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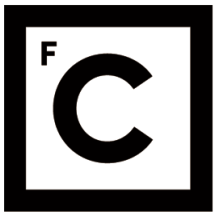

Ciências
ULisboa

Salema, *Sarpa salpa* (Linnaeus 1758): stock structure in the eastern Atlantic and biological characterization off the Portuguese coast

Doutoramento em Biologia

Especialidade em Biologia Marinha e Aquacultura

Rafaela Barros Paiva

Tese orientada por:

Prof. Doutor Leonel Serrano Gordo e pela Prof.ª Doutora Maria José Costa

Documento especialmente elaborado para a obtenção do grau de doutor

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Rafaela Carla Viseu Barros Paiva

2015

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Resumo e palavras-chave

Resumo: Esta tese contempla o primeiro estudo multidisciplinar de salema, *Sarpa salpa*, no sul do Nordeste Atlântico porque analisa a estrutura do seu manancial e carateriza alguns dos parâmetros biológicos da espécie. Para analisar a estrutura do manancial, estudaram-se indivíduos de três regiões de Portugal continental e do Arquipélago da Madeira, complementados com informações de indivíduos do Mar Mediterrâneo para a análise genética, enquanto a análise biológica da espécie foi baseada em exemplares capturados na região centro do País. Considerando a análise da estrutura do manancial, técnicas morfo-geométricas aplicadas à forma do corpo e a análise elíptica de Fourier aplicada à forma do otólito indicaram alguma sobreposição dos indivíduos de Portugal continental, especialmente entre o centro e o sul, e uma forte separação dos indivíduos do Arquipélago da Madeira. Ambas as técnicas indicaram que devem existir pelo menos dois mananciais, um na Madeira e outro em Portugal continental. No entanto, os resultados não foram claros quanto à existência de mais um manancial no norte do país deixando em aberto a questão se realmente existe um manancial no norte e outro que contempla o centro e sul de Portugal. A existência de mananciais, colocada em evidência pelas análises morfológicas, indica que os indivíduos foram sujeitos à acção de divergência genética ou à adaptação em locais com condições ambientais particulares, sendo este último o mais plausível no presente caso. Em relação à genética, a análise dos dados mitocondriais e nucleares suportam as seguintes hipóteses: a) não há evidência de estruturação do manancial entre as amostras obtidas no Oceano Atlântico e no Mar Mediterrâneo; b) existe uma

redução actual no fluxo de genes entre os locais de amostragem; c) há um claro sinal de expansão dos possíveis locais de refúgio na costa de Africa, com uma acumulação de mutações *in situ*; d) a introgressão de ADN, encontrada nos dados mitocondriais e nucleares, parece ser antiga, existente muito provavelmente antes da expansão. Relativamente ao estudo da biologia da espécie em Portugal continental, foram obtidas novas informações no âmbito das análises da idade, crescimento e reprodução, onde não existiam dados prévios. Foram estimados os parâmetros de história de vida que são particularmente importantes pois podem ser usados em modelos ecológicos, para informar, avaliar e melhorar a gestão dos mananciais pesqueiros. Os parâmetros de história de vida estimados incluem a duração da época de reprodução (Setembro a Novembro), o padrão de crescimento (médio-baixo), o comprimento de primeira maturação dos machos (25 cm), a determinação do tipo de fecundidade (determinado) e o seu cálculo. Foram encontradas estruturas císticas nos ovários e os resultados obtidos indicaram que estas estruturas podem fazer parte da estratégia reproductiva de *S. salpa*.

Palavras-chave: *Sarpa salpa*, Oceano Atlântico, morfometrias do corpo e do otólito, análise genética, idade e reprodução

Abstract and keywords

Abstract: This thesis comprises the first multidisciplinary study of *Sarpa salpa* in the southern Northeast Atlantic Ocean because it analyzes its stock structure and characterizes the biological parameters of the specimens off the Portuguese coast. To achieve those goals, specimens from three areas of mainland Portugal and Madeira were collected and supplementary samples from Mediterranean Sea were obtained for genetic analysis. Considering the analysis of the stock structure, the body geometric morphometric techniques and the elliptical Fourier analysis indicated some overlap in the mainland specimens, especially between the central and south areas, and a clear separation of Madeira individuals. Both techniques indicated that at least two stocks may occur, one in Madeira and the other in mainland waters but were not clear regarding to the existence of an additional stock in mainland waters, separating the northern region from the central and south areas. Evidence of stocks through morphometric analysis indicated that individuals have been underlying some genetic divergence or adaptation to local conditions, being the latter the most applicable to the present case. Regarding genetics, the analysis of mitochondrial and nuclear data of *S. salpa* support the hypothesis that: a) there is no evidence of stock structure between Atlantic and Mediterranean samples; b) exists a reduction of the current gene flow among sampling sites; c) there is a clear expansion signal from the possible refuges in the coast of Africa with accumulation of mutation *in situ*; d) the mitochondrial and nuclear DNA introgression detected seems to be ancient, probably existing before the expansion took place. For the analysis of age, growth and

reproduction new information was given for the Portuguese continental coast, where no information was available. This new information is particularly valuable because life history parameters of *S. salpa* were estimated and that can be used to provide baseline data to be applied in ecological modelling, to inform, improve and assess the fisheries towards a sustainable management. The information determined on *S. salpa* life history parameters, captured in the Portuguese waters, included: the duration of the spawning season (from September to November); the determination of growth pattern (medium-slow); the estimation of L_{50} for males (25 cm); the clarification of the fecundity type (determinate) and its estimation. Cystic structures have been documented in *S. salpa* ovaries and the results indicated that the cysts may be part of the species reproductive strategy.

Keywords: *Sarpa salpa*, Atlantic Ocean, body and otolith morphometrics, genetic analysis, age and reproduction

Resumo alargado

A salema, *Sarpa salpa* (Linnaeus 1758), pertence à Família Sparidae e encontra-se amplamente distribuída pelo Mar Mediterrâneo, Mar Negro, Oceano Atlântico Oriental (desde a Baía da Biscaia até ao Cabo da Boa Esperança) e Oceano Índico Ocidental (desde Moçambique até ao Cabo da Boa Esperança). É explorada comercialmente ao longo de toda a sua área de distribuição sendo capturada quer pela pesca artesanal quer pelo arrasto. Esta espécie é largamente consumida, principalmente devido ao seu baixo custo e, devido a este facto, é frequentemente procurada por pessoas de classes económicas mais baixas. Até ao momento, os trabalhos efectuados na presente espécie foram realizados principalmente no Mar Mediterrâneo e no Oceano Índico Ocidental, e tiveram como principais objectivos o estudo dos hábitos e conteúdos alimentares, da distribuição espacial, do crescimento e da biologia da reprodução. Pelo contrário, poucos estudos foram publicados para o Oceano Atlântico Oriental. Apesar de a espécie ser explorada comercialmente, não existem dados sobre as diferentes populações que podem existir ao longo da sua área de distribuição e também não existem medidas de gestão tendo em vista a sua sustentabilidade pesqueira.

Deste modo, o presente estudo teve como principais objectivos definir a estrutura do manancial de *S. salpa* no sul do Nordeste Atlântico e caracterizar a biologia da população de Portugal continental. Esta tese é composta por seis capítulos, quatro dos quais referentes a artigos científicos, publicados ou em revisão em revistas internacionais de arbitragem científica.

No capítulo 1, introdução geral, é apresentado o enquadramento do tema da presente tese, destacando os principais aspectos sobre o conhecimento actual em relação à biologia, ecologia e pesca de *S. salpa*.

De forma a cumprir os objectivos propostos, a presente tese foi dividida em duas Partes: Parte I - Estrutura do manancial e Parte II – Biologia. Os capítulos 2 (morfometria do corpo e do otólito) e 3 (análise genética) estão incluídos na Parte I, e os capítulos 4 (idade, crescimento e reprodução) e 5 (estruturas císticas) estão incluídos na Parte II.

Para a Parte I, foram escolhidos diferentes métodos para detectar a estruturação do manancial de *S. salpa* no sul do Nordeste Atlântico, de modo a implementar uma abordagem holística para responder ao primeiro objectivo da tese.

No capítulo 2 foram usados indivíduos capturados ao longo de Portugal continental (norte, centro e sul) e no Arquipélago da Madeira, e encontram-se descritas as técnicas morfo-geométricas aplicadas à forma do corpo e a análise elíptica de Fourier aplicada ao contorno do otólito. Ambas as técnicas indicaram alguma sobreposição dos indivíduos de Portugal continental, especialmente entre o centro e o sul, e uma forte separação dos indivíduos da Madeira. Os resultados indicados por ambas as técnicas sugerem que devem existir pelo menos dois mananciais, um na Madeira e outro em Portugal continental; no entanto, os resultados não foram claros quanto à existência de um segundo manancial no norte do país. A existência de mananciais, colocada em evidência pelas análises morfológicas, indica que os indivíduos foram sujeitos à acção de divergência genética ou à adaptação em locais com condições ambientais particulares, sendo esta última hipótese a mais aplicável no presente caso.

No capítulo 3 foram analisados indivíduos de 11 locais de amostragem distintos distribuídos pelo Oceano Atlântico nordeste [quatro regiões de Portugal Continental (Apúlia, Peniche, Setúbal, Algarve), Marrocos, Açores e Madeira] e pelo Mar Mediterrâneo (Espanha, Itália, Croácia e Grécia). O padrão filogeográfico foi analisado através de 109 sequências da região de controlo do ADN mitocondrial e através de 86 sequências do primeiro intrão da proteína ribossomal S7 do gene nuclear. A análise dos dados mitocondriais e nucleares encontra-se descrita no capítulo 3 e os principais resultados obtidos indicaram que: a) não há evidência de estruturação do manancial entre os indivíduos amostrados no Oceano Atlântico e no Mar Mediterrâneo; b) actualmente existe uma redução no fluxo de genes entre os locais de amostragem; c) há um sinal de expansão dos possíveis locais de refúgio na costa de Africa, com uma acumulação de mutações *in situ*; e d) a introgressão de ADN mitocondrial e nucleares aparenta ser antiga, existente muito provavelmente antes da expansão ter ocorrido. O facto da análise genética não ter evidenciado nenhum nível de estruturação do manancial entre os indivíduos amostrados no Oceano Atlântico e no Mar Mediterrâneo pode ser devida ao pequeno número de indivíduos utilizados ou ao poder dos marcadores genéticos seleccionados não ser apropriado para detectar subdivisões em *S. salpa*.

A Parte II corresponde à resposta ao segundo objectivo proposto, caraterizando-se pela descrição dos parâmetros biológicos da população de *S. salpa* de Portugal continental.

Para o capítulo 4 foram obtidas amostras comerciais de *S. salpa*, capturadas em Portugal continental, num total de 904 indivíduos. As idades estimadas, pela leitura

directa de otólitos inteiros, variaram entre os zero (5.2 cm) e os 14 anos (41.4 cm). A análise do padrão de crescimento dos incrementos detectou um incremento falso, entre os 1.08 e os 1.25 mm, que foi designado por anel juvenil; este anel foi encontrado em todos os indivíduos, inclusive nos indivíduos de menor dimensão (que variaram entre 5.2 e 9.8 cm), e deve corresponder à migração dos juvenis para uma área de berçário. Os parâmetros de crescimento de von Bertalanffy foram estimados pela leitura directa e pelo retrocálculo e, de acordo com o valor informativo de Akaike, o segundo método foi o que melhor descreveu o crescimento de *S. salpa*. Esta espécie é hermafrodita protândrica, tendo o processo de mudança de sexo dos indivíduos amostrados ocorrido entre os 28.6 e os 40.9 cm. Foram determinados alguns parâmetros de história de vida tais como a época de reprodução (que decorreu entre Setembro e Novembro), o comprimento de primeira maturação para os machos (estimado em 24.5 cm, o que corresponde a uma idade de primeira maturação de 2 anos); o tipo de fecundidade (determinada) e a fecundidade relativa anual (variou entre 462 e 2 662 oócitos por grama de fêmea eviscerada). Este capítulo contém informações fundamentais sobre os parâmetros de história de vida de *S. salpa* capturada em Portugal continental, onde até ao momento, não existia nenhuma informação deste tipo. Por exemplo, o tamanho de captura mínimo permitido em águas portuguesas, para *S. salpa*, é de 18 cm, inferior ao comprimento de primeira maturação estimado para os machos, o que significa que existe uma necessidade real de modificar o actual tamanho de captura mínimo permitido.

No capítulo 5 encontra-se descrita a presença de estruturas rígidas, facilmente observáveis a olho nu na análise dos ovários. Tendo em conta que não existia

nenhuma referência para tal ocorrência nos ovários de *S. salpa*, estas estruturas foram descritas morfologicamente e analisadas estatisticamente. A análise histológica sugeriu que estas estruturas correspondem a oócitos hidratados remanescentes que aparecem isoladamente ou em grupo, formando uma estrutura cística e que podem corresponder a um evento de desova incompleto ou sem sucesso. As estruturas císticas foram encontradas frequentemente (em todos os meses excepto em Outubro) e com valores médios de prevalência mais elevados nos meses que antecedem a época de reprodução. A presença de oócitos hidratados remanescentes em ovários de peixes, documentados como isolados ou agregados em cistos, é considerada rara e encontra-se deficientemente documentada mas, no presente caso, os resultados sugerem que as estruturas císticas possam fazer parte da estratégia reproductiva de *S. salpa*.

Finalmente, no capítulo 6 é apresentada uma discussão geral que inclui os principais resultados obtidos nos capítulos anteriores e, como conclusão, também são apresentadas algumas propostas de linhas de investigação futura.

List of papers

This thesis is comprised by the papers listed below, each corresponding to a chapter, from 2 to 5. The author of this thesis is the first author in all papers and was responsible for conception and design of the work, field surveys, sample collection and processing, laboratory analytical procedures, data analysis and manuscript writing of all the papers. Remaining authors collaborated in some or several of these procedures. All papers published were included with the publishers' agreement.

CHAPTER 2 - Morphometric analysis

Body geometric morphometric and otolith shape analysis: Complementary tools for stock discrimination of *Sarpa salpa* in the Northeast Atlantic Ocean

Rafaela Barros Paiva, Vera Sequeira, Ana Neves, Ana Rita Vieira, Maria José Costa and Leonel Serrano Gordo

Submitted to Helgoland Marine Research

CHAPTER 3 - Genetic analysis

Post glacial expansion and current reduced gene flow in salema population *Sarpa salpa* (Linnaeus 1758) (family Sparidae)

Rafaela Barros Paiva, Ana Sofia Rodrigues, Ana Neves, Vera Sequeira, Ana Rita Vieira, Carla Ribeiro Da Silva, Francisco Pina-Martins, Maria José Costa, Leonel Serrano Gordo and Octávio S. Paulo

Submitted to Journal of Biogeography

CHAPTER 4 - Age, growth and reproduction

Age, growth and reproduction of the protandrous hermaphrodite fish, *Sarpa salpa*, from the Portuguese continental coast

Rafaela Barros Paiva, Ana Neves, Vera Sequeira, Ana Rita Vieira, Maria José Costa and Leonel Serrano Gordo

Accepted in the Journal of the Marine Biological Association UK

CHAPTER 5 - Cystic structures

Cystic structures in fish ovaries: more common than we think - the case study *of Sarpa salpa* (Sparidae)

Rafaela Barros Paiva, Ana Neves, Ana Rita Vieira, Vera Sequeira, Catarina Vendrell, Maria José Costa, Maria Peleteiro and Leonel Serrano Gordo

Published in Cybium (2014) 38(2): 158 - 160.

CHAPTER 1

General introduction

Objectives and thesis structure

General introduction

Sarpa salpa (Linnaeus 1758) (Figure 1) belongs to the Family Sparidae that consists of approximately 115 species in 33 genera. This Family is widely distributed in tropical and temperate coastal waters, includes a large number of species of high economic value (Herrán *et al*., 2001) and constitutes one of the most important fish resources in the Atlantic Ocean and in the Mediterranean Sea. *S. salpa* is the only species present in the Genus *Sarpa* and is commonly known as salema, salema porgy, cow bream or goldline (Russell *et al*., 2014). Morphologically is characterized by an oval to oblong body, is laterally compressed, with greenish to blue colors back , silvery on the sides and a dozen of longitudinal lines yellow (Vitale *et al.*, 2002).

Figure 1. *Sarpa salpa* individuals.
(Available at: www.skaphandrus.com)

S. salpa is a benthopelagic oceanodromus marine fish that lives in shallow waters (up to 70 meters) and inhabits predominantly rocky areas with algal or seagrass coverage, such as *Posidonia oceanica* and *Cymodocea nodosa*, and also sandy bottoms (Villamil *et al*., 2002; Criscoli *et al*., 2006; Pallaoro *et al*., 2008). The young fish are carnivorous and the adults are almost exclusively herbivorous, being considered a species with an age related mix diet (Havelange *et al*., 1997). In the Mediterranean Sea

this species is responsible for up to 75 % of the total herbivorous consumption (Jadot *et al*., 2006) and the daily requirements of vegetable food is about 6 - 20 % of its body weight (Criscoli *et al*., 2006). In addition, its ecological importance is well recognized while the economic value of *S. salpa* in fisheries is quite limited (Abecasis *et al*., 2012).

As most species of Sparidae family, *S. salpa* presents a hermaphroditic behavior. Two patterns of functional hermaphroditism are recognized in fishes, simultaneous, when both eggs and sperm mature at the same time, and sequential, when fish functions as either male or female at any one time (Price, 1984). Sequential hermaphrodites may be either protogynous, individuals mature as females and then transform into males, or protandrous, males are the initial sex and females the terminal sex (Mitcheson and Liu, 2008). Initially, this species was described as both protandrous (D'Ancona, 1949; Lissia-Frau, 1966) and as a rudimentary hermaphrodite (Michèle and Lafaurie, 1974; Joubert, 1981) but nowadays is characterized by protandric hermaphroditism (Walt and Mann, 1998).

It is a widely distributed species (Figure 2), occurring along the Mediterranean (Jadot *et al*., 2006) and Black Sea (Pashkov and Reshetnikov, 2012), in the eastern Atlantic (from the Bay of Biscay to Cape of Good Hope) and in the Western Indian Ocean (from Mozambique to Cape of Good Hope) (Walt and Mann, 1998). Additionally, this species is gregarious, sometimes forming sizeable schools, often reaching hundreds of individuals (Gera *et al*., 2013; Russell *et al*., 2014). *S. salpa* is used for human consumption by coastal fishery populations being captured by artisanal fisheries in the west coast of Africa (Walt and Beckley, 1997), Canary Islands (Villamil *et al*., 2002) and Azores archipelago (INE, 2011), and by trawl and artisanal fisheries in

Portuguese mainland (INE, 2011) and in the Mediterranean Sea (Criscoli *et al*., 2006). In Portugal, the species has a low commercial value which reflects their low importance in the fisheries statistics, being the maximum landings obtained in 1991 with a value of 364 tonnes (Figure 3) (DGRM, 2015). These values are much lower when compared with landings in the Mediterranean Sea where Food and Agriculture Organization (FAO) landing statistics show a steady increase over the last 50 years, with a peak in the early 1990s at around 4 000 tonnes and stabilizing at around 2 000 tonnes during the period from 1996 to 2005 (Russel *et al*., 2014).

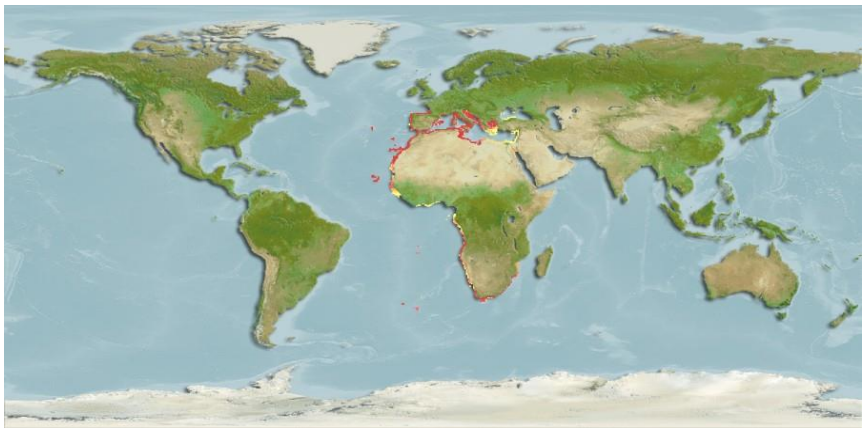

Figure 2. Distribution map of *Sarpa salpa*. (Available at: www.fishbase.com)

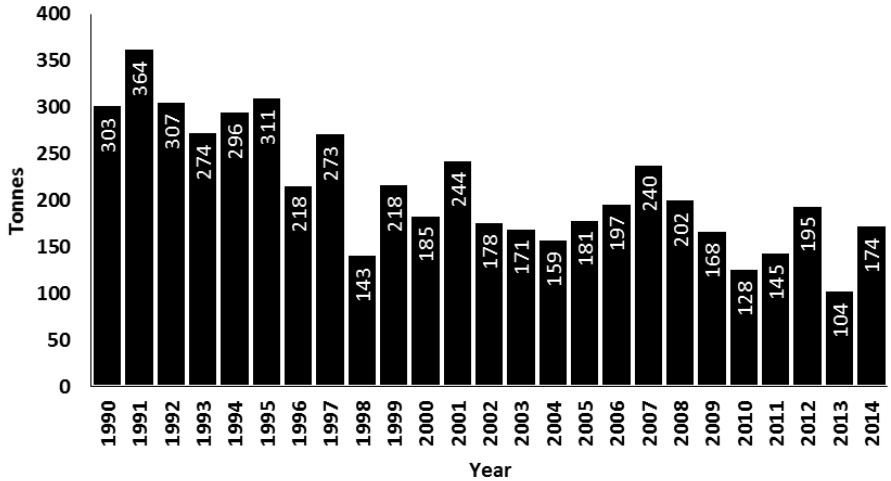

Figure 3. Annual landings of *Salpa salpa* in the Portuguese waters. (DGRM, 2015)

Due to its low cost, *S. salpa* is frequently found in the menu of the lower income classes (Bellassoued *et al*., 2014). Furthermore, some human intoxication cases have been described after their consumption (Spanier *et al*., 1989) because the species may ingest *P. oceanica* as part of their diet, which can contain ciguatera toxins (Bellassoued *et al*., 2014) originating the so-called ciguatera fish poisoning (CFP). However it must be said that these cases are extremely rare. Fish contaminated with ciguatera toxins were first captured in 2004 at the Canary Islands, then in 2008 at Selvagens Islands (Madeira Archipelago), and again in Canary Islands (Vale, 2011). Ciguatera toxins affect the central nervous system and cause a hallucinatory syndrome (Bellassoued *et al*., 2014).

Until now, investigation on *S. salpa* occurred mainly on the Mediterranean sea and in the Indian Ocean focusing on feeding habits (Gerking, 1984; Antolic *et al*., 1994; Havelange *et al*., 1997), spatial distribution (Dulčić *et al*., 1997; Ruitton *et al*., 2000; Jadot *et al*., 2006), and growth and reproductive biology (e.g. Walt and Beckley, 1997; Walt and Mann, 1998; Criscoli *et al*., 2006; Pallaoro *et al*., 2008; El-Etreby *et al*., 2015). On the contrary, little information has been published from Atlantic Ocean: Villamil *et al*. (2002) gave some information on its growth and reproductive biology in the Canary Islands, and behavior studies were made in a coastal lagoon in the south of Portugal by Abecasis *et al*. (2012).

The essential population parameters, such as abundance, age, growth, survival, reproduction, maturity and recruitment, are also called life history parameters (Begg, 2005) and their estimation can be used to provide baseline data to be applied in ecological modelling, to inform and improve the fisheries management and for stock

assessments. For stock identification, in addition to the life history parameters, other different methods can be used: natural markers (body and otolith morphometrics, meristics, genetics, parasites, otolith elemental composition, and fatty acid profiles) and applied marks (internal and external tags, electronic tags, and otolith thermal marking) (Cañás *et al*., 2012). The expression of these methods are under the control of environmental and genetic factors or by the combination of both factors; environmental factors tend to influence phenotypic characteristics within stock although, phenotypic differences do not provide direct evidence of genetic isolation between stocks (Begg and Waldman, 1999). This is because many individual characteristics are phenotypically plastic whereby a single genotype can develop different phenotypes in different environments (Heino, 2014).

The incorporation of multiple stock identification methods (holistic approach) has several advantages to single approach studies (Begg and Waldman, 1999): one of these techniques may detect stock structure where others fail to do so; greater confidence is obtained when contrasting approaches provide congruent results; additional levels of stock structure may be observed with approaches that offer different sensitivities; and finally, multiple applications of discrete approaches, to stock identification problems, provide more empirical information on the relative merits of alternative approaches to this still developing field (Waldman, 2005). Begg and Waldman (1999) recommended the use of at least a genetic procedure and at least one phenotypic-based approach for an integrated stock identification. And the ideal approach is to apply multiple stock identification methods to the same sample of fish and considerer the results using a multidisciplinary perspective (Cadrin and Secor,

2009). In the present thesis, three techniques were selected: body and otolith morphometrics and genetics. Body and otolith morphometrics are the two most frequently employed and cost-effective methods (Begg and Waldman, 1999; Burke *et al*., 2008; Sajina *et al*., 2011). There was a great improvement from the body traditional morphometric methods to the actual technique of landmark-based geometric morphometric, which enables a more powerful stock identification allowing better data collection, suitable descriptions of shape and the potential of new analytical techniques (Cadrin and Friedland, 1999; Cadrin, 2000). The otolith morphology is considered species-specific and the most routinely method used to describe it involve elliptical Fourier series analysis (Leguá *et al*., 2013; Stransky, 2014). The utilization of elliptical Fourier analysis surpasses some of the limitations of conventional Fourier analysis: equal divisions over the interval sampled; dependency of the coordinate system; and the difficulty of dealing with the outlines that curve back on themselves (Lestrel, 1989). Genetic markers are ideal stock discriminators because they are: a) independent of environmental changes during the course of an individual's lifetime; b) composed of discrete units of information (so that population differences can be readily quantified); c) encoded the universal language of DNA (which is applicable to all forms of life); d) measurable with reasonable efforts and costs; and e) analyzed with statistical procedures that provide estimates of error associated with the process (Antoniou and Magoulas, 2014).

Despite the commercial exploitation of the fishery, no information is available on the different populations that may exist along the distribution area, being the species treated, for managing purposes, as one stock unit and no management

measures have ever been implemented towards the sustainable use of this resource. Furthermore, a comprehensive study of *S. salpa* life history from the Atlantic Ocean does not exist. Taking into account the poor knowledge on the biology of *S. salpa* in the eastern Atlantic, it becomes imperative to develop studies in order to understand the species dynamics towards the understanding of its stock structure.

Objectives and thesis structure

This thesis has two main goals: G1) to define the stock structure of *S. salpa* in the eastern Atlantic, and G2) to characterize the biology of the Portuguese mainland population. The thesis includes four scientific papers published, in review or submitted in peer reviewed international journals, each corresponding to a chapter.

Chapter 1 comprises the present general introduction, highlighting the main aspects regarding the present knowledge on *S. salpa* biology, ecology and fisheries.

To accomplish G1 the application of different methodological approaches in different sampling sites, covering the distribution area, were proposed: body and otolith morphometrics, and genetic analysis. These approaches are included in PART I - Stock structure and comprise two chapters: CHAPTER 2, Morphometric analysis, and CHAPTER 3, Genetic analysis.

G2 will be achieved through the analysis of biological parameters of *S. salpa* from Portuguese mainland and were included in PART II – Biology. This second part comprises two chapters: CHAPTER 4, Age, growth and reproduction, and CHAPTER 5, Cystic structures.

Finally, Chapter 6 encompasses a general discussion, covering the results attained throughout the thesis and some future research objectives are considered.

The results expected in this thesis will allow the elaboration of a database with the most relevant information for future assessment of the species through the identification of stock units. In Portuguese mainland, this database will benefit from information from both the fishery (available from Portuguese directorate of fisheries and aquaculture) and the biological parameters. This database will allow the implementation of reliable assessment models, which can lead to the proposal of specific and adequate management measures.

