th degree is equivalent to a resolution of 6 – 7.5 km for the regions included between 46N and 30N of latitude. The model is composed of 50 vertical layers, ranging from 1 m in the surface, to 450 m at the bottom. The considered bathymetry is based on the GEBCO-08 database (MEDIMAP bathymetry, http://gebco.net version 20081212). The full description of the model is provided in Madec (2012). The model is composed by a set of bi- and three-dimensional variables as reported in Table 5 and Table 6 while in Table 7 are reported the main parameters of the NEMOMED12 model.

Larval dispersal model

The larval dispersal model was run using the free Java tool Ichthyop 3.2 (Lett *et al.*, 2008), a Lagrangian platform for three-dimensional particle-tracking model, where an Individual Based Model (IBM) is forced by the 3D hydrodynamic model NEMOMED12. A full description of the larval transport model can be found in Lett *et al.* (2008). This tool is particularly useful for the description of the fish early-life dispersal patterns, since it implements some of the crucial processes affecting the dispersal dynamics such as spawning, active behaviour and fish growth, mortality and settlement. It has been developed to study the interactions between physical (e.g., ocean currents, temperature) and biological (e.g., growth, mortality) factors (Lett *et al.*, 2008).

According to the objectives of the study, we used backward simulations, where the three sampled settlement areas were considered as the release areas of the particles in the simulations while the final positions of each propagule after the simulations were used to identify the source areas thus corresponding to the spawning grounds.

We ran simulations from 15/07 to 07/08, corresponding to the observed 17 settlement events, and coinciding with the settlement dates revealed by PLD (Table 2). For each settlement event, 1000 virtual propagules were released from the settlement areas. The number of propagules was chosen in order to allow a representative visualization of potential larval trajectories. The propagules (i.e. virtual settlers) were released on coastal shallow waters of the three settling sites.

Simulations were based on daily information of zonal and meridional velocity, temperature and salinity from the previously described NEMOMED12 model. The bio-ecological parameters used were date of settlement, the estimated PLD and the considered larval competency period of the settlement. During the simulations, different conditions determined the various outputs of the model. Propagules crossing the boundaries of the domain were considered lost while the ones intercepting the interface between sea and land were retained in place by the standstill option.

The propagules were tracked in space and time over all the simulations and the geographic positions of each virtual larva (latitude, longitude) were acquired every 6 hours of simulation until the spawning day. For each simulation, the propagule trajectories characteristics were recorded in a NetCDF file containing the instantaneous information, such as the time of the simulation, the longitude, latitude, and depth of each propagule at each time step, and its arrival zone.

Analyses

The final position and trajectories of each propagule were handled and mapped from the NetCDF file using the R software (R Development Core Team, 2011). The number of propagules exported, imported and retained in each release site (i.e. settlement site) was calculated using the selection by location function in ArcDesktop® 10.2. The travelled distances of each propagule from the spawning site until the settling area were calculated using the Geospatial Modelling Environment tool (GME, www.spatial.ecology.com). To describe the dispersal patterns of *M. barbatus* larvae, we firstly investigated the relationship between PLD and the travelled distance (expressed as decimal degrees DD). Then the dispersal kernels were calculated for each site as function of the propagule abundance against the final distance from the settlement release point.

To illustrate the spatial dispersal pattern, we computed a connectivity matrix (Figure 4). The probability of local retention (LR), described by the diagonal of the matrix, was calculated as

the proportion of propagules produced at each site that remain in this same site (Burgess *et al.*, 2014). Self-recruitment (SR) was calculated as the proportion of larvae settling at each site compared to the total number of larvae released from this site that settled everywhere (Berumen *et al.*, 2012).

The export probability (EP) was calculated as the proportion of larvae released (i.e. settlers) at each site (lines of the connectivity matrix) that disperse over other sites (columns of the connectivity matrix). Import probability (IP) was defined as the proportion of all larvae released from each site which remain within the site (Munguia-Vega *et al.*, 2014).

In order to investigate the source – sink dynamics, the spatial distribution of the potential spawning areas were mapped by means of a kernel density function (Silverman, 1998) as implemented in the Spatial Analyst toolbox of ArcDesktop® 10.2 suite. Using the final position of each propagule at the end of the simulations, this function calculates the density of point features, fitting a symmetric, smoothly curved surface (kernel surface) around each point. The values of this surface decrease inversely to the distance from the final point position. The final outputs are raster features, where the densities values are overlapped to the raster cell centre, and thus mapped to highlight the potential provenience of the larvae settling at each site.

RESULTS

Evaluating the potential larval dispersal of Mullus barbatus

During the 17 simulations, a total of 17000 propagules were released from the 3 settling areas considered. The larval dispersion was simulated from 15/07/2011, the earliest hatching date identified from otoliths analyses, and the trajectories are reported in Figure 2. The mean distances travelled by the larvae for each settlement site are reported in Table 3. The mean dispersal distance ranged from a maximum of 1.39 ± 0.55 DD for the site CB to a minimum of 0.24 ± 0.18 DD for PO. The highest values of dispersal distances were registered by the propagules released from CB on 10th of August (PLD = 37 days), equal to 1.50 DD (sd = 0.82). More particularly, over the 28 days of simulation for the site CB, larvae travelled daily 0.048 ± 0.03 DD. PP showed lower values, respectively 0.52 ± 0.23 DD and 0.66 ± 0.11 DD, for PLD of 28 and 30. The lowest values were registered for the 26 days simulation ran on 31th of July from PO (0.17 ± 0.02 DD). Considering the daily distance travelled by the propagules, the lowest values were registered also in this case for the site PO, with a daily distance included between 0.006 ± 0.002 DD (PLD = 29) and 0.013 ± 0.003 DD (PLD = 36).

Table 3. Mean distances and standard deviation calculated per each site and each PLD estimations

SITE	PLD	Overall distance (Decimal Degrees)		Daily distance (Decimal Degrees)	
		mean	devst	mean	devst
PO	25	0.231	0.184	0.009	0.007
	26	0.174	0.017	0.007	0.001
	28	0.235	0.188	0.008	0.007
	29	0.187	0.063	0.006	0.002
	30	0.249	0.245	0.008	0.008
	34	0.196	0.085	0.006	0.003
	36	0.474	0.115	0.013	0.003
	tot	0.241	0.178		

SITE	PLD	Overall distance (Decimal Degrees)		Daily distance (Decimal Degrees)	
		mean	**devst**	**mean**	**devst**
PP	28	0.518	0.226	0.018	0.008
	30	0.663	0.107	0.022	0.004
	tot	0.566	0.207		
CB	28	1.353	0.077	0.048	0.003
	29	1.289	0.133	0.044	0.005
	37	1.502	0.823	0.041	0.022
	42	1.425	0.683	0.034	0.016
	tot	1.392	0.546		

A dispersal kernel was produced for each settlement site (Figure 2), reporting the distance frequencies distribution of larvae dispersed from the spawning areas.

Each site was characterized by a different dispersal kernel shape. At PO, the density of the propagules decreased very fast with the distance from the release area (i.e. the initial point of the simulation), and just the 18.27% of the propagules reached a distance of more than 0.275 DD. Larvae settling in this site showed the maximum frequencies (81.7%) at the distances ranging from 0 to 0.225 DD. CB showed the highest distance frequencies (59,3%) within a range between 1.3 and 1.5 DD. PP show a bimodal distribution with frequencies of 11,5% for distances below the 0.150 DD and a peak for the 0.550-0.650 DD category (55.6%). Overall, for this site, about 90 % of the released propagules dispersed over a range of 0.750 DD.

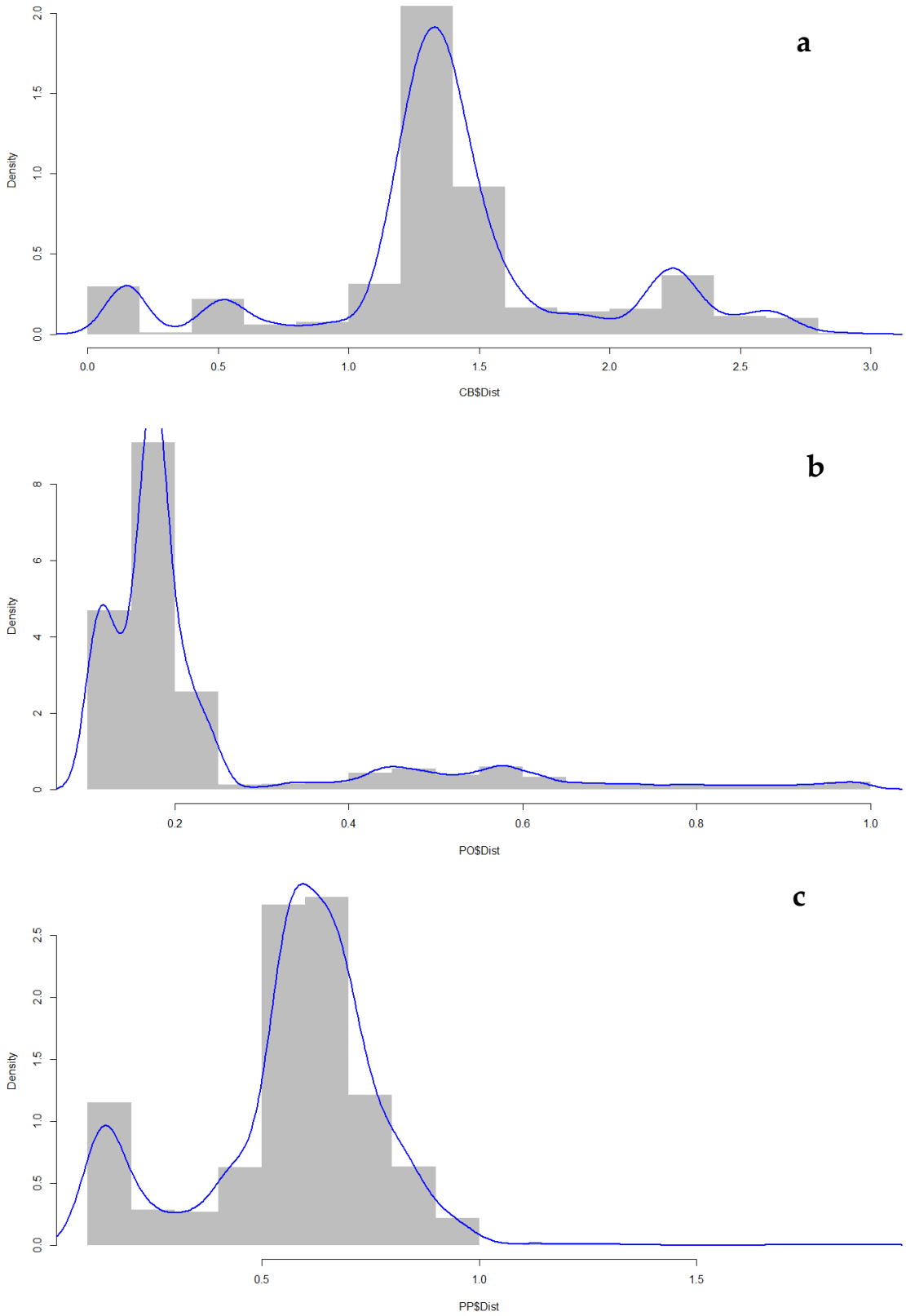

Figure 2. Dispersal Kernels. Dispersal distance frequencies of *M. barbatus* propagules settling in (a) Capo Boi; (b) Poetto; (c) Porto Pino.

To graphically visualize the modelled dispersal processes throughout the study area in relation to the dominant ocean currents, the trajectories of the propagules released from the settlement sites were plotted (Figure 3). Looking at the larval trajectories is observable a wide dispersal range for the propagules released from CB. The propagules released from CB ran alongshore the east coast of Sardinia. Conversely, the propagules from PO tend to stay around the release-settlement site. Propagules released from PP tend to spread eastward (11/08/2014) and westward (26/07/2011), with a dense network of passageways in the Gulf of Cagliari, located within the PO boundaries.

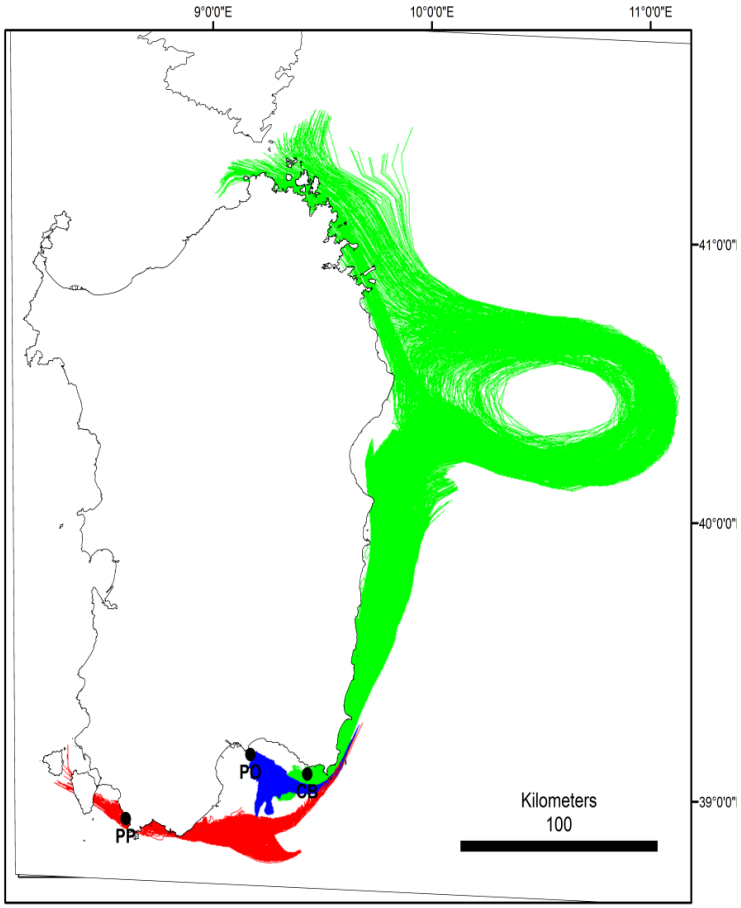

Figure 3. Trajectories of larvae released from the three settlement site obtained by the backward simulation. Red, blue and green represent respectively the trajectories of the larvae released from PP, PO and CB

Describing the patterns of larval exchange: source–sink dynamics

We analysed the distribution of the potential spawning areas over the whole study domain

and looked at the source–sink dynamics through the design of a connectivity matrix presenting the connection probabilities calculated among the spawning and the settlement sites. As observable in Figure 4, the fraction of larvae retained was very high for PO, with a LR value of 0.885 and a very low IP (0.114). The site CB showed the highest IP (0.93), with more than 90% of the larvae coming from other sites. CB dispersed propagules over the three settlement sites, with a frequency of respectively 9% (PP), 11% (PO) and 7% (CB). As well, PP showed low rates of local retention (0.166) and high IP (0.883), being mainly replenished by PO (73.7 %) and CB (about 10%) larvae. Nonetheless, this site presented the highest values of self-recruitment (SR = 1), since it was able to furnish larvae just locally.

