# PHYLOGEOGRAPHY AND HISTORICAL DEMOGRAPHY OF THE WARM WATER COSTAL FISH OF THE AZORES IN THE CONTEXT OF THE RECENT EVOLUTION OF THE ATLANTIC AND MEDITERRANEAN

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# PREVIOUS NOTE

This dissertation is the final product of my PhD work that aimed at understanding the evolutionary relationships of the inshore fish fauna of the northeastern Atlantic and Mediterranean, placing a particular emphasis on the Azores islands. My research focused on the study of twelve coastal fish species using molecular markers and applies phylogeographic and historical demography approaches.

This dissertation in composed of an Introduction, a collection of ten papers produced in collaboration with my advisors and colleagues and a Discussion that integrates the results obtained for all species. Nine of these papers are already published or accepted for publication in scientific journals, while one of them is submitted and waits editorial decision.

# NOTA PRÉVIA

Esta dissertação é o produto final da minha tese de doutoramento que visa a compreensão das relações evolutivas da fauna piscícola costeira do Atlântico nordeste e do Mediterrâneo, dando particular ênfase às ilhas dos Açores. O meu trabalho foca o estudo de doze espécies de peixes costeiros utilizando marcadores moleculares, e emprega métodos filogenéticos e de demografia histórica.

Esta dissertação é composta por uma Introdução, uma colecção de dez artigos produzidos em colaboração com os meus orientadores e colegas, e uma Discussão que integra os resultados obtidos para todas as espécies. Nove destes artigos estão já publicados ou aceites para publicação em revistas científicas, enquanto que um deles está submetido e aguarda uma decisão editorial.

# ABSTRACT

In this thesis the evolutionary relationships of the inshore fish fauna of the northeastern Atlantic and Mediterranean were assessed. Twelve coastal fish species from six families: Blenniidae, Labridae, Pomacentridae, Scaridae, Sparidae and Tripterygiidae, were studied using mitochondrial and nuclear molecular markers. Results were analyzed applying phylogeographic and historical demography approaches.

Species revealed four distinct phylogeographic patterns that were supported by genetic diversity and demographic parameters of the different populations: i) two distinct groups of populations (sometimes considered different species), one including the Mediterranean and the Atlantic coast of western Europe, and another including the Atlantic archipelagos of Canaries, Madeira and Azores (*Chromis chromis/ C. limbata*, *Parablennius sanguinolentus/ P. parvicornis* and the two lineages of *Tripterygion delaisi*); ii) no appreciable genetic differentiation between any of the populations (*Sparisoma cretense*, *Thalassoma pavo* and *Diplodus sargus*); iii) marked differentiation of the Azorean population (*Lipophrys pholis* and *Coryphoblennius galerita*) and a clear divergence between Mediterranean and western European locations as well as Madeira and Canaries (*Coryphoblennius galerita*); and iv) one form in the Mediterranean and in the northeastern Atlantic coast (*Parablennius gattorugine*) and another one in the Atlantic islands and European coasts (*P. ruber*), thus in sympatry with *P. gattorugine.* These distinct phylogeographic patterns can be explained by a combination of differential effects of the Pleistocene glaciations in several areas of the Atlantic and Mediterranean and the particular thermal tolerances and dispersal capabilities of the species. The species conforming to the first pattern are warm water species that would not have been able to survive the colder glacial periods in the more affected areas such as western Europe, eastern Canaries, the Azores and most of the Mediterranean. These species might have survived the cold periods in warmer refuges such as Madeira, the western Tropical coast of Africa and some southern pockets of the Mediterranean. After warmer conditions were restored, fishes surviving the glaciations in the western Tropical coast of Africa would have expanded northwards colonizing the northern coast of Africa and the Macaronesian islands, while fishes from the south of Mediterranean invaded the entire Sea and the adjacent European Atlantic coast. Isolation between the two refuges might have promoted divergence and eventually speciation. Colonization of the Azores would have been possible by fishes that survived in Madeira, and also in the western coast of Africa, with the intermediate islands of Canaries and Madeira acting as stepping stones. Species that conform to the pattern of no genetic differentiation among the populations are species with higher dispersal ability, which might have promoted a very fast mixing of the populations after warmer conditions were restored, erasing the signs of population differentiation. The third pattern was depicted for the two cold-water species studied. These species might have persisted during the Pleistocene cooling episodes in the less affected areas, among which are the Azores. The long term persistence of these species coupled with their limited dispersal ability

would have promoted the genetic differentiation of the more isolated locations such as the Azores and the Mediterranean. The fourth pattern pointed to a speciation in the Azores or Madeira followed by an invasion of the European shores.