PART I

Stock structure

CHAPTER 2

Morphometric analysis

Body geometric morphometric and otolith shape analysis: Complementary tools for stock discrimination of *Sarpa salpa* in the Northeast Atlantic Ocean

SUMMARY

Understanding the stock structure of the species is fundamental to define appropriate management strategies in fisheries. Body geometric morphometric techniques and elliptical Fourier analysis were applied on salema, *Sarpa salpa*, individuals from three areas along the mainland coast of Portugal (north, central, and south regions) and from Madeira waters, in order to clarify the stock structure. Both techniques indicated some overlapped in the mainland specimens, especially between the central and south areas, and a clear separation of Madeira individuals. Concerning the body geometric morphometric analysis, individuals were highly correctly classified to geographic areas (86.7 %), being specimens from Madeira and the north mainland well differentiated from the other areas; on the contrary, specimens from central and south mainland which are geographically closer showed some overlap. For the otolith shape data analyses, size-corrected normalized elliptic Fourier descriptors showed significant effect of area on otolith contour shape. An overall classification success of 70.2 % on the canonical discriminant analyses was achieved suggesting populations discrimination on Portuguese waters, although an overlap between the three areas along the mainland coast, particularly between central and south areas, was present. The results support the existence of two or three stocks of *S. salpa* in the southern Northeast Atlantic.

INTRODUCTION

Modern fisheries management is moving towards a precautionary approach to ensure sustainable use of the marine resources (ICES, 1998; Sajina *et al*., 2011). To achieve this goal, the knowledge of stock structure is essential for designing appropriate management regulations in fisheries where multiple stocks are differentially exploited (Waldman, 2005). According to Begg and Waldman (1999), the term stock is defined as

a population or portions of a population, in which all members are characterized by similarities, and which may include members of several different subpopulations.

Stock identification employs many techniques, such as, traditional tags, parasites as natural tags, otolith microchemistry, genetic markers, meristics, morphometric characters and otolith shape, being the latter two most frequently employed and cost-effective methods (Begg and Waldman, 1999; Burke *et al*., 2008; Sajina *et al*., 2011). There was a great improvement from the traditional morphometric methods to the actual technique of landmark-based geometric morphometric, which enables a more powerful stock identification allowing better data collection, suitable descriptions of shape and the potential of new analytical techniques (Cadrin and Friedland, 1999; Cadrin, 2000). Morphometric expression is simultaneous under the control of genetic and environmental factors (Begg and Waldman, 1999), such as temperature, salinity, radiation, dissolved oxygen, water depth and current flow (Turan *et al*., 2006). A large number of authors successfully implemented this technique discriminating populations of fish species, e.g. sea bream (*Sparus aurata*) (Loy *et al*., 1999), topsmelt silverside (*Atherinops affins*) (O'Reilly and Horn, 2004), Pacific sardine (*Sardinops sagax*) (García-Rodríguez *et al*., 2011), bluemouth (*Helicolenus dactylopterus*) (Rodríguez-Mendoza *et al*., 2011; Sequeira *et al*., 2011), beaked redfish (*Sebastes mentella*) (Valentin *et al*., 2014), acadian redfish (*Sebastes fasciatus*) (Valentin *et al*., 2014).

Otoliths are hard, calcium carbonate structures located directly behind the brain of teleost fish, and they help with balance, orientation, and sound detection-much like the inner ear of mammals (Wright *et al*., 2002). Otolith shape is considered to be species-specific and the mechanisms causing intraspecific variation in otolith shape are

thought to be related to variation in environmental conditions, food supply and also genetic dissimilarities (Arechavala-Lopez *et al*., 2012; Hamer *et al*., 2012). Despite these influences, it cannot be infer between genetic and environmental differences but otolith shape can provide a phenotypic basis for stock separation (Pothin *et al*., 2006). Its use in distinguishing stocks of the same species have been applied in diverse studies (Wakefield *et al*., 2014), with levels of classification success ranging from 60 to 95 % (Burke *et al*., 2008). Stock identification is a central theme in fisheries science and is a prerequisite for the tasks of stock assessment and fishery management (Cadrin *et al*., 2014), because high exploitation combined with ineffective fisheries management, may result in depletion of fish stocks (Turan, 2006).

Salema, *Sarpa salpa* (Linnaeus 1758), is an eurytherm fish that lives in shallow waters and predominantly inhabits sandy bottoms and seagrass beds between 0 and 70 meters (Villamil *et al*., 2002). It is a widely distributed species, occurring throughout the Mediterranean (Jadot *et al*., 2006) and Black Sea (Pashkov and Reshetnikov, 2012), and in eastern Atlantic from the Bay of Biscay to South Africa (Walt and Mann, 1998). This species is a protandrous hermaphrodite, changing from male to female through a nonfunctional intersexual phase (Criscoli *et al*., 2006). Until now, investigation on *S. salpa* occurred mainly on the Mediterranean region focusing on feeding habits (Gerking, 1984; Antolic *et al*., 1994; Havelange *et al*., 1997) and spatial distribution (Dulčić *et al*., 1997; Ruitton *et al*., 2000; Jadot *et al*., 2006). In the Atlantic, information is only available for the Canary Islands regarding life history pattern (Villamil *et al*., 2002).

The Sparidae family constitutes one of the most important fish resources in the Atlantic Ocean and in the Mediterranean Sea. In Portuguese waters alone, the annual

landings of sparid fishes have reached 5 917 tonnes during 2014, in which 227 tonnes are of *S. salpa* (INE, 2015). This species is used for human consumption by coastal fishery populations being captured by artisanal fisheries in the west coast of Africa (Walt and Beckley, 1997), Canary Islands (Villamil *et al*., 2002) and Azores archipelago (INE, 2011), and by trawl and artisanal fisheries in Portuguese mainland (INE, 2011) and in the Mediterranean Sea (Criscoli *et al*., 2006). Despite the commercial exploitation of the fishery, no information is available on the different populations that may exist along the distribution area, being the species treated, for managing purposes, as one stock unit and no management measures have ever been implemented towards the sustainable use of this resource.

The present study investigates the stock structure of *S. salpa* population in Portuguese waters analyzing individuals from three areas along the mainland coast of Portugal (north region - Esposende, central region - Setúbal, and south region - Algarve) and from Madeira waters, using geometric morphometric techniques and elliptical Fourier analysis which could be useful to further develop a management strategy.

MATERIALS AND METHODS

Sampling

A total of 166 individuals were sampled: 131 from de Portuguese mainland (32 from the north region, 54 from the central region, and 45 from the south region) and 35 from Madeira (Figure 4; Table 1). Adult individuals were obtained from commercial andings caught by local artisanal fisheries from June to November 2013, mainly during

the spawning season.

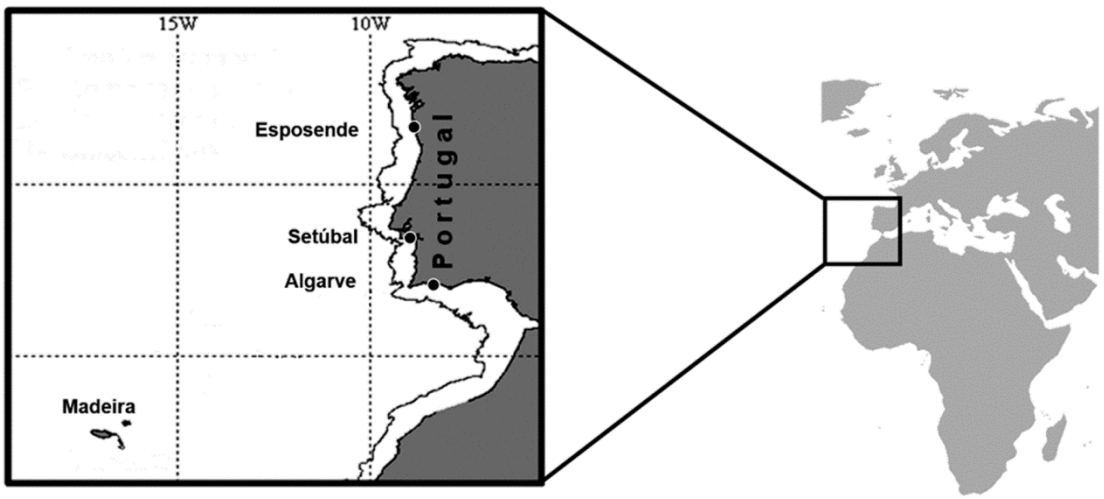

Figure 4. Map of the sampling area with the sampling sites in the Northeast Atlantic: three areas along the mainland coast of Portugal (north mainland - Esposende, central mainland - Setúbal, and south mainland - Algarve) and other in Madeira.

Table 1. Details of *Sarpa salpa* individuals analyzed in each study area.

	N_{bgm}	N_{os}	Size range (TL, cm)	Mean TL (cm) ± standard deviation
North mainland	32	32	35.9 - 44.2	40.2 ± 2.19
Central mainland	54	52	29.3 - 35.6	32.8 ± 1.18
South mainland	45	45	29.3 - 40.0	35.2 ± 3.07
Madeira	35	32	18.2 - 28.9	21.9 ± 5.68
Total	166	161		

TL, total length; N_{bgm}, total number of individuals used in the body geometric morphometric analysis, N_{os}, total number of otoliths used in the otolith shape analysis

All samples were placed individually into plastic bags, deep-frozen (-20ºC) and stored in a horizontal position to avoid any deformation of the body until the time of the analysis. Total length (TL, ± 0.1 cm) was recorded for each fish. As suggested by Tuset *et al*. (2006), an analysis by sex within the geographic areas was not performed because the present species is a protandrous hermaphrodite.

Body geometric morphometric analysis

Landmark capture

To take digital images, the individuals were placed on a box with sand to overcome the body roundness. The fins were erected to make the origin and insertion points visible and each individual was labeled with a specific identification code. A total of 17 anatomic landmarks were selected (Figure 5) to provide an adequate coverage of body shape, and they were defined based on previous works made on Sparidae family species (Loy *et al*., 2000; Palma and Andrade, 2002; Costa and Cataudella, 2007; Antonucci *et al*., 2009). The left side of each fish was photographed using a Canon EOS 1100D 12.2 Mega Pixels digital camera on a tripod, with a fixed focal length of 53 mm to avoid optic distortions of the images, and a ruler was placed next to each specimen to obtain scaling information. Individuals from the four areas were randomly selected to image acquisition to avoid any bias related to geographical area and in the way the operator performed his tasks (Fruciano *et al*., 2012). The Cartesian coordinates of each anatomical mark were obtained using the software tpsDig version 2.10 (Rohlf, 2006).

Geometric morphometrics

The body shape was analyzed using landmark-based geometric morphometric methods described by Rohlf (1990) and Bookstein (1991). A superimposition method based on generalized Procrustes analysis to unit centroid size (Rohlf, 1990; Zelditch *et al*., 2004; Valentin *et al*., 2014) was used to remove differences attributed to the position, orientation and scale between homologous landmarks of all individuals, using

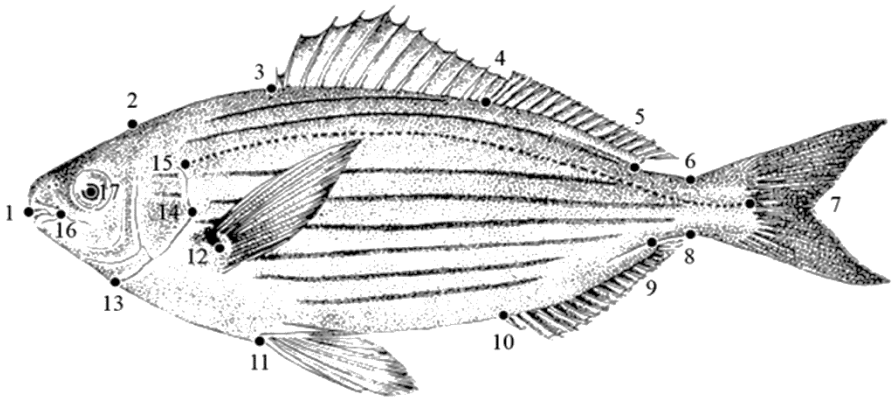

Figure 5. Landmarks used on *Sarpa salpa* geometric morphometric analysis. (1) anterior tip of the snout; (2) posterior point of the neurocranium: (3) and (5) origin and posterior end of the dorsal fin; (4) first soft ray of the dorsal fin; (6) and (8) points of maximum curvature of caudal peduncle; (7) end of lateral line; (9) and (10) insertion and origin of the anal fin; (11) origin of the pelvic fin; (12) mid-point of pectoral fin insertion; (13) insertion of the operculum on the lateral profile; (14) posterior limit of the operculum; (15) origin of lateral line; (16) posterior extremity of pre-maxillary; (17) centre of the eye. (Source: Food and Agriculture Organization of the United Nations)

the Morpho J software package (Klingenberg, 2011). The new Procrustes coordinates were used for the subsequent analysis.

Since the size composition was different among geographical areas (Table 1), a multivariate regression of the Procrustes coordinates on centroid size (described in detail by Monteiro (1999)) was used to estimate and remove the effect of size on the shape of the individuals. A percentage of the total variation around the sample mean was chosen to show the amount of shape variation for which each regression accounted. To test the null hypothesis of independence between shape and size a permutation test using 10,000 runs was applied (Good, 1994), using the Morpho J software package. The residuals of this regression were then used as 'size-free' variables in subsequent statistical analysis.

Statistical analyses

Multivariate analysis of variance (MANOVA) was calculated to test the interaction between geographic areas and TL, using the software IBM SPSS Statistics 22.0 (SPSS Inc.). The multivariate measures used as indicator of significance were Wilks' lambda (λ) (Wilks, 1932) and Pillai's trace (Pillai, 1955).

A canonical variate analysis (CVA) was performed to detect morphometric differences in body shape of *S. salpa* between areas and to study the possible use of body shape in the classification of individuals regarding their origin. Procrustes distances were used in pairwise comparisons of mean shapes from different locations. To test the null hypothesis of no difference between samples, a permutation test with 10,000 runs was performed using the Morpho J software package. Visualization of mean shapes from different locations was executed using warped outline drawings, performed with the wireframe function of Morpho J software package.

Discriminant analysis was performed using the software IBM SPSS Statistics 22.0 (SPSS Inc.) to calculate the jackknife cross-validation procedure (described by Ripley (1996)).

To access the discriminatory effectiveness of the analysis Wilks' λ and the Cohen's Kappa (Κ) (Cohen, 1960) were calculated.

A significance level of 0.05 was used for all the statistical analysis.

Otolith shape analysis

Otolith extraction and image acquisition

Sagittae otoliths (thereafter referred as otoliths) were extracted, cleaned in distilled water, dried and kept in tubes. A total of 161 otoliths were used in the analysis.

To maintain consistency and avoid asymmetric effects, only the left otolith was used for shape analysis. Each otolith was systematically placed on a dark background, immersed in a 1:1 glycerin-alcohol solution, positioned with the *sulcus acusticus* down and the anterior region to the left, and was examined under a Wild Heerbrugg stereomicroscope connected with a high-resolution camera (Leica DFC 290) linked to a computer. Digital images of each otolith were then acquired with the free image acquisition software Irfan View 32. The magnification was kept constant at 9× for all the photographed otoliths.

Elliptical Fourier descriptors (EFDs)

The shape of each otolith was assessed with the elliptic Fourier descriptors (EFDs), proposed by Kuhl and Giardina (1982). EFDs can define any type of shape with a closed two-dimensional contour and it consists of the decomposition of a curve into a sum of harmonically related ellipse.

Each harmonic yields four coefficients that are used as input variables for standard multivariate statistics, such as discriminant analysis (Baylac and Frieβ, 2005). SHAPE software version 1.3 (Iwata and Ukai, 2002) was used to extract the contour shape of

the otoliths and to evaluate biological contour shapes based on the EFDs. The coefficients of the EFDs were subsequently normalized to be invariant with respect to size, rotation and starting point, with the procedure based on the ellipse of the first harmonic, which causes the degeneration of the first three coefficients (a1=1, b1=c1=0). A total of 100 harmonics were generated for each individual. In order to be able to use multivariate analysis the number of harmonics must be reduced. To determine the appropriate number of harmonics to adequately describe the otolith shape, a sub-sample of 60 otoliths (15 otoliths from each area) were randomly chosen; the threshold of 99 % of accumulated variance, suggested by Lestrel (1997), was used to select the minimum number of harmonics for the best reconstruction of the otolith outline. To visualize the shape differences between the geographic areas, average otolith shapes were plotted for each group by means of the reproduced outlines of the averaged normalized EFDs (NEFDs) within a group; these visualizations were made in the PrinPrint package of SHAPE software version 1.3.

Statistical analyses

The relationship between the NEFDs and fish TL was analyzed using Pearson's correlation coefficient (r) in all geographic areas. Following the example of Cañás *et al*. (2012), the standard residuals from a linear regression of each significantly correlated NEFD between fish TL were calculated for the whole data to eliminate the effect of the fish TL on the NEFDs. The normality and homoscedasticity of variance of the size-corrected NEFDs were tested ($p > 0.05$). The size-corrected NEFDs were then used as

'size-free' variables in subsequent statistical analysis. MANOVA was used to test for significant differences in size-corrected NEFDs between geographic areas. Only the size-corrected NEFDs that met the assumptions of normality and homoscedasticity were included in the MANOVA analysis; the geographic areas were used as independent variables and the normalized EFDs as the dependent variables. The multivariate measures used as indicator of significance were Wilks' λ and Pillai's trace.

Canonical discriminant analyses (CDA) were performed with the stepwise method and the jackknife cross-validation procedure was used to calculate an unbiased estimation of classification success. To access the discriminatory effectiveness of the analysis the Wilks' λ and the Cohen's Κ were calculated. All statistical analyses were performed using IBM SPSS Statistics 22.0 (SPSS Inc.) and a significant level of 0.05 was used.

RESULTS

Body geometric morphometric analysis

Despite obtaining different size compositions among geographical areas (Table 1), only a small amount of shape variation (7.2 % of total shape variation, P = 0.002), estimated by multivariate regression of the Procrustes coordinates on centroid size, was related to the size of individuals. Furthermore and according to MANOVA, shape differences between the interaction geographical area and TL were not significant (Wilks' λ = 0.043, $F_{(330.000,\ 1036.928)}$ = 1.113, p value = 0.111, η_p^2 = 0.248; Pillai = 2.540, $F_{(330.000,\ 1177.000)}$ = 1.071, p value = 0.212, η_p^2 = 0.231), which showed that different TL do not interfere in the analysis of body shape.

The analysis of mean body shapes of *S. salpa* from the four geographical areas showed statistical differences (Figure 6; Table 2). The first two canonical variates of the CVA explained 60.9 % and 27.2 % of the variance, respectively (Figure 6). The plot showed that north mainland and Madeira are well differentiated from the other locations while central and south mainland are geographically close showing some overlap.

Table 2. Procrustes distances between mean body shapes of *Sarpa salpa* individuals from the four study areas and the corresponded P value (in brackets) obtained from permutation tests (10,000 permutation runs).

	South mainland	**North mainland**	**Madeira**
North mainland	0.0301 (< 0.0001)	-	-
Madeira	0.0424 (< 0.0001)	0.0423 (< 0.0001)	-
Central mainland	0.0136 (< 0.0001)	0.0312 (< 0.0001)	0.0364 (< 0.0001)

Illustration of mean body shapes for *S. salpa* from the different locations (Figure 7) present marked differences in the head and pectoral region, affecting the position of snout, pre-maxillary, operculum and pectoral fin. Furthermore, individuals from central and south mainland presented a similar mean body shape (almost all the landmarks were overlapped) having only a slight difference in the insertion and origin of the anal fin.

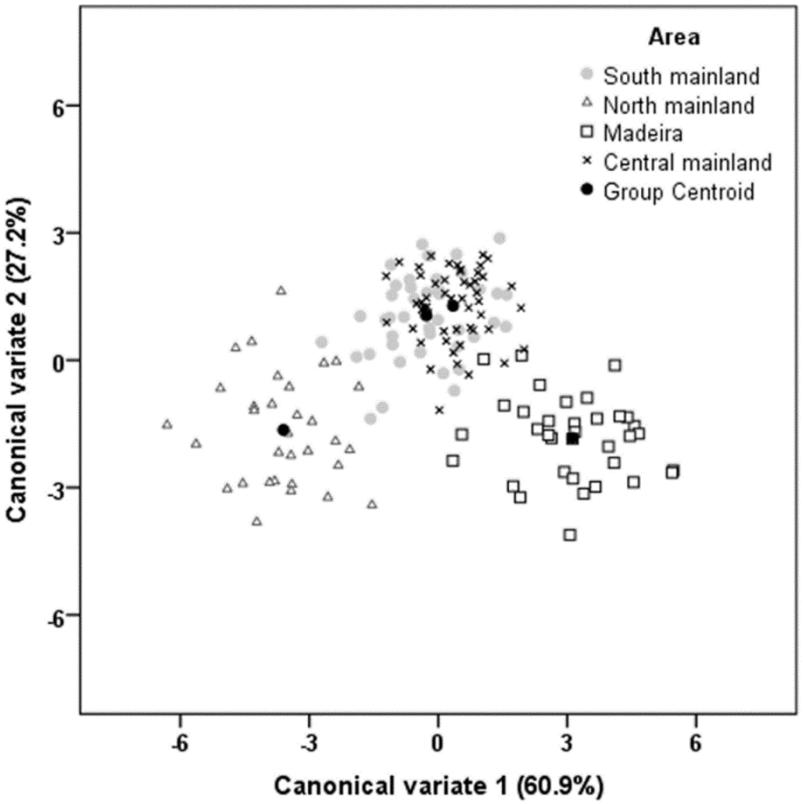

Figure 6. Two-dimensional ordination of *Sarpa salpa* individuals from the four locations based on canonical variate analysis (CVA).

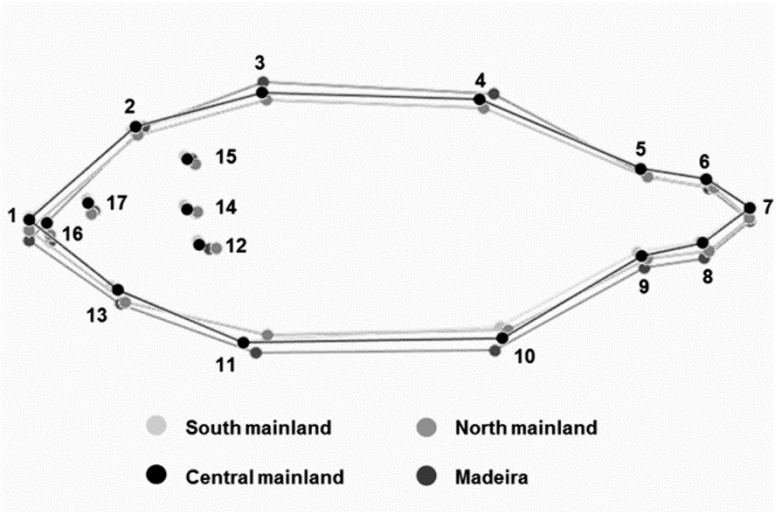

Figure 7. Illustration of mean body shapes for *Sarpa salpa* from the four locations, represented by a warped outline drawing.

An overall classification success of 86.7 % was achieved (Cohen's Κ = 0.820) (Table 3). High correct classifications rates were obtained with north mainland showing the highest (90.6 %) and south mainland the lowest (80.0 %) values. The percentage of individuals incorrectly classified between central and south mainland was considered low (< 17.8 %), even being geographically close.

Table 3. Jackknife classification matrix of the discriminant analysis between geographic areas performed on *Sarpa salpa* body shape analysis.

	N	South mainland	North mainland	Madeira	Central mainland
South mainland	45	**80.0** (36)	2.2 (1)	0.0 (0)	17.8 (8)
North mainland	32	6.3 (2)	**90.6** (29)	3.1 (1)	0.0 (0)
Madeira	35	0.0 (0)	0.0 (0)	**88.6** (31)	11.4 (4)
Central mainland	54	7.4 (4)	0.0 (0.0)	3.7 (2)	**88.9** (48)

Percentages in rows represent the classification into the areas given in columns (correct classification in bold). Number of individuals (N) allocated in each area are given in brackets. Overall classification success: 86.7 %, Wilks'λ = 0.029, Cohen's Κ = 0.820.

Otolith shape analysis

To visualize differences in average shapes, the reproduced outlines of the mean of NEFDs by geographic areas were plotted in Figure 8. It was determined that 13 harmonics were needed to represent the complex morphology of the otolith of *S. salpa*, as they were responsible for over 99 % of

Figure 8. Illustration of the average shapes for *Sarpa salpa*, from the different geographic areas, represented by the outlines of the mean normalized elliptic Fourier descriptors (NEFDs). (south mainland – light grey solid line; north mainland – dark grey solid line; Madeira – black solid line; central mainland - dark grey dot line)

the shape variation (Figure 9). So, 13 harmonics and thus 53 Fourier coefficients (one coefficient from the first harmonic and 52 coefficients from harmonics 2-14) were used for the data analyses.

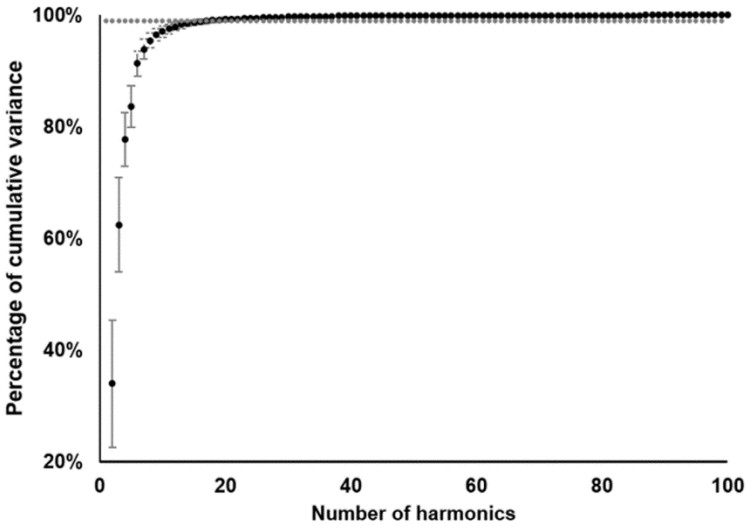

Figure 9. Mean and standard deviation of the cumulative variance of 100 harmonics for 60 otoliths randomly selected.

The relationship between the NEFDs and fish TL was analyzed using Pearson's correlation coefficient (r) in all geographic areas. From a total of 53 NEFDs used in the analysis, only two were significantly correlated ($p < 0.05$) with TL in North mainland and Madeira areas; one NEFDs in central mainland and ten in south mainland. MANOVA results to compare otolith size-corrected NEFDs from the four areas showed a significant effect of area on otolith contour shape differences (Wilks' $\lambda = 0.022$, $F_{(159.000, 315.688)} = 5.081$, p value < 0.01, $\eta_p^2 = 0.719$; Pillai = 1.954, $F_{(159.000, 321.000)} = 3.773$, p value < 0.01, $\eta_p^2 = 0.651$). The differences between the otolith contour shape among areas evidenced by the MANOVA were also indicated by the CDA. The variables used in the discriminant analysis are shown in Table 4. The results of CDA show that

differences in otolith contour were found between individuals from all areas although some overlap was observed. The CDA highlighted a significant discrimination between the mainland coast of Portugal and from Madeira waters (Figure 10). The first two axes of the CDA explained 81.0 % and 13.7 % of the variance, respectively; in general, these two axes accounted for > 90 % of the total dispersal of the groups. The CDA showed some overlap between the three areas along the mainland coast of Portugal, although north mainland area was more separated from the others. The overlap was more evident between central and south mainland's which are geographically closer.

Table 4. Jackknife classification matrix of the discriminant analysis between geographic areas performed on *Sarpa salpa* otolith contour shape.