Table 4. Connectivity matrix (relative frequencies), local retention (LR) and self-recruitment (SR) values for the considered sites

		Settlement Sites				
		PP	**PO**	**CB**	**LR**	**SR**
Spawning sites	**PP**	0.166	0	0	0.166	1
	PO	0.737	0.885	0	0.885	0.545
	CB	0.096	0.114	0.070	0.070	0.249

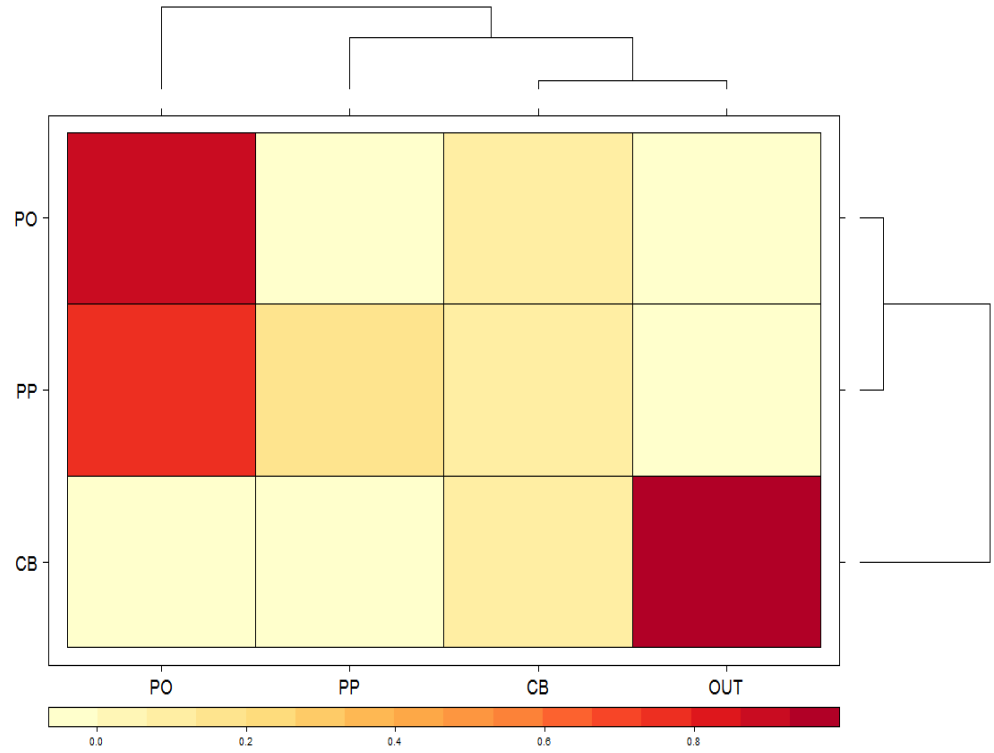

Figure 4. Connectivity matrix presenting the proportion of larvae dispersed from the spawning areas (x axis) and recruited in the settling areas (y axis).

Identifying the potential spawning areas

The maps presented in the following figures (5, 6, 7) report the spatial distribution of the larvae, expressed as kernel densities, based on the final localization of the propagules at the end of the simulations. Differences in the dispersal range are evident for all the three predicted scenarios, varying for wide dispersal, as it's the case of larvae settled in CB, to very short movements carried on by the larvae settled in PO. The larvae settled in CB were found to be spawned on the east coast of the island (Figure 5), where propagules disperse southward until reach the settlement ground located in the south of the island. The model simulations revealed also other possible small spawning areas located in the northern part of Sardinia, precisely in the Strait of Bonifacio, being the farthest estimated spawning area at about 200 km away of the settling ones.

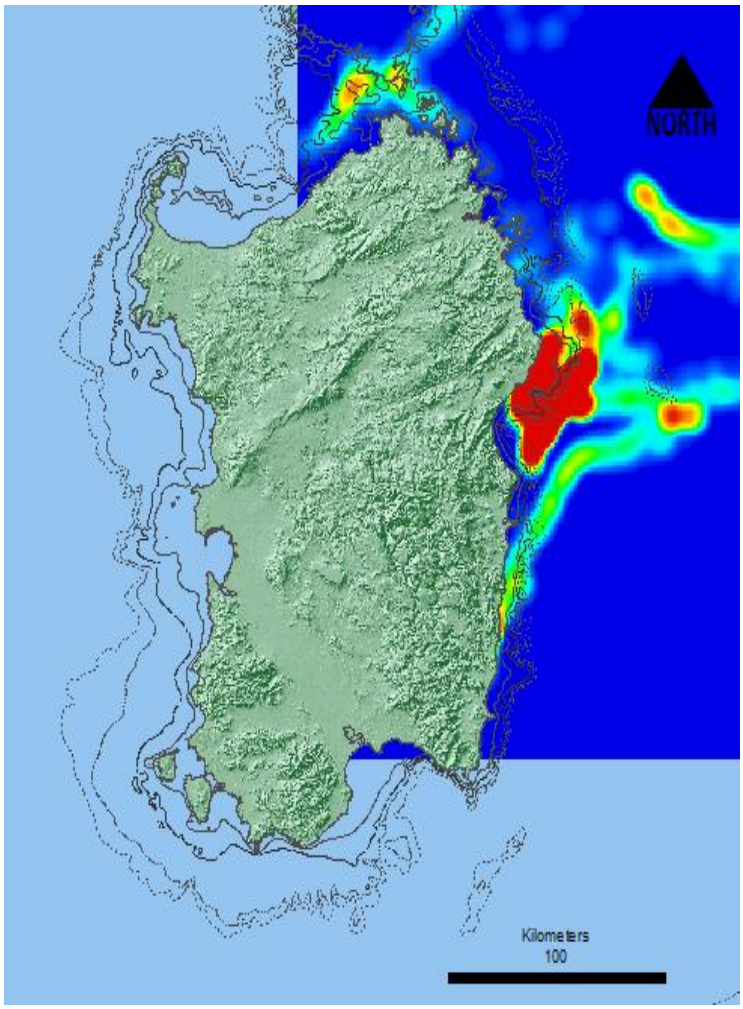

Figure 5. Potential spawning areas identified for larvae settling in CB (kernel density based on back-track larval dispersal simulations)

The site PO (Figure 6) showed a strong local retention, and the main potential spawning areas were identified inside the gulf, at less than 30 km from the settling areas (0-400 m of depth). Due to the peculiar hydrographical regime characterizing this area, larvae settled in PO showed a very limited dispersal, and just a small fraction of the propagules came from other sites, more precisely from areas located in the east of Sardinia, inside the boundaries of CB.

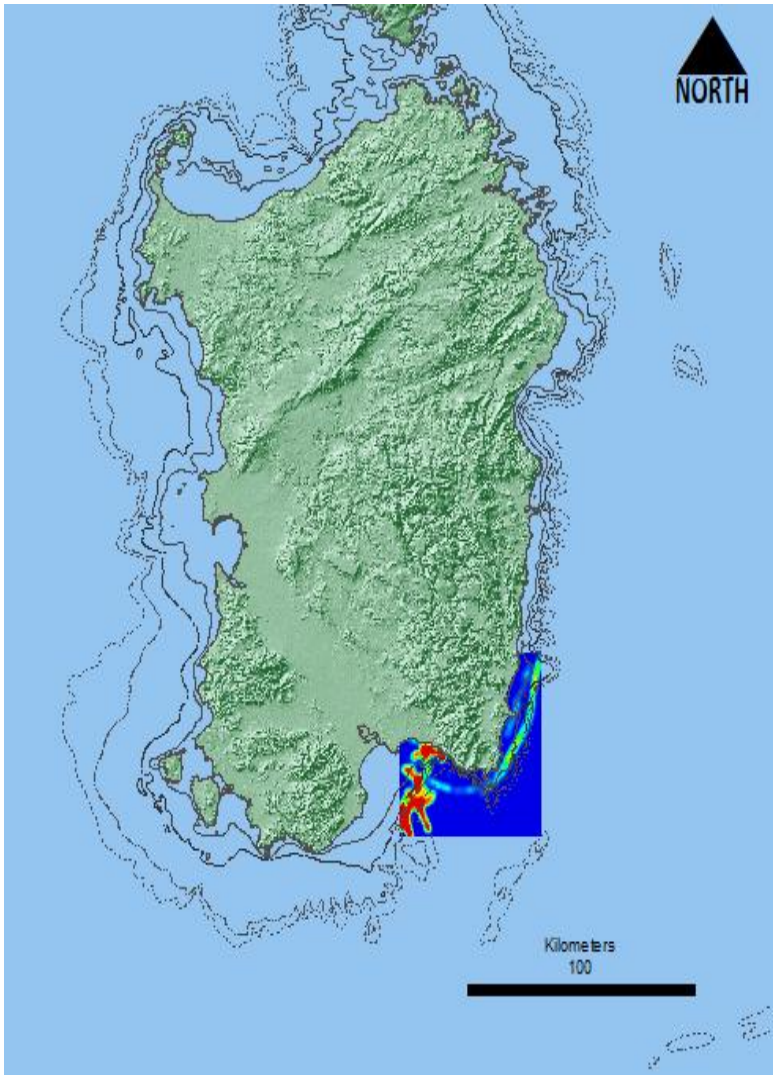

Figure 6. Potential spawning areas identified for larvae settling in PO (kernel density based on back-track larval dispersal simulations)

For the larvae which settled in the PP site, the main larval sources were represented by the spawning areas located in the Gulf of Cagliari within the PO site, where the bathymetry ranges from 20 to 200 m (Figure 7). Small possible spawning areas were also found inside the PP areas boundaries, which were found to be able to furnish 16% of the larvae settling

at PP.

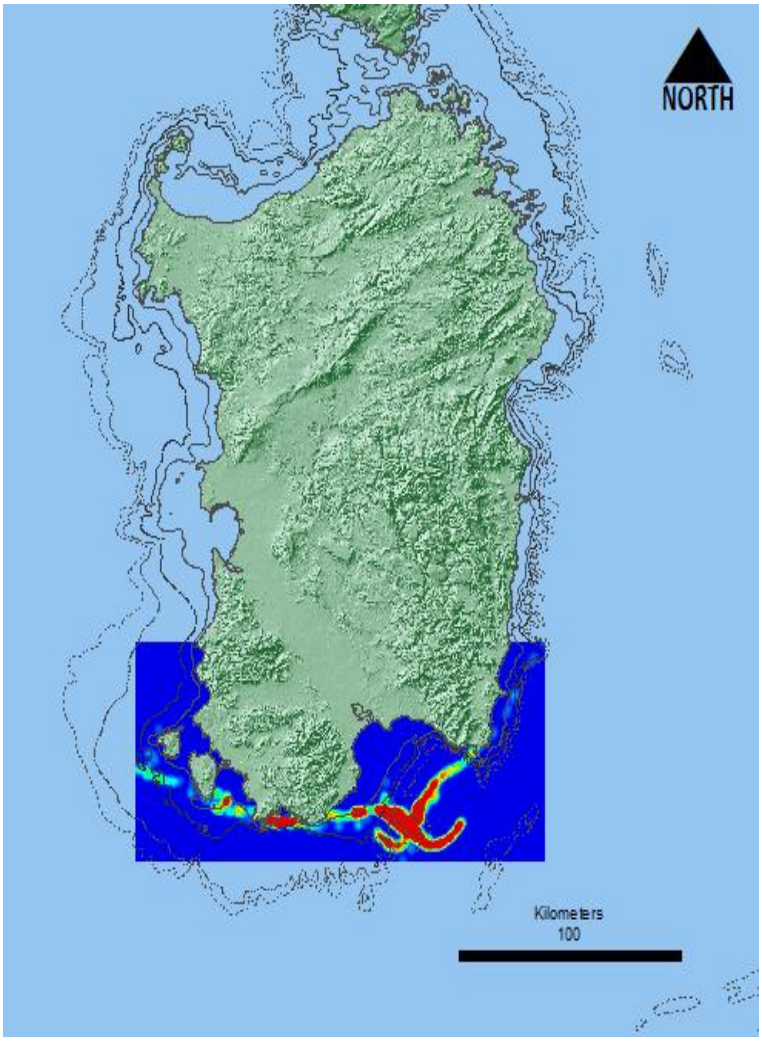

Figure 7. Potential spawning areas identified for larvae settling in PP (kernel density based on back-track larval dispersal simulations)

DISCUSSION

The model here reported embodied a reliable representation of the potential larval dispersal patterns observed in the Sardinia region, identifying potential larval sources (spawning areas) by the use of empirical information concerning settlement sites. The connections' network has been investigated among different sites, revealing a main propagule-flow oriented from the north-east of Sardinia till the south-west of the region.