Concerning the Atlantic-Mediterranean transition, only one species, the blennniid *Coryphoblennius galerita*, showed a clear and strong genetic differentiation between the two basins, that was accompanied by morphological differentiation. Historical isolation caused by sea level lowering at the Gibraltar Strait during the Pleistocene glaciations might have promoted the divergence between the two basins. The complex pattern of gyres and eddies of the Alboran sea can also constitute an effective physical barrier between the two regions. Other factors such as larval behavior and the superficial currents during *C. galerita*'s spawning season my also have influenced the segregation of the two divergent lineages.

Within the Mediterranean *Thalassoma pavo* and *Chromis chromis* showed a restriction to gene flow south of the Greek Peloponnese, where a permanent anticyclonic gyre has been identified.

This study contributes to further our knowledge on the evolutionary relationships of the coastal fauna of the Atlantic-Mediterranean, pointing out that features like thermal tolerances and dispersal ability of the species are amongst the important forces shaping the phylogeographic patterns of the species.

# RESUMO

Nesta tese são analisadas as relações evolutivas da fauna piscícola costeira do Atlântico nordeste e do Mediterrâneo. Foram estudadas doze espécies de peixes costeiros pertencentes a seis famílias: Blenniidae, Labridae, Pomacentridae, Scaridae, Sparidae e Tripterygiidae, utilizando marcadores moleculares mitocondriais e nucleares. Os resultados foram analisados através de métodos filogeográficos e de demografia histórica.

As espécies revelaram quatro padrões filogeográficos distintos, suportados pelas diversidades genéticas e demografias históricas das diferentes populações: i) dois grupos distintos de populações (por vezes considerados espécies diferentes), um incluindo o Mediterrâneo e a costa oeste europeia, e outro incluindo os arquipélagos atlânticos das Canárias, Madeira e Açores (*Chromis chromis/ C. limbata*, *Parablennius sanguinolentus/ P. parvicornis* e as duas linhagens de *Tripterygion delaisi*); ii) ausência de diferenciação genética entre as populações (*Sparisoma cretense*, *Thalassoma pavo* e *Diplodus sargus*); iii) acentuada diferenciação da população dos Açores (*Lipophrys pholis* e *Coryphoblennius galerita*) e uma divergência clara entre o Mediterrâneo e o oeste europeu, bem como a Madeira e Canárias (*Coryphoblennius galerita*); e iv) uma forma no Mediterrâneo e costa atlântica nordeste (*Parablennius gattorugine*) e outra nas ilhas atlânticas e na costa europeia (*P. ruber*), em simpatria com *P. gattorugine*. Estes padrões filogeográficos distintos podem ser explicados pela combinação dos efeitos diferenciados das glaciações do Pleistocénio em várias áreas do Atlântico e do Mediterrâneo com as tolerâncias térmicas e capacidades de dispersão das diferentes espécies. As espécies que se enquadram no primeiro padrão são espécies de água quente que durante os períodos glaciares mais frios não poderiam ter sobrevivido nas áreas mais afectadas como o oeste europeu, as ilhas este das Canárias, os Açores e a maior parte do Mediterrâneo. Estas espécies devem ter sobrevivido os períodos frios em refúgios mais quentes como a Madeira, a costa Tropical oeste de Africa e algumas bolsas de água mais quente a sul do Mediterrâneo. Após as condições mais quentes terem sido repostas, os peixes que sobreviveram às glaciações na costa Tropical oeste de África, ter-se-ão expandindo para norte, colonizando a costa norte de África e as ilhas da Macaronésia, enquanto que os peixes do sul do Mediterrâneo terão invadido todo este mar e a costa atlântica europeia adjacente. O isolamento dos dois refúgios deverá ter promovido divergência e eventualmente especiação. A colonização dos Açores deverá ter sido possível por peixes que sobreviveram na Madeira e também na costa oeste Africana, com as ilhas intermédias das Canárias e Madeira a actuar como *stepping stones*. As espécies que se enquadram no padrão de inexistente diferenciação populacional são espécies com maior capacidade de dispersão, o que terá permitido uma mistura rápida das populações após as condições mais quentes terem sido repostas, eliminando quaisquer sinais de diferenciação populacional. O terceiro padrão foi identificado para os duas espécies de água fria estudados. Estas espécies deverão ter persistido nas áreas menos afectadas, incluindo os Açores, durante os períodos frios do Pleistocénio. A persistência prolongada deste peixes, bem como a sua reduzida capacidade

de dispersão terão promovido a diferenciação genética das regiões mais isoladas como os Açores e o Mediterrâneo. O quarto padrão aponta para um fenómeno de especiação nos Açores ou na Madeira, e posterior invasão das costas europeias.