	N	South mainland	North mainland	Madeira	Central mainland
South mainland	45	**51.1** (23)	8.9 (4)	2.2 (1)	37.8 (17)
North mainland	32	21.9 (7)	**68.8** (22)	0.0 (0)	9.4 (3)
Madeira	32	0.0 (0)	0.0 (0)	**100.0** (32)	0.0 (0)
Central mainland	52	21.2 (11)	7.7 (4)	1.9 (1)	**69.2** (36)
Variables		b2, a3, b3, b4, c4, d4, a5, b5, d5, a6, b6, c6, a7, d8, b9, c9, c12, c13, b14			

Percentages in rows represent the classification into the geographic areas given in columns (correct classification in bold). Number of individuals (N) allocated in each area are given in brackets. Overall classification success: 70.2 %, Wilks' λ = 0.063, Cohen's Κ = 0.596

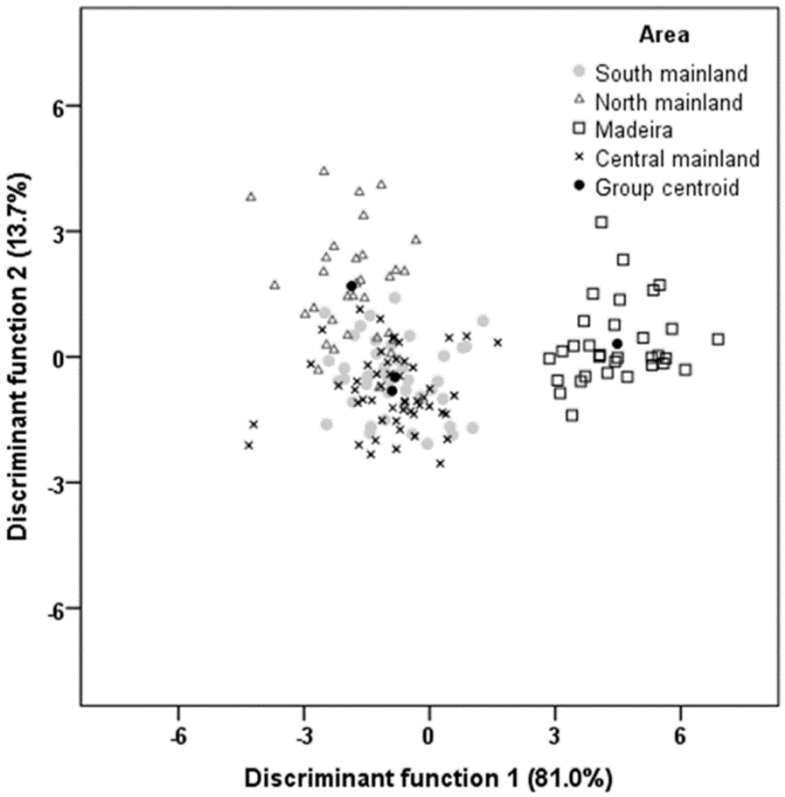

Figure 10. Two-dimensional ordination plot of *Sarpa salpa* individuals from the four geographic areas based on canonical discriminate analysis (CDA).

Overall jackknifed classification success was 70.2 %, ranging from 51.1 % correct classification for south mainland to 100 % for Madeira (Table 4). Differences between areas were significant (Wilks' $\lambda = 0.063$, $p < 0.001$) and the results from the Cohen's Κ procedure confirmed the classification success obtained by the discriminant analysis (Table 4).

DISCUSSION

Both techniques proved to be a powerful tool in stock identification as other authors have already shown (e.g. Rodríguez-Mendoza *et al*. (2011), Sequeira *et al*. (2011), Stransky *et al*. (2014)), providing a fast, reliable, and sustainable method of identifying

stocks, with lower costs associated (Burke *et al*., 2008). The expression of both techniques are under the simultaneous control of genetic and environmental factors, such as water temperature, salinity, food availability and type of substrate (Begg and Waldman, 1999; Vignon, 2012). Even in the absence of genetic differences, various environmental factors, (e.g. water temperature and food availability) (Vignon, 2012) are thought to influence fish growth rate that in turn can affect the body fish growth and the otolith growth, affecting the otolith shape, and producing different expressions.

Body form in fishes is a product of their ontogeny (Cadrin, 2005). It is affected by the genetic makeup of an individual, but it also reflects adaptations to the environmental characteristics of their ecological niches since associations between body shape and ecological variables are commonly observed among populations of related fishes (Swain *et al*., 2005). Shape analysis has become an efficient and powerful tool in stock identification (Stransky *et al*., 2014). It is less time-consuming, has lower running costs (with the software for carrying out the analysis once images are taken being freely available) and the procedure is also far less destructive to otoliths (only images of whole otoliths are used) (Burke *et al*., 2008). Therefore, and as stressed by Cardinale *et al*. (2004), otolith shape analysis can be seen as a complement technique towards an effective fisheries management.

The differences observed in the present study between the mean body shapes of the four areas, particularly in the head area, can also be due to particular adaptations to environmental conditions where each population lives. *S. salpa* is considered to be an herbivorous species, although it has an age-related mixed diet: the young fish are

mainly carnivorous (crustacean) while adults are almost exclusively herbivorous (Havelange *et al*., 1997). This adjustment in diet is certainly affected by the food resources available in the studied areas and the variations in mouth shape and position (anterior tip of the snout and posterior extremity of pre-maxillary) can be related to differences in diet (Cadrin, 2005). Love and Chase (2009) studied the scup (*Stenotomus chrysops*) (family Sparidae) populations from the north-western Atlantic Ocean and found that most morphological differences were related to forehead shape, which may not be related to important growth or reproductive characteristics of interest in stock assessments. For other species of the family Sparidae (e.g., squirefish *Chrysophrys auratus*), forehead shape naturally differs among populations and sexes (Love and Chase, 2009).

Both body shape and otolith shape analysis results showed differences in the individuals from the four geographic areas, although some overlap was observed between individuals from the three mainland areas, which was more evident between central and south areas, those that are geographically closer. It is known that fish distribution and recruitment rates are intrinsically linked to hydrographic conditions where the species lives (Keating *et al*., 2014), so the principal cause of these results may be the hydrological conditions that occur in the Portuguese coast. The Portuguese continental shelf is reached by a diversity of hydrological and oceanographic features characterized by narrow and large continental platform areas, and deep submarine canyons and valleys (Gomes, 2009). Canyons, in particular, potentially disrupt and influence the general faunal relationships between depth, latitude and surface productivity (King *et al*., 2008). From the overlap observed between the three areas

along the mainland coast of Portugal, for both analysis, specimens from the north area are more separated from those of center and southern areas and this fact can be explained by the presence of Nazaré canyon (39º 30' 36''N, 9º 55 12''W), a very deep canyon northwards Setúbal, which is coincident with the latitude of Merrett's faunal division (Merrett, 1987), being important in the separation of fish communities limiting the mixture of northern and central-southern communities (King *et al*., 2008).

Considering the Nazaré canyon as a latitudinal boundary that determines the distribution of fish assemblages from the north and central-southern areas of the Portuguese mainland, then the closest affinities between the specimens from the central and south mainland areas can be explained by the larval drift and the presence of the Portuguese current (the result of the combination of North Atlantic Drift Current and the Azores current) that has a characteristic movement north to south reaching the Canary Current (Neves *et al*., 2008). *S. salpa* is a marine fish that spawn pelagic eggs (Strydom *et al*., 2014), and had already been described that planktonic eggs and larvae can be dispersed over large areas due to transport by ocean currents, leading to a mixing of early life stages among spawning locations, which results in less stock structure (Hare, 2005). Pattrick and Strydom (2009) tested the swimming abilities of late-stage larvae, wild-caught in temperate South Africa, of cape white seabream (*Diplodus capensis*) and *S. salpa* in controlled swimming chambers and stated that the late-stage larvae of these Sparidae are strong swimmers. The observed overlap among the geographically closer areas, central and south mainland, can be explained by the mixing of early life stages, or by adult's migration, or some specimens from these two areas can be exposed to similar environmental force, or share genetic traits (Farias *et al*., 2009).

Regarding the results obtained for Madeira, the presence of natural geographical barriers may contribute largely to the differences between populations (Palma and Andrade, 2002). The strong separation between Madeira and the Portuguese mainland, obtained by the appliance of the two techniques, suggest that Madeira specimens must constitute a distinct stock. In fact, the unique environmental characteristics of Madeira, such as a volcanic island surrounded by large abyssal profundities (Torres and Andrade, 2010) inserted in a subtropical environment (the mean temperature of the surface water is 18°C in the winter months, and 21.8°C in the summer) (Moreira, 1988), might have contributed for the isolation of Madeira population.

In conclusion, the present results indicate the possibility of the existence of local populations of *S. salpa* that should be considered in the future management options. At least two stocks may occur, one in Madeira and the other in mainland waters however, and due to the low affinities between the northern population when compared with the individuals from central and southern areas, the presence of two distinct stocks in mainland waters may also be considered. Nevertheless, other studies should be implemented to confirm this hypothesis.

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CHAPTER 3

Genetic analysis

Post glacial expansion and current reduced gene flow in salema population *Sarpa salpa* (Linnaeus 1758) (family Sparidae)

SUMMARY

The oceanographic conditions may affect the genetic structure of marine populations and knowledge of their structure is fundamental to adequately manage marine fishes. In the present study, phylogeographic pattern of *Sarpa salpa* was analyzed using sequences from the mitochondrial control region and the first intron of the nuclear S7 ribosomal protein gene. The high haplotype and nucleotide diversity detected in the sampling areas probably resulted from an expansion from the possible refuges in the coast of Africa with accumulation of mutation *in situ*. The results suggest that *S. salpa* population is large, has the capacity to retain new mutations locally but currently there is no evidence of extensive gene flow between the populations as suggested by significant F_{ST} values. The analysis of molecular variance (AMOVA) indicated that no significant population genetic structure exists in the study areas. Additionally mitochondrial DNA showed introgression between *S. salpa* and Sparidae species, and this introgression seems to be very ancient, probably before the expansion. The results also showed a signal of nuclear introgression only in the Murcia individuals.

INTRODUCTION

The knowledge of population structure is fundamental to adequately manage marine fishes. The genetic structure of marine populations can be affected by the oceanographic conditions (Silva *et al*., 2010) mainly because the larval phase is extremely vulnerable to the environmental flux (Strydom *et al*., 2014). Newly hatched larvae are transported passively in the plankton, increasing the potential of dispersal over a broad geographical area (Gilg and Hilbish, 2003; Strydom *et al*., 2014).

A high potential for dispersal and an absence of barriers to migration are believed to cause high connectivity even between distant populations, preventing long term population subdivision in the marine environment; on the other hand, individuals with a short larval phase is expected to have a low dispersal ability and limited genetic exchange between populations (Palumbi, 1994; Bargelloni *et al*., 2003).

The potential for dispersion of larvae along the Atlantic Ocean can be affected by the Northeast Atlantic Current system, which is dominated by the Gulf Stream and is divided into two main branches, the North Atlantic Current (flowing north) and the Azores Current (flowing east) (Domingues *et al*., 2006). The combination of these two currents causes the Portuguese Current, which has a characteristic movement from north to south, meeting the Canary Current (Neves *et al*., 2008). This multibranch system is even more complex due to seasonal variations (Domingues *et al*., 2006): in winter the prevailing ocean currents have to shift north, due to the influence of winds originated from south and southwest, with very active and energetic characteristics; on the contrary, in summer, less energetic currents are prevalent throughout the inner shelf, and water masses are transported to the platform through streams of water in direction of the coast (Gomes, 2009).

The potential for dispersion of larvae along the Atlantic Ocean can also be affected by the water movements to or off the Mediterranean. The Strait of Gibraltar is the frontier between southwestern Europe and northwestern Africa, and the Atlantic water enters in the surface of Mediterranean through the Strait, because is less dense, and a semi-permanent anticyclone gyre is formed in the Alboran Sea (González-

Wangüemert *et al*., 2011). This mass of water flows to the East, following the North African coast with eddies near the Libyan coast (González-Wangüemert *et al*., 2011).

The Sparidae fish family consists of approximately 115 species in 33 genera, is widely distributed in tropical and temperate coastal waters, and occasionally occurs in estuaries (Chiba *et al*., 2009). This family includes a large number of species of high economic value (Herrán *et al*., 2001) and constitutes one of the most important fish resources in the Atlantic Ocean. In Portuguese waters, the annual landings of sparid fishes has reached 5 917 tonnes during 2014, of which 227 tonnes are of *Sarpa salpa* (Linnaeus 1758) (INE, 2015). Salema, *S. salpa*, is an eurytherm fish that lives in shallow waters, between 0 and 70 meters, and inhabits predominantly sandy bottoms and seagrass beds, such as *Posidonia oceanica* and *Cymodocea nodosa* (Villamil *et al*., 2002; Criscoli *et al*., 2006). As an herbivore *S. salpa* has a strong impact in seagrass meadows, counting for up 75 % of the total herbivorous consumption (Jadot *et al*., 2006; Abecasis *et al*., 2012). It is a widely distributed species, occurring throughout the Mediterranean (Jadot *et al*., 2006) and Black Sea (Pashkov and Reshetnikov, 2012), and in the eastern Atlantic from the Bay of Biscay until South Africa (Walt and Mann, 1998).

Until now, the main focus on the species biology has been on feeding habits (Havelange *et al*., 1997; Peirano *et al*., 2001), distribution (Bariche *et al*., 2004), reproduction (Walt and Mann, 1998; Criscoli *et al*., 2006; Pallaoro *et al*., 2008) and growth (Walt and Beckley, 1997; Criscoli *et al*., 2005; Pallaoro *et al*., 2008). No data on stock structure exists and until now, no genetic information on *S. salpa* is available on the different populations that may exist along the distribution area and no

management measures have ever been implemented towards the sustainable use of this resource, despite the commercial exploitation of the fishery.

In the present study we want to analyze the genetic structure of *S. salpa* considering 11 sampling sites distributed across the Northeastern Atlantic Ocean and the Mediterranean Sea. To achieve this goal, fragments of the mitochondrial control region and the first intron of the nuclear S7 ribosomal protein gene were analyzed. These genes have been successfully applied in marine fish's phylogeographical studies: e.g. *Diplodus sargus* (Domingues *et al*., 2007a), *Tripterygion delaisi* (Domingues *et al*., 2007b), *Taurulus bubalis* (Almada *et al*., 2012), *Halobatrachus didactylus* (Robalo *et al*., 2013).

MATERIALS AND METHODS

Sampling and DNA extraction

Samples of *S. salpa* were obtained from commercial landings caught by local artisanal fisheries. Fin clips were cut immediately after collection of the individuals and stored at room temperature in 96 % ethanol. Figure 11 shows the location of the 11 sample sites and Tables 5 and 6 identifies the number of samples analyzed. Total DNA extraction was carried out according to standard protocols, using EZNA - tissue DNA Kit (Omega).

Figure 11. Map with sampling locations for *Sarpa salpa*. The dashed line represents the distribution area. (Available at: http://upload.wikimedia.org).

Table 5. Sampling localities of *Sarpa salpa*, with respective sample sizes (N), number of polymorphic sites, number of haplotypes, and genetic diversity indices for mitochondrial control region. * - included the individuals with DNA introgression

Sampling area	ID code	N	Number of polymorphic sites	Number of haplotypes	Haplotype diversity (h)	Nucleotide diversity (π)	% of private haplotypes	Number of private haplotypes
Azores	AZO	9	49	7	0.917 ± 0.092	0.049 ± 0.027	100	7
Azores*	AZO*	11	192	9	0.946 ± 0.066	0.209 ± 0.110	100	9
Algarve	ALG	11	37	11	1.000 ± 0.039	0.044 ± 0.024	91	10
Apulia	APU	11	57	11	1.000 ± 0.039	0.052 ± 0.029	100	11
Croatia	CRO	11	49	11	1.000 ± 0.039	0.048 ± 0.027	91	10
Greece	GRE	10	32	9	0.978 ± 0.054	0.028 ± 0.016	100	9
Italy	ITA	10	37	10	1.000 ± 0.045	0.033 ± 0.019	90	9
Italy*	ITA*	11	187	11	1.000 ± 0.039	0.127 ± 0.068	91	10
Madeira	MAD	11	39	9	0.946 ± 0.066	0.048 ± 0.026	100	9
Morocco	MOR	10	39	10	1.000 ± 0.039	0.043 ± 0.024	60	6
Morocco*	MOR*	11	186	11	1.000 ± 0.039	0.135 ± 0.072	64	7
Murcia	MUR	1	0	1	1.000 ± 0.000	0.000 ± 0.000	0	0
Peniche	PEN	9	52	9	1.000 ± 0.052	0.060 ± 0.033	100	9
Peniche*	PEN*	10	190	10	1.000 ± 0.045	0.157 ± 0.084	100	10
Setúbal	SET	11	45	11	1.000 ± 0.039	0.044 ± 0.024	82	9
TOTAL		**104**	107	94	0.998 ± 0.002	0.047 ± 0.023	86	125
TOTAL*		**109**	228	99	0.998 ± 0.002	0.091 ± 0.044	86	94

Table 6. Sampling localities of *Sarpa salpa*, with respective sample sizes (N), number of polymorphic sites, number of haplotypes, and genetic diversity indices for the first intron of the S7 ribosomal protein gene. * - included the individuals with DNA introgression

Sampling area	ID code	N	Number of gene copies	Number of polymorphic sites	Number of sequences	Number of Heterozygotes	Number of Homozygotes	Haplotype diversity (h)	Nucleotide diversity (π)	% of private haplotypes	Number of private haplotypes
Azores	AZO	3	6	9	6	3	0	1.000 ± 0.096	0.064 ± 0.044	67	4
Algarve	ALG	1	2	2	2	1	0	1.000 ± 0.500	0.037 ± 0.045	50	1
Apulia	APU	4	8	15	7	4	0	0.964 ± 0.077	0.088 ± 0.055	14	1
Croatia	CRO	5	10	16	7	5	0	0.911 ± 0.077	0.095 ± 0.057	43	3
Greece	GRE	4	8	16	7	4	0	0.964 ± 0.077	0.087 ± 0.054	14	1
Italy	ITA	5	10	19	8	5	0	0.956 ± 0.059	0.113 ± 0.066	50	4
Madeira	MAD	4	8	12	4	3	1	0.786 ± 0.113	0.091 ± 0.057	0	0
Morocco	MOR	5	10	20	8	5	0	0.933 ± 0.077	0.109 ± 0.064	50	4
Murcia*	MUR*	5	10	2	2	5	0	0.556 ± 0.075	0.021 ± 0.017	100	2
Peniche	PEN	3	6	12	5	3	0	0.933 ± 0.122	0.095 ± 0.062	40	2
Setúbal	SET	4	8	12	4	2	2	0.643 ± 0.184	0.067 ± 0.043	0	0
TOTAL		**38**	**76**	31	30	35	3	0.913 ± 0.020	0.092 ± 0.050	26	20
TOTAL*		43	86	56	32	40	3	0.927 ± 0.016	0.176 ± 0.090	26	22

DNA amplification and sequencing

Polymerase chain reaction (PCR) amplification of mitochondrial control region and of the first intron of nuclear S7 ribosomal protein gene, were performed with the following pairs of primers: dloop – LPro1 (5'- ACTCT CACCC CTAGC TCCCA AAG - 3') and HDL1 (5'- CCTGA AGTAG GAACC AGATG CCAG - 3'), and the first intron of the S7 ribosomal protein gene – S7RPEX1F (5'- TGG CCT CTT CCT TGG CCG TC - 3') and S7RPEX2R (5'- AAC TCG TCT GGC TTT TCG CC - 3'). PCR amplification reactions were performed in a final volume of 20 µl, with 2.0 µl of DNA, 0.8 µl of each primer, 1.6 µl of dNTPs, 4.0 µl of 5x reaction buffer, 0.9 µl of $MgCl_2$, 1.2 µl of BSA, 0.2 µl of Taq DNA polymerase (PROMEGA) and including 8.5 µl ddH_2O. A programmable thermal cycler (Applied Biosystems Gene Amp ® PCR System 2700) was used for amplifications.

The PCR thermal cycling conditions consisted in an initial denaturation step of 95 ºC for 5 minutes (min) followed by 35 cycles with the profile: denaturation at 95 ºC for 45 seconds (sec), annealing at 68 ºC for 35 sec for mitochondrial control region and 69 ºC for nuclear gene, extension at 72 ºC for 1 min, and a final extension step of 72 ºC for 10 min. All PCR products were checked for the presence of right sized products on 1 % agarose gels, purified with PCR Sure Clean Plus kit following the manufacturer's protocol (BIOLINE), and were sequenced at Macrogen (http://dna.macrogen.com).

DNA Analyses

Phylogenetic analysis

Sequences obtained were verified and edited using Sequencher, version 4.0.5 (http://www.genecodes.com) and BioEdit, version 7.0.9.0 (Hall, 1999). Subsequently, the sequences were aligned using Clustal X, version 2.0.10 (Thompson *et al*., 1997) or MAFFT, version 7 (Katoh and Standley, 2013) and converted to appropriate format with Concatenator, version 1.1.0 (Pina-Martins and Paulo, 2008). For the nuclear locus heterozygous indels were resolved using Champuru, version 1.0 (Flot, 2007) and haplotypes were infer using PHASE, version 2.1 (Stephens *et al*., 2001; Stephens and Scheet, 2005), allowing for recombination within haplotypes and using a cutoff default value of 0.9.

For the mitochondrial DNA (mtDNA) dataset a sequence from the Carangidae Family, *Trachurus murphyi* (GenBank accession number: FJ946812), and a sequence from the Sebastidae Family, *Sebastes borealis* (GenBank accession number: DQ678609), were used as outgroups, while for the nuclear locus a sequence from the Trypterygiidae Family, *T. delaisi* (GenBank accession number: EF484644.1), was selected. Because preliminary analysis show that five samples were clearly distinct from the remained mtDNA samples, a new dataset with additional Sparidae sequences, for the same primer used in the present study were selected in GenBank (http://blast.ncbi.nlm.nih.gov) [*Acanthopagrus schlegeli* (GenBank accession numbers: DQ314765.1 and KJ586516.1), *Dentex dentex* (GenBank accession numbers: AY014743.1 and AY014744.1), *Diplodus puntazzo* (GenBank accession numbers: AF373431.1 and AF373430.1), *D. sargus* (GenBank accession numbers: EF468622.1 and

EF428560.1), *Diplodus vulgaris* (GenBank accession numbers: HQ997833.1 and HQ997832.1), *Lithognathus mormyrus* (GenBank accession numbers: GQ924861.1 and GQ924860.1), *Pagrus major* (GenBank accession numbers: AB299265.1 and AB713839.1), *Sparus aurata* (GenBank accession numbers: JN801247.1 and JN801246.1), *Spicara smaris* (GenBank accession numbers: HM038522.1 and HM038521.1) and *Spicara maena* (GenBank accession numbers: HM038519.1 and HM038518.1)], and using the previous *T. murphyi* and *S. borealis* as outgroups was also analyzed. For the nuclear locus, due to the observed genetic distance of the Murcia samples these sequences were blasted in GenBank to find the most similar sequences. Consequently a new dataset was formed with seven additional Sparidae GenBank sequences [*Gymnocrotaphus curvidens* (GenBank accession number: JX282377.1), *Pachymetopon blochii* (GenBank accession number: JX282378.1), *Cymatoceps nasutus* (GenBank accession numbers: KM257466.1 and KM257298.1), *D. vulgaris* (GenBank accession number: JQ624693.1) and *D. sargus* (GenBank accession numbers: EF467764.1 and EF467729.1)] and using the previous *T. delaisi* as outgroup.

The evolutionary history of the four datasets was inferred using the Maxima Parsimony method, implemented by the software Mega, version 6.06 (Tamura *et al*., 2013), and optimal trees were found using a heuristic search with Subtree-Pruning-Regrafting (SPR) (Nei and Kumar, 2000) as the branch-swapping algorithm. Initial trees were obtained via stepwise addition with 10 replicates of random addition sequence. Bootstrapping with 1000 pseudo-replicates was performed to evaluate the robustness of the nodes of the phylogenetic trees. Only the trees resulted from the new datasets are presented.

Additionally, haplotype network was obtained for both locus, using Network version 4.6 (http://www.fluxus-engineering.com), running a median joining algorithm (Bandelt *et al*., 1999).

Genetic Data Analyses

For both mitochondrial control region and nuclear gene of *S. salpa***,** the software Arlequin, version 3.5.1.2 (Excoffier and Lischer, 2010) was used to calculate the number of polymorphic sites, number of haplotypes, haplotype and nucleotide diversities and percentage of private haplotypes. The uncorrected pairwise-distances were calculated in Mega, version 6.06.

An analysis of molecular variance (AMOVA) was also performed in Arlequin to examine hierarchical population structure, pooling the sample sites into two groups, Atlantic and Mediterranean. Population differentiation (F_{ST}) was estimated using Arlequin. Mismatch distributions of pairwise differences between all individuals (without introgression) were analyzed with Arlequin to explore the demographic history of populations. The time of population expansion was determined by converting the expansion time parameter TAU to time in years using the formula TAU = 2ut, where u is the mutation rate per nucleotide per year multiplied by sequence length (*i.e.* number of nucleotides) and t is the time, in years, since the population expanded. The demographic changes were also examined by calculating the Harpending's raggedness index (RAG) (Harpending *et al*., 1993) and the sum of squared deviations (SSD) between the observed and expected mismatch for each of the populations were calculated using the methods of Schneider and Excoffier (1999) using Arlequin. The

demographic history was also assessed with Tajima's D test (Tajima, 1989) and Fu's F statistics (Fu, 1997) using the same software.

RESULTS

Mitochondrial control region

A total of 109 *S. salpa* mitochondrial control region sequences (315 base pairs) were analyzed, representing a total of 99 haplotypes, and whose sequences were registered in GenBank (accession numbers: KU186669 - KU186767). Only eight haplotypes were shared among different individuals and the other 91 haplotypes were singletons. Five of the shared haplotypes were represented in more than one sampling site, while the other three were only shared between individuals restricted to the same sampling sites. The number of haplotypes per sampling site ranged from 9 to 11 (Table 5). The overall values of haplotype diversity and nucleotide diversity were 0.998 ± 0.002 and 0.091 ± 0.044, respectively.