The simulations highlighted that exchanges can occur between areas distant of more than 200 km. The oceanographic processes determined the large observed dispersal range and each considered site was characterized by a peculiar transport dynamics. As reported for other species (Alemany *et al.*, 2006; Cowen *et al.*, 2006), the physical environmental factors represent an important feature in shaping the larval dispersal range, the distances and thus, they are essential parameters explaining the connectivity patterns. Indeed, the identified patterns of dispersal and the consequent exchanges among sites reflected the general circulation occurring in the studied area (Malanotte-Rizzoli, 2001).

The strength of connectivity depended on the site (and the related hydrographic conditions) and the PLD (Werner *et al.*, 2007). Overall, high levels of connectivity were found and the 62% of the settled larvae came from other areas. The southward current that flows along the east coast of Sardinia helps in provoking a wide dispersion of propagules and connecting geographically very distant areas. In fact, along-shore circulation was found to have a determinant role in the larval supply to the south-east part of Sardinian (site CB), determining high levels of connectivity between the identified spawning areas (located in the east coast of Sardinia) and the known settlement sites. Moreover, a westward oriented flux is recognizable in the south of Sardinia, and the CB and PO sites were found accountable for the replenishment of larvae coming from the PP site (Figure 8).

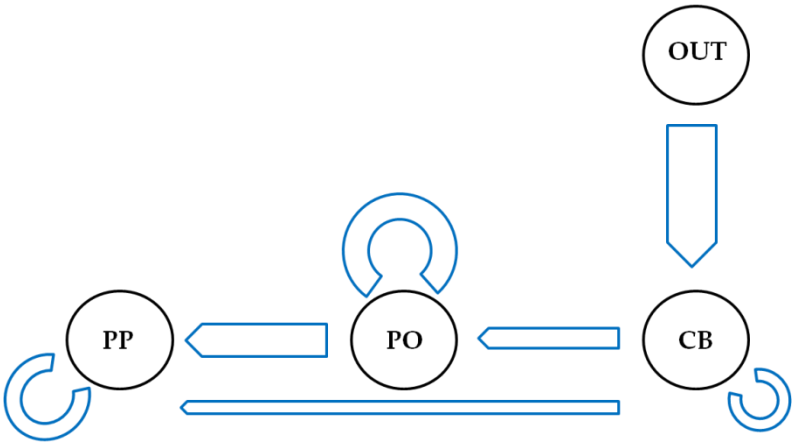

Figure 8. Scheme of the connections between the study sites. Arrows are proportional to the amount of the exchange

Inversely, the local and small-scale circulation patterns in the Gulf of Cagliari determined high retention rates for the PO site located at the centre of the Gulf. Larvae produced inside the Gulf of Cagliari (PO site), due to the relatively enclosed circulation patterns observed in the Gulf, have a high probability to settle within the same site boundaries (above 88%). However, more than 10% of the larvae settling in PO came from spawning areas located within the borders of CB, close to the Villasimius - Capo Carbonara MPA, located in the south-east cape of the island. Overall, spawning areas located in the Gulf of Cagliari were found to have a strong importance in replenishing the south of Sardinia, precisely PO and PP sites.

Most of the probable spawning locations highlighted by the back-tracking simulations were located on bathymetries included in the range 200 - 400m, mainly on soft-bottom (mud or sand) of the continental shelf. Such habitat is known as particularly favourable for *M. barbatus* and the information obtained by the simulation are in agreement with what were reported by previous studies about the spawning areas of this species (Garofalo *et al.*, 2008).

It is also to consider that most of the exchanges were identified in proximity of some protected areas, located along the Sardinian coasts (International Park of the Strait of Bonifacio, Tavolara-Punta Coda Cavallo marine national park and Villasimius-Capo Carbonara MPA, from north to south respectively, Figure 9), that may contribute in maintaining *M. barbatus* populations connectivity among the considered areas.

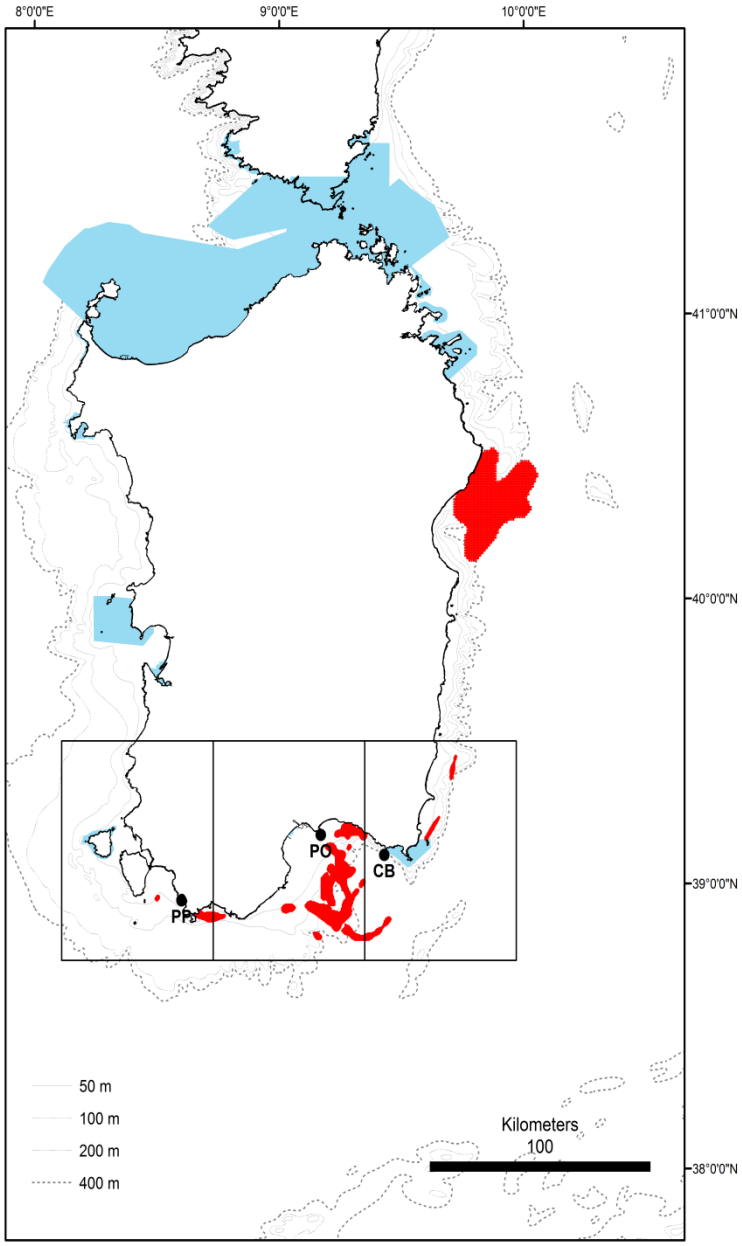

Figure 9. Potential identified *M. barbatus* spawning areas (in red) and existing protected areas along the Sardinian coasts (light blue areas)

Long-distance dispersers, such as *M. barbatus*, may have a significant impact on the demographic and genetic structure, and tend to homogenize the population structure over large geographic scale. Anyway, even if the PLD is a primary parameter in the definition of the dispersal dynamics, the dispersal capabilities of the considered species are not controlled just by it and some behavioural and biological aspects (such as the larvae vertical migrations, swimming capabilities and mortality) must be, when known, taken into account during the simulation processes

(Macpherson and Raventos, 2006). The dispersal pathways here described have to be considered one type of potential dispersal patterns for the *M. barbatus* larvae in South Sardinia. Dispersal distances and patterns may however change in function of several factors such as the proper larvae behaviour, like possible diel vertical migrations, its swimming velocity, and its mortality during the planktonic phase, which could add strong variability in the recruitment processes (Leis, 2007; Werner *et al.*, 2007). Mortality for example, which can be caused by numerous factors (starvation, predation, lethal temperatures, etc.) with different magnitudes, may affect the transport success from the spawning to the nursery areas (Bailey *et al.*, 1997; Rankin and Sponaugle, 2014). Moreover, estimates on local retention are tricky to achieve because of the difficulties in obtaining direct measurement of the reproductive success (number of larvae released) and mortality rates at early life stages for the majority of fish species.

Modelling approaches that integrate key physical dynamic parameters and biological traits provide an effective tool to investigate dispersal and connectivity of marine populations. However, results from our modelling approach are in part constrained by some limitations encountered in modelling larval dispersal. Our analyses, as previously reported in the Mediterranean sea (Andrello *et al.*, 2013), are limited by the resolution scale of the biophysical model grid size (7 km spatial resolution). It cannot take into account the small-scale hydrographic processes, which may cause an underestimation of the near-shore retention (Garavelli *et al.*, 2012), particularly relevant for demersal species.

For all these reasons, validating dispersal modelling approaches with other comparative multidisciplinary approaches integrating different methods for characterizing connectivity (e.g. population genetics analysis, large scale larval tagging, microchemistry or microstructures of otoliths) is necessary to improve the reliability of the information obtained, as confirmed by numerous studies (Gilg and Hilbish, 2003; Baums *et al.*, 2006; White *et al.*, 2010; Leis *et al.*, 2011; Alberto *et al.*, 2011; Crandall *et al.*, 2012; Foster *et al.*, 2012; Soria *et al.*, 2012; Munguia-Vega *et al.*, 2014).

The approaches used in our study demonstrate the reliability of the results obtained by the integration of local biological empirical information such as otolith sclerochronology, and by using mechanistic bio-physical models for modelling dispersal patterns.

By the simulation of different scenarios, it allowed to estimate the potential contribution of different spawning areas to the empirically observed settling areas and to the spatial extension of the exchanges, improving the ecosystem basis knowledge of *M. barbatus* populations.

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ANNEXES

Table 5. NEMOMED12 2D variables

2D variables	
sea surface temperature	(SST - C)
sea surface salinity	(SSS - PSU)
sea surface density	(kgm-3)
sea surface height	(SSH-m)
net upward water ux	(kg m-2 s-1)
concentration/dilution water ux	(kg m-2 s-1)
surface salt ux	(kg m-2 s-1)
net downward heat ux	(W m-2)
shortwave radiation	(W m-2)
turbocline depth	(m)
mix layer depth	(< 0 01 - m)
ice fraction	([0; 1])
surface heat flux: damping	(W m-2)
surface water flux: damping	(kg m-2 s-1)
surface salt flux: damping	(kg m-2 s-1)
Bowl index	
climatological SST	(C)
climatological SSS	(PSU)
zonal wind stress	(taux - N m-2)
meridional wind stress	(tauy - N m-2)
lateral eddy diffusivity	(m2 s-1)

Table 6. NEMOMED12 3D variables

3D variables	
salinity	(PSU)
potential temperature	(C)
potential density	(kg m-3)
zonal component of the current	(m s-1)
meridional component of the current	(m s-1)
vertical velocity	(m s-1)
vertical eddy diffusivity	(m2 s-1)
vertical eddy viscosity	(m2 s-1)

Table 7. MED12 parameters (Lebeaupin Brossier *et al.*, 2011).

Horizontal resolution	1/12°
Time step	$\Delta t = 720$ s
Number of vertical levels	50
Horizontal eddy diffusivity for tracers	$A^{hT} = 100$ m²/s
Horizontal viscosity for velocities	$A^{hm} = 1.25 \times 10^{10}$ m⁴/s²
Vertical diffusivity enhancement coefficient	$A_{EVD}{}^{vm} = 10$ m²/s²
Bottom drag coefficient	CD = 0.001
Bottom friction energy	eb = 0.0025 m2/s2
SST relaxation dQ	dQ/dt = -10 W/m²/s
SSS relaxation	$\Delta z_0/\tau_S$ ¼ 16 m/day

Description of the regional connectivity patterns

The previous chapter highlighted the potential contribution of different spawning areas and the spatial extension of the exchanges, showing that connectivity may occur at a regional scale, within a maximum range of about 200 km. By this work has been possible to identify the potential spawning areas, revealing the source-sink relationship, by evaluating the dynamics of larval exchange among different spawning and settlement areas. These findings improve the ecosystem basic knowledge of the red mullet populations, demonstrating at the same time the reliability of the simulation larval dispersal simulations obtained by the integration of local biological empirical information, as otolith sclerochronology, and mechanistic bio-physical models.

The next step

Thus, this chapter reinforces the need for further examination of how dispersal influence genetically the populations on a broader scale, argument of the next chapter, for accomplishing and validating the information here acquired.

CHAPTER IV

GENETIC CONNECTIVITY AND EARLY LIFE HISTORY STAGES: THE CASE OF RED MULLET (*MULLUS BARBATUS*, L. 1758) IN THE WESTERN MEDITERRANEAN SEA

GENETIC CONNECTIVITY AND EARLY LIFE HISTORY STAGES: THE CASE OF RED MULLET (*MULLUS BARBATUS*) IN THE WESTERN MEDITERRANEAN SEA

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ABSTRACT

Many coastal species are characterized by a bi-partite life cycle, where the dispersal of pelagic larvae is accountable for the maintenance, with different degrees, of gene flow and exchange among different units. Here, by the use of 10 microsatellite markers, we investigated the genetic connectivity across the western Mediterranean Sea of the red mullet (M*ullus. barbatus*), a highly targeted species by the fisheries, focusing on the early life history stages. Our results suggest that the genetic distribution of *M. barbatus* is represented by a metapopulation composed by local demes, with high connections between them. This work enhance the informative connectivity background for the Mediterranean Sea, evidencing the importance of early life-history stages in shaping population dynamics and structuring.

KEYWORDS

Connectivity, dispersal, settlers, microsatellites, *Mullus barbatus*, western Mediterranean Sea.

INTRODUCTION

The assessment of population's structure is a crucial step in maintaining and managing marine resources. Proper management and conservation strategies require to know about the precise frame of the spatial interactions among population units to adapt the management strategies (Palumbi, 2003).