No que respeita à transição entre o Atlântico e o Mediterrâneo, apenas uma espécie, o blenio *Coryphoblennius galerita*, mostrou uma clara e forte diferenciação genética entre as duas bacias, acompanhada por diferenciação morfológica. O isolamento histórico causado pela redução do nível do mar no Estreito de Gibraltar durante as glaciações do Pleistocénio, poderá ter promovido a divergência entre as duas bacias. O padrão complexo de redemoinhos do Mar Alboriano pode também constituir uma barreira física efectiva entre as duas regiões. Outros factores como o comportamento larvar e as correntes superficiais durante a época de reprodução de *C. galerita*, podem ter também influenciado a segregação das duas linhagens divergentes.

Dentro do Mediterrâneo, *Thalassoma pavo* e *Chromis chromis* revelaram a existência de restrição ao fluxo genético a sul da Peloponésia grega, onde um *gyre* anticiclónico foi identificado.

Este estudo contribui para alargar o nosso conhecimento acerca das relações evolutivas da fauna costeira do Atlântico-Mediterrâneo, e aponta características como a tolerância térmica e capacidade de dispersão das espécies, como forças importantes para o delinear de padrões filogeográficos das espécies.

# INTRODUCTION

## 1. A brief history of the northeastern Atlantic and the Mediterranean

Early history

For long geological periods a single and immense sea, the Tethys Sea, existed separating continents into a southern and a northern group. The Tethys Sea connected up what are today the east Pacific, the central Atlantic, the Mediterranean, the Indian Ocean and the west Pacific. During the Paleocene, India pivoted away from the African coast and its northern margin contacted Asia. In the Eocene India completely fused with Asia eliminating the northern section of the Tethys Sea. By that time, Africa had moved closer to Spain leaving only a narrow passage for Tethys Sea to reach the Atlantic Ocean. In the early Miocene, African and Eurasian plates eventually contacted via the Arabian Peninsula, eliminating the Tethys Sea and creating the Mediterranean (Briggs 1995). At the same time, Central America was in place except for a small gap between Panama and Columbia. In late Miocene, Africa kept getting closer to Eurasia, causing the formation of the Alps and other mountains to the east of that chain. These elevations separated the Paratethys basin from the Mediterranean. By that time the Atlantic fauna, especially the Mediterranean one, showed a clear tropical Indo-West-Pacific character (Ekman 1967). A not yet fully understood tectonic movement closed the connection between the Mediterranean and the Atlantic. This event triggered a major desiccation event of the Mediterranean, the Messinian Sality Crisis, between 5.96 and 5.33 million years ago (Mya) (Krijgsman et al. 1999; Duggen et al. 2003). The isolation from the Atlantic caused a significant fall in the Mediterranean water level followed by erosion and deposition of non-marine sediments (Krijgsman et al. 1999). Following this stage, the Paratethys broke through to the Mediterranean basin, creating a series of freshwater lakes (Briggs 1995). Except for a few species capable of living in brackish or hypersaline lagoons, the marine fauna of the Mediterranean became extinct during this desiccation episode. Following the formation of the Strait of Gibraltar the marine conditions of the Mediterranean were restored with an abruptly refill from the Atlantic.

At the Pliocene, several events of biogeographical and evolutionary importance took place. The flooding of Beringia allowed the intermix of the marine faunas of the North Pacific and Arctic-North Atlantic (Briggs 1995). At about 3 Mya the northern hemisphere experienced a glaciating event, which originated a dramatic high-latitude cooling. The tropical character of the central Atlantic changed considerably. The northern marine fauna migrated southwards, the boreal marine biota of the Arctic went extinct or moved to the North-Atlantic and a new cold biogeographic region began (Ekman 1967; Briggs 1995). By the upper Pliocene, the Mediterranean had attained its present warm-temperate regime. Another relevant event was the rise of the Isthmus of Panama (3.1-3.5 Mya, Coates and Obando 1996) that separated the Atlantic and the Pacific Oceans. At about the same time, the opening of the Bering Strait between Alaska and Siberia, allowed the extremely rich

marine fauna of the Pacific to invade the Arctic and subsequently the Atlantic Ocean (Briggs 1995).