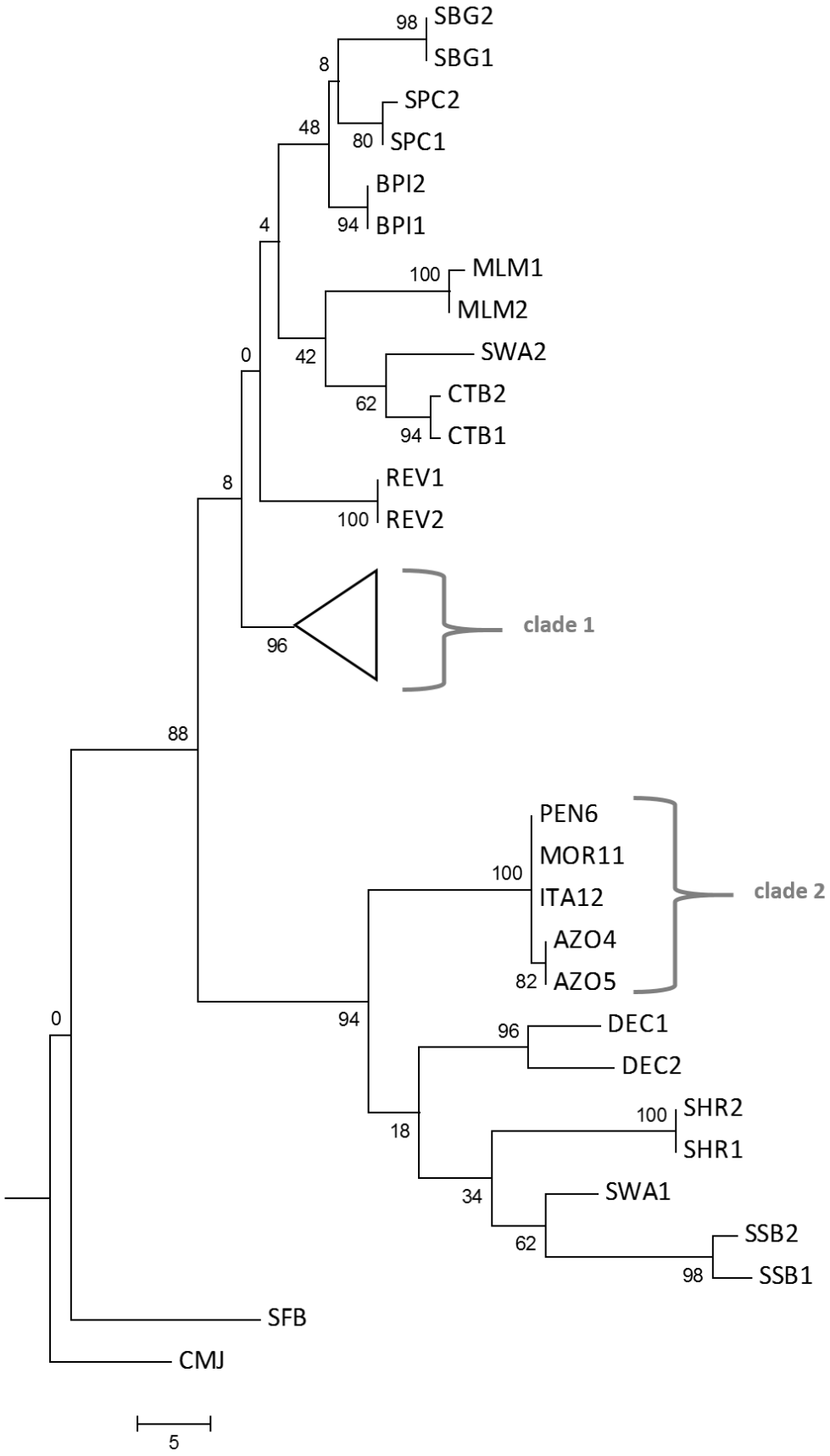
Phylogenetic tree showed no separation between the Atlantic and Mediterranean samples (Figure 12 A). The *S. salpa* samples were divided into two major clades, designated as clade 1 and clade 2; the clade 1 contained the majority of the samples and clade 2 is formed by five individuals from different origins. These five individuals corresponded to one specimen from Peniche, one from Morocco, one from Italy and two from Azores, and consequently the nucleotide diversity values for these four sampling areas are greater than the others (Table 5).

These individuals increased substantially the overall nucleotide diversity value (with: 0.091 ± 0.044; without: 0.047 ± 0.023). The additional samples of *D. dentex*, *D. puntazzo*, *L. mormyrus* and one sample of *D*. *sargus* from GenBank were positioned closer to clade 2 and the remaining samples were positioned closer to clade 1. The haplotype network from clade 1 did not show a phylogeographic structure among the sampling sites (Figure 12 B); the network is characterized by several haplotypes at low frequency and showed two distinct lineages that are not geographically separated because each lineage is composed by samples from different sampling sites. Additionally a maximum value of uncorrected pairwise distances of 8 % among the individuals of the clade 1, and a maximum value of 57 % among individuals of *S. salpa* of the two clades were obtained.

The AMOVA using mitochondrial control region data considered two groups, Atlantic (Algarve, Apulia, Peniche, Setúbal, Azores, Madeira, Morocco) and Mediterranean (Greece, Croatia, Italy, Murcia), showed lack of population structure, other approaches with different group combinations showed the same result. The AMOVA analysis calculated for the individuals of clade 1 showed that a high percentage (92 %) of the variance derived from within the populations variance and a small variance component was attributable to variation between the Atlantic and the Mediterranean groups (4 %), while the calculations including samples from clades 1 and 2 indicated that a small variance component was attributable to variation between the Atlantic and the Mediterranean groups (1 %) (Table 7 A). The range of F_{ST} values, calculated between sampling sites, including samples from clade 1 (Supplementary Material 1 A), was from zero to 0.537, and the maximum differentiation was found between Murcia and

Greece (F_{ST} = 0.537), followed by Murcia and Italy (F_{ST} = 0.510), Italy and Morocco (F_{ST} = 0.333), and Greece and Morocco (F_{ST} = 0.313).

A)



(Fig. 12)

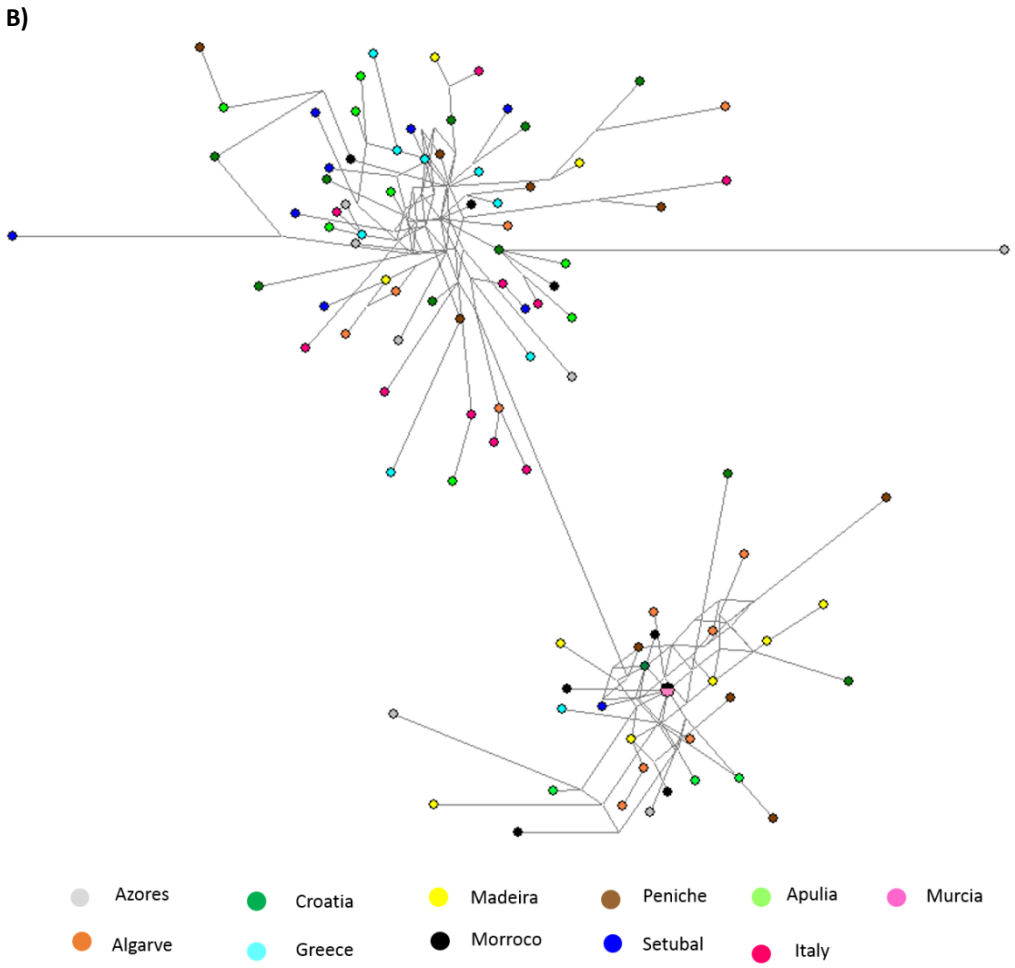


Figure 12. A) Maxima Parsimony tree of *Sarpa salpa* for the mitochondrial control region sequences, using *Trachurus murphyi* (CMJ) and *Sebastes borealis* (SFB) as outgroups. The bootstrap values are indicated at the nodes. The length of each branch is proportional to the number of nucleotide substitutions.
(Azores: AZO, Italy: ITA, Morocco: MOR, Peniche: PEN, *Acanthopagrus schlegeli*: MLM, *Dentex dentex*: DEC, *Diplodus puntazzo*: SHR, *Diplodus sargus*: SWA, *Diplodus vulgaris*: CTB, *Lithognathus mormyrus*: SSB, *Pagrus major*: REV, *Sparus aurata*: SBG, *Spicara smaris*: SPC, *Spicara maena*: BPI)
B) Median-joining haplotype network based on the mitochondrial control region sequences from *Sarpa salpa*. The size of each circle is proportional to the number of individuals carrying each haplotype (the smallest circles corresponded to one individual) and the length of each line is proportional to the number of substitutions.

Mismatch distribution based on mitochondrial control region sequences were estimated and SSD tests were performed (Table 8). The RAG under the demographic expansion model was also calculated, resulting that all individuals from clade 1 had non-significant raggedness index which indicates that data has relatively good fit to a model of population expansion (Harpending, 1994) (Table 8). The expansion time

parameter TAU = 5.119 (95 % confidence interval = 2.877 - 34.689) were used to calculate the time of population expansion. Mitochondrial control region mutations rates in fish are widely variable (Almada *et al*., 2012) and in the absence of a specific calibrated mutation rate for the mitochondrial control region of *S. salpa*, two very distinct rates were assumed. If we assumed a divergence rate of 11 % per million years (Myr) for the mitochondrial control region in teleosts (McMillan and Palumbi, 1997), we estimated the time needed for these expansion events to occur was approximately 73 867 (95 % confidence interval = 41 515 - 500 563) years before present (BP). Applying a conventional mitochondrial control region rate of 2 % per Myr (Bargelloni *et al*., 2003), the time estimated is 406 270 (95 % confidence interval = 228 333 - 2753 095) years BP. Tajima's D value was negative (Tajima's D = -0.918; P = 0.194) for the calculation for all individuals from clade 1 (Table 8), indicating an excess of rare nucleotide site variants, compared to what would be expected under a neutral model of evolution (Joshi *et al*., 2013). The results of Fu's Fs test showed a negative value for all individuals from clade 1 indicating an excess of rare haplotypes.

Table 7. Results of hierarchical analysis of molecular variance (AMOVA) for **A)** mitochondrial control region and **B)** first intron of the S7 ribosomal protein gene.

A)	**Source of variation**	**d.f.**	**Sum of squares**	**Variance components**	**% of variation**
Without introgression: clade 1	Among groups	1	24.017	0.326 Va	4
	Among populations within groups	9	84.286	0.260 Vb	4
	Within populations	93	645.995	6.946 Vc	92
	Total	103	754.298	7.532	
With introgression: clade 1 + clade 2	Among groups	1	25.564	0.193 Va	1
	Among populations within groups	9	148.273	0.259 Vb	2
	Within populations	98	1367.282	13.952 Vc	97
	Total	108	1541.119	14.404	

B)	**Source of variation**	**d.f.**	**Sum of squares**	**Variance components**	**% of variation**
Without introgression: clade 1	Among groups	1	2.067	-0.022 Va	-1
	Among populations within groups	8	22.295	0.044 Vb	2
	Within populations	66	162.625	2.464 Vc	99
	Total	75	186.987	2.486	
With introgression: clade 1 + clade 2	Among groups	1	40.578	0.336 Va	6
	Among populations within groups	9	209.832	2.759 Vb	52
	Within populations	75	167.625	2.235 Vc	42
	Total	85	418.035	5.330	

Table 8. Parameters for the mismatch distribution of *Sarpa salpa* determined for mitochondrial control region (DLOOP) and for the first intron of the S7 ribosomal protein gene (S7). Tajima's D, Fu's F-statistics and their statistical significance are also presented. Numbers in parenthesis are the upper and lower bound of the 95 % confidence interval (1000 bootstrap replicates).

		Mismatch distribution analysis							**Neutrality tests**			
		Goodness-of-fit tests				Parameters			Tajima's D test		Fu's test	
		Sum of Squared Deviation (SSD)	P_{SSD}	Harpending's Raggedness index (RAG)	P_{RAG}	Teta 0	Teta 1	TAU	Tajima's D	P	F	P
Without introgression: clade 1	**DLOOP**	0.008	0.470	0.002	0.670	12.672	99999.000	5.119 (2.877 - 34.689)	-0.918	0.194	-24.111	0.001
	S7	0.015	0.100	0.034	0.110	0.005	26.094	5.969 (3.576 - 8.117)	-0.685	0.279	-12.727	0.000

First intron of the nuclear S7 ribosomal protein gene

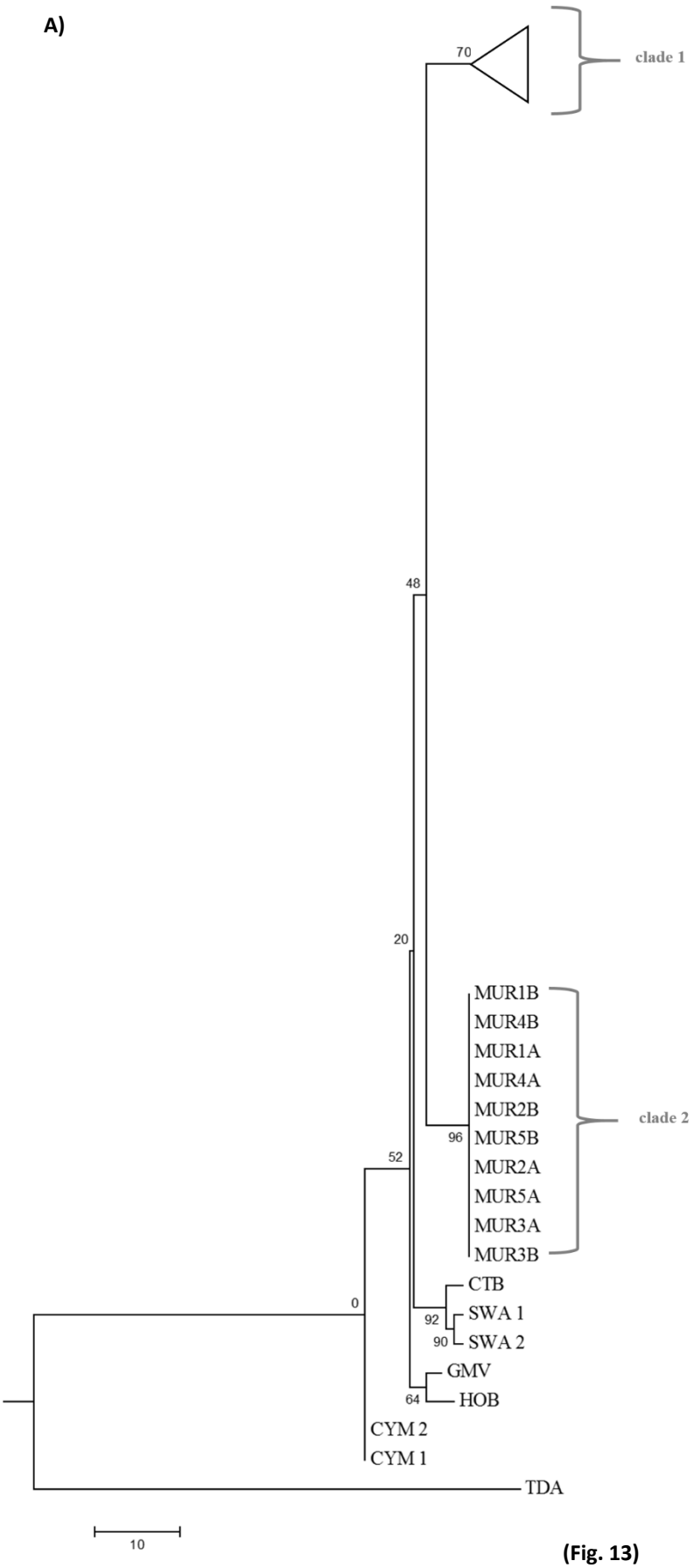
A total of 43 *S. salpa* individuals were analyzed for the first intron of the nuclear S7 ribosomal protein gene, the fragments were 336 base pairs long and the sequences were registered in GenBank (accession numbers: KU186768 - KU186799). Two sequences per individual were analyzed because the individuals were heterozygotes, giving a total of 86 sequences analyzed, and representing a total of 32 haplotypes and a total of 33 genotypes. Twenty haplotypes were singletons and the other 12 haplotypes were shared among different individuals. Ten of the shared haplotypes were represented in more than one sampling sites, while the other two were only shared between individuals restricted to the same sampling site. The number of haplotypes for each sampling site ranged from 2 to 8 (Table 6). The most common haplotype (h2 - KU186769) was found in seven sample sites (Apulia, Croatia, Greece, Italy, Madeira, Morocco and Setúbal). Murcia presented only two haplotypes (h31 and h32, KU186798 and KU186799 respectively), which were not shared with the others sampling sites. All sampling sites showed high haplotype diversity (ranging between 0.643 and 1.000), except Murcia which presented a slightly lower value of 0.556 ± 0.075.

Phylogenetic tree showed no separation between the Atlantic and Mediterranean samples (Figure 13 A). The *S. salpa* samples were divided in clade 1, which contained all the individuals except Murcia, and clade 2, which was formed for all Murcia individuals. All the additional samples from GenBank presenting the behavior of an outgroup. The Murcia sampling site influenced the overall nucleotide diversity value (0.176 ± 0.090), and the overall nucleotide diversity calculated only for the clade 1 is lower 0.092 ± 0.050.

The haplotype network for clade 1 did not show a phylogeographic structure among the sampling sites (Figure 13 B); the network is characterized by several haplotypes at low to low-moderate frequency and no separation appears to be present between Atlantic and Mediterranean individuals, and between sampling sites. The uncorrected pairwise-distances had a maximum value of 7 % when the Murcia individuals are compared with the others, and only 3 % when the comparison is made with all the individuals except Murcia.

The AMOVA analysis showed that 99 % of the variance is explained by within population's variation and 2 % of the variance component was attributable to variation among population within the Atlantic and

A)



(Fig. 13)

the Mediterranean groups (Table 7 B). F_{ST} values, calculated for all the sampling sites, including samples from clade 1, are shown in Supplementary Material 1 B. The range of F_{ST} values ranged from zero to 0.418. F_{ST} values indicated no significant difference ($p > 0.05$) in each pairwise combination of these 10 sampled areas, except in the next pairs: Azores - Algarve (F_{ST} = 0.418), Algarve - Peniche (F_{ST} = 0.302) and Azores - Peniche (F_{ST} = 0.249).

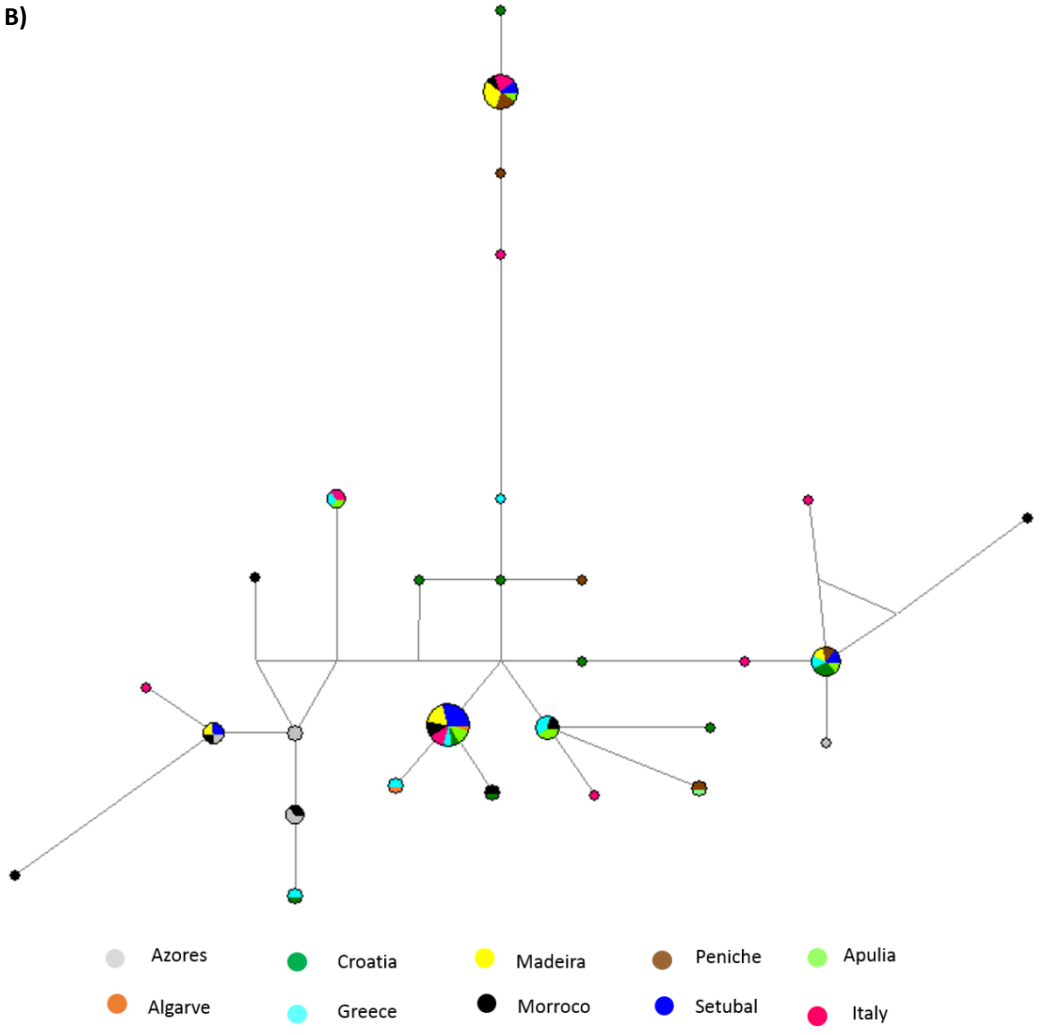


Figure 13. A) Maxima Parsimony tree of *Sarpa salpa* for the first intron of the S7 ribosomal protein gene sequences, using *Tripterygion delaisi* (TDA) as outgroup. The bootstrap values are indicated at the nodes. The length of each branch is proportional to the number of nucleotide substitutions.
(Murcia: MUR, *Gymnocrotaphus curvidens*: GMV, *Pachymetopon blochii*: HOB, *Cymatoceps nasutus*: CYM, *Diplodus vulgaris*: CTB, *Diplodus sargus*: SWA)
B) Median-joining haplotype network based on the first intron of the S7 ribosomal protein gene from *Sarpa salpa*. The size of each circle is proportional to the number of individuals carrying each haplotype (the smallest circles corresponded to one individual) and the length of each line is proportional to the number of substitutions.

Mismatch distribution was estimated and SSD tests were performed (Table 8). The Tajima's D and Fu's Fs values calculated were -0.685 (P = 0.279) and -12.727 (P = 0.000), respectively. The expansion time parameter TAU = 5.969 (95 % confidence interval = 3.576 - 8.117) was used to calculate the time of population expansion. In the absence of a specific calibrated mutation rate for the first intron of the nuclear S7 ribosomal protein gene in the Family Sparidae the mutation rate estimated for the Family Centrarchidae, 0.34 % (Near *et al*., 2011) is assumed; we estimated the time needed for these expansion events to occur was approximately 2 612 482 (95 % confidence interval = 1 565 126 - 3 552 608) years BP.

DISCUSSION

Evolutionary and demographic history

During the Pliocene, the Northeastern Atlantic experienced severe climatic and sea level fluctuations (Domingues *et al*., 2007a). Most studies assume that the Pleistocene and specially the Last Glacial Maximum (LGM) were critical times (Robalo *et al*., 2013) because the marine fauna was extremely affected in terms of geographic distribution and therefore influenced in genetic shaping patterns (Domingues *et al*., 2007a). The Northeastern Atlantic, particularly during the penultimate glaciation of the Iberian margin, had an average summer sea surface temperatures (SSTs) of 14 ºC, reaching a maximal values of 19 ºC (Abreu *et al*., 2003). On the other hand, during the last glaciation period cooling events seem to have been more extreme, being the lowest summer SST values estimated at 4.8 ºC (Abreu *et al*., 2003). The reconstructions of Mediterranean SST, during the glacial summer, ranged from 14 ºC in the Alboran Sea

to 23 ºC in the south east of the basin (Hayes *et al*., 2005). The warmer eastern basin had a maximum temperature of ~16 ºC, with cooler (13 ºC) surface water conditions observed in the Aegean Sea extending southwards into the Levantine basin (Hayes *et al*., 2005). Although the west-east temperature gradient was significantly stronger during the glacial winter than today, the Aegean Sea was much cooler having a 5 ºC decrease during the glacial winter (Hayes *et al*., 2005).

Current research suggested that the glacial period could cause an expansion of cold water species, while it could also cause a contraction for some warm water species (Almada *et al*., 2012). A study on a more tolerant cooler waters species, *Liphophris pholis*, suggested that the present population of Azores would have survived there, eventually adapting to long cold periods during the LGM (Stefanni *et al*., 2006). Also, previous studies on warm water species, *Chromis limbata* and *T. delaisi* , suggested that during the postglacial recolonization they may have followed two routes: fish reached the Azores from Madeira, which in turn is connected to the western African coast, while the southwestern European shores were colonized from Mediterranean refuges (Domingues *et al*., 2006; Domingues *et al*., 2007b).

Due to climate changes it's fundamental to understand the role of the different thermal tolerances of each species in terms of their response to the SST oscillations. As referred in the Introduction, *S. salpa* is a widely distributed species and has a great spectrum of thermal tolerance, varying from 12 to 30 ºC (www.noaa.gov). Assuming that ancestral populations of *S. salpa* have a similar thermal tolerance than present populations, it is probable that during glacial peaks *S. salpa* might have disappeared from the Atlantic coast of Europe and Mediterranean, and persisted in large tropical

and subtropical refuges. It is also probable that during the interglacial events these refuges must have acted as propagation sources for the recolonization of areas to the North (Almada *et al*., 2012), allowing *S. sarpa* to disperse from the tropical and subtropical areas to the Iberian and Mediterranean coasts and consequently, *S. sarpa* in temperate areas expanded from southern populations already with high levels of genetic diversity, as indicated by the results.

A compilation of several works with mitochondrial values of genetic diversity from different fish species, related with their spectrum of thermal tolerance, was made (Supplementary Material 2). For each native range of species (www.fishbase.org), the values of thermal tolerance accordingly to the SST data available in the National Oceanic and Atmospheric Administration (www.noaa.gov) were obtained. It was observed that inside the Sparidae Family, *S. salpa* presented the highest nucleotide diversity value when compared to the values calculated for *D. sargus* (nucleotide diversity = 0.016, obtained for González-Wangüemert *et al*. (2010); nucleotide diversity = 0.024, obtained for González-Wangüemert *et al*. (2011)). This compilation seems to suggest that thermal tolerance and nucleotide diversity could be related indicating that higher thermal tolerance allow large species range and probably higher effective population size. A more detailed study, with different marine species and from different Family's, must be performed to test this pattern. However a direct comparison of the mtDNA results obtained for *S. salpa* with other fish species, could be problematic since this species is a protandrous hermaphrodite (changing from male to female through a nonfunctional intersexual phase) (Criscoli *et al*., 2006), implying that all the individuals, being in the male or female phase, will pass copies of mtDNA, which influences the variation observed in the mtDNA results.

The overall pattern of the phylogenetic trees, haplotype networks and all the AMOVA results confirm the hypothesis that *S. salpa* lacks population structure. Moreover, results of the AMOVA detected no significant differences in all hierarchical levels, indicating that no significant population genetic structure exists in the study areas due to sharing older haplotypes. Most of the individuals analyzed had different haplotypes, although the same haplotypes were observed in different sampling sites, and this also provides no support for the existence of genetic structure in *S. salpa* off Atlantic and Mediterranean.

The lack of Atlantic and Mediterranean differentiation are not new in phylogeographic studies because several contrasting patterns have already been described in the area, from restricted gene flow, with a clear divide, to high levels of gene flow levels between areas (Domingues *et al*., 2007b). These contrasting patterns may be due to different biological characteristics of the species and it is well known that the larval ecology affects the extent of the gene flow (Domingues *et al*., 2007b). Lower levels of differentiation between marine fish populations are attributed to higher dispersal potential during planktonic egg, larval or adult life-history stages, due to transport by ocean currents (Grant and Bowen, 1998; Hare, 2005). This higher dispersal potential combined with an absence of physical barriers to movement between ocean basins, leads to a mixing of early life stages among spawning locations which results in less stock structure (Grant and Bowen, 1998; Hare, 2005). It is known that *S. salpa* is a marine fish that spawn pelagic eggs (Strydom *et al*., 2014) but no data is available to prove the duration of the larval stage neither the influence that ocean currents may have on them. Pattrick and Strydom (2009) tested the swimming abilities of late-stage

larvae, wild-caught in temperate South Africa, of cape white seabream (*Diplodus capensis*) and *S. salpa* in controlled swimming chambers and stated that the late-stage larvae of these Sparidae are strong swimmers.