The scales of connectivity depend on the degree to which individuals of a same species (at the larvae, juvenile or adults phases) from different population units are exchanged (Sale, 2004; Cowen and Sponaugle, 2009). For many marine organisms with sedentary adult stages, the larval stage plays a crucial role in the maintenance of the interconnections (i.e. gene flow) across populations through the pelagic larval dispersal (Kinlan and Gaines, 2003; Shanks *et al.*, 2003; Taylor, 2003; Pineda *et al.*, 2007)

However, the realized dispersal results from the interaction of numerous biological and environmental factors (Galarza, 2007; Galarza *et al.*, 2009a; Nanninga and Berumen, 2014) . Physical barriers as currents (Machado-Schiaffino *et al.*, 2009), salinity (Sabatés and Olivar, 1996), temperature (O'Connor *et al.*, 2007) or other geographical constraints as fronts (Galarza *et al.*, 2009a), act directly on the dispersal processes regulating the genetic exchanges and contribute significantly to the definition of the connectivity patterns and population boundaries (Cowen and Sponaugle, 2009). Life history traits such as reproductive strategies (Selkoe *et al.*, 2006), pelagic larval duration (PLD), swimming capabilities and larval behaviour can also strongly affect the levels of realized dispersal and thus of connectivity (Leis, 2002; Leis and McCormick, 2002).

Despite its importance, measuring connectivity patterns remains a major challenge for marine ecologists and fisheries scientists (Armsworth, 2002; Kinlan and Gaines, 2003; Cowen *et al.*, 2006; Siegel *et al.*, 2008; Cowen and Sponaugle, 2009). For defining marine fish population structure, genetic tools are widely used to quantify genetic and demographic connectivity over different spatial scales (Hellberg *et al.*, 2002; Weersing and Toonen, 2009; Salas *et al.*, 2010). In the recent years, using these techniques, several studies have revealed complex structures of spatial genetic differentiation for several fish populations at macro- and micro spatial scale (Hedgecock *et al.*, 2007). In particular, polymorphic nuclear markers such as microsatellites (STRs), which present a high mutation rate (DeWoody and Avise, 2000), are known suitable to detect the presence of fine

genetic structuration in many population (Hauser and Carvalho, 2008), furnishing reliable information on the contemporary genetic connectivity (Selkoe and Toonen, 2006; van der Meer *et al.*, 2012).

Due to its biological characteristics, the red mullet (*Mullus barbatus,* Linnaeus, 1758) has potentially high dispersal capabilities (Chapter III). This species has pelagic eggs (Suquet and Person-Le Ruyet, 2001), large-size pre-settlement stages (up to 6 cm of total length) and relative long pelagic larval duration and competency settlement period (25-42 days and 17 days, respectively). Because of its high relevance in the fisheries, this species has been highly studied in the Mediterranean context, and several aspects of its biology and ecology have been evaluated, such as its growth, reproduction (Bianchini and Ragonese, 2011; Esposito *et al.*, 2013; Fiorentino *et al.*, 2013), diet (Esposito *et al.*, 2013), stock-recruitment dynamics (Levi *et al.*, 2003), distribution patterns of adults (Lombarte *et al.*, 2000; Tserpes *et al.*, 2002; Maravelias *et al.*, 2007), nursery areas distribution (Carlucci *et al.*, 2009), larval distribution (Sabatés and Palomera, 1987) and early life history traits (pelagic larval duration, Chapter II and III).

Additionally, various research focused on the definition of genetic population structure and variability across Mediterranean areas, mainly through the use of microsatellites (Garoia *et al.*, 2004; Galarza *et al.*, 2007, 2009b; Maggio *et al.*, 2009; Félix-Hackradt *et al.*, 2013), RFLP - Restriction Fragment Length Polymorphism - (Mamuris *et al.*, 2001) and allozymes (Mamuris *et al.*, 1998a, 1998b; Arculeo *et al.*, 1999). These studies provided some evidences about the complex genetic structure characterizing red mullet (meta)populations along the Mediterranean coasts. Separated stock units were detected in Atlantic and Mediterranean Sea (Galarza *et al.*, 2009b). Isolated populations were found in the Adriatic Sea (Garoia *et al.*, 2004; Maggio *et al.*, 2009) while Félix-Hackradt and colleagues (2013) showed a micro-scale genetic homogeneity in Southern Spain using post-larval individuals. Nevertheless, while connectivity through larval dispersal has been investigated by these works, large-scale patterns of genetic variability during the settlement phase has been evaluated just by one local scale study (Félix-Hackradt *et al.*, 2013). This stage is particularly relevant for shaping population dynamics. Genetic structures can be also found between pre-settlers (i.e. post-larvae) and recruits as a result of natural selection on genetic variability during the settlement stages (Vigliola *et al.*, 2007) and the effect of pre-settlers or juvenile migration may be reflected in the genetic diversity at different scales (Di Franco *et al.*,

2012).

In order to improve the knowledge on the connectivity patterns and the genetic flows through early life-history stages of *M. barbatus* over the western Mediterranean Sea scale, we conducted a large-scale study on its post-larval and post-settlers populations using 10 microsatellites markers. Our general aim was to determine the extent of the gene flow and reveal the connectivity patterns across the western Mediterranean basin. The specific goals were to:

(i) characterize the genetic pool of the settlers' population of *M. barbatus*

(ii) detect significant genetic variability among different sites

(iii) investigate the most probable populations source

(iv) assess the genetic differences related to the spatial distance between sites

MATERIAL AND METHODS

Ethic statement

No protected nor endangered species were used in the study. Specimens were immediately sacrificed by adding 70% ethanol in the water to reach a ratio of 1/3 of ethanol and 2/3 of sea water, acting like an anaesthetic. After the death, individuals were stored in ethanol (96%) for conservation before DNA extraction.

Sampling scheme

Individuals around settlement phase (i.e. pre and post-settlers) of *Mullus barbatus* were collected using light traps (CARE^{RM}, Ecocean, Montpellier, France) and hand nets in 2013 from 4 locations in the western basin of the Mediterranean Sea (Figure 1): Balearic Islands (BAL), Murcia (MUR), Sardinia (SAR) and Sicily (SIC). Individuals have been caught during the settlement period, between June and September (Suquet and Person-Le Ruyet, 2001).

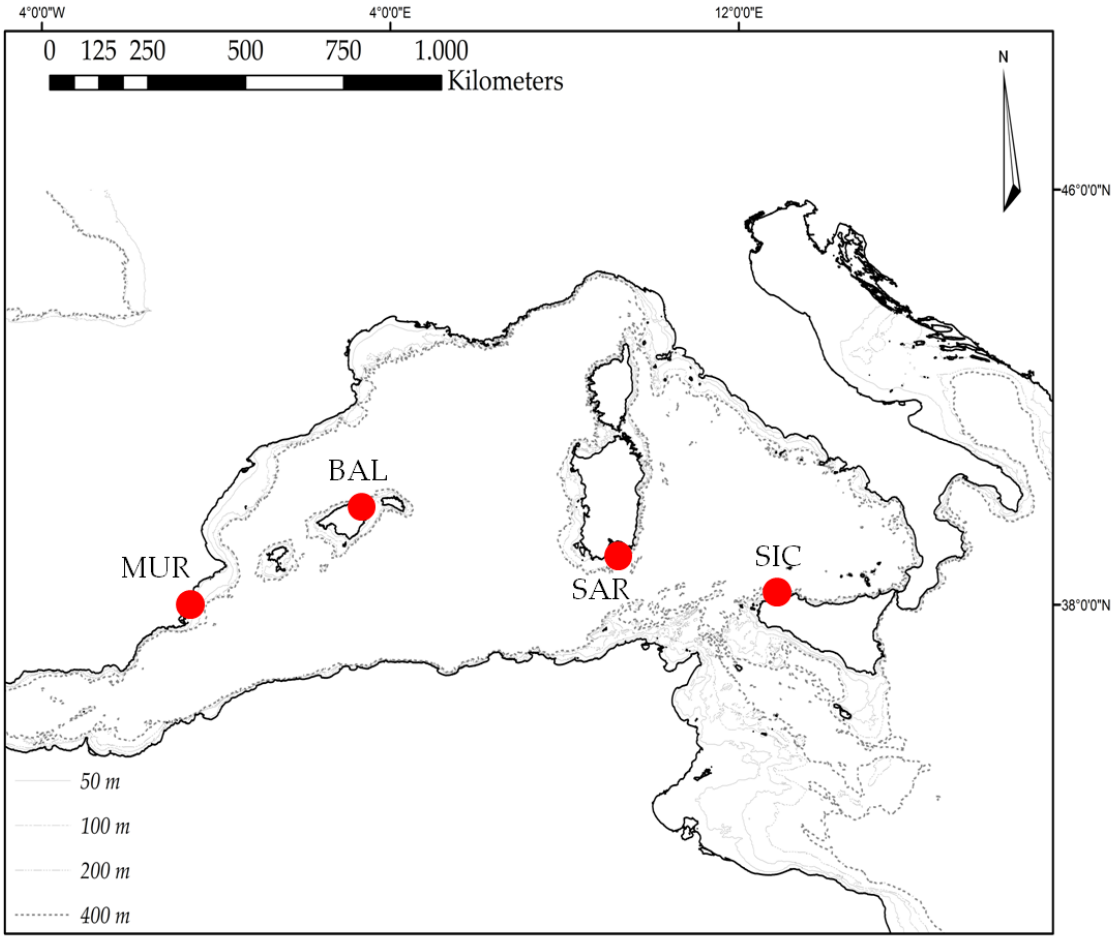


Figure 1. Sampling sites

Genetic analyses

Using a phenol–chloroform protocol (Green and Sambrook, 2012), total DNA was extracted from 10-20 mg of tissue of the caudal fin following steps: (i) cellular lysis was obtain by adding 600 µl of lysis buffer (0.5 M Tris, 0.1 M EDTA, 2% SDS, pH 8.8), (ii) protein denaturation was induced by adding 5 µl of proteinase K (25 mg/ml), (iii) 400 µl 5M ammonium acetate (-20ºC, pH 8, autoclaved) was included to allow protein precipitation, obtained by centrifugation at 13000 rpm for 15 minutes, (iv) the DNA was precipitated by addition of 600 µl of isopropanol (-20 °C) to the solute previously obtained and centrifuged for 30 minutes at 13000 rpm, (v) after removing the solute, 1 ml of 70% ethanol was added and centrifuged 15 minutes at 13000 rpm, (vi) the supernatant was removed and the tubes let to dry until all the ethanol evaporated, and finally (vii) total DNA were suspended in ultrapure water at a concentration of 50 ng/µL.

Individuals were genotyped with 10 microsatellites (Mbar003, Mbar011, Mbar014, Mbar028, Mbar046, Mbar055, Mbar063, Mbar130, Mbar132 and Mbar133), referring to the set of markers developed by (Galarza *et al.*, 2007) for *M. barbatus*. The amplification was conducted by a multiplex PCR in 10 μl total volume, which included 50 ng of DNA, 1× reaction buffer, 2 mM of $MgCl_2$, 0.2 μm of each primer, 0.3 mM dNTPs, 1 mg/ml BSA and 0.75 U Taq polymerase (Biotools™, Madrid, Spain). The amplification was performed at 95 °C for one 5-min cycle, 30 cycles of 92 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s and 72 °C for a 30-min cycle. The PCR products were visualized by capillary electrophoresis using an ABI™ 3730 DNA analyzer (Applied Biosystems, Foster City, CA, USA) and sized with an internal size-standard (Servei Central de Suport a la Investigacio Experimental, University of Valencia, Spain). Alleles and genotype were subsequently scored using GENEMAPPER™ v. 3.5 software (Applied Biosystems).

Statistical analyses

All the statistical analyses were performed on R statistical software (R Development Core Team, 2011).

(i) characterize the genetic pool of the settlers' population of *M. barbatus*

The total number of alleles, alleles frequencies, allelic richness, such as the average number of alleles per locus, (Leberg, 2002), private alleles, null alleles, observed (HO) and expected heterozygosity (HE) were calculated for each locus and location. A Bartlett's test was performed to test the presence of statistically significant differences between HO and HE variances under the null hypothesis of no differences.

A rarefaction curve was plotted in order to evaluate the relationship between the allelic richness and the sampling size. Deviations from Hardy-Weinberg equilibrium (HWE) and estimates of linkage-disequilibrium (LD) per locus and per locality were determined. Polymorphic loci were tested for conformity to Hardy-Weinberg expectations using X2 tests. LD was tested by the use of the Index of Association (IA) (Brown *et al.*, 1980) and a one-side permutation tests was used to test the null hypothesis that alleles observed at different loci were not associated.

(ii) detect significant genetic variability among different locations

A Fisher's exact test for significant allelic differentiation among subpopulations, was conducted to determine if alleles in sub-populations were drawn randomly from a larger population.

The genetic differentiation between sampling localities was explored using the Nei's pairwise Fixation Indexes (F_{ST})(Nei, 1973; Weir and Cockerham, 1984). The associated *p-values* were obtained using Monte Carlo simulations with 999 permutations. Moreover, the fixation index in a group of populations (F_{IT}) and the average in each population (F_{IS}) were calculated for each locus.

A hierarchical cluster analysis was performed on the set of pairwise F_{ST} among localities and a dendrogram was produced to illustrate the genetic divergence using the unweighted pair-group method by using arithmetic means.

Then, to investigate the genetic relationships among localities, a pairwise distance matrix (i.e. dissimilarity matrix) was obtained basing on Bruvo distances among samples. The matrix was then used to construct a principal component analysis (PCA). In order to give a measure of the gene flow, the number of migrants (N_m) exchanged among localities were estimated. Values of N_m were calculated from the pairwise F_{ST} values, according the formula $N_m = (1/F_{ST})-1)*4^{-1}$ (Wright, 1969).

(iii) investigate the most probable populations source

Discriminant Analysis of Principal Components (DAPC) was used to identify and describe clusters of genetically related individuals (Jombart *et al.*, 2010). We defined not-assigned individuals as those having no more than 0.5 probability of membership to any group.

(iv) assess the genetic differences related to the spatial distance of the locations

The genetic-spatial distances relationships were investigated by the correlation of Nei's F_{ST} values and the site geographical distance matrices, under the isolation by distance hypothesis. Geographical distance was measured as the most direct linear maritime route (km). A Mantel's test (Mantel, 1967) was performed for estimating the correlation between spatial and genetic diversity.

RESULTS

Characterize the genetic pool of the settlers' population of Mullus barbatus

A total of 325 settlers of *Mullus barbatus* were analysed (Table 1). All microsatellite loci were polymorphic, specially six microsatellite loci (number of alleles ≥ 14). The mean number of alleles per locus was 15.97 ± 8.75 with a maximum mean value registered for the site Sardinia (SAR), that showed an average value of 17.50 ± 9.45 alleles per locus. Overall, Mbar014 was the highest polymorphic marker (36.00 ± 2.70 alleles) while Mbar028 showed on average a reduced variability, with 4.00 ± 0.81 alleles. Specifically, the total number of alleles per locus ranged from 3 (locus Mbar028, Balearic) to 40 (locus Mbar014, Sardinia) and the number of private alleles was 8 for Balearic and Murcia, 14 for Sardinia and 6 for Sicily. The highest value of allelic richness per locus was recorded for Murcia (37.72, Mbar014) and the average allelic richness per location ranged from 15.318 (Balearic) to 13.930 (Sicily).

The HE per locus ranged from 0.039 (locus Mbar028, Sardinia) to 0.955 (locus Mbar014, Balearic and Sardinia). HO varied from 0.039 (locus Mbar028, Sardinia SAR) to 0.951 (locus Mbar014, Sardinia). The genetic diversity per location estimated as mean HE ranged from 0.728 (Sardinia) to 0.762 (Murcia).

Overall, the highest differences in observed and expected mean heterozygosity was 0.15 (sd = 0.02). Each population showed a deficit in the observed number of heterozygotes (Figure 2 and Table 2). The maximum difference was observed in the Balearic (0.176), while the minimum difference value was reported in Murcia (0.120). Bartlett's test performed to test the differences between observed and expected heterozygosity, revealed a significant *p-value* (<0.01) for each considered location.

Table 1. Summary table

		Mbar 003	***Mbar 011***	***Mbar 014***	***Mbar 028***	***Mbar 046***	***Mbar 055***	***Mbar 063***	***Mbar 130***	***Mbar 132***	***Mbar 133***	***Overall***
BAL (n=72)	*Number of alleles*	26	12	35	3	9	15	19	14	12	21	16,6
	Allelic Richness	24.735	11.132	31.924	2.625	8.462	14.054	16.695	13.522	11.202	18.831	15.318
	HO	0.528	0.676	0.944	0.042	0.623	0.606	0.676	0.250	0.764	0.690	0.579
	HE	0.936	0.684	0.955	0.041	0.803	0.884	0.860	0.778	0.747	0.871	0.756
MUR (n=59)	*Number of alleles*	23	15	34	4	7	15	12	18	9	13	15
	Allelic Richness	22.362	14.128	32.719	3.963	7	14.455	11.673	17.919	8.979	12.575	14.57
	HO	0.678	0.797	0.893	0.119	0.725	0.678	0.649	0.509	0.684	0.695	0.642
	HE	0.926	0.727	0.949	0.114	0.796	0.888	0.827	0.842	0.728	0.826	0.762
SAR (n=126)	*Number of alleles*	21	15	40	5	11	15	20	15	11	22	17.5
	Allelic Richness	19.161	11.86	31.954	2.861	9.459	13.665	15.312	11.999	9.966	17.215	14.345
	HO	0.590	0.592	0.951	0.040	0.567	0.508	0.589	0.321	0.702	0.786	0.564
	HE	0.877	0.635	0.955	0.039	0.778	0.881	0.860	0.718	0.707	0.834	0.728
SIC (n=68)	*Number of alleles*	23	8	35	4	8	14	15	13	10	18	14.8
	Allelic Richness	21.911	7.701	32.073	3.688	7.924	13.691	13.567	12.85	9.467	16.435	13.930
	HO	0.574	0.606	0.896	0.118	0.750	0.677	0.574	0.403	0.731	0.779	0.610
	HE	0.935	0.635	0.948	0.113	0.816	0.891	0.852	0.801	0.703	0.836	0.752

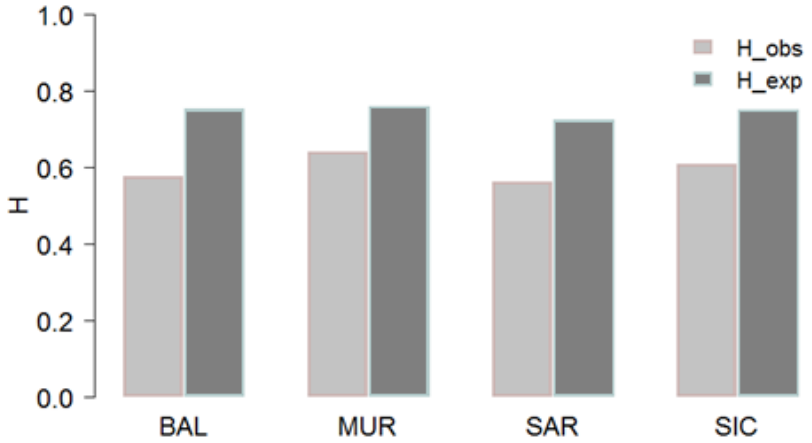


Figure 2. Mean observed (light grey) and expected heterozygosity (dark grey) of *M. barbatus* populations at each location

Table 2. Results of the T test performed to evaluate the differences between mean observed and expected heterozygosity

	t	**df**	***p-value***	**mean of the differences**
BAL	2.989	9	0.008	0.176
MUR	3.064	9	0.007	0.120
SAR	3.226	9	0.005	0.164
SIC	2.865	9	0.009	0.142

In Figure 3, the rarefaction curve shows the amount of variation of the allelic richness depending on the sample size, showing the adequacy of the samples for representing the genetic variability of the population.

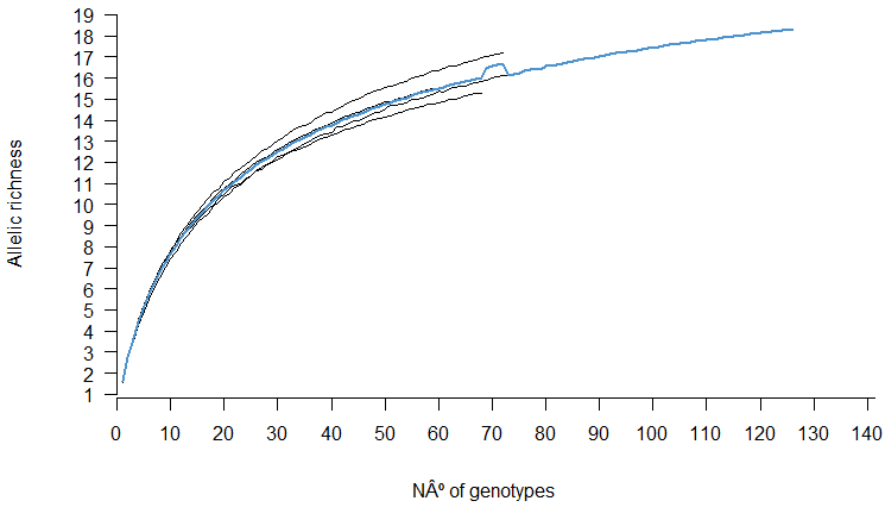


Figure 3. Rarefaction curve for each population (dark lines) and related mean values (blue line)

All the populations revealed a significant departure from the HWE for the loci Mbar003, Mbar046, Mbar055 and Mbar130 (Table 3). Balearic showed a significant value for Mbar133 (*p-value* = 0.001). Mbar011 departed significantly from the HWE for Murcia (*p-value* = 0.001) and Sardinia (*p-value* = 0.001). Overall, 7 markers for Sardinia depart from the HWE.

Table 3. Departures from the Hardy-Weinberg equilibrium at all the loci (*p-values* < 0.001*)

	Mbar 003	***Mbar 011***	***Mbar 014***	***Mbar 028***	***Mbar 046***	***Mbar 055***	***Mbar 063***	***Mbar 130***	***Mbar 132***	***Mbar 133***
BAL	0*	0.371	0.972	0.999	0.344	0*	0*	0*	0.998	0*
MUR	0*	0.010*	0.947	1.000	0.988	0*	0.045*	0*	0.208	0.365
SAR	0*	0.001*	0.363	1.000	0.001*	0*	0*	0*	0.025*	0.914
SIC	0*	0.064	0.069	1.000	0.494	0*	0*	0*	0.604	0.811

Null allele frequencies were calculated for each locus. Highest values were reported for Mbar003 and Mbar132, with frequencies major of 0.2, presented the highest frequencies of null alleles (Figure 4).

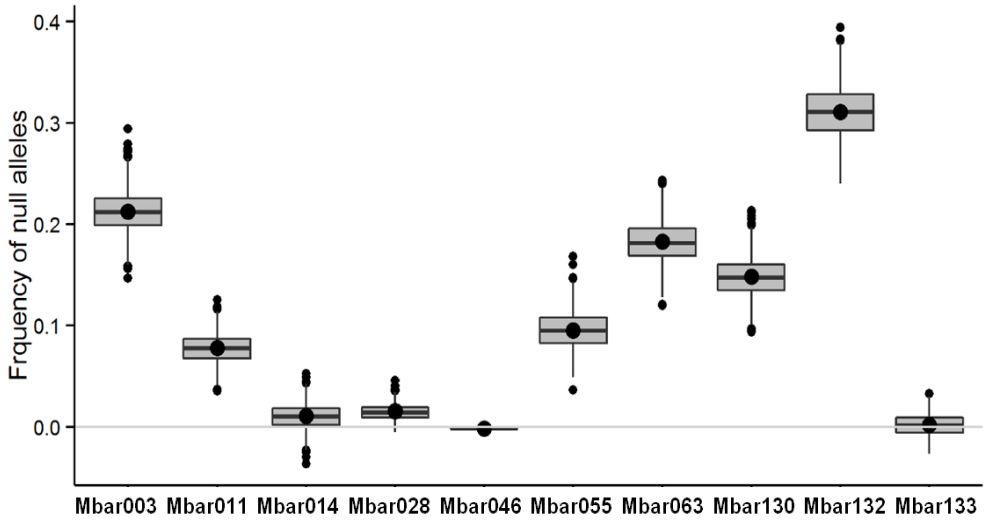


Figure 4. Boxplot of null allele frequencies for each locus

In order to test the linkage disequilibrium (LD), the index of association (IA) was calculated for each location and tested under the null hypothesis that observed alleles at different loci were not linked. The overall IA (Table 4) was low (0.090). Balearic showed the highest value (0.136) while inversely, Sicily presented the lowest values (0.015). Any value was found to be significant (*p-values* > 0.01) and all the populations can be considered in a linkage-equilibrium state.

Table 4. Association index and respective *p-values* obtained by performing 999 permutations

	IA	***p-value***
BAL	0.136	0.34
MUR	0.075	0.59
SAR	0.072	0.70
SIC	0.015	0.79
OVERALL	0.090	0.69

Detect significant genetic structuring among different sites

Fisher's exact test was conducted to determine if alleles in sub-populations were drawn randomly from a larger population (test for homogeneity allelic differentiation among sub-populations), indicating a significant departure from the null hypothesis of panmixia (*p-value* = 0.001) for the loci Mbar003, Mbar046, Mbar130 and Mbar133 and a slightly spatial heterogeneity.

In the Table 5 are provided the results of F statistics for all genotypes and all loci among populations. The overall value of F_{ST} was significantly different from zero (F_{ST} = 0.008; *p-value* = 0.001). Overall F_{IT} and F_{IS} values were 0.22 and 0.21, respectively (*p-values* < 0.01). Pairwise F_{ST} ranged from 0.004 (Murcia - Sicily) to 0.012 (Murcia - Sardinia). F_{ST} values were significant for Balearic - Sardinia (F_{ST} = 0.007, *p-value* = 0.01), Murcia - Sardinia (F_{ST} = 0.012, *p-value* = 0.001) and Sardinia - Sicily (F_{ST} = 0.011, *p-value* = 0.001).

Table 5. Spatial distance (km) and F_{ST} values for the sampled populations (*p-values* for 999 permutations = 0,001** and 0,01*)

	BAL	**MUR**	**SAR**	**SIC**
BAL		440	490	850
MUR	0.005		820	1200
SAR	0.007*	0.012**		340
SIC	0.005	0.004	0.011**	

The hierarchical analyses was performed on F_{ST} values. The cluster dendrogram (Figure 5) resumes graphically the genetic distances among localities examined. Sardinia clustered apart from the other sites. On the lowest level of the dendrogram, Murcia and Sicily showed less heterogeneity, clustering together with a F_{ST} of 0.004.