According to Grant and Bowen (1998) and Aboim *et al*. (2005) the pattern of high haplotype diversity and low nucleotide diversity, observed in the present study, is attributed to expansion after a period of low effective population size, caused by founder events or bottlenecks, or by a rapid population growth that enhances the retention of new mutations. This pattern of genetic diversity can be caused by shifts in climate or in oceanographic conditions (Grant and Bowen, 1998). The present results support the hypothesis that high haplotype diversity in the sampling areas resulted from an expansion from the possible refuges in the coast of Africa, during the current interglacial period, from a population that maintained high levels of genetic diversity, due to an accumulation of mutation *in situ*. There is a clear expansion signal, because the species has the capacity to retain new mutations locally what can suggest the *S. salpa* population is apparently large. Additionally many of the derived allele are private alleles, and are not shared among sampled sites. This high number of private alleles, also suggests that nowadays there's no extensive gene flow between populations which also influences the F_{ST} values between the sampling areas, generating a significant genetic difference between some sample sites. Ancestral haplotypes with origin in the refuge population are common and shared among sampling sites and contributing for the lack of structure, and most of the derived alleles are private alleles and are locally distributed contributing to some local differentiation, and suggesting the reduction of current gene flow among sampling sites. This hypothesis of expansion

of *S. salpa* is also supported by the mismatch distribution results for both genes, and the non-significant results in goodness-of-fit distribution suggested that population expansion occurred recently (Rogers, 1995).

According to the results Morocco coastal area, geographically closer to the possible refuges from the coast of Africa, could have received migrants from the southern populations after the Pleistocene glaciations and acted later as the source for the northern colonization (Domingues *et al*., 2007a). Domingues *et al*. (2007a) suggested that Saharan upwelling filaments are capable of transporting larvae from the African neritic zone into oceanic areas and towards the Canary archipelago. Indeed, *S. salpa* from Morocco showed high genetic diversity and the lower percentage of private haplotypes for mitochondrial gene (Table 5). The Morocco data from the nuclear gene does not present the lowest percentage of private haplotypes comparatively to other sampling sites, because the nuclear genes generally tend to evolve more slowly than mitochondrial ones, and consequently there was not enough time to locally recover the genetic variability as it happened for the mtDNA (Lin and Danforth, 2004).

DNA introgression

The mitochondrial results suggest that there was a mtDNA introgression between *S. salpa* and another Sparidae species. The mitochondrial introgression seems to be very old, probably existing before the expansion took place, because the clade 2, containing the individuals with introgression, is distinct from the clade 1, formed by the remaining samples of *S. salpa*. Chow and Kishino (1995) was the first study to report molecular evidence for introgressive hybridization in marine pelagic species. Introgression of the

mitochondrial genome from one species to another, by hybridization, is more common in fishes than in other vertebrates (Chow and Kishino, 1995; Rognon and Guyomard, 2003; Sullivan *et al*., 2004). This may be due to the fact that fishes present external fertilization, weak ethological reproductive barriers, weak gametic specificity and high susceptibility to secondary contacts between recently evolved forms (Rognon and Guyomard, 2003). Additionally, the viable hybrids are often fertile and gene introgression could normally occur after natural or man-induced secondary contacts (Rognon and Guyomard, 2003). Natural introgressive hybridization between species seems relatively widespread, and has an important evolutionary significance in adaptation and speciation, has it provides favorable conditions to major and rapid evolution, and has a significant contribution in species gene diversity (Rognon and Guyomard, 2003).

Aboim *et al*. (2010) verified that mitochondrial markers from one species can sometimes be transferred into another as a result of hybridization events, and found that more than a third of the analyzed individuals with mtDNA introgression did not present any evidence of nuclear introgression. This can be applied to the present results because the mitochondrial marker showed signals of introgression and the nuclear marker used, only detected a signal in one single sampling site, in Murcia. The nuclear results suggest that there could be a nuclear DNA introgression between *S. salpa* and the close Sparidae species, *G. curvidens*, *P. blochii*, *C. nasutus*, *D. vulgaris*, and *D. sargus*. In fact, three of the five species (*G. curvidens*, *P. blochii* and *C. nasutus*) only exist in the south Atlantic. This might indicate that the nuclear DNA introgression occurred in a refuge in the coast of Africa and when *S. salpa* dispersed, the individuals

already contained the DNA introgressed. Other studies with different nuclear markers must be performed in order to confirm, if signals of nuclear introgression exist or not.

The DNA introgression could be more common than shown by previous studies in fish population genetics, due to the weak isolation barriers among species, but further studies are necessary to confirm this putative role of species introgression in fish evolution.

The present work reveals no signs of differentiation between the Atlantic and Mediterranean populations or any genetic population structure of *S. salpa* for mitochondrial and nuclear markers. The applied genetic techniques showed that the species have a higher level of genetic variability when compared with other Sparidae species but weren't able to distinguish stock populations.

Additionally to the lack of evident structure, there was a strong signal for demographic expansion, potentially from an already genetic diverse, glacial refuge, from an African coastal population. The high number of private alleles suggests the current low levels of gene flow among sampled sites what contrast with our previous expectations. Marine fishes are generally viewed as resistant to extinction because there are no barriers in marine waters (Grant and Bowen, 1998) however, oscillations in the SST, caused by climate change events, conditions the survival of a species according to their thermal tolerance, moreover biotic and abiotic factors seems limiting the dispersal among geographical areas, at least at an ecological scale.

Finally, the detection of mitochondrial and nuclear introgression is a very important aspect of studying the phylogenetic history (Bossu and Near, 2009) although the causes and consequences of these processes raise new questions for future works. It was also verified that fishes with lower temperature ranges have lower nucleotide diversity.

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PART II

Biology

CHAPTER 4

Age, growth and reproduction

Age, growth and reproduction of the protandrous hermaphrodite fish, *Sarpa salpa*, from the Portuguese continental coast

SUMMARY

Sarpa salpa is a commercial exploited species in the Atlantic Ocean with little available information for the essential population parameters, such as age, growth and reproduction. The present study aims to describe the above parameters of *S. salpa* obtained off the coast of Portugal. Ages were estimated from the whole otolith readings; the minimum and the maximum ages observed were 0 and 14 years, respectively, corresponding to 5.2 and 41.4 cm of total length (TL). Whole otolith readings and backcalculation approaches were used to estimate the parameters of the von Bertalanffy growth function and the Akaike's information criterion value suggested that the second approach was the best one to describe the growth of *S. salpa*: L_∞ = 45.07 cm, k = 0.14 year^{-1} and t_0 = -1.43 year.
The species is a protandric hermaphroditic and the sex changed process occurred between 28.6 and 40.9 cm TL. A short spawning season was identified, extending from September to November. The estimated length at first maturity for males was 24.5 cm TL, corresponding to an age at first maturity of 2 years. This species exhibited a determinate fecundity type and the relative annual fecundity varied between 462 and 2 662 oocytes per gram of gutted weight.

INTRODUCTION

Salema, *Sarpa salpa* (Linnaeus 1758), is a benthopelagic gregarious sparid marine fish that sometimes forms sizeable schools (Russell *et al*., 2014), and lives in shallow waters (up to 70 meters) inhabiting predominantly littoral waters near rock with algal or seagrass coverage, such as *Posidonia oceanica* and *Cymodocea nodosa*, as well as sandy bottoms (Villamil *et al*., 2002; Criscoli *et al*., 2006; Pallaoro *et al*., 2008). It is a widely distributed species, occurring along the Mediterranean (Jadot *et al*., 2006) and

Black Sea (Pashkov and Reshetnikov, 2012), in the eastern Atlantic (from the Bay of Biscay to Cape of Good Hope) and in the Western Indian Ocean (from Mozambique to Cape of Good Hope) (Walt and Mann, 1998). Initially, *S. salpa* was described as a rudimentary hermaphrodite (Joubert, 1981) but nowadays is characterized by protandric hermaphroditism: the male gonadic tissue matures first and the female tissue develops later (Walt and Mann 1998; Villamil *et al.,* 2002). More recently, Paiva *et al*. (2014) showed the presence of hard structures in *S. salpa* ovaries, easily seen macroscopically and suggested to be masses of hydrated oocytes by histological analysis; these structures appear either isolated or in groups, forming a cystic structure, and showed a higher prevalence in months preceding the spawning season suggesting to be related with the reproductive strategy of the species.

S. salpa become important in Portuguese fishery landings (DGRM, 2015) given an increasing interest in new potential resources as a consequence of overexploitation of many marine fish stocks (FAO, 2014). Fish consumption tends to be based on locally and seasonally available products and over exploitation allowed a diversification of the consumers feeding habits based on new available local fish species. *S. salpa* fits in this category and have reached an increasing importance among other Sparidae species representing 4 % of the total landed in Portuguese waters (INE, 2015). This increasing interest justifies the acquisition of new biological information, particularly regarding their life history patterns, including essential population parameters such as abundance, age and growth, survival, reproduction, maturity and recruitment (Begg, 2005). This information is important to provide baseline data on population dynamics and productivity rates needed for stock assessments (Begg, 2005).

Until now, investigation on *S. salpa* occurred mainly on the Mediterranean Sea and in the Indian Ocean focusing on feeding habits (Gerking, 1984; Antolic *et al*., 1994; Havelange *et al*., 1997), spatial distribution (Dulčić *et al*., 1997; Ruitton *et al*., 2000; Jadot *et al*., 2006), and growth and reproductive biology (e.g. Walt and Beckley, 1997; Walt and Mann 1998; Criscoli *et al*., 2006; Pallaoro *et al*., 2008; El-Etreby *et al*., 2015). The information on the species from Atlantic Ocean is restricted to one work from the Canary Archipelago (Villamil *et al*., 2002) focused on the biology, and age and growth.

The main objective of the present study was to describe age, growth, and reproduction of *S. salpa* captured off the coast of Portugal. These findings will be useful to improve the existent fishing regulation and propose future management measures on the species exploitation.

MATERIALS AND METHODS

Sampling

Sampled individuals were obtained monthly between January 2012 and December 2012 from artisanal fisheries (namely trammel nets) in the central region of the Portuguese coast. Due to the gear selectivity individuals with total length (TL) smaller than 28 cm were poorly represented and those under 17 cm TL were not represented at all. To overcome this issue, samples were also obtained in Óbidos lagoon (the largest coastal lagoon located in the central coast of Portugal), using a beach seine net, which allowed catching individuals smaller than 10 cm TL.

For each individual the following parameters were taken: TL (to the nearest 0.1 cm), total weight (TW, to the nearest 0.01 g), gutted weight (GW, to the nearest 0.01 g), and gonad and liver weights (W_{gon} and W_{liv}, respectively, to the nearest 0.01 g).

Gonads were stored in a 10 % buffered formaldehyde solution. Tissue samples were dehydrated in a graded ethanol series (70 - 96%) and embedded in methacrylate resin following standard procedures. Sections of 3 μm were stained with toluidine blue. Individuals were sexed histologically as undifferentiated, male, female and bisexual (gonads in a nonfunctional intersexual phase with both ovarian and testicular tissues present).

Length-weight relationship

A total of 904 individuals were obtained: 853 individuals with TL ranging from 17.2 to 45.1 cm were obtained from commercial landings, and 51 individuals with TL ranging from 5.2 to 9.8 cm were caught in Óbidos lagoon (Figure 14). The Mann-Whitney test was applied to evaluate the existence of significant differences between males and females according to TL. The relationship between TL and TW was calculated using a power function ($TW = aTL^b$) and the *t*-test was used to analyze differences in allometric coefficients (Zar, 1999).

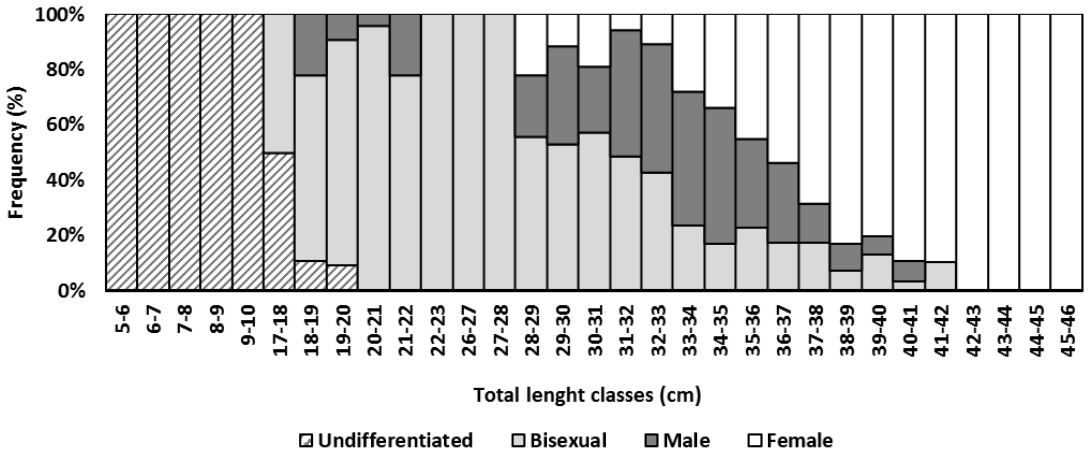

Figure 14. Frequency of undifferentiated, male, female and bisexual individuals of *Sarpa salpa*.

Age and growth

The sagittal otoliths were removed, cleaned and stored dry in plastic vials. Right otoliths were immersed in a 1:1 glycerine-alcohol solution, with *sulcus acusticus* placed downwards, and the posterior region was observed under a stereomicroscope using reflected light, a dark background and an 18x magnification; a total of 579 otoliths were used for age determination.

To analyze the trends in growth pattern, the radius and the distances between the nucleus and the successive growth increments were measured for a total of 487 otoliths, using a micrometer eyepiece.

To analyze the consistency among readers, a subsample of 115 otoliths, covering all available length classes, was read by two of the authors to establish a reading and interpretation criteria, assuming birth date as October 1st (Matić-Skoko *et al*., 2004). Reproducibility between readers was analyzed using the average percent error (APE) (Beamish and Fourier, 1981), the coefficient of variation (CV) (Chang, 1982) and the

index of precision D (Chang, 1982). A test of symmetry (Bowker, 1948) was used to detected systematic differences between the ages assigned by the two readers.

The marginal increment ratio analysis (MIR) (Fowler and Short, 1998) was based on 175 otoliths and was used to test the existence of an annual growth pattern. The MIR was based on individuals ranging from 18.7 to 42.5 cm TL.

To estimate the parameters of the von Bertalanffy growth function (VBGF) (von Bertalanffy, 1938), two approaches were used: whole otolith readings and backcalculation. Backcalculation allowed the estimation of lengths at ages that were rarely observed (Francis, 1990), particularly between 11 and 28 cm TL. The relationship between otolith radius (OR) and TL was established by power regression equation to back calculate the mean length-at-age (Francis, 1990; Folkvord *et al.,* 2002). According to Tuset *et al.* (2004), the standardized residuals of the regression were used to identify which type of estimate (biological or mathematical) should be used and to determine which age class should be considered in the backcalculation analysis. The geometric mean regression was used to calculate the mean length-at-age at lower ages.

VBGF curves were fitted to length-at-age data by nonlinear regression. To choose the best approach, the Akaike's information criterion (AIC) (Akaike, 1973) was used. These analyses were performed using the R routine stats software package (R-3.1.0 for Windows).

Reproduction

Gonad characterization

To test the homogeneity of the oocyte distribution within and between ovaries, six spawning capable females (ranging between 35.4 and 39.2 cm TL) were analyzed. Slices from anterior, middle and posterior regions of the right ovary, and from middle region of left ovary were taken and oocyte size distribution were compared within and between the two ovaries using the analysis of variance (ANOVA).

Digitized images of histological sections were used to characterize each stage of spermatogenesis and oogenesis (maximum and minimum diameters were measured and the average was used). Measurements were performed on 346 male cells and on 624 oocytes with a visible nucleus using the software ImageJ (Schneider *et al*., 2012).

Sexual cycle

The sexual maturity phase of each individual was histologically assigned according to the standardized terminology proposed by Brown-Peterson *et al*. (2011), which divides the microscopic maturity phases into immature (1), developing (2), spawning capable (3 SC) and its actively spawning subphase (3 AS), regressing (4) and regenerating (5). Furthermore, the gonadosomatic index ($GSI = (W_{gon}/GW) \times 100$) of sexually mature individuals was used to identify the spawning season of *S. salpa*. The hepatosomatic index ($HSI = (W_{liv}/GW) \times 100$) and the Fulton condition factor ($K = (GW/TL^3) \times 100$) were also estimated.

Simple regression analysis was used to investigate relationships between GSI with HSI and K. The non-parametric Kruskal-Wallis test was used to investigate the significance of differences in GSI, and K between monthly samples.

Length and age at first maturity

The size at which 50 % of the fish were mature was estimated for 160 males, collected during the spawning season, adjusting the following logistic function: $Y = 1/(1+\exp^{(-1 \times (a+b \times TL))})$, where Y was percentage of mature individuals as a function of size class (TL), and *a* and *b* are constants. Length at first maturity (L_{50}) was estimated as: $L_{50} = -(a/b)$. The substitution of L_{50} in the von Bertalanffy growth function corresponded to the age at first maturity (A_{50}). Due to the inexistence of immature females, it was not possible to calculate their L_{50} or A_{50}.

Fecundity

To investigate the fecundity type of *S. salpa*, the four lines of evidence suggested by Hunter *et al*. (1992), Greer-Walker *et al*. (1994), and Murua and Saborido-Rey (2003) were examined: *(i)* oocyte size-frequency distribution; *(ii)* seasonal variation in the percentage of different oocyte classes during spawning season (*i.e.* previtellogenic and early vitellogenic vs. advanced vitellogenic oocytes); *(iii)* seasonal variation in the mean diameter of the advanced vitellogenic oocytes; and *(iv)* incidence of atresia through the spawning season.

To evaluate line *(i)*, digitized images of histological sections of 32 females in the actively spawning subphase (ranging between 30.5 and 45.1 cm TL) during the spawning season were analyzed, being maximum and minimum oocytes diameters measured using the software ImageJ.

To evaluate lines *(ii) and (iii)*, the gravimetric method (Hunter *et al*., 1989) was applied to 41 ovaries in the actively spawning subphase sampled throughout the spawning season (ranging between 30.5 and 45.1 cm TL). From each ovary, one sample varying between 0.03 - 0.04 g (to the nearest 0.01 g) was taken, placed into a mesh sieve with 125 µm and sprayed with pressure water in order to separate the secondary growth oocytes (comprised between the cortical alveolar and hydrated oocytes stages) from primary growth oocytes (the mesh size sieve was chosen based on the mean diameter value of the cortical alveolar oocytes, estimated in histological sections, please see Results section, Reproduction: Gonad characterization). Then, the oocytes were collected in a watch glass, and separated using a needle and tweezers, in order to be photographed with a digital camera coupled to a stereomicroscope. Subsequently, each image was analyzed by ImageJ software with the ObjectJ plugin (available at: https://sils.fnwi.uva.nl/bcb/objectj) and the diameter threshold for each oocyte stage was defined by previous histological sections analysis. ANOVA was applied to investigate the differences between the mean number of previtellogenic and early vitellogenic oocytes, and advanced vitellogenic oocytes throughout the spawning season. The seasonal variation in the mean diameter of the advanced vitellogenic oocytes throughout spawning season was also analyzed by ANOVA.

To evaluate line *(iv)*, the relative intensity of atresia in vitellogenic oocytes, the percentage of alpha atresia stage oocytes in the total number of oocytes present in an individual ovary (Hunter and Macewicz, 1985), was calculated using digitized images of histological sections from 15 females (varying between 30.5 and 42.9 cm TL) at the actively spawning subphase, throughout the spawning season. ANOVA was used to detect significant statistical differences in the relative intensity of atresia.

The results indicated that *S. salpa* presented determinate fecundity, which implies that the standing stock of vitellogenic oocytes is fixed prior to the onset of the spawning period (Ganias *et al*., 2014) (see Results section, Reproduction: Fecundity). For species with determinate fecundity, the number of vitellogenic oocytes measured prior to the beginning of spawning is considered equivalent to the potential annual fecundity (FP_a) (Hunter *et al*., 1992; Murua *et al*., 2003). Therefore, the gravimetric method (Hunter *et al*., 1989) was applied to estimate FP_a in 26 females at the spawning capable phase (ranging between 33.8 and 42.5 cm TL) without post ovulatory follicles (histologically confirmed). Three subsamples (varying between 0.03 - 0.04 g, to the nearest 0.01 g) were taken from each ovary and no significant differences were found among subsamples of the same female (chi-squared test, $\chi^2 = 66.01$, df = 77, p = 0.81). The same methodology used before to analyze fecundity type lines of evidence *(ii)* and *(iii)* was followed to estimate the fecundity of *S. salpa*. The realized annual fecundity (FR_a) was estimated after discounted atretic losses from FP_a (Murua *et al*., 2003) and the relative annual fecundity was also calculated as the number of oocytes per gram of GW.

Before each ANOVA, the assumptions were checked. Statistical analysis was performed using Statistica software (version 12) and IBM SPSS Statistics (version 22) with a significance level of 0.05.

RESULTS

Length-weight relationship

The males ranged between 18.7 - 40.9 cm TL and females between 28.6 - 45.1 cm TL; the Mann-Whitney test revealed significant differences between sexes ($U = 17123.50$; $p < 0.001$), being the females larger than males (Figure 14).

The relationship between TL and TW was: $TW = 0.06\ TL^{2.64}$, $R^2 = 0.85$ for males and $TW = 0.02TL^{2.91}$, $R^2 = 0.81$, for females. Males presented a negative allometry ($t = -4.24$; $p < 0.05$) while females presented an isometric growth ($t = -1.14$; $p > 0.5$).

Age and growth

Trends in growth pattern

The analysis of the growth increments detected a false increment between 1.08 and 1.25 mm; this false increment was designated as juvenile band since does not correspond to a yearly growth increment because it was already present in smaller individuals (between 5.2 and 9.8 cm TL) captured in the Óbidos lagoon. The truly yearly growth increments were well individualized being the mean and standard deviation of the first 12 increments as follows: 2.02 ± 0.03 mm, 2.81 ± 0.02 mm, 3.21 ± 0.03 mm,

3.51 ± 0.03 mm, 3.76 ± 0.44 mm, 3.99 ± 0.32 mm, 4.16 ± 0.40 mm, 4.31 ± 0.61 mm, 4.53 ± 0.54 mm, 4.78 ± 0.04 mm, 4.98 ± 0.03 mm and 5.19 ± 0.03 mm.

Consistency among readers

The indexes of precision between readers were 1.74 %, 1.37 % and 1.23 % for CV, APE and D, respectively. A total agreement of 79.1 % and an agreement within one year of 97.4 % were achieved. Analyzing the agreement plot which compares the age assignment between readers (Figure 15), reader 2 tended to assign slightly higher ages until age 11. Nevertheless, there was no evidence of systematic disagreement between readers, as demonstrated by the test of symmetry (test of symmetry: χ^2 = 12.33, df = 10, p = 0.26).

As a good agreement between readers was achieved, the remaining otoliths were read twice by the first author, and those estimates that did not differ in more than 1 year were accepted and used to assign a modal age.

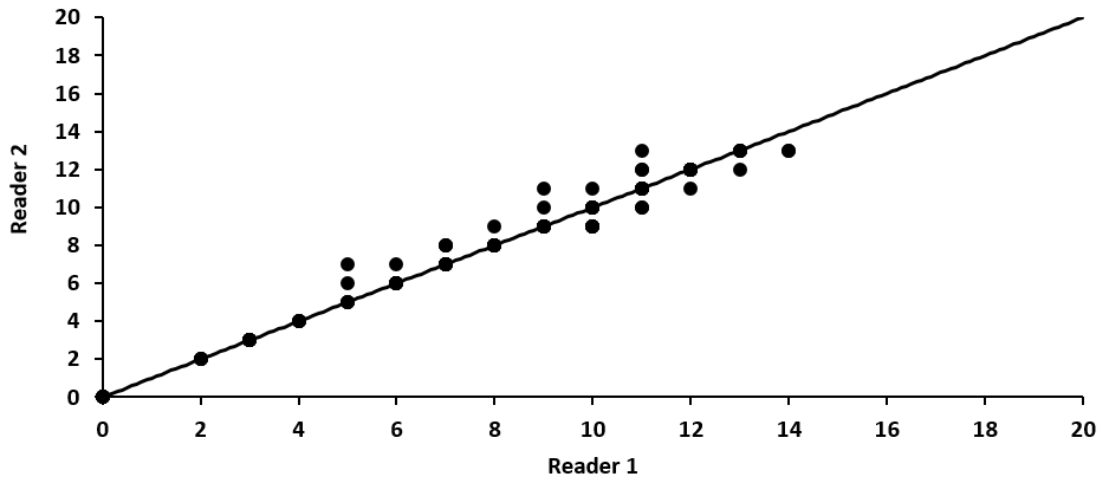

Figure 15. Agreement plot for comparisons between ages assigned by readers 1 and 2 in whole otoliths of *Sarpa salpa*.

MIR, age determination and growth comparisons

MIR showed that there is a clear annual pattern of growth increment formation, with highest values found between May to November and being the lowest values found between December and April, suggesting that the growth increment is formed in the latter period (Figure 16).

For age determination a total of 579 individuals were used, ranging between 5.2 and 44.6 cm TL. Since this species is hermaphrodite and present sex reversal, the estimation of age by sex was not considered (Walt and Beckley, 1997). Table 9 presents the age-length key for all individuals; the minimum age observed was 0 years (5.2 cm TL) and the maximum age observed was 14 years (41.4 cm TL). Individuals with age 1 were not sampled.

Regarding backcalculation approach, a good relationship between TL and OR was obtained for all individuals ($TL = 99.42\ OR^{1.22}$, $R^2 = 0.87$). The backcalculation was only applied until age class 11, since the analysis of the standardized residuals of the regression showed that otolith growth was allometric by age class 12.

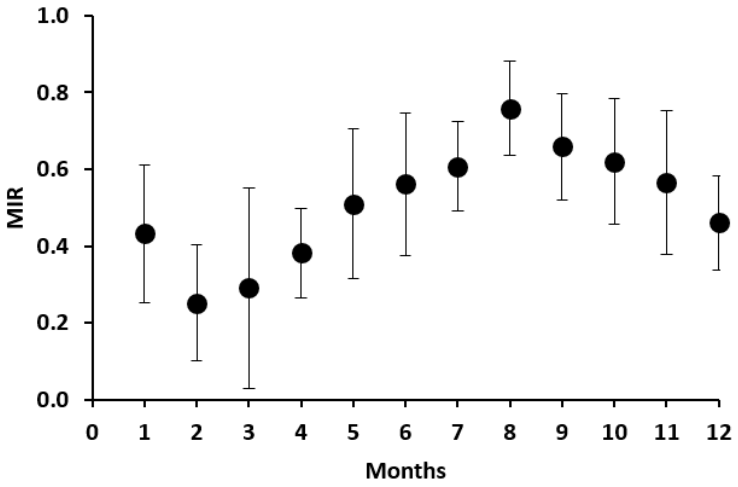

Figure 16. Monthly evolution of marginal increment ratio analysis (MIR) in whole otoliths of *Sarpa salpa*.

Table 9. Age-length (in cm) key obtained by direct reading of the otoliths of *Sarpa salpa*; SD, standard deviation.