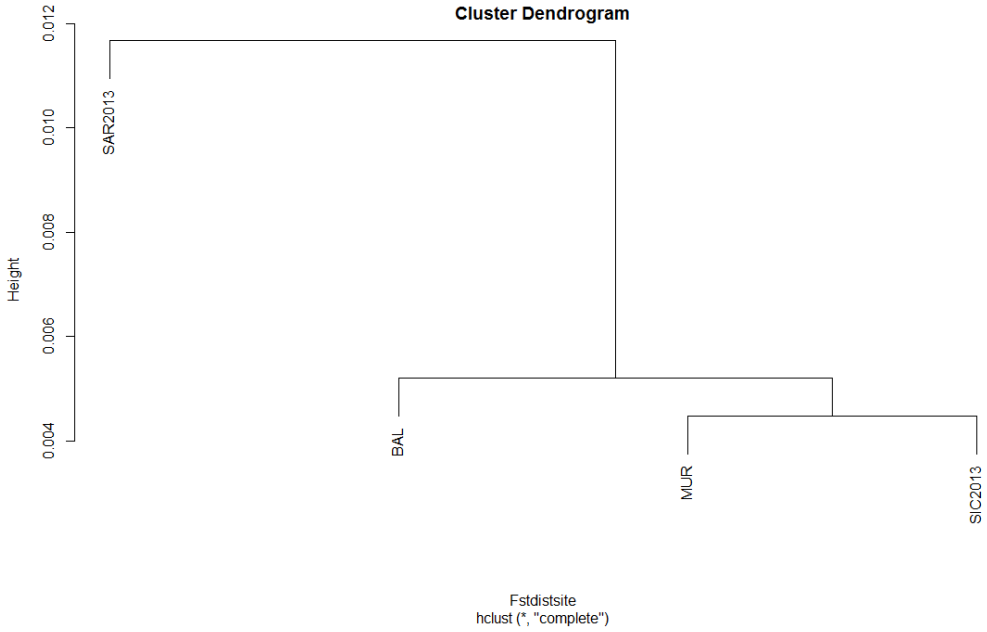
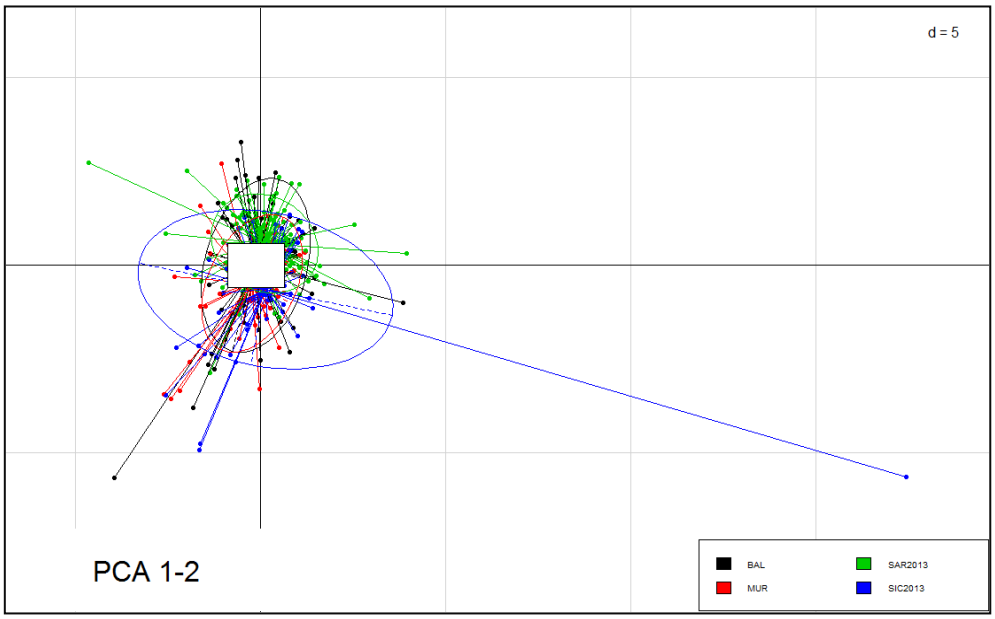


Figure 5. Cluster of Fixation Indexes F_{ST} differences

The results plotted in the Principal Coordinates Analysis (PCA) performed on standardized alleles frequencies, did not reveal any clustering pattern. In Figure 6 is presented the PCA plotted with 95% inertia ellipses of populations. The first 52 principal components of PCA were retained in the preliminary data transformation step, and contain more than 90% of the total genetic variation. The eigenvalues plot did not show any predominant component, indicating the absence of strong variation explained by a particular component. No separated clusters were observed from the plot, and the samples overlapped showing no particular pattern.



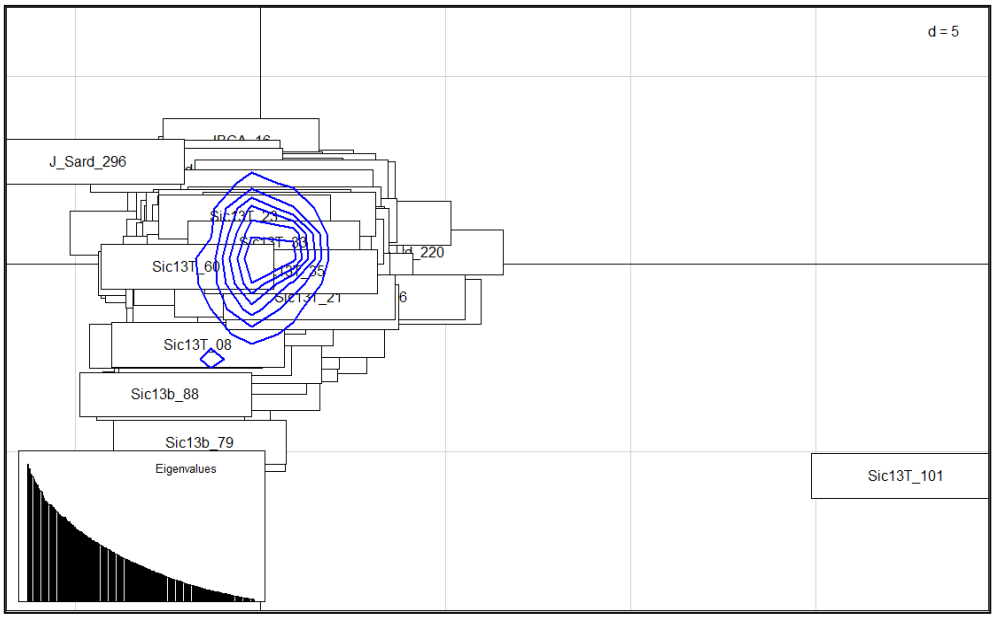


Figure 6. PCA results

The AMOVA performed considering all localities showed significant value (*p-value* < 0.001). The 98.5% of total genetic variance was explained within locations (Table 6).

Table 6. AMOVA results

	Degrees of freedom	**Sum of Squares**	**Mean square**	**Sigma**	**Explained (%)**	***p-value***
Among locations	3	35.89	11.97	0.08	1.5	0.001
Among samples	321	1741.81	5.43	5.42	98.5	0.001
Total	324	1777.70	5.49	5.51	100	

Investigate the most probable populations source

DAPC was used to investigate the pattern of genetic diversity of the populations, identifying clusters of genetically related individuals. The results showed that 63.25% of the individuals was successfully reassigned to the sampling original population.

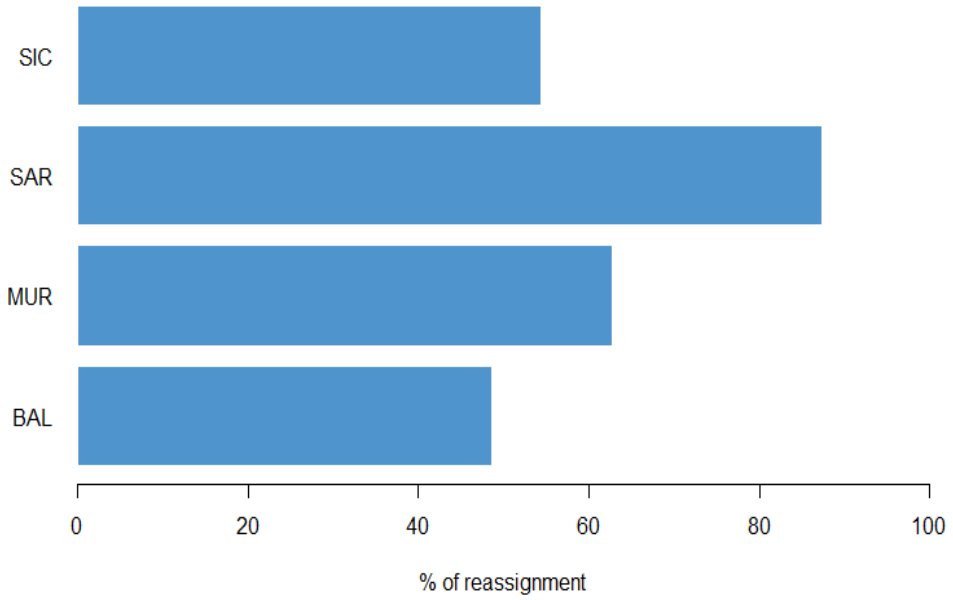


Figure 7. Percentage of correct assignments per population

Sardinia (Figure 7) presents the highest re-assignment values (87.30%) followed by Murcia (62.71%). The minimum value was displayed by Balearic samples, with a percentage of re-assignment equal to 48.61%. In Sardinia, individuals showed low probabilities (< 0.50) to be assigned to other population, particularly in respect of Murcia (Figure 8).

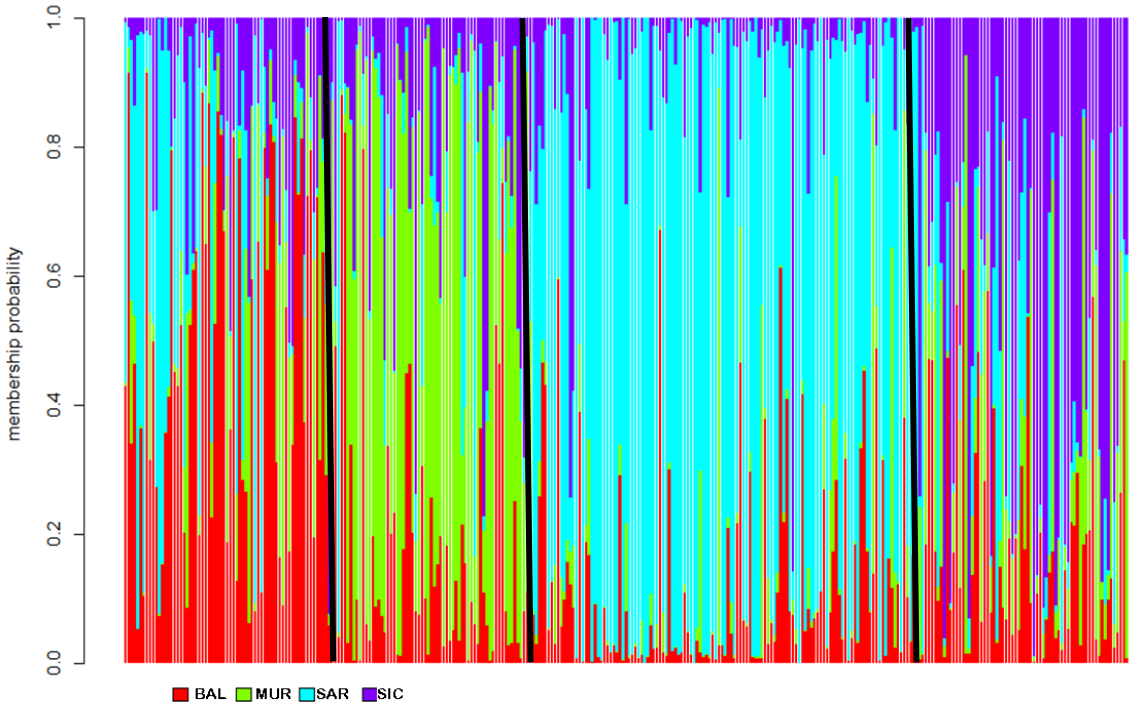


Figure 8. Map of the membership probabilities

The number of migrants was calculated on the F_{ST} values (Table 7). The highest exchanges rate were found for Murcia-Sicily (56.63) and Balearic-Sicily (52.50). Lowest values were registered between the sites Murcia-Sardinia and Sardinia-Sicily, with a number of migrants of about 20 individuals.

Table 7. Number of migrants (N_{em}) per locality

	BAL	**MUR**	**SAR**
MUR	47.84	Inf	
SAR	36.95	21.17	Inf
SIC	52.50	55.63	22.02

Assess the genetic differences related to the spatial distance of the sites

No correlation was found between the spatial distance and the F_{ST} among different populations (Pearson r = -0.37, R^2 = 0.143, *p-value* = 0.45, y = -4E-06x + 0,01; reported in Figure 9).

The Mantel test performed did not show significant values (*p-value* = 0.755). Based on these results, we cannot reject the null hypothesis that spatial and genetic distances are unrelated (alpha = 0. 05). The observed correlation highlighted the low dependency of the genetic divergence in respect of the geographical distance.

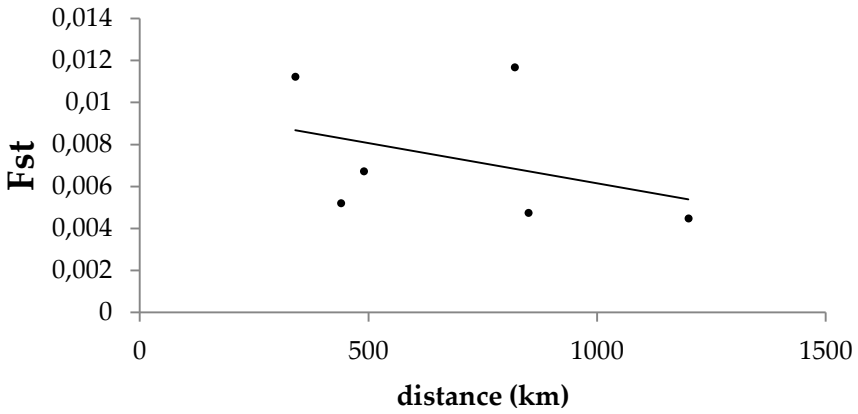


Figure 9. Relationship between F_{ST} and geographical distance

DISCUSSION & CONCLUSION

In general, the results showed a high genetic variation. Each location was characterized by a high allelic richness and genetic diversity (HE) with a variable number of loci that departed from HWE. As reported by several authors (Mamuris *et al.*, 1998b; González-Wangüemert *et al.*, 2004; Maggio *et al.*, 2009; González-Wangüemert and Pérez-Ruzafa, 2012) departures from Hardy-Weinberg expectations may be due to several factors such as inbreeding, population sub-structuring (i.e Wahlund effect) or the presence of null alleles caused by technical issues. The same causes may explain the significant heterozygote deficit systematically found for each location. The analyses performed without the loci presenting the highest null alleles frequencies (Mbar003 and Mbar132; preliminary analyses not reported) did not show different patterns, excluding the influence of the presence of null alleles in the HWE deviations and heterozygotes deficit.

All the loci were established to be in linkage equilibrium, suggesting the presence of frequent recombination event. The overall F_{ST} value (0.088) indicates slight but significant differences among localities, pointing out the presence of a structured population. Fisher's exact test indicated a significant departure from the null hypothesis of panmixia. AMOVA results confirm the presence of a subtle but significant, genetic structure, supported by the re-assignment rates showed by the membership probabilities provided by the DAPC. Overall, the population is represented by a group of single demes locally and moderately separated and that cannot be considered as a single panmictic homogeneous population. (Figure 10)

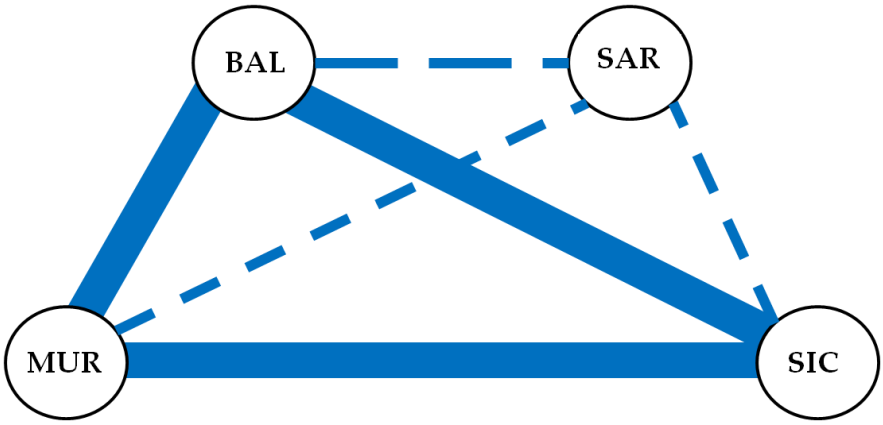


Figure 10. Synthetic scheme of the connection among different sites. Lines represent, proportionally, the level of connection, dotted-lines represent low levels of connection

Nonetheless, Mantel's test did not indicated isolation by distance and no significant correlation was found between the genetic diversity and the spatial distance among localities. The number of migrants, with a mean values of 39.35 ± 15.14, suggest a high exchange of individuals among different localities (demes). It is important to consider that inferring on the number of migrants can be difficult when F_{ST} is low (< 0.05), as often observed for marine species with pelagic larvae (Hedgecock, 2010), because of the high number of larvae exchanges involved.

Results here obtained are in agreement with previous works conducted on this species. At local scale, Félix-Hackradt and colleagues (2013), found low levels of genetic heterogeneity and no significant genetic differences amongst *M. barbatus* samples on the southern Spanish coasts. As well, RLFP and allozymes used in the Greek waters (Mamuris *et al.*, 1998b, 2001), showed no genetic structure and did not revealed any relation to geographic distances. Genetic structuring at relative small scale was found by the use of RAPDs (Mamuris *et al.*, 1998a) while large scale studies with allozymes showed a structured population across the western and eastern Mediterranean Sea (Arculeo *et al.*, 1999). Other works, such as Garoia *et al.* (2004) and Maggio *et al.* (2009), detected low structured isolated population in the Adriatic Sea, while lower differentiation rates for the samples of other western Mediterranean sites (Tyrrhenian Sea, Ionian Sea, Sicily Straits and the Gulf of Lions) was reported. Galarza *et al.* (2009b), investigating on the structure and genetic distribution of the adult population across the Mediterranean Sea, found a stronger heterogeneity if compared with our resutls, resembling that of a metapopulation composed by independent, self-recruiting subpopulations with some connections between them.

All these studies support the hypothesis about the presence of different demes at a regional scale, characterized by a high level of exchange. Considering the presence of a structured population in the western Mediterranean Sea, the gene flow among different sites seems to be maintained mainly by early life stages.

The presence of different, connected demes found in this work can be explained by *M. barbatus* history traits, such as its small-scale reproductive migration and the long larval pelagic phase, that promote and determine the genetic flow among different units. This species is mainly found on sandy or/and muddy habitats (Tserpes *et al.*, 2002) at depths ranging from 10 to 300 m, where individuals perform small-scale reproductive migration toward deeper aggregation grounds during the summer season and also display a more general behaviour to live deeper

while they grow (Machias and Labropoulou, 2002). These areas summon a great number of reproductive individuals from surroundings areas, acting as regional hot-spot of genetic admixture (Selkoe *et al.*, 2006). Moreover, eggs and larvae are pelagic and the larvae spends between 25 to 42 days in the water column until finding a suitable habitat to settle (Sabatés and Palomera, 1987; Macpherson and Raventos, 2005) (Chapter II) thus constituting an important source of genetic homogenization (Schunter *et al.*, 2011). These dynamics are also affected by several oceanographic processes such as currents, winds or eddies, that have a strong influence on larval dispersal and consequently on population connectivity (Macpherson and Raventos, 2006; Galarza *et al.*, 2009a; Palumbi, 2003; Schunter *et al.*, 2011) (Chapter III).

Low values of F_{ST} and consequently the relative high of number of migrants found in this study can be explained the coupled effect of hydro-geographical and biological characteristics, but not by the spatial distance. Sardinian samples, for example, presented the most accentuate differences (7 of the 10 loci departured from the Hardy-Weinberg equilibrium), indicating a high inbreeding rate and a large differentiation from the other groups ($F_{ST} > 0.007$). This result reflects the peculiar dispersal dynamic of the Gulf of Cagliari characterized by a semi-enclosed hydrographic circulation pattern that can determine high levels of self-recruitment and/or local retention.

Since few individuals per generation can maintain genetic homogeneity among populations (Wright, 1951; Mamuris *et al.*, 2001) the migration rate observed is difficult to be realized by individuals moving among sites in one generation (i.e. Murcia-Sicily are at a distance of 1200 km) during their pelagic phases. These findings support the hypothesis of intermediate populations along the western Mediterranean coasts that gradually bring to the spatial divergence estimated, particularly in the south part of the western basin (i.e. north African coasts), where no site were sampled. However, it is also to consider that inferring on connectivity with the number of migrants is particularly challenging to estimate when F_{ST} is low (< 0.05), as is often the case for marine species with pelagic larvae (Hedgecock, 2010).

The genetic distribution of *M. barbatus* is represented by a metapopulation composed by local demes, with high connections between them, mainly maintained by early life history stages. From a management perspective, since different demes may have different responses to environmental and human induced stressors (i.e. fishing pressure) these findings represent a

reliable and useful information for marine spatial planning and MPAs or other resource management measures enforcements.

Overall, genetic structure and population connectivity patterns in the marine dominion are strongly linked with hydro-geographic and bio-ecological factors. These factors contribute to gene flow dynamics, influencing the evolutionary processes of the species and stabilizing the populations (e.g. preventing isolation or extinction). In this context, larval dispersal reduces the level of structuring of marine populations by homogenizing genetic pools (Riginos and Victor, 2001; Selkoe *et al.*, 2006; Hedgecock *et al.*, 2007) .

This research aimed to determine the extent of the gene flow and the connectivity patterns of the settlers populations of *M. barbatus* in the western Mediterranean basin. The results of this work enhance the informative connectivity background for the Mediterranean Sea, evidencing the importance of early life-history stages in shaping population dynamics and structuring. Our findings confirm the suitability of microsatellites, characterized by high mutation rates, for elucidating fine scale and recently derived population structure. Analyses on SSR allows to obtain empirical estimates of genetic connectivity that can be also employed to validate theoretical modelling and spatial distribution forecasting, providing a more consistent support for the management of the common fish resources.

Further studies are required to achieve an accurate informative background on the population structure and on factors influencing the dispersal processes, particularly on the mechanisms that regulate the gene flow between early and adult stages.

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CONCLUSIONS

Starting point

In benthic marine fishes, the bi-partite cycle divide the life of individuals in two main stages: the pelagic and the benthonic phase. The pelagic larval period, accountable for the larval dispersal, represent a fundamental and crucial step in the population dynamics, and the success of the reproductive processes may depend from the correct carrying out of this phases until the settlement processes. Thus, the magnitude and the geographical profile of population's connectivity patterns are, at least in part, determined by the extent to which environmental attributes interact with these species traits.

The challenge

The challenge of this research was to provide, by the implementation of a multi-disciplinary integrated approach, a picture of the realized and potential population connectivity at sub-regional scale (Sardinia) and regional scale (Western Mediterranean Sea) for the red mullet. The general objectives of this research were to acquire the basic information on *M. barbatus* life history traits (PLD, competency phase estimation) and on the dispersal processes and the connectivity patterns.

Thesis structure and basic questions

The thesis has been articulated in four main questions, corresponding to the four question at the base of the research:

Which is the best otolith microstructure for ageing post-larval individuals?

Do spatial variations exist in the settlement patterns?

What are the patterns of the potential larval dispersal?

What are the patterns of the genetic connectivity?

Acquisition of empirical information on ELHT

In the first part of the thesis, the Chapters I and II were centred on the acquisition of the empirical information about early life history traits, such as PLD, spawning date and settlement date, providing some insights into the

species traits in Mediterranean context, trying to explain the timing of settlement dynamics.

Connectivity assessment

The second part of the thesis (Chapters III and IV), was specifically dedicated on the definition of connectivity patterns, potential and realized. In the chapter III, potential larval dispersal was simulated on sub-regional scale (Sardinia) by the integration in the dispersal model of the empirical information acquired in the first part of the thesis, while the Chapter IV dealt with the definition of the genetic connectivity.

CHAPTERS SYNTHESIS

CHAPTER I. SAGITTAE *VS* LAPILLI: COMPARING RED MULLET SETTLERS OTOLITHS MICROSTRUCTURES

Which is the best otolith microstructure for ageing post-larval individuals?

Question background and purposes

Designing the proper structure for obtaining reliable daily-age information is a crucial issue in schlerochronogy studies. Moreover, processing otoliths requires time and economical and human resources. Indeed, as first step of the work in this chapter was defined the best structure to obtain daily age information on first life stages of *M. barbatus*. We compared sagittae and otolith reading's precision, time invested for processing otolith and the capabilities of each otolith type in describing the growth of early stages.

Lapilli as the best solution for aging *M. barbatus* settlers

Our conclusions showed that, even if sagittae and lapilli provided meaningful estimation of the age and the growth with very similar precision levels, lapilli offer an easier and faster processing for ageing *Mullus barbatus* settlers. The high agreement between the age estimations obtained from different otolith, with 67.3% of the samples that presented differences smaller than 1 day, exclude the presence of allochronic deposition of the daily increments between the two otoliths during the ontogenetic development (e.g. pre- or post-hatching

or first feeding increment formation) reported for other species (Fey *et al.*, 2005; Islam *et al.*, 2009). Because of the more homogeneous deposition ring rate, younger individuals (< 50 mm TL) were easier to process and to read than bigger individuals.

Otolith length as growth predictor

If compared with the age, the otolith length was a better predictor of the total length of the fish, showing the suitability of both otoliths for growth studies. The moderate relationship of the age and the total length of the fish, for each otolith type, confirm the variability in the somatic growth rate at the early life history stages (Hare and Cowen, 1995; American Fisheries Society, 2004).

Chapter findings and relevance

Thus, considering overall the results, lapilli were elected as the best solution for ageing red mullet settlers. However, in case of doubtful readings, we suggested the mutual validation of the estimates obtained by both otoliths, particularly for lager individuals (> 50 mm). By these findings, this chapter furnished the methodological support for the next one, where we conducted a more specific evaluation of the settlement patterns across the western Mediterranean Sea.

CHAPTER II. EARLY LIFE HISTORY TRAITS OF *MULLUS BARBATUS*: EVIDENCES FROM OTOLITH SCLEROCHRONOLOGY

Do spatial variations exist in the settlement patterns?

This chapter describes the information on the settlement phase obtained through the analyses of otoliths. Using post-larval otolith sclerochronology, different early life history traits (timing of settlement, competency period, PLD, spawning date) were defined across different areas of the Western Mediterranean sea with a particular focus on Sardinian settlement dynamics.

M. barbatus presented an extended competency period (20 days), that let larvae the possibility to delay the settlement settle in a temporal window ranging from 25 to 45 days. By comparing the 3 studied areas, it has been possible to

identify a clear spatial variability of larval duration of *M. barbatus* in the Western Mediterranean Sea that was not observed, at a smaller spatial scale, among Sardinian sites. Moreover, the information obtained on the settlement events, where larvae from different reproductive pulses settle in the same period, suggested the presence of patches of post-larval individuals with different ages.

CHAPTER III. DESCRIBING FISH CONNECTIVITY PATTERNS: INTEGRATION OF OTOLITH SCLEROCHRONOLOGY INFORMATION IN LARVAL DISPERSAL MODELS

What are the patterns of the potential larval dispersal?

Question background and purposes

To answer this question we implement the empirical information obtained in the previous chapters for the calibration of a larval dispersal model. The purpose of this frame of the work was to investigate, at relatively small scales (about 200 km), the potential dispersal patterns, connectivity rates and the source-sink dynamics of *Mullus barbatus*, assessing the effect of PLD, spawning date and hydrographical circulation on the potential larval dispersal. To achieve this goal we performed a backward simulation to model the potential sources of the settlers from three sites located in the south of Sardinia, basing on the information previously acquired in the Chapter II about PLD, spawning date and settlement date.

Simulation results

The simulations revealed a particle flow oriented from the spawning areas located in north-east of Sardinia to the south-west settlement areas of the island. On average high levels of connectivity were found among the sites, with the 62% of the total number of settled larvae are produced in other sites. Different scenarios were revealed for each site, varying from high self-recruitment rates (site PP, SR = 1) or high local retention (site PO, LR = 0.855) to sites characterized by high levels of larval supply from external spawning areas, as the case of site CB (93%), where particle dispersed also for more than 200 km.