Classes (cm)	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	Total
5-6	15															15
6-7	23															23
7-8	7															7
8-9	2															2
9-10	4															4
17-18			2													2
18-19			2	7												9
19-20			5	14	2											21
20-21			8	8	8											24
21-22			1	3	4	1										9
22-23						1										1
26-27				1	1											2
27-28						1	2									3
28-29					1	2		5	1							9
29-30					3	4	5	4	1							17
30-31					1	3	7	6	2	1						20
31-32						4	4	7	6	1	3					25
32-33						1	9	18	15	12	3	2				60
33-34						2	4	11	11	6	3	1				38
34-35						1	2	4	14	9	5	1				36
35-36						1	1	3	11	15	7	4				42
36-37							1	3	13	10	11	4	4	1		47
37-38							1	4	4	9	12	7				37
38-39								5	4	11	13	7	5	1		46
39-40								1	5	4	5	4	5	1	1	26
40-41									3	3	5	8	2	3	1	25
41-42										2	4	2	4	5	1	18
42-43										1	3	2	2			8
43-44											1		1			2
44-45												1				1
Total	51	0	18	33	20	21	36	71	90	84	75	43	23	11	3	579
Mean	6.66		19.72	19.92	23.15	30.17	31.86	33.15	34.83	36.04	37.37	38.31	39.63	40.32	40.50	
SD	1.14		1.11	1.48	4.03	3.40	2.23	2.80	2.61	2.60	2.77	2.64	2.05	1.60	1.00	

Table 10 presents the von Bertalanffy growth parameters obtained from the two approaches used. The value of L_∞ obtained for the whole otolith readings was slightly lower than the maximum observed length, and value of L_∞ obtained for the backcalculation approach was similar to the maximum observed length. The AIC values suggested that the backcalculation approach was the best approach to describe the growth of *S. salpa* (Table 10).

Table 10. Parameters of the von Bertalanffy growth equation calculated for *Sarpa salpa* in the present study and in other studies. Comparisons between backcalculation, determined in the present study, and other studies were presented.

	Method	Study area	L_∞ (cm)	K (year^{-1})	t_0 (year)	AIC	*n*	Size range TL (cm)	Age group	Likelihood comparison between B and other studies		
										χ^2	df	significance
Present study	WOR	Portugal	44.19 (0.70)	0.17 (0.01)	-0.94 (0.07)	2753.00	579	5.2 - 44.6	0 - 14			
Present study	B	Portugal	45.07 (1.84)	0.14 (0.01)	-1.43 (0.17)	27.99	390	7.5 - 38.4	0 - 11			
Villamil *et al*. (2002)	WOR	Canary archipelago	48.00	0.21	-0.97	3937.72	960	12 - 45	0 - 11	91.64	3	*
Pallaoro *et al*. (2008)	WOR	Croatia	36.62	0.22	-0.92	2451.63	756	16 - 44	2 - 15	12.59	3	*
Walt and Beckley (1997)	WOR	east coast of South Africa	22.44	0.55	-0.51	1954.88	575	4 - 27	0 - 6	51.73	3	*

(WOR, whole otolith reading; B, backcalculation; Standard deviation in brackets; AIC, Akaike information criterion; *n*, number of individuals; df, degrees of freedom; $\alpha = 0.05$ and * = $P < 0.05$).

Reproduction

Gonads characterization

Of the 904 specimens analyzed, 55 were undifferentiated, 255 were males, 346 were females and 248 were bisexuals (Figure 14). The individuals ranging between 5.2 and 9.8 cm TL were undifferentiated as well as a small percentage of individuals between 17.6 and 19.70 cm TL (0.4 % of the total number of individuals). The bisexual

individuals appeared between 17.2 and 41.5 cm TL, the males between 18.7 and 40.9 cm TL, and the females between 28.6 and 45.1 cm TL.

In this species, the oocyte development is group-synchronous, since in the maturing ovary at least two cohorts of oocytes can be distinguished: one constituted by small oocytes to be spawned in future breeding seasons, and another formed by larger oocytes to be spawned during the current breeding season (Murua *et al*., 2003). There were no significant differences in oocyte size distribution among and between ovaries (ANOVA, $p > 0.05$), so samples were collected from the middle region of the right ovary.

Table 11 summarizes the range size, mean and standard deviation of different sexual cells present in male and female tissues. All the analyzed males presented inactive female tissue (Figure 17 A and B). When males enter in actively spawning subphase different sexual cells were present (Figure 17 C) and the spermatic duct was full of spermatozoa (Figure 17 A and B). Spawning capable females presented tertiary vitellogenic oocytes (Figure 17 D) and in the actively spawning subphase yolk coalescent (Figure 17 E) and hydrated oocytes (Figure 17 F) were observed. In the case of females two different types of ovaries were found: ovaries with residual male tissue (occupying a small part of the ovary) (Figure 17 G and H) and ovaries with no longer visible male tissue, indicating a complete regression of it; in bisexuals gonads both tissues were present and always in an inactive form (Figure 17 I).

Table 11. Summary of the range size, mean and standard deviation of the different *Sarpa salpa* sexual cells present in male and female tissues.

			Diameter (µm)			
			Min	**Max**	**Mean**	**SD**
Male	Spermatogonia	Sg	3.92	5.87	4.72	0.55
	Primary spermatocysts	Sc1	3.20	4.36	3.68	0.25
	Secondary spermatocysts	Sc2	1.77	2.87	2.17	0.21
	Spermatids	St	1.62	2.18	1.82	0.16
	Spermatozoa	Sz	1.26	1.88	1.67	0.14
Female	Primary growth	Pg	30.51	112.48	69.81	17.69
	Cortical alveolar	Ca	90.52	160.78	125.39	15.05
	Primary vitellogenic	Vtg1	139.69	227.74	167.93	17.53
	Secondary vitellogenic	Vtg2	175.32	308.31	237.10	30.87
	Tertiary vitellogenic	Vtg3	320.26	506.92	425.54	45.28
	Germinal vesicle migration	GVM	339.73	501.29	436.51	35.63
	Germinal vesicle breakdown & Yolk coalescent	GVBD & Yc	358.43	592.69	468.84	47.05
	Hydrated	H	527.08	907.24	599.20	89.52

Sexual cycle

The distribution of the maturity phases of males and females throughout the year is presented in Figure 18. Immature males were rare, being only detected in January and November, and developing males occurred from June to November. Males spawning season started in September, with more than 50 % being in the actively spawning subphase, and ended in November. No males were sampled in May. Regarding females, no immature specimens were sampled. Females spawning season coincided with males, starting in September and ending in November, and more than 50 % of females in the regenerating phase was observed from December to July. Males and females GSI followed the annual sexual cycle pattern (Figure 18 A and B): in males, the GSI reached their maximum value in October and in the case of females in September. The monthly evolution of the HSI and K is presented in Figure 19 A for males and in Figure 19 B for females. The maximum male HSI value was observed in April and the minimum value in December while in the case of females, the maximum value was

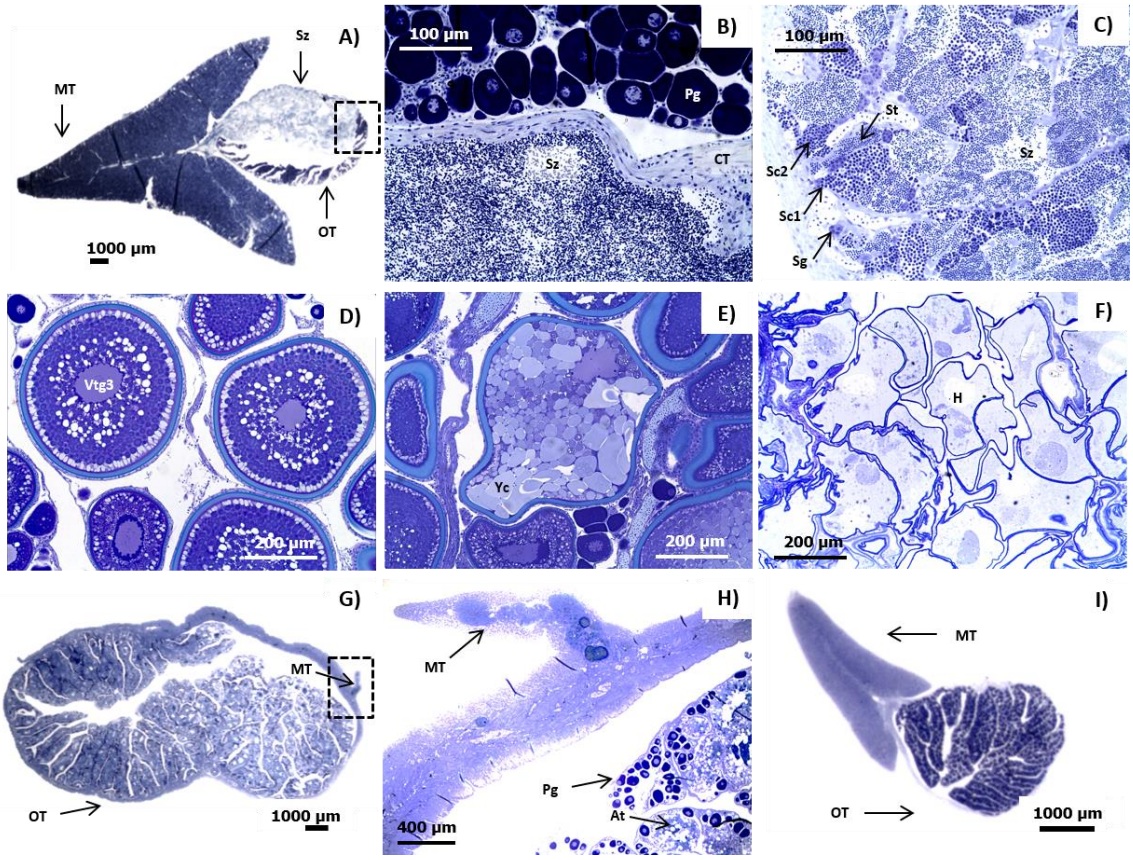

Figure 17. Cross sections of *Sarpa salpa* gonads: **A)** male at the actively spawning phase; **B)** detailed of figure A) assigned by a dashed square; **C)** different sexual cells present in male at the actively spawning subphase; **D)** tertiary vitellogenic oocyte in a female at the spawning capable phase; **E)** yolk coalescence oocyte in a female at the actively spawning subphase; **F)** hydrated oocytes of a female at the actively spawning subphase; **G)** female at the regressing phase; **H)** detailed of figure G) assigned by a dashed square; **I)** bisexual individual with both inactive tissues, male and female. At, atresia; CT, connective tissue; H, hydrated oocyte; MT, male tissue; OT, ovarian tissue; Pg, primary growth oocyte; Sc1, primary spermatocytes; Sc2, secondary spermatocytes; Sg, spermatogonia; St, spermatids; Sz, spermatozoa; Vtg3, tertiary vitellogenic oocyte; Yc, yolk coalescence oocyte.

observed in September and the minimum value in March. The mean K values remain relatively low (varying between 1.0 – 1.4 %) throughout the year for both sexes, in the case of males it reached their maximum value in July and in the case of females in August. Males and females GSI, HSI and K were significantly different throughout the year, as showed by Kruskal-Wallis test (H_{GSI} = 104.11, H_{HSI} = 65.65, H_K = 98.15 for males and H_{GSI}= 200.76, H_{HSI} = 121.75, H_K = 178.41, for females, $p < 0.001$). GSI and HSI for both males and females were significantly related (males: R = 0.16 and p = 0.01; females: R = 0.27 and $p < 0.001$). The relationship between GSI and K was significantly

correlated for females (R = 0.25 and p < 0.001) but not significant for males (R = 0.06 and p = 0.32).

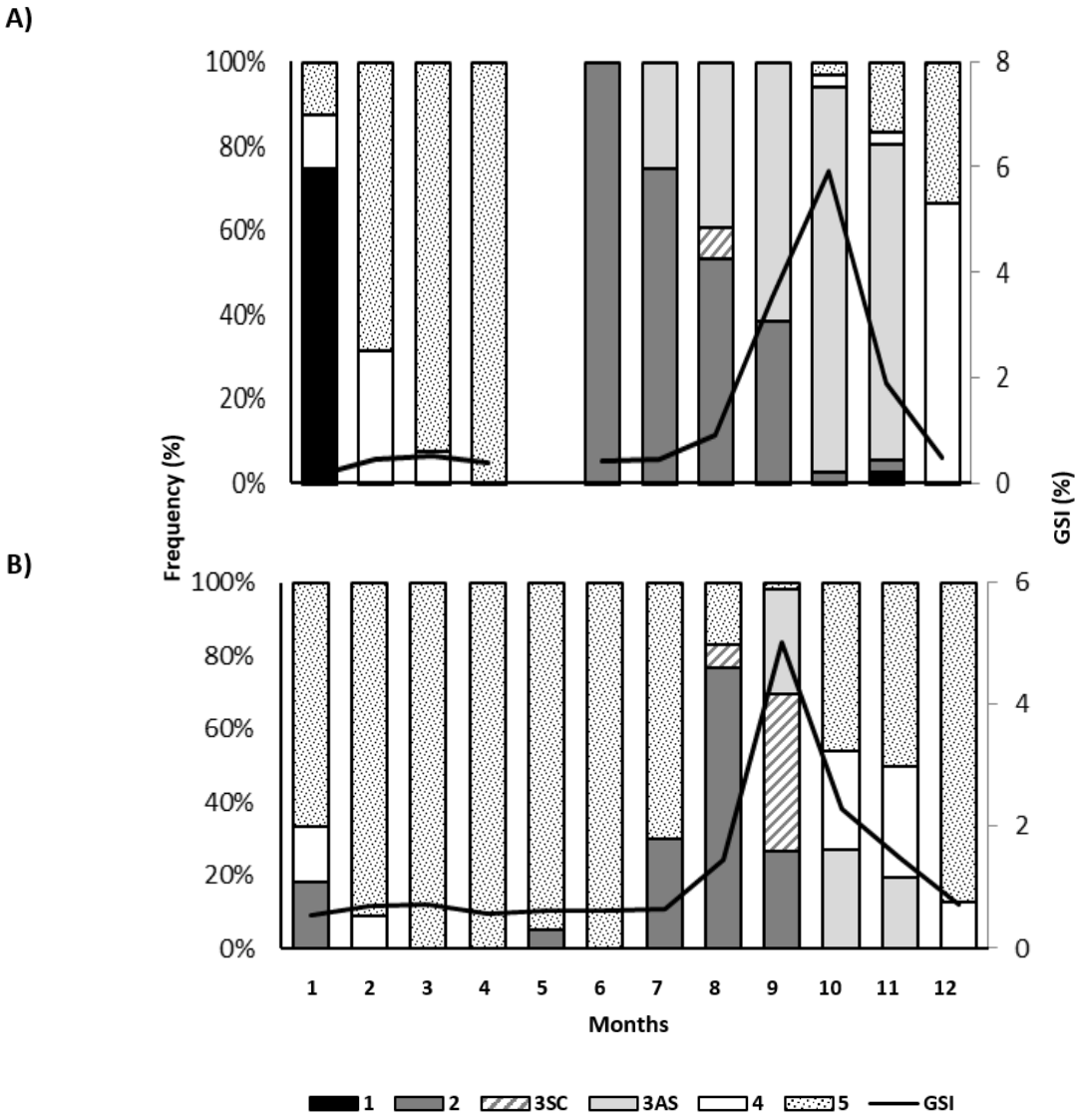

Figure 18. Monthly variation of the maturity phases and the gonadosomatic index (GSI) for *Sarpa salpa* **A)** males and **B)** females. 1, immature; 2, developing; 3 SC, spawning capable; 3 AS, actively spawning; 4, regressing; 5, regenerating.

Length and age at first maturity

The following logistic function $Y = 1/(1+\exp^{(-1 \times (173.63+7.09 \times TL))})$ was adjusted to 160 males collected during the spawning season. The estimated L_{50} was 24.5 cm TL and, based on the previous age determination, the length at first maturity corresponded to age 2. All females sampled were sexually mature and the smallest had 28.6 cm TL.

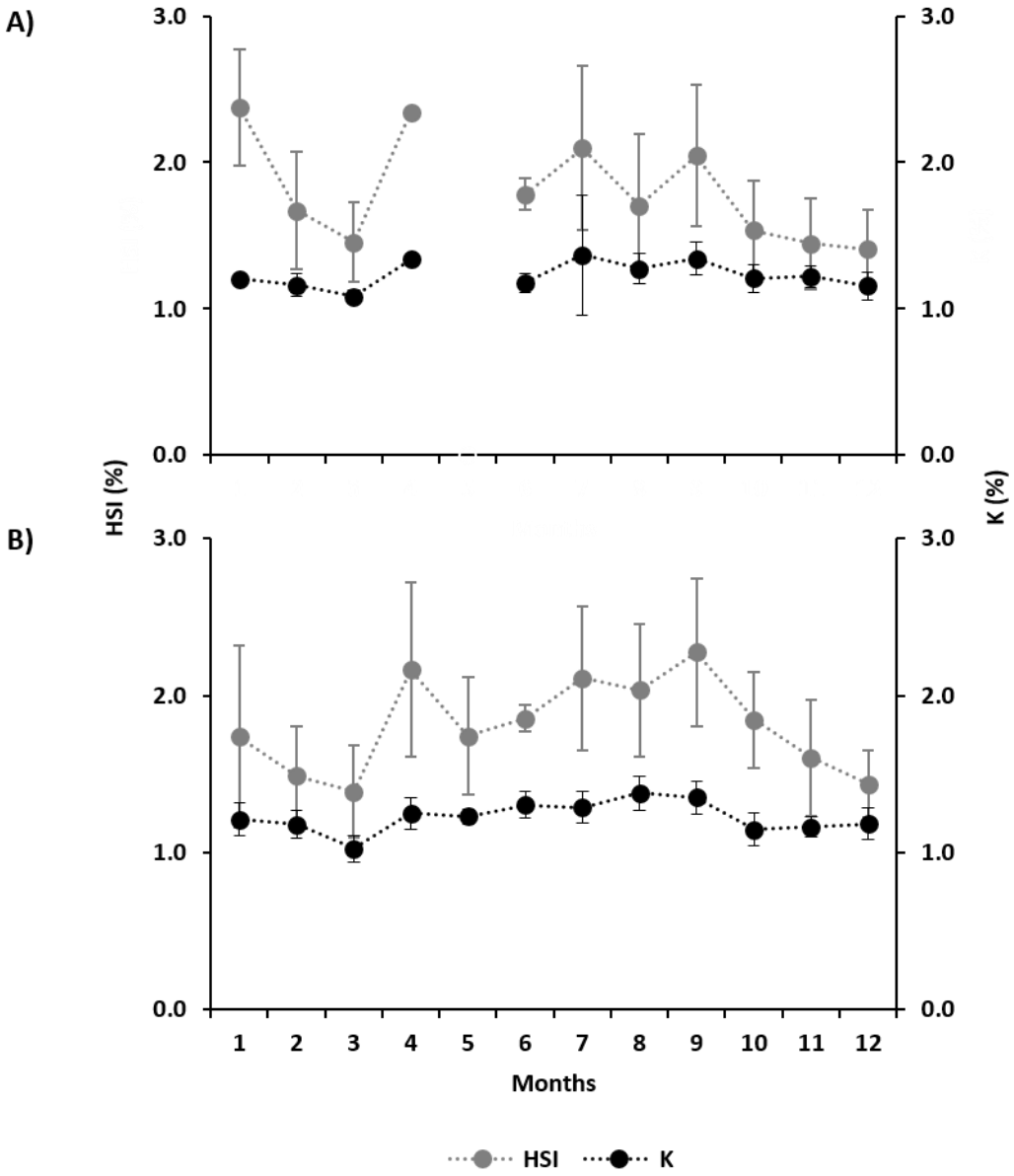

Figure 19. Monthly variation of the mean and standard error of, hepatosomatic index (HSI) and Fulton's condition factor (K) for *Sarpa salpa* **A)** males and **B)** females.

Fecundity

In this study four lines of evidence were analysed in order to classify the fecundity type of *S. salpa*. For the *(i)* line of evidence, the oocyte size-frequency distribution, of the actively spawning females during the spawning season, was continuous between primary and secondary oocytes for September and October but at the end of the spawning season, in the period between 1 - 15 November, a gap starts to form between 125 and 375 µm, separating the less developed oocytes from the more developed ones, and in the period 15 - 30 November only oocytes lower than 125 µm and oocytes larger than 475 µm were present (Figure 20). Regarding the *(ii)* line, the mean oocyte number of previtellogenic and early vitellogenic oocytes showed a decreasing statistical significant trend (ANOVA: $p < 0.05$) throughout the spawning season (Figure 21 A); the mean oocyte number of advanced vitellogenic oocytes throughout the spawning season also showed a decreasing statistical significant trend (ANOVA: $p < 0.05$). For the *(iii)* line, the mean oocyte diameter of the advanced vitellogenic oocytes increased during the spawning season but it was not statistically significant (ANOVA: $p = 0.06$) (Figure 21 B). Finally for the *(iv)* line of evidence, the relative intensity of alpha atresia was low throughout the spawning season, varying between 7 and 14 % (monthly mean values), and no significant differences between months were found (ANOVA: $p = 0.17$).

The FP_a ranged between 273 424 and 2 453 753 oocytes and FR_a ranged between 266 133 and 2 453 753 oocytes and no significant differences were found between these estimations (t-test: $p = 0.92$). A significant power relation of FR_a with W_{gon}, GW and TL was observed, although was stronger with W_{gon} (FR_a vs W_{gon}: $r = 0.91$, $p < 0.05$; FR_a vs

GW: r = 0.69, p < 0.05; FR_a vs TL: r = 0.67, p < 0.05). The relative annual fecundity varied between 462 oocytes g^{-1} GW (37.5 cm TL and 591.19 g GW) and 2 662 oocytes g^{-1} GW (41.2 cm TL and 921.62 g GW) with a mean and standard deviation of 1336 ± 571 oocytes g^{-1} GW.

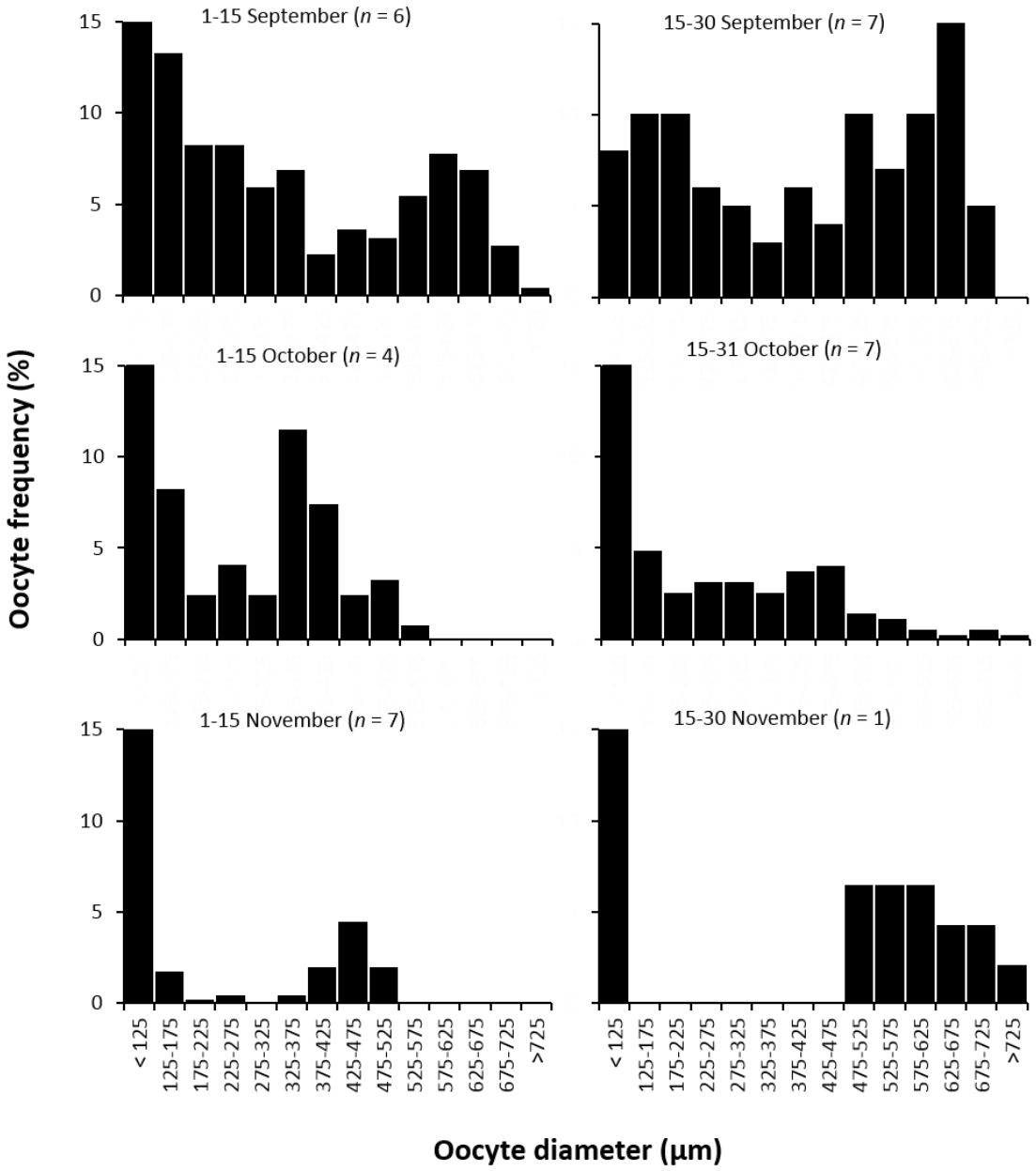

Figure 20. *Sarpa salpa* oocyte size-frequency distribution per 50 µm diameter class through the spawning period. *n*, number of females used in each sampling period.

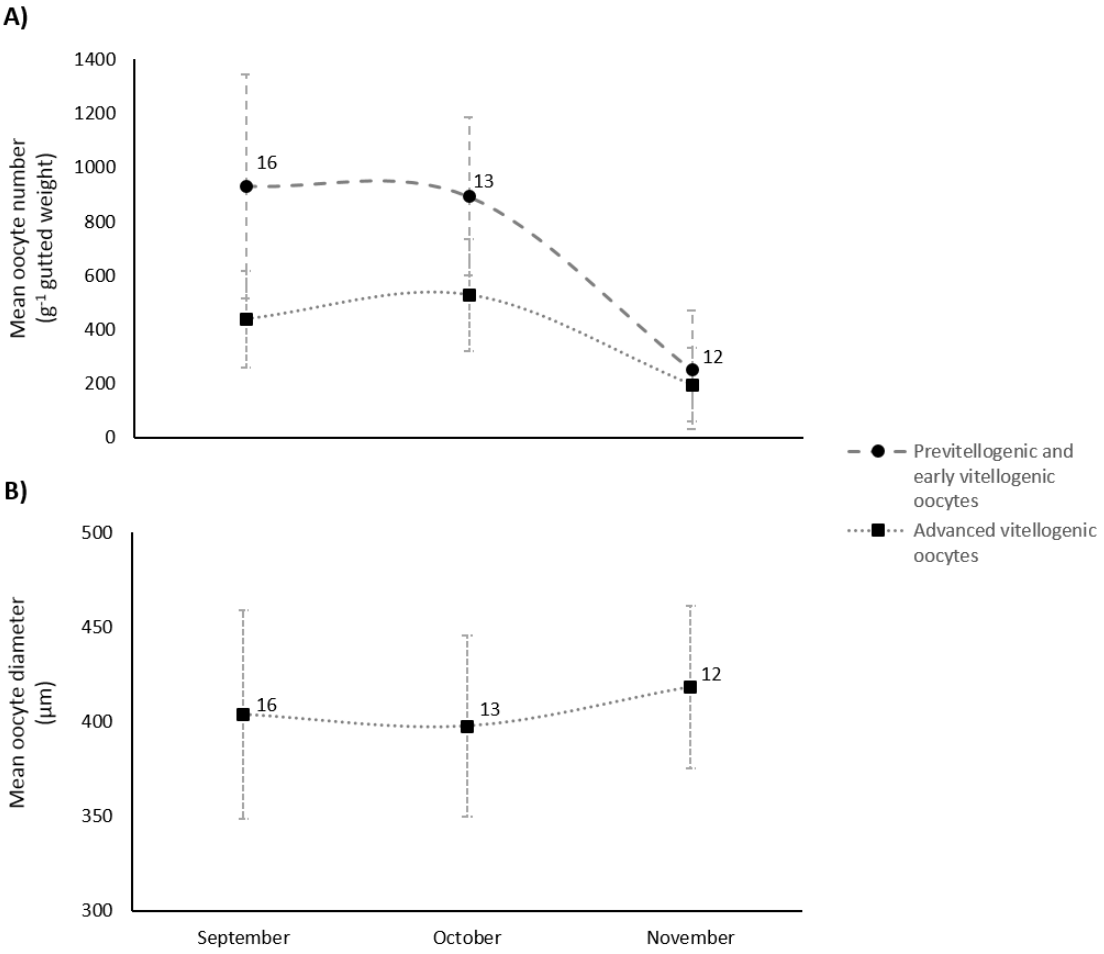

Figure 21. Variation of the **A)** mean and standard error of different oocyte classes, and the **B)** mean and standard error of the diameter of advanced vitellogenic oocytes along the spawning season of *Sarpa salpa*. The number of females used are given in the chart for each month.

DISCUSSION

Length-weight relationship

This study presents the greatest length range ever published on the species. Females attained higher lengths than males as already referred by other authors such as Criscoli *et al*. (2006), in the central western coasts of Italy, and Villamil *et al.* (2002), in the Canary archipelago. This species is a protandrous hermaphrodite, and therefore the

difference in length distribution by sex is due to the hermaphrodite condition and cannot be considered as sexual dimorphism.

Age and growth

Otoliths of *S. salpa* showed the common increment deposition pattern of Teleostei, with translucent rings alternating with opaque rings, attributed to slow and fast growth periods, respectively. The presence of a juvenile band must be most certainly the result of an early migration to nursery areas like other species, such as European seabass, *Dicentrarchus labrax* (Linnaeus, 1758) (Gordo, 1989). Although the species presents a good individualization of the growth increments until age 12, the first two are better defined and separated which may be related to the age at first maturity obtained for males. In fact, it is known that after maturation occurs, the available energy must be first channeled to the reproductive effort and secondly to growth, leading to narrow growth increments in the forthcoming years (Wootton, 1992).

Whole otolith readings were compared among two readers to minimize the error associated with the subjectivity of age estimation. The APE measures the amount of variation between ages (Walt and Beckley, 1997) and the values estimated in the present study are below the APEs calculated for the Canary archipelago (3.1 %, Villamil *et al*. (2002)) and for the east coast of South Africa (3.9 %, Walt and Beckley (1997)), indicating a good reproducibility of age determinations for the present study. The ranges of the precision estimates for the two readers were well below the average values reported in the literature (Campana, 2001). The high precision indices found in

this work also suggested that whole otoliths are adequate for age and growth studies of this species and no further techniques for ring enhancement are needed, avoiding expensive and long analysis processes (Paiva *et al*., 2013).

These results suggest that *S. salpa* is a medium-lived species since the oldest individual was estimated to be 14 years old. The maximum age obtained is similar to the one obtained in the Adriatic Sea (15 years, 44 cm TL) (Pallaoro *et al*., 2008), but is higher than the age estimated in the east coast of South Africa (6 years, 27 cm TL) (Walt and Beckley, 1997), in the central western coasts of Italy (7 years, 33 cm TL) (Criscoli *et al*., 2006) and in the Canary archipelago (11 years, 45 cm TL) (Villamil *et al*., 2002).

The backcalculation approach was chosen as the best one to describe the growth of *S. salpa* from Portuguese waters and significant differences were obtained in the comparison of the growth parameters estimated using this technique and all other areas (Table 10). These discrepancies may be due to different criteria in growth pattern interpretation used in the several laboratories. To overcome this issue, an interchange otolith program should be implemented between all laboratories involved in this fishery. Different environmental/habitat characteristics from each area may also contribute to explain the differences found in the growth pattern.

Reproduction

Analysis of gonad organization and development confirmed that *S. salpa* is a protandrous hermaphrodite. The existence of bisexual gonads between 17.2 and 41.5 cm TL showed that the sex change process is a gradual phenomenon (Criscoli *et al*.,2006). This had already been reported for the species in the central-east Atlantic

(Canary Archipelago) (Villamil *et al*., 2002) where similar results (sex changed between 22.1 and 38.0 cm TL) were obtained.

Different spawning periods have been described along the distribution area and in the present study *S. salpa* spawns from September to November, which is similar with the results described for the Mediterranean Sea [Adriatic: September to October (Pallaoro *et al*., 2008); western central coast of Italy: March to May and September to November (Criscoli *et al*., 2006); Libyan coast: October to December (El-Etreby *et al*., 2015)] but is different for the results obtained for the Canary Archipelago which described a maximal gonadal activity between December and January (Villamil *et al*., 2002). Nevertheless, in the east coast of South Africa the spawning season of *S. salpa* extended from March to September, with a reproductive peak from April to August (Walt and Mann, 1998). These differences may be a result of different biotic (mainly nutritional) and environmental factors (Sarkar and Upadhyay, 2011), or by the combination of both (Falcón *et al*., 2003).

The estimated L_{50} for males (24.5 cm TL) was bigger than the values recorded in the east coast of South Africa (14.5 cm fork length) (Walt and Mann, 1998), in central western coasts of Italy (19.5 cm TL) (Criscoli *et al*., 2006) and in the Adriatic (20.6 cm TL) (Pallaoro *et al*., 2008), but was lower than the value recorded in the Canary archipelago (26.6 cm TL) (Villamil *et al*., 2002). Our results did not allow to estimate the females L_{50} since no immature females were found. This means that, after the sexual inversion all females mature in the following season. However, and for comparison purposes, the smallest mature female obtained in the present study (28.6cm TL) was similar to the estimated L_{50} for females (29.4 cm TL), recorded in the

Canary archipelago (Villamil *et al*., 2002). The estimated A_{50} for males (2 years) is in agreement with those reported by Villamil *et al*. (2002) as these authors pointed out that *S. salpa* attained sexual maturity between the end of the first and the second year of life.

According to Gordo *et al*. (2008), fecundity estimates are essential to calculate the spawning stock biomass, so the clarification of the fecundity type is crucial because the fecundity calculations varied with the fecundity type. Two types of fecundity can be defined: determinate and indeterminate (Hunter *et al*., 1992; Murua and Saborido-Rey, 2003). In fishes with determinate fecundity, oocyte recruitment is completed before onset of spawning and hence the number of advanced oocytes in the ovary corresponds to the FP_a; in contrast, in fishes exhibiting indeterminate fecundity, oocyte recruitment and spawning period overlaps, *i.e.* potential fecundity is not fixed before the beginning of spawning (Ganias *et al*., 2014). This is the first study to investigate the fecundity type of *S. salpa* based on the four lines of evidence, as suggested by Hunter *et al*. (1992), Greer-Walker *et al*. (1994), and Murua and Saborido-Rey (2003), and synthetized in Ganias *et al*. (2014).

For the *(i)* line of evidence no distinct hiatus could be observed between the primary and secondary oocytes growth stages during the spawning season; although a distinct gap could be observed near the end of spawning season in November. A distinct hiatus indicates that annual fecundity is determinate whereas the lack of a hiatus may indicate that annual fecundity is indeterminate. However, the lack of a hiatus does not necessarily indicate that fecundity is indeterminate (Murua and Saborido-Rey, 2003) *Merlangus merlangus* (Hislop and Hall, 1974), *Scomber scombrus* (Greer-Walker *et al*., 1994), *Solea solea* (Witthames and Greer-Walker, 1995) and *Trisopterus luscus*

(Alonso-Fernández, 2011) did not present a distinct hiatus but had a determinate fecundity. Regarding the *(ii)* line of evidence, a statistically significant decrease in the mean number of previtellogenic and early vitellogenic oocytes and in the mean number of advanced vitellogenic oocytes was found during spawning season, which is a clear evidence for determinate fecundity where there is no replacement of oocytes after each spawning event (Murua and Saborido-Rey, 2003). Regarding the *(iii)* line of evidence, an increase pattern in the mean diameter of advanced vitellogenic oocytes was found along the spawning season although with no statistical significance. In fishes with determinate fecundity a seasonal increase in the mean diameter of secondary growth oocytes may be expected along the spawning season (Murua and Saborido-Rey, 2003). However, the mean diameter of secondary growth oocytes remains constant or declines as the spawning season progresses in some species with determinate fecundity, for example in *S. scombrus* (Greer-Walker *et al*., 1994) and in *Gadus morhua* (Kjesbu *et al*., 1990), respectively. The results obtained in this line of evidence would be improved if a larger number of actively spawning females could have been sampled during the spawning season, which reduces the reliability of this analysis. Finally for the *(iv)* line of evidence, the relative intensity of alpha atresia calculated was low through the spawning season, which favors the criterion of determinate fecundity. These lines of evidence suggested that the fecundity of *S. salpa* can be considered as determinate and fecundity estimation was made accordingly.

El-Etreby *et al*. (2015) studied the fecundity of *S. salpa* in Libya and found that the potential fecundity ranged from 22 952 to 15 123 096 oocytes and the relative fecundity ranged from 568 to 12 287 oocytes g^{-1} GW; these estimated ranges of both potential and relative fecundities were much larger than the ones obtained in the

present study. These differences may be caused by the method of counting and by the different environmental conditions existing in each area where the individuals inhabit.

As mentioned in the Introduction section, *S. salpa* has an unusual reproductive strategy associated with cystic structures in fish ovaries; these structures could correspond to remains of hydrated oocytes that can appear either isolated or in groups, forming a cystic structure (Paiva *et al*., 2014). Their duration in the ovaries and their role in the reproductive strategy is not fully understood and further studies are necessary to clarify these issues but their presence may result in a reduction of the fecundity estimates.

Life history parameters can be used to provide baseline data to be applied in ecological modeling, to inform and improve the fisheries management and for stock assessments. The knowledge on life history parameters of *S. salpa*, such as medium-slow growth, short spawning season, L_{50} around 25 cm, and fecundity estimates could be used in future assessment models in order to implement sustainable management measures. For example, in the Portuguese waters, 18 cm TL is the minimal length allowed to catch *S. salpa* (DGRM, 2015) but the estimated L_{50} in the present work was higher, suggesting the need to modify the present value. So, the determination of the life history parameters is particularly valuable to improve effective and caution management plans.

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CHAPTER 5

Cystic structures

Cystic structures in fish ovaries: more common than we think - the case study of *Sarpa salpa* (Sparidae)

SUMMARY

Ovaries of salema *Sarpa salpa* presented hard structures easily seen macroscopically and histological analysis suggested that these structures correspond to masses of hydrated oocytes, which are remnants of an incomplete or unsuccessful spawning event. The generalised linear model (GLM) showed that the presence of cysts was significantly affected by month. The fact that these structures appear frequently and in high prevalence may suggest that they are related to or associated with the reproductive strategy of this species.

INTRODUCTION

The morphological characteristics of follicular atresia in the fish ovary have been known for more than three decades (Babin *et al*., 2007), but the cellular and molecular aspects of follicular atresia involving the degenerative processes of the oocyte and the follicular wall is not completely understood (Miranda *et al*., 1999). The presence of atretic follicles in the teleost ovary can be associated with stress, fasting, biocidal agents, light, temperature, confinement and inadequate hormone levels (Miranda *et al*., 1999). Atresia is present in all species and may affect all types of oocytes. Its intensity is usually low throughout the year, although it may increase during spawning season, especially in species with indeterminate fecundity that usually present mass atresia events at the end of that period (Murua and Saborido-Rey, 2003). Changes in the atretic process caused by an inflammation of the follicular envelope, leading to the formation of cysts at different stages of follicular development have been reported

recently (Domínguez-Petit *et al*., 2011), although atresia of hydrated oocytes is considered rare and is poorly documented (Rideout and Burton, 2000; Brown-Peterson *et al*., 2007; Alonso-Fernández, 2011; Madureira *et al*., 2011).

This study focuses on an eurytherm sparid fish, salema *Sarpa salpa* (Linnaeus, 1758), a protandrous hermaphrodite (Criscoli *et al*., 2006). Information on reproduction of *S. salpa* is available only for the Canary Islands and is based only on macroscopic analysis of the gonads (Villamil *et al*., 2002). The present work is the first study to describe the morphology of cystic structures in *S. salpa* ovaries and to analyze the prevalence of aggregated cystic structures throughout the year and its relation to specimens' size and sexual maturity stage.

MATERIALS AND METHODS

Sampled individuals were obtained on a monthly basis between January 2012 and July 2013 and captured with artisanal fisheries in the central region of the Portuguese coast.

Because this species is a protandric hermaphrodite, only individuals above 28 cm of total length (TL) (females) were considered. The TL (to the nearest 0.1 cm) and the gutted weight (GW, to the nearest 0.01 g) were registered. Ovaries were removed, weighed (W_{ova}, to the nearest 0.01 g) and stored in a 10 % formaldehyde solution buffered at pH = 7 with carbonates (CH2O; M = 30.03; D = 1.02 g/cm^3). Tissue samples were dehydrated in a graded ethanol series (70 - 96 %) and embedded in a methacrylate resin and in paraffin, following standard histological techniques. Methacrylate resin and paraffin sections of 3 µm were cut and stained with toluidine

blue and haematoxylin and eosin, respectively. The sexual maturity stage of each individual was histologically assigned according to Brown-Peterson *et al*. (2011). The gonadosomatic index (GSI) was calculated according to the following expression: GSI = (W_{ova}/GW) × 100. Also Fulton condition factor (K) was estimated as K = (GW/TL^3) × 100.

The cyst prevalence, defined as the percentage of females with aggregated cystic structure in the ovary, relatively to the total number of sampled mature females, was estimated by month and TL of females. Analyses of variance using the generalised linear model (GLM) were conducted to test the influence of month, GSI, K and TL on cyst presence; the analyses were performed in the software Statistica 11.

RESULTS

A total of 239 females were sampled, ranging from 28.6 to 44.6 cm TL (Table 12) and the histological analysis showed that all females were sexually mature. Macroscopically, the ovaries revealed the presence of hard structures with a yellow to brown colour (Figure 22 A) in 24.3 % of the females sampled. Histological analysis suggested that these structures correspond to remains of hydrated oocytes that can appear isolated or in groups that join together forming a cystic structure (Figure 22 B and C) corresponding to an incomplete or unsuccessful spawning event. Cysts seemed to be formed by numerous *zona radiata* membranes folded over one another and surrounded by a stratified epithelium (Figure 22 D). Macrophages in the cyst envelope were also observed (Figure 22 C).

These cysts were found in all months (Table 12), except in October, and the mean prevalence value was higher (ranging between 40 and 67 %) in months that precede the spawning period, August - November (R. B. Paiva, personal observation). The greater prevalence of these structures (45.2 %) was found in the 37 - 38 cm TL class. Regarding the maturity stages, the cysts were only observed in the regenerating stage (according to the classification of Brown-Peterson *et al*. (2011)), affecting 36.0 % of females in this stage. The GLM showed that the presence of cysts was significantly affected by month (GLM: F = 2.921; d. f. = 11; $p < 0.05$) and was not affected by the TL of the females (GLM: F = 0.007; d. f. = 1; p = 0.93), the GSI (GLM: F = 0.013; d. f. = 1; p = 0.90) or K (GLM: F = 0.307; d. f. = 1; p = 0.58).

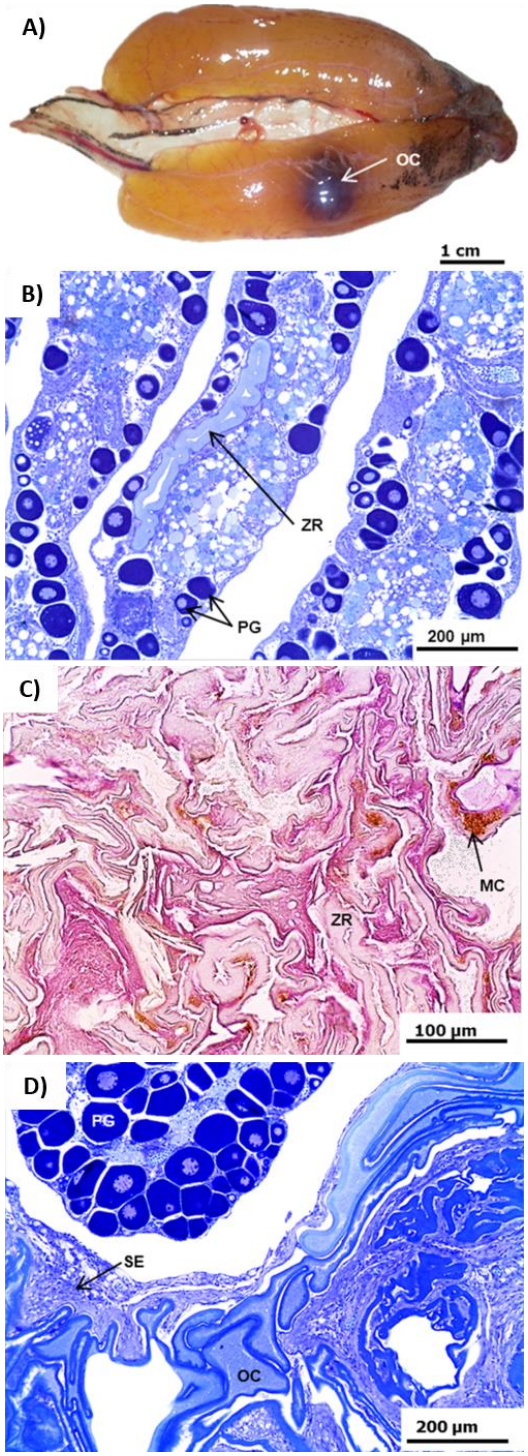

Figure 22. Ovary images of *Sarpa salpa*: **A)** Ovarian cyst observed macroscopically; **B)** Isolated cystic structure stained with toluidine blue; **C)** Aggregated cystic structure stained with haematoxylin and eosin; **D)** Detail of an aggregated cystic structure stained with toluidine blue. MC: macrophages; OC: ovarian cyst; PG: oocyte primary growth; SE: stratified epithelium; ZR: *zona radiata*.

Table 12. Mean ± standard deviation (S.D.) of the total length (TL) and gonadosomatic index (GSI), cyst prevalence and number of *Sarpa salpa* females (*n*).

Month	Mean TL (cm) ± S.D.	Mean GSI ± S.D.	Cyst prevalence (%)	*n*
January	35.69 ± 3.64	0.55 ± 0.10	3.30	33
February	36.20 ± 2.78	0.68 ± 0.20	26.09	23
March	35.43 ± 1.86	0.71 ± 0.20	43.75	16
April	36.29 ± 2.10	0.56 ± 0.10	41.67	12
May	36.09 ± 1.90	0.62 ± 0.10	44.44	18
June	33.70 ± 1.84	0.62 ± 0.10	50.00	2
July	36.79 ± 2.58	0.66 ± 0.20	66.67	15
August	36.88 ± 1.97	1.18 ± 0.50	19.05	21
September	37.11 ± 2.15	2.68 ± 0.50	5.26	19
October	36.61 ± 1.81	1.15 ± 0.60	0.00	17
November	36.42 ± 1.80	0.79 ± 0.50	17.95	39
December	38.10 ± 2.65	0.71 ± 0.20	33.33	24
Total				**239**

DISCUSSION

The presence of remains of hydrated oocytes in fishes, isolated or aggregated in the ovary, has been reported sporadically, e.g., zebrafish *Danio rerio* (Hamilton, 1822) (Madureira *et al*., 2011), atlantic cod *Gadus morhua* Linnaeus, 1758 (Rideout and Burton, 2000), atlantic blue marlin *Makaira nigricans* Lacépède, 1802 (Brown-Peterson *et al*., 2007) and pouting *Trisopterus luscus* (Linnaeus, 1758) (Alonso-Fernández, 2011), and has been related to abnormal atresia due to changes in the energy requirement of females (Rideout *et al*., 2005), availability of food (Burton and Idler, 1984), environmental conditions (e.g., temperature, pH or the presence of pollutants) and population factors (e.g., changes in the proportion of sexes) (Domínguez-Petit *et al*.,

2011). The presence of these cystic structures was also observed in several other species along the Portuguese coast such as boarfish *Capros aper* (Linnaeus, 1758) (V. Sequeira, personal observation), Senegal seabream *Diplodus bellottii* (Steindachner, 1882) (C. Vendrell, personal observation) and forkbeard *Phycis phycis* Linnaeus, 1766 (A. R. Vieira, personal observation). However, the presence of a great prevalence of cysts (not sporadic) has never been reported in any species. Although the reasons that lead to this new type of degeneration are still unknown, the fact that these structures appear frequently (all months except in October) and in higher values in the months preceding the GSI highest values (Table 12), suggest that this process may be part of the reproductive strategy for *S. salpa*. Furthermore, no environmental perturbation or anthropogenic disturbance occurred in the sampling area that could be associated to this phenomenon in the sampling years. These structures showed to be relatively loose inside the ovary and were observed very close to the genital opening, being easily released after a slight pressure on the ovary. This suggests the hypothesis that the cysts can be released before spawning begins, allowing for wider space in the ovary for future feasible oocytes. Further studies are necessary to clarify this issue and determine how long the cysts remain in the ovaries.

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CHAPTER 6

General discussion

Conclusions

General discussion

The present study aimed to clarify the stock structure of *Sarpa salpa* in the eastern Atlantic Ocean, and to characterize the biology of the Portuguese mainland population. To accomplish the first objective, three techniques were used: body and otolith morphometrics, and genetics. For the first two methodologies, a total of 166 individuals, from three areas along the mainland coast of Portugal (north region - Esposende, central region - Setúbal, and south region - Algarve) and from Madeira waters, were collected and studied using landmark-based geometric morphometric methods and the elliptic Fourier descriptors (EFDs), respectively. In the genetic study, *S. salpa* individuals were collected from 11 sampling sites distributed across the Northeastern Atlantic Ocean and the Mediterranean Sea (Apulia, Peniche, Setúbal, Algarve, Morocco, Azores, Madeira, and Murcia, Italy, Croatia, Greece, respectively). The phylogeographic pattern of *S. salpa* was analyzed using 109 sequences from the mitochondrial control region and 86 sequences from the first intron of the nuclear S7 ribosomal protein gene.

For the second objective, the age, growth, and reproduction parameters of *S. salpa* captured off the coast of Portugal were described. To accomplish this goal, commercial samples of *S. salpa* were obtained on a monthly basis, reaching a total of 579 individuals for the age determination and a total of 904 individuals for the reproduction analysis. For age and growth studies supplementary samples of smaller individuals were taken from beach seine net, because individuals with total length (TL)

under 17 cm were not captured due to the gear selectivity of the trammel nets used by artisanal fisheries. The analysis of the ovaries of *S. salpa* revealed the presence of hard structures, easily seen macroscopically. These structures were morphologically described and statistically analyzed since there is no previous reference of these structures in *S. salpa* ovaries'.

PART I - STOCK STRUCTURE

Morphometric analysis

Both morphometric techniques indicated some overlap in the mainland specimens and a clear separation of Madeira individuals. Concerning the body geometric morphometric analysis, individuals were highly correctly classified to geographic areas (86.7 %), being specimens from Madeira and the north mainland well differentiated from the other areas; on the contrary, specimens from central and south mainland, which are geographically closer, showed some overlap. For the otolith shape data analyses, size-corrected normalized EFDs showed significant effect of area on otolith contour shape. An overall classification success of 70.2 % on the canonical discriminant analyses was achieved suggesting populations discrimination on Portuguese waters, although an overlap between the three areas along the mainland coast, particularly between central and south areas, was present. The results support the existence of local populations of *S. salpa* that should be considered in the future management options. The results indicated that at least two stocks may occur, one in Madeira and the other in mainland waters. However, and due to the low affinities

between the northern population when compared with the individuals from central and southern areas, one additional stock could be considered in northern mainland. Nevertheless, other studies should be implemented to confirm this hypothesis.

Genetic analysis

The genetic analysis of *S. salpa* detected 99 haplotypes and 32 haplotypes for the mitochondrial control region and the first intron of the nuclear S7 sequences, respectively. For the mitochondrial control region 91 haplotypes were singletons, while for the first intron of the nuclear S7 20 haplotypes were singletons; this high number of singletons or private haplotypes suggests the reduction of current gene flow among sampling sites. A lack of structuration between the Atlantic Ocean and Mediterranean Sea samples was evidenced by both markers and has already been found in other phylogeographic studies (Domingues *et al*., 2007b); the degree of genetic differentiation and species structuration is influenced by certain factors as the homing or movement of adults to localized spawning areas or the larval retention due to shifting ocean currents and gyres (Shaklee, 1984; Domingues *et al*., 2007b). It has already been described that *S. salpa* spawn pelagic eggs (Strydom *et al*., 2014), having the capacity to be transported by ocean currents, but no data to prove the duration of the larval stage neither the influence that ocean currents may have on them exist. In addition to the lack of evidence structure, there was a strong signal for demographic expansion, potentially from an already genetic diverse population; the high haplotype diversity obtained in the sampling areas resulted from an expansion of a population that maintained high levels of genetic diversity, from the possible refuges in the coast

of Africa with accumulation of mutation *in situ*. This hypotheses of expansion of *S. salpa* is also supported by the mismatch distribution results for both markers. The applied genetic techniques showed that *S. salpa* has a higher level of genetic variability when compared with other Sparidae species but the genetic results are of little value to resolve their stock structure.

An integrated approach combining morphological characters and fish genetics was applied to *S. salpa* individuals in order to analyze their stock structure. This multidisciplinary approach compared characteristics that are unstable on different time-scales: the morphological characters, also designated as quantitative or phenotypic traits, can change rapidly on a shorter time scale (Valentin *et al*., 2014) and can be due to demographic, genetic, or environmental differences, or any combination of these (Heino, 2014); the genetic markers are independent of environmental changes during the course of an individual's lifetime and represent a deeper and more permanent source of separateness than those due to demography and the environment factors (Antoniou and Magoulas, 2014; Heino, 2014). Any consistent morphological difference between groups, based on phenotype methods, can be used to separate stocks (Waldman, 2005) however, genetic methods may not be sufficient for defining the geographical limits of stocks (Grant *et al*., 1999) because a single genotype can develop different phenotypes in different environments due to the plasticity of the phenotypic traits (Heino, 2014). Therefore, care must be used in the interpretation of data because morphometric analysis provides information on phenotypic stocks, and indicate that at least two stocks may occur, one in Madeira

and another in mainland waters, but theresults obtained for genetic analysis showed no evidence of stock structure. The results indicate that stock separation may not be due to genomic differences but by local environmental conditions (Begg and Waldman, 1999), that include but are not limited to temperature, salinity, radiation, dissolved oxygen, food availability, water depth and current flow (Turan *et al*., 2006). However the stock separation, indicated by the morphometric analysis, can be caused by local adaptation which has a genetic basis and can only be identified by adaptive genes involved in the adaptation process but in the present thesis only neutral genes were selected. In addition, it is possible that the sample size or the power of the selected genetic markers are not appropriate for the detection of subdivisions in this species (Abaunza *et al*., 2008).

To successfully manage a species fishery, it is crucial to understand their stock structure to be able to design appropriate management regulations in fisheries (Begg and Waldman, 1999) and this is particularly important when the target species is commercial exploited with a broad geographical range, as is the case of *S. salpa*. But when the results of different stock identification methods are not consonant, such as in the case of *S. salpa*, the default management option should be to use a precautionary approach to ensure resource sustainability and maintenance of genetic biodiversity (Begg and Waldman, 1999). More stock identification methods must be applied on *S. salpa* individuals in order to provide strong evidence on the species population structure.