Chapter findings

The strength of connectivity depended on the site, the related hydrographical

conditions and the PLD, and larvae spawned at a particular point could have a different fate from larvae spawned at the same point at a different time. PLD is a primary parameter in the definition of the dispersal dynamics but on the other side is to consider that the dispersal capabilities are not a simple function of the time spent in the pelagic environment and some behavioural and biological aspects (as vertical migrations, swimming capabilities, mortality) should be, when available, taken into account during the simulation processes (Macpherson and Raventos, 2006).

Relevance of the study

The results represent a reliable illustration of the potential contribution of different spawning areas and the spatial extension of the exchanges, showing that connectivity is likely to occur at a regional scale, within a maximum range of about 200 km. These findings improve the ecosystem basic knowledge of the *M. barbatus* populations and demonstrating the reliability of the simulation obtained by the integration of local biological empirical information, as otolith sclerochronology, and mechanistic bio-physical models in modelling dispersal patterns. Nevertheless, integrated modelling in this study reinforces the need for further examination of how dispersal influence genetically the populations for accomplishing and validating the information here reported, argument of the next chapter.

CHAPTER IV. GENETIC CONNECTIVITY AND EARLY LIFE HISTORY STAGES: THE CASE OF RED MULLET (*MULLUS BARBATUS*) IN THE WESTERN MEDITERRANEAN SEA

What are the patterns of genetic connectivity?

Question background and purposes

This chapter provided an overview of the genetic connectivity in the Western Mediterranean Sea for *M. barbatus* settlers. The population structure was investigated using 10 microsatellite markers.

Main results

In general, the results showed a high genetic variability, a variable number of loci per site that departed from Hardy-Weinberg equilibrium, and a systematic

heterozygote deficit found for each site. All the loci were in linkage equilibrium, symptomatic of frequent recombination events. The overall FST value (0.088) indicates slight but significant differences among localities, pointing out the presence of a structured population with a high number of migrants ($N_m = 39,35 \pm 15,14$), suggesting a high exchange of individuals among different sites. No isolation by distance and no significant correlation was found between the genetic diversity and the spatial distance among sites was found. The results confirm the presence of a subtle but significant, structure and a significant departure from the null hypothesis of panmixia. Genetic divergence can be explained the coupled effect of hydro-geographical and biological characteristics, but not by the spatial distance.

Chapter findings

Overall, the population is represented by a group of single demes locally and moderately separated and that cannot be considered as a single panmictic homogeneous population. Considering the presence of a structured settlers population in the western Mediterranean Sea, the gene flow among different sites seems to be mainly preserved by early life history stages, avoiding drifting or isolation of population, thus maintaining low values of F_{ST} trough larval dispersal.

Relevance of the study

The results of this work enhance the informative connectivity background for the Mediterranean Sea, evidencing the importance of early life-history stages in shaping population dynamics and structuring. Our findings confirm the suitability of microsatellites for elucidating fine scale and recently derived population structure. Analyses on SSRs allows to obtain empirical estimates of connectivity that can be also employed to validate theoretical modelling and spatial distribution forecasting, providing a more consistent support for the management of the common fish resources.

GENERAL DISCUSSION

Overall, connectivity patterns in the marine dominion are strongly linked with environmental and bio-ecological factors. These factors contribute to

determine the amount of the exchanges among different units, influencing both demographically and genetically the populations and their structures.

Environmental factors

Coupled with human activities (overfishing, translocation, ecosystem degradation, not considered in this work), other environmental factors concurred in the determination of the genetic and demographic connectivity. These factors include water masses circulation (ocean currents, gyres, eddies, upwelling processes) (Chapter III; Palumbi, 2003; Carlsson *et al.*, 2004; Grant, 2005; Zardoya *et al.*, 2004; Raventos and Macpherson, 2005b; Galarza *et al.*, 2009a; Schunter *et al.*, 2011), habitat discontinuities (bottom topography, substrate distribution) and geographical distances (Dawson, 2001; Riginos and Nachman, 2001; Shaw *et al.*, 2004; Beldade *et al.*, 2006; Hemmer-Hansen *et al.*, 2007; Medina *et al.*, 2007), and environmental stressors (temperature and salinity gradients) (Cimmaruta *et al.*, 2005; Foll and Gaggiotti, 2006; Florin and Höglund, 2007).

Bio-ecological factors

Biotic factors include those referring to the particular biology of *M. barbatus*. The presence of different, connected demes found in this work can be explained by *Mullus barbatus* history traits, such as its small-scale reproductive migrations and the long larval pelagic phase, that promote the flow, both demographic and genetic, among different units. This species is mainly found on sandy or/and muddy habitats (Tserpes *et al.*, 2002) at depths ranging from 10 to 300 m, where individuals perform small-scale reproductive migration toward deeper aggregation grounds during the summer season (Machias and Labropoulou, 2002). Habitat preferences of adult individuals (Lombarte *et al.*, 2000; Tserpes *et al.*, 2002) coupled with regional geomorphological characteristics, such as the topography of the continental shelf, may be responsible for the patterns observed, as previously hypothesized by Garofalo and colleagues (2008). In agreement with these observations, spawning locations highlighted by our simulations, were located on bathymetries included in the range 200-400 m, mainly on soft-bottom (mud or sand) of the continental shelf. These areas aggregate large numbers of reproductive individuals from surroundings areas, and may act as regional spots of genetic admixture (Selkoe *et al.*, 2006).

Eggs and larvae spends between 25 to 45 days in the pelagic environment until finding a suitable habitat to settle (Chapter II), characterized by rapid growth rates. In fact, *M. barbatus* presents large competent larvae (50 - 60 mm TL), gifted of good swimming capabilities, that are able to perform active migrations both horizontal or vertical (personal observation) depending on trophic, ontogenetic incipits or determined by prey-predator interactions. Generally, larger size-at-hatching, faster larval growth rate larger size-at-settlement, higher condition at metamorphosis and faster early juvenile growth may enhance survival of juvenile fishes and is consistent with the growth-mortality hypothesis (Anderson, 1988; Meekan and Fortier, 1996; Searcy and Sponaugle, 2001; Shima and Findlay, 2002; Vigliola and Meekan, 2002; McCormick and Hoey, 2004; Raventos and Macpherson, 2005a; Sponaugle and Grorud-Colvert, 2006; Hamilton *et al.*, 2008). Moreover, long-distance dispersers, as *M. barbatus*, may have a significant impact on population structure over large geographic scale, ensuring the exchange among areas with (Riginos and Victor, 2001; Selkoe *et al.*, 2006; Hedgecock *et al.*, 2007; Schunter *et al.*, 2011).

This work can be also viewed in respect of the limitations emerged during the research. The limitations mainly regard the general lack of information of the environmental factors and bio-ecological traits previously described.

Modelling limitations and data resolution

Results from our simulation are in part constrained by the resolution scale of the NEMOMED12 grid size, as previously underlined by Andrello *et al.* (2013), when modelling larval dispersal of *Epinephelus marginatus* in the Mediterranean sea's MPAs network. The grid size of 7 km used in this work, lacks of the small-scale hydrographic processes and may cause an underestimation of near-shore retention, which increasingly seems to affect the connectivity patterns of coastal fishes (Garavelli *et al.*, 2012b). This lack of knowledge also regards the information, both spatial and temporal, quantitative and qualitative of the main human-induced threats (i.e. overfishing, habitat deterioration, global warming and other disturbances). Such stressors, may have a broad potential effect on the dispersal and the survival rates, and thus, on connectivity.

Limitations from missing species traits

Moreover, behaviour, directional active swimming, orientation mechanisms are proved to affect the patterns of larval dispersal predicted, particularly under the assumption of passive transport. However, our understanding of behavioural patterns are still limited. Studies to evaluate swimming capabilities in terms of velocity, resistance and energy requirements, would furnish precious information for modelling purposes.

Genetic connectivity and high exchange rates

In presence of large size populations, that present low levels of differentiation ($F_{ST} < 0.05$), inferring on the demographical connectivity it's particularly challenging, because of the difficulties in discerning the temporal scale of the differences revealed (Allendorf and Luikart, 2009; Hedgecock, 2010).

However the implementation of other complementary methods, such as otolith chemistry, would add some important information about the natal sources, the habitat transition, enriching the results obtained in this study.

Implication for conservation

Currently, the availability of data on the magnitude of dispersal and levels of population connectivity in many marine organisms is limited for most of the species (Halpern and Warner, 2003; Gaines *et al.*, 2010b), particularly for the Mediterranean Sea. Thus, this informative lack may represent an impediment in the implementation of ecological relevant management strategies and in the prediction of the effects and the consequences of such management, particularly for threatened species, as *M. barbatus* that has been recently considered as in a overexploitation condition (Scientific, Technical and Economic Committee for Fisheries, 2013). It has been widely claimed that proper protection of such diversity will depend on the extent to which we understand the processes that generate and maintain the range, the diversity and the resilience of populations (Palumbi, 2003). Open populations, where the dynamics of the exchange (and thus, connectivity) could be regulated by non-local factors, require a broad scale management, while contrarily, structured populations need local management measures, able at the same time, to preserve the interconnection of populations within demes encompassing the species' range. Thus, since different demes may have different responses to environmental and human induced stressors (i.e.

fishing pressure) the findings of this thesis represent a reliable and useful information for marine spatial planning (as MPAs or other management measures aimed to the safeguard of exploited species, as red mullet. For example, from the simulations obtained in the Chapter III, most of the exchange were identified in proximity of some protected areas located from the north to the south of Sardinia (International Park of the Bonifacio Strait, Tavolara-Punta Coda Cavallo marine national park and Villasimius-Capo Carbonara MPA), that may contribute in maintaining *M. barbatus* populations connectivity among the considered areas and the relatives demes.

OUTLOOK REMARKS

Mullus barbatus larval dispersal can reach a range of 200 km, as found for several fish species (Kinlan and Gaines, 2003; Palumbi, 2004; Botsford *et al.*, 2009; Di Franco *et al.*, 2012a). The differences of early life traits (i.e. PLD, spawning and settlement date) recorded at western Mediterranean scale suggest a certain level of larval patchiness, as observed for other species (Paris and Cowen, 2004). This is likely due to the occurrence of different spawning pulses during the spawning period recorded for the species in this study. The dispersal of individuals across distant areas, even not significant in demographic terms, is accountable for the maintenance of genetic flow among different demes. Fluctuations in the level of exchange among different areas, due to the variability of the source - sink dynamics, and habitat fragmentation due to human or environmental changes could have major implications in the population connectivity patterns and thus, in management and conservation needs.

In conclusion, following a multidisciplinary approach, this thesis give a considerable contribution to the comprehension of the connectivity dynamics of the red mullet both at local and regional scale, and on the processes and the relationships between physical and biological properties governing dispersal, settlement and connectivity in coastal fishes. Synthetically, the study provides: (i) empirical and theoretical data on early life history traits of this species; (ii)

baseline data on the utility of the integration of different methods (otolith sclerochronology, modelling and microsatellite markers), for determining levels of population connectivity and (iii) new insights on the importance of ELHS in shaping the connectivity patterns.

PERSPECTIVES

This thesis provides a more detailed understanding of connectivity patterns at sub-regional and regional scale, offering a baseline for future research.

Some work is still needed to deeply and comprehensively understand the mechanisms of connectivity. (My) Future research should include an improved spatial resolution of the dispersal processes, taking into account coastal oceanographic regimes and small scale dynamics, where competent larvae, endued of high sensorial and behavioural capabilities characterizing the late pelagic phases, are able to chose the best place to settle. To enhance our knowledge on these small-scale dynamics, it is necessary i) to acquire additional empirical information on swimming capabilities and physiological (i.e. sensorial) and behavioural aptitudes of larvae, and ii) to improve the resolution of the hydrographical knowledge on the other side.

Therefore, it would be worthy to replicate this kind of studies on a higher number of species displaying different life history traits (life stages, reproductive strategies, etc.) to further the understanding of population dynamics of demersal fishes. Moreover, field experiments should be thus undertaken to investigate the effects of presently occurring climate changes (e.g. temperature, pH and other environmental parameters) on the dispersal, settlement, growth and reproduction of temperate coastal fish species.

Since dispersal processes and the consequent connectivity patterns may vary in space and time, the acquisition of reliable information on seascape connectivity (i.e. the combined spatial pattern metrics such as habitat type, patch size, distance to patch and dispersal abilities of fish species) should be implemented in

a form of adaptive management. Such kind of management need to be implemented to maintain the ecological integrity, basing on the concept of using management practices that have the ability to be modified based on new experience and insights (Pahl-Wostl, 2006). Adaptive management aims to identify uncertainties in the management of an ecosystem while using hypothesis-testing to further understand the system functioning; this requires to learn from the outcomes of the on-going scientific evidences and thus to adapt the management strategies, until successfully meeting the ecosystem ecological needs.

In the next future, the additional knowledge obtained from our efforts to understand the seascapes functioning and dynamics will aid to develop new perspectives regarding the marine ecological awareness and to implement proper adaptive management strategies.

THE THESIS IN PILLS

Chapter I

I. *Lapilli present some advantages (high precision, good capabilities in describing growth, time saving) in studying early life history traits of M. barbatus*

Chapter II

II. *The pelagic larval duration of M. barbatus ranged from 25 to 45*

III. *Red mullet displaying an extended competency period, of about 20 days*

IV. *Individuals from different reproductive pulses can be patchy distributed to settle at the same time*

Chapter III

V. *M. barbatus present wide potential dispersal capabilities, with a maximum dispersal range of about 200 km.*

VI. *Patterns of connectivity are defined locally with different scenarios, ranging from high self-recruitment or local retention rate to wide larval dispersal.*

VII. *Oceanographic conditions, spawning date and PLD are crucial in the definition of the patterns of connectivity.*

VIII. *PLD is a primary parameter in the definition of the dispersal dynamics, but the dispersal capabilities are not controlled just by it and some behavioural and biological aspects may affect the larval dispersal*

Chapter IV

IX. *Across the Mediterranean Sea ,this species is organized in different demes, characterized by a high number of connections (migrants) among different demes.*

X. *Genetic differences not explained by the spatial distance*

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