PART II - BIOLOGY

Age, growth and reproduction

New information was given for the Portuguese continental coast, where no information was previously available. The first increment identified in the whole otoliths appeared between 1.08 - 1.25 mm and was already present in individuals with TL varying between 5.2 and 9.8 cm. This first increment was designated as juvenile band since did not correspond to a yearly growth band and should be a result of an early migration to nursery areas as already described for other species, European seabass, *Dicentrarchus labrax* (Linnaeus, 1758) (Gordo, 1989). The minimum age observed was 0 years and the maximum age observed was 14 years with 5.2 and 41.4 cm TL, respectively. The maximum age obtained in the present study is similar to the results obtained in the Adriatic Sea (15 years, 44 cm TL) (Pallaoro *et al*., 2008), but is higher than the results obtained in the east coast of South Africa (6 years, 27 cm TL) (Walt and Beckley, 1997), in the central western coasts of Italy (7 years, 33 cm TL) (Criscoli *et al*., 2006) and in the Canary archipelago (11 years, 45 cm TL) (Villamil *et al*., 2002). Whole otolith readings and backcalculation approaches were used to estimate the parameters of the von Bertalanffy growth function (VBGF). The parameters of the VBGF estimated were: L_∞ = 44.19 cm, k = 0.17 $year^{-1}$ and t_0 = -0.94 year for the whole otolith readings, and L_∞ = 45.07 cm, k = 0.14 $year^{-1}$ and t_0 = -1.43 year for the backcalculation approach. The Akaike's information criterion value suggested that the last approach was the best one to describe the growth of *S. salpa*.

For the reproduction study, 55 undifferentiated, 255 males, 346 females and 248 bisexuals were analyzed. *S. salpa* is a protandric hermaphroditic, the sex changed process occurred between 28.6 and 40.9 cm TL, and presented a short spawning season, extended from September to November. The length at first maturity (L_{50}) estimated for males was 24.5 cm and, based on the previous age determination, this length correspond to age 2. The estimated L_{50} was greater than the values recorded for males in the east coast of South Africa (14.5 cm fork length) (Walt and Mann, 1998), in central western coasts of Italy (19.5 cm TL) (Criscoli *et al*., 2006) and in the Adriatic (20.6 cm TL) (Pallaoro *et al*., 2008), but was lower than the value recorded in the Canary archipelago (26.6 cm TL) (Villamil *et al*., 2002). Due to the inexistence of immature females, it was not possible to calculate their L_{50} but the smallest mature female obtained was 28.6 cm TL, which was very similar to the estimated L_{50} recorded for females in the Canary archipelago (29.4 cm TL) (Villamil *et al*., 2002).

In order to investigate the fecundity type of *S. salpa* four lines of evidence, suggested by Hunter *et al*. (1992), Greer-Walker *et al*. (1994), and Murua and Saborido-Rey (2003), were investigated. For the first line of evidence no distinct hiatus could be observed between the primary and secondary oocytes growth stages during the spawning season although a distinct gap could be observed near the end of spawning season in November. A distinct hiatus indicates that annual fecundity is determinate whereas the lack of a hiatus may indicate that annual fecundity is indeterminate, but, the lack of a hiatus does not necessarily indicate that fecundity is indeterminate (Murua and Saborido-Rey, 2003). Regarding the second line of evidence, a statistically significant decrease in the mean number of previtellogenic and early vitellogenic oocytes and in the mean number of advanced vitellogenic oocytes

was found during spawning season, which is a clear evidence for determinate fecundity since there is no replacement of oocytes after each spawning event (Murua and Saborido-Rey, 2003). Regarding the third line of evidence, an increase pattern in the mean diameter of advanced vitellogenic oocytes was found along the spawning season although with no statistical significance. In fishes with determinate fecundity a seasonal increase in the mean diameter of secondary growth oocytes is expected along the spawning season (Murua and Saborido-Rey, 2003) although in some species with determinate fecundity the mean diameter of secondary growth oocytes remained constant (Greer Walker *et al*., 1994) or declined (Kjesbu *et al*., 1990) as the spawning season progresses. For the last line of evidence, the relative intensity of alpha atresia calculated was low (varying between 7 and 14 %) through the spawning season, indicating that fecundity is determinate. These lines of evidence suggested that *S. salpa* exhibited a determinate fecundity type, which indicated that the oocyte recruitment is completed before onset of spawning (Ganias *et al*., 2014), and all fecundity calculations were made accordingly. The realized annual fecundity (FR_a) ranged between 266 133 and 2 453 754 oocytes and the relative annual fecundity varied between 462 and 2 662 oocytes per gram of gutted weight (GW). According to the size-advantage model, the sequential hermaphroditism may be advantageous (Ghiselin, 1969) for the species because an individual may change sex if it would result in an increase in its expected future fitness. Males are generally capable of producing similar amounts of gametes regardless of body size and in the females, the fecundity increases with body size (Oldfield, 2005); in the present study, a significant power relation of FR_a with gonad weight, GW and TL was observed.

Cystic structures

Macroscopically analysis of *S. salpa* ovaries revealed the presence of hard structures with a yellow to brown color in 24.3 % of the females sampled. Histological analysis suggested that these structures correspond to remains of hydrated oocytes that can appear isolated or in groups that join together forming a cystic structure, corresponding to an incomplete or unsuccessful spawning event. Cysts seemed to be formed by numerous *zona radiata* membranes folded over one another and surrounded by a stratified epithelium. The cystic structures appear frequently (all months except in October) and with higher mean prevalence values in the months preceding the spawning period, suggesting that this process may be part of the reproductive strategy for *S. salpa*. Furthermore, this phenomenon could not be associated with environmental perturbation or anthropogenic disturbance occurred in the sampling area. These structures showed to be relatively loose inside the ovary and were observed very close to the genital opening, being easily released after a slight pressure on the ovary. This suggests that the cysts can be released before spawning begins, allowing for wider space in the ovary for future feasible oocytes.

It must be noticed that the presence of cystic structures has never been documented before in *S. salpa* ovaries. The presence of remains of hydrated oocytes in fishes, isolated or aggregated in the ovary, has been reported sporadically in zebrafish *Danio rerio* (Hamilton, 1822) (Madureira *et al*., 2011), atlantic cod *Gadus morhua* Linnaeus, 1758 (Rideout and Burton, 2000), atlantic blue marlin *Makaira nigricans* Lacépède, 1802 (Brown-Peterson *et al*., 2007) and pouting *Trisopterus luscus* (Linnaeus, 1758) (Alonso-Fernández, 2011), and was also observed in several other

species along the Portuguese coast such as boarfish *Capros aper* (Linnaeus, 1758) (V. Sequeira, personal observation), Senegal seabream *Diplodus bellottii* (Steindachner, 1882) (C. Vendrell, personal observation) and forkbeard *Phycis phycis* Linnaeus, 1766 (A. R. Vieira, personal observation). One of the reasons that may be influencing the short report of evidence of cysts can be the fact that the authors may consider them as an artifice, or in other words as something that has no meaning, because they appeared sporadically and decided to not report them. In the case of *S. salpa* this is not applicable because a great prevalence of cysts, not sporadic, was found. Additional studies are required to clarify the role of the cystic structures in the reproduction and to determine how long they remain in the ovaries.

Conclusions

The present thesis gave new information of *S. salpa*, on the stock structure and on the biological parameters, for the Portuguese waters where there is no previous available data. This thesis constitutes a working base for future investigation on fisheries management and stock identification although, there are some aspects that were not completely exploited due to sampling limitations, time constraints or due to financial restrictions that should be considered in future works. For example: it will be important to increase the number of individuals and the number of sampling areas for the morphometric studies; to use different genetic markers and increase the number

of individuals for genetic analyzes; and to improve the knowledge of the cystic structures with complementary studies on biochemistry structure and physiological processes. All this information will be necessary to improve the knowledge of the population in order to better define the stock structure to, in the future, properly assess and manage this emerging resource.

CHAPTER 7

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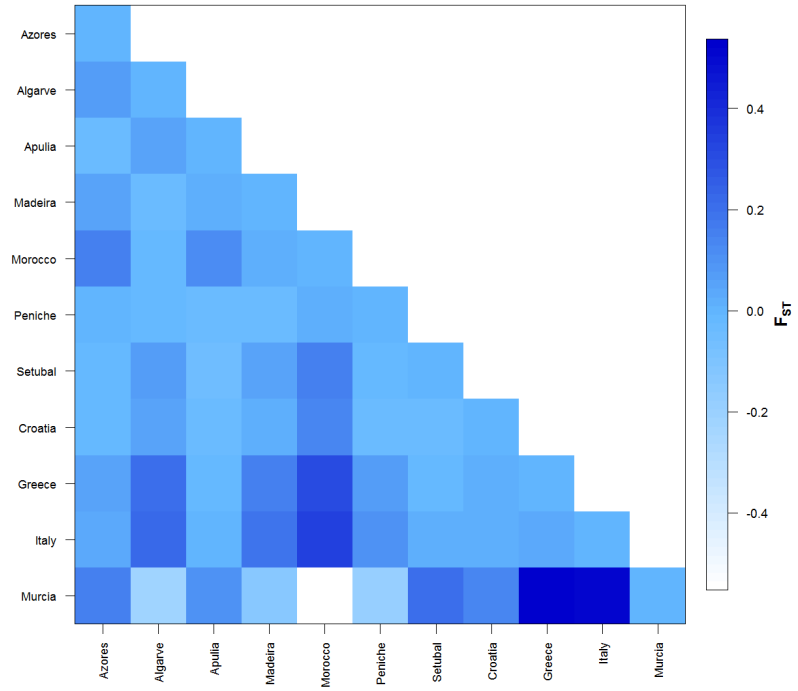
CHAPTER 8

Supplementary Material

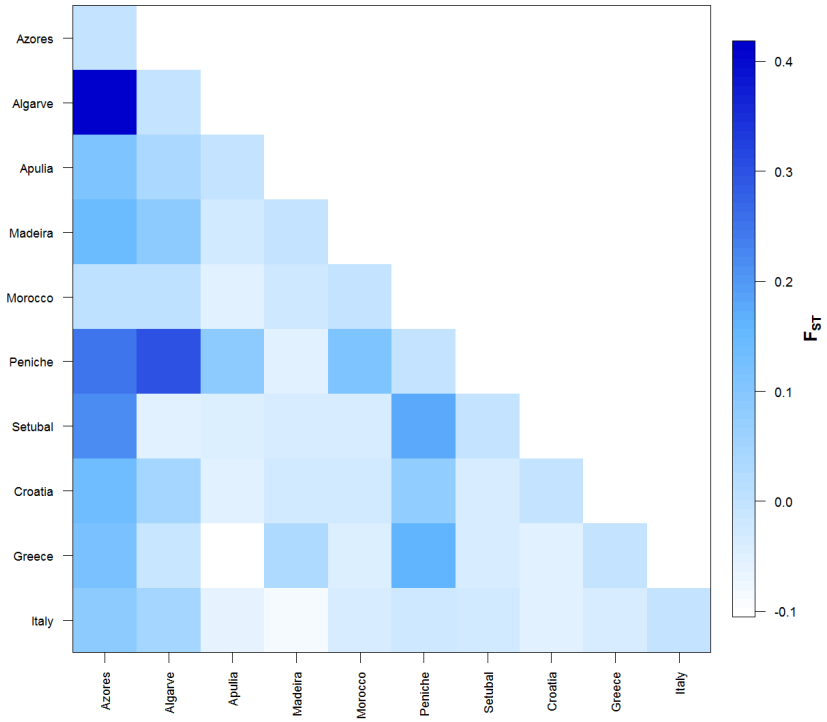
Supplementary Material

Supplementary Material 1. Pairwise F_{STs} for *Sarpa salpa* individuals without DNA introgression calculated for the **A)** mitochondrial control region sequences and **B)** first intron of the S7 ribosomal protein gene.

A)



B)



Supplementary Material 2. List of marine fish species with the respective temperature range, study areas and, haplotype and nucleotide diversities.

Family	Specie	Thermal amplitude (º C) (according distribution area)		Study areas	Country	Number of individuals	Haplotype diversity (h)	Nucleotide diversity (π)	Gene	Author
		T_{min}	T_{max}							
Sparidae	*Sarpa salpa*	12	30	Atlantic and Mediterranean	Portugal, Spain, Italy, Croatia, Greece, Morocco	109	0.998 ± 0.002	0.091 ± 0.044	DLOOP	present work
						104	0.998 ± 0.002	0.047 ± 0.023		present work, without the five individuals with signals of mitochondrial introgression
Sparidae	*Diplodus sargus*	6	24	Azores	Portugal	18	0.850	0.027	DLOOP	Domingues *et al*. (2007)
				Madeira	Portugal	20	0.968	0.035		
				São Pedro	Portugal	21	1.000	0.036		
				Canaries	Spain	15	0.990	0.034		
				Mau	Morocco	17	1.000	0.033		
				Barcelona	Spain	27	0.971	0.024		
				Naples	Italy	6	1.000	0.026		
				Kea	Greece	9	1.000	0.028		
				Sifnos	Greece	8	1.000	0.033		
Sparidae	*Diplodus sargus*	6	24	Azores	Portugal	20	0.977	0.023	416-bp fragment of the control region	González-Wangüemert *et al*. (2011)
				Canary	Spain	20	0.987	0.025		
				Galicia	Spain	23	0.993	0.029		
				Faro	Portugal	20	1.000	0.023		
				Murcia	Spain	20	0.966	0.021		
				Banyuls	France	21	0.943	0.024		
				Mallorca	Spain	22	0.993	0.022		
				Tunisia	Tunisia	22	1.000	0.027		
				Castellamare	Italy	20	1.000	0.018		
				Total		188	0.987	0.024		

Sparidae	*Diplodus sargus*	6	24	Azores	Portugal	20	0.638	0.001	Cyt-b	González-Wangüemert *et al.* (2010)
				Canary	Spain	20	0.200	0.000		
				Galicia	Spain	23	0.551	0.001		
				Faro	Portugal	20	0.552	0.001		
				Murcia	Spain	20	0.838	0.002		
				Banyuls	France	21	0.889	0.002		
				Mallorca	Spain	22	0.765	0.002		
				Tunisia	Tunisia	22	0.688	0.001		
				Castellamare	Italy	20	0.709	0.002		
				Total		188	0.720	0.016		
Sparidae	*Pagrus pagrus*	12	24	Faro	Portugal	42	0.420	0.006	DLOOP	Bargelloni *et al*. (2003)
				Spain	Spain	35	0.360	0.003		
				Italy	Italy	31	0.350	0.003		
				Iraklion	Greece	54	0.530	0.006		
Sparidae	*Lithognathus mormyrus*	12	30	Faro	Portugal	19	0.620	0.006	DLOOP	Bargelloni *et al*. (2003)
				Spain	Spain	30	0.680	0.012		
				Italy	Italy	25	0.900	0.016		
				Iraklion	Greece	23	0.530	0.017		
Sparidae	*Dentex dentex*	8	24	Faro	Portugal	30	0.490	0.049	DLOOP	Bargelloni *et al*. (2003)
				Spain	Spain	28	0.560	0.008		
				Italy	Italy	23	0.670	0.008		
				Greece	Greece	45	0.660	0.018		
Sparidae	*Pagellus bogaraveo*	6	15	Azores	Portugal	29	0.069	0.000	DLOOP	Bargelloni *et al*. (2003)
				Spain	Spain	48	0.520	0.005		
				Italy	Italy	25	0.420	0.004		
				North Aegean Sea	Greece	29	0.200	0.002		
Sparidae	*Spondyliosoma cantharus*	6	24	Faro	Portugal	18	0.940	0.016	DLOOP	Bargelloni *et al*. (2003)
				Spain	Spain	19	0.980	0.019		
				North Aegean Sea	Greece	14	0.980	0.018		
Tripterygiidae	*Tripterygion delaisi*	12	24	Azores	Portugal	13	0.150		DLOOP	Domingues *et al*. (2007b)

				Madeira	Portugal	4	0.250				
				Canaries	Spain	16	0.500				
				Arrabida	Portugal	16	0.060				
				Italy	Italy	12	0.420				
				Croatia	Croatia	2	1.000				
				Cyprus	Cyprus	3	1.000				
Pomacentridae	*Chromis limbata*	15	24	Azores	Portugal	25	0.480		0.036		DLOOP	Domingues *et al*. (2006)
				Madeira	Portugal	18	0.940		0.058		
				Canaries	Spain	19	1.000		0.050		
Cottidae	*Taurulus bubalis*	3	15	Oslo	Norway	14	0.791	± 0.073	0.007	± 0.041	control region	Almada *et al*. (2012)
				Gullmars fjord	Sweden	30	0.694	± 0.059	0.004	± 0.003	
				Egersund	Norway	14	0.571	± 0.132	0.004	± 0.003	
				Helgoland	Germany	30	0.733	± 0.058	0.004	± 0.002	
				Roscoff	France	32	0.571	± 0.129	0.003	± 0.002	
				Galicia	Spain	21	0.562	± 0.126	0.004	± 0.002	
				Portugal	Portugal	24	0.605	± 0.111	0.006	± 0.004	
Sebastidae	*Helicolenus dactylopterus*	0	30	Cape Verde	Cape Verde		0.976		0.033		control region	Aboim *et al*. (2005)
				South Carolina	USA		0.911		0.006		
				Madeira	Portugal		1.000		0.031		
				Peniche	Portugal		1.000		0.029		
				Azores	Portugal		0.996		0.029		
				Total		208					
Sebastidae	*Helicolenus dactylopterus*	0	30	Cape Verde	Cape Verde		0.788		0.006		Cyt-b	Aboim *et al*. (2005)
				South Carolina	USA		0.426		0.002		
				Madeira	Portugal		0.936		0.007		
				Peniche	Portugal		0.860		0.007		
				Azores	Portugal		0.865		0.005		
				Total		212					
Scorpaenidae	*Pontinus kuhlii*	10	24	Seine seamount (Madeira)	Portugal	10	0.978		0.016		control region	Catarino *et al*. (2013)
				Madeira	Portugal	4	1.000		0.017		

				Cape Verde	Cape Verde	10	0.978	0.019		
				Voador seamount (Azores)	Portugal	10	0.956	0.019		
				Condor seamount (Azores)	Portugal	10	0.933	0.017		
Scorpaenidae	*Pontinus kuhlii*	10	24	Seine seamount (Madeira)	Portugal	10	0.933	0.008	Cyt-b	Catarino *et al*. (2013)
				Madeira	Portugal	4	0.833	0.005		
				Cape Verde	Cape Verde	10	0.956	0.006		
				Voador seamount (Azores)	Portugal	10	0.689	0.004		
				Condor seamount (Azores)	Portugal	10	0.911	0.007		
Blenniidae	*Ophioblennius atlanticus*	15	24	Azores	Portugal	9	0.417	0.001	Cyt-b	Muss *et al*. (2001)
				Cape Verde	Cape Verde	16	0.992	0.012		
				São Tomé	São Tomé e Príncipe	14	0.891	0.005		
				St. Paul's Rocks	Brazil	12	0.803	0.005		
				Ascencion Island	an integral part of the British overseas territory	13	0.974	0.007		
				Saint Helena	an integral part of the British overseas territory	12	0.970	0.005		
				Northeast Brazil	Brazil	15	1.000	0.011		
				Trindade Island	Brazil	10	0.933	0.011		
Labridae	*Symphodus melops*	8	15	Algarve	Portugal	27	0.830 ± 0.070	0.550 ± 0.360	control region	Robalo *et al.* (2012)
				Galicia	Spain	29	0.790 ± 0.070	0.480 ± 0.320		
				Plymouth	UK	28	0.780 ± 0.080	0.700 ± 0.430		

				Belfast	Ireland	24	0.780	±	0.080	0.630	±	0.400		
				Roscoff	France	16	0.700	±	0.130	0.340	±	0.260		
				Lisbon	Portugal	35	0.670	±	0.090	0.430	±	0.290		
				Oslo	Norway	30	0.250	±	0.100	0.070	±	0.090		
				Egersund	Norway	21	0.190	±	0.110	0.050	±	0.080		
				Kristiansand	Norway	23	0.090	±	0.080	0.020	±	0.050		
				Gullmars fjord	Sweden	30	0.070	±	0.060	0.020	±	0.040		
Batrachoididae	*Halobatrachus didactylus*	10	24	Tagus river	Portugal	30	0.000	±	0.000	0.000	±	0.000	control region	Robalo *et al*. (2013)
				Sado river	Portugal	19	0.000	±	0.000	0.000	±	0.000		
				Mira river	Portugal	8	0.250	±	0.180	0.076	±	0.108		
				Algarve	Portugal	34	0.672	±	0.046	0.279	±	0.220		

Supplementary Material 3. Haplotype list from the mitochondrial control region and from the first intron of the S7 ribosomal protein gene.

Sampling area	mitochondrial control region			first intron of the S7 ribosomal protein gene			
	Individual code	Haplotype	Acession number	Individual code	Genotype	Haplotype	Acession number
Azores	AZO 1	h1	KU186669	AZO 3 A	1	h8	KU186775
	AZO 2	h2	KU186670	AZO 3 B		h30	KU186797
	AZO 3	h3	KU186671	AZO 4 A	2	h7	KU186774
	AZO 4	h4	KU186672	AZO 4 B		h14	KU186781
	AZO 5	h5	KU186673	AZO 5 A	3	h3	KU186770
	AZO 6	h6	KU186674	AZO 5 B		h4	KU186771
	AZO 8	h1	KU186669				
	AZO 9	h7	KU186675				
	AZO 10	h1	KU186669				
	AZO 11	h8	KU186676				
	AZO 12	h9	KU186677				
Algarve	ALG 7	h10	KU186678	ALG 15 A	4	h1	KU186768
	ALG 8	h11	KU186679	ALG 15 B		h27	KU186794
	ALG 9	h12	KU186680				
	ALG 10	h13	KU186681				
	ALG 11	h14	KU186682				
	ALG 12	h15	KU186683				
	ALG 13	h16	KU186684				
	ALG 14	h17	KU186685				
	ALG 15	h18	KU186686				
	ALG 16	h19	KU186687				
	ALG 18	h20	KU186688				
Apulia	APU 1	h21	KU186689	APU 1 A	5	h6	KU186773
	APU 2	h22	KU186690	APU 1 B		h10	KU186777
	APU 3	h23	KU186691	APU 2 A	6	h2	KU186769
	APU 4	h24	KU186692	APU 2 B		h11	KU186778
	APU 5	h25	KU186693	APU 4 A	7	h2	KU186769
	APU 7	h26	KU186694	APU 4 B		h18	KU186785
	APU 8	h27	KU186695	APU 5 A	8	h12	KU186779
	APU 9	h28	KU186696	APU 5 B		h21	KU186788
	APU 10	h29	KU186697				
	APU 11	h30	KU186698				
	APU 12	h31	KU186699				
Croatia	CRO 2	h32	KU186700	CRO 2 A	9	h11	KU186778
	CRO 3	h33	KU186701	CRO 2 B		h21	KU186788
	CRO 4	h34	KU186702	CRO 3 A	10	h2	KU186769
	CRO 5	h35	KU186703	CRO 3 B		h21	KU186788

	CRO 6	h36	KU186704	CRO 4 A	11	h5	KU186772
	CRO 7	h37	KU186705	CRO 4 B		h9	KU186776
	CRO 8	h38	KU186706	CRO 5 A	12	h19	KU186786
	CRO 9	h39	KU186707	CRO 5 B		h21	KU186788
	CRO 10	h40	KU186708	CRO 6 A	13	h2	KU186769
	CRO 11	h41	KU186709	CRO 6 B		h28	KU186795
	CRO 12	h42	KU186710				
Greece	GRE 1	h43	KU186711	GRE 1 A	6	h2	KU186769
	GRE 3	h44	KU186712	GRE 1 B		h11	KU186778
	GRE 4	h45	KU186713	GRE 3 A	14	h11	KU186778
	GRE 5	h44	KU186712	GRE 3 B		h24	KU186791
	GRE 6	h46	KU186714	GRE 5 A	15	h1	KU186768
	GRE 7	h47	KU186715	GRE 5 B		h21	KU186788
	GRE 9	h48	KU186716	GRE 6 A	16	h6	KU186773
	GRE 10	h49	KU186717	GRE 6 B		h9	KU186776
	GRE 11	h50	KU186718				
	GRE 12	h51	KU186719				
Italy	ITA 1	h52	KU186720	ITA 1 A	17	h2	KU186769
	ITA 2	h53	KU186721	ITA 1 B		h13	KU186780
	ITA 3	h54	KU186722	ITA 2 A	18	h21	KU186788
	ITA 6	h55	KU186723	ITA 2 B		h22	KU186789
	ITA 7	h56	KU186724	ITA 3 A	19	h2	KU186769
	ITA 8	h57	KU186725	ITA 3 B		h25	KU186792
	ITA 9	h58	KU186726	ITA 6 A	20	h15	KU186782
	ITA 10	h59	KU186727	ITA 6 B		h18	KU186785
	ITA 11	h60	KU186728	ITA 7 A	21	h6	KU186773
	ITA 12	h61	KU186729	ITA 7 B		h18	KU186785
	ITA 13	h62	KU186730				
Madeira	MAD 6	h63	KU186731	MAD 6 A	22	h18	KU186785
	MAD 7	h64	KU186732	MAD 6 B		h21	KU186788
	MAD 8	h65	KU186733	MAD 7 A	7	h2	KU186769
	MAD 9	h66	KU186734	MAD 7 B		h18	KU186785
	MAD 10	h65	KU186733	MAD 8 A	23	h14	KU186781
	MAD 12	h65	KU186733	MAD 8 B		h18	KU186785
	MAD 13	h67	KU186735	MAD 11 A	24	h2	KU186769
	MAD 14	h68	KU186736	MAD 11 B		h2	KU186769
	MAD 15	h69	KU186737				
	MAD 16	h70	KU186738				
	MAD 17	h71	KU186739				
Morocco	MOR 2	h72	KU186740	MOR 2 A	25	h16	KU186783
	MOR 4	h73	KU186741	MOR 2 B		h20	KU186787
	MOR 5	h74	KU186742	MOR 4 A	26	h2	KU186769
	MOR 6	h75	KU186743	MOR 4 B		h17	KU186784
	MOR 7	h76	KU186744	MOR 5 A	27	h2	KU186769
	MOR 8	h10	KU186678	MOR 5 B		h29	KU186796

	MOR 9	h41	KU186709	MOR 6 A	6	h2	KU186769
	MOR 10	h77	KU186745	MOR 6 B		h11	KU186778
	MOR 11	h78	KU186746	MOR 7 A	28	h8	KU186775
	MOR 12	h79	KU186747	MOR 7 B		h14	KU186781
	MOR 13	h80	KU186748				
Murcia	MUR 1	h74	KU186742	MUR 1 A	29	h31	KU186798
				MUR 1 B		h32	KU186799
				MUR 2 A	29	h31	KU186798
				MUR 2 B		h32	KU186799
				MUR 3 A	29	h31	KU186798
				MUR 3 B		h32	KU186799
				MUR 4 A	29	h31	KU186798
				MUR 4 B		h32	KU186799
				MUR 5 A	29	h31	KU186798
				MUR 5 B		h32	KU186799
Peniche	PEN 1	h81	KU186749	PEN 2 A	30	h21	KU186788
	PEN 2	h82	KU186750	PEN 2 B		h26	KU186793
	PEN 4	h83	KU186750	PEN 4 A	31	h18	KU186785
	PEN 5	h84	KU186752	PEN 4 B		h23	KU186790
	PEN 6	h85	KU186753	PEN 5 A	32	h12	KU186779
	PEN 8	h86	KU186754	PEN 5 B		h18	KU186785
	PEN 9	h87	KU186755				
	PEN 10	h88	KU186756				
	PEN 11	h89	KU186757				
	PEN 12	h90	KU186758				
Setúbal	SET 3	h91	KU186759	SET 3 A	22	h18	KU186785
	SET 4	h92	KU186760	SET 3 B		h21	KU186788
	SET 5	h93	KU186761	SET 4 A	24	h2	KU186769
	SET 6	h94	KU186762	SET 4 B		h2	KU186769
	SET 7	h95	KU186763	SET 5 A	24	h2	KU186769
	SET 8	h59	KU186727	SET 5 B		h2	KU186769
	SET 9	h96	KU186764	SET 7 A	33	h2	KU186769
	SET 10	h97	KU186765	SET 7 B		h14	KU186781
	SET 11	h98	KU186766				
	SET 12	h99	KU186767				
	SET 13	h75	KU186743				