Pleistocene glaciations

The Pliocene glaciation event was the begging of a series of glacial-interglacial cycles in the Northern Hemisphere that extended through the Pleistocene culminating in the Last Glacial Maximum (LGM) 30-19 thousand years ago (kya) (Lambeck et al. 2002). Adams et al. (1999) reviewed studies of ice cores, deep ocean cores and sediments and proposed a consensus picture for the climate changes of the last few million years. According to these authors, ice sheets started to develop in the Pliocene, between 4 and 2.5 Mya. Cycles of glacial-interglacial occurred every 41 thousand years (ky) in the beginning of the Pleistocene and every 100 ky after the mid Pleistocene. Two interglacial periods with climate analog to the present one occurred about 420-390 kya and 130-115 kya. The last half of the Pleistocene was characterized by series of extremely cold and arid periods, the so-called Heinrich events, interleaved with rapid warmer periods, the Dansgaard-Oeschger events (Adams et al. 1999). The last Heinrich event occurred around 17-15 kya just after the LGM. After the beginning of the deglaciation a sudden and brief cold event (Younger Dryas) similar to the Heinrich events took place at 12,9-11,5—kya (Adams et al. 1999). Both the onset of the Younger Dryas and the retreat of the ices that ended the event were extremely rapid phenomena (Bard et al. 1987; Dansgaard et al. 1989).

In addition to extreme climate changes, these glacial/interglacial cycles have also been implicated in severe sea level fluctuations, salinity fluctuations and changes in sea surface hydrology (Cortijo et al. 1997; Chapman et al. 2000). The waxing and waning of ice sheets over the last 800 ky caused major sea-level shifts occurring at intervals of approximately 100 ky, with maximum amplitudes of 120-140m (Lambeck et al. 2002).

Studies on foraminiferal assemblages situated the North Atlantic polar front during the LGM at 42-46°N extending in an east-west direction (CLIMAP 1976). Chapman et al. (2000) showed that the southward expansion of the polar conditions reached as far as 40°N on at least ten separate occasions during the last glacial-interglacial cycle, having considerable environmental impact in regions located far away from the area of iceberg melting. According to the same authors, the magnitude of sea surface temperature (SST) decreases ranged from 3 to 6°C. The northeastern Atlantic area located between 38° and 45°N experienced therefore a steep south-north SST gradient. The magnitude of the cooling as well as the SST gradient during the LGM are given in Table 1.

**Table 1** Present day and Pleistocene (18 kya) sea surface temperatures (winter and summer) estimated for several Atlantic and Mediterranean locations. Data from CLIMAP (1976).

| **Location** | **Latitude N** | **Temperatures (°C)** | | | |
|---|---|---|---|---|---|
| | | **Feb. present** | **Feb. 18Kya** | **Aug. present** | **Aug.18Kya** |
| Atlantic Coast | | | | | |
| N Scotland | 59-55° | 7.8-8.6 | ice | 13.1-13.5 | ice |
| Irish Sea | 53-51° | 8.6-9.7 | -0.2-ice | 15-15.3 | 4.9-ice |
| W English Ch | 49-47° | 9.5-10.3 | 0.7-2.2 | 16.4-17 | 6.1-6.3 |
| Biscay | 45-41° | 10.8-12.8 | 0.2-5.6 | 18.3-19.7 | 6.4-9.9 |
| Lisbon | 39° | 13.6 | 7.2 | 20 | 11.5 |
| Cp S Vincent | 37° | 14.2 | 9 | 20.3 | 13.3 |
| Gibraltar St | 35-31° | 14.2-16.7 | 9-11.4 | 20.3-22.8 | 13.3-15.3 |
| Cp Juby | 29° | 17 | 11.7 | 21.7 | 16.8 |
| Cp. Bojador | 27-21° | 18.1-19.2 | 9.8-11.5 | 22-23.1 | 14.8-16.6 |
| Cp. D'Arguin | 19-17° | 18.9 | 9.9-10 | 24.2-25.8 | 15.2-17.3 |
| Senegal | 15-14° | 20.3-27.8 | 13.1-26.2 | 24.2-28.1 | 19.5-23.5 |
| Atlantic Islands | | | | | |
| Azores | 39° | 15 | 12 | 22.2 | 17.4 |
| Madeira | 33° | 17 | 15.1 | 22.8 | 20.3 |
| Canaries | 27° | 18.3 | 13 | 22.5 | 17.8 |
| Cape Verde | 15° | 21.7 | 18.2 | 25.6 | 23.1 |
| Mediterranean coast | | | | | |
| Spain (NW Med) | 41° | 12.2 | 7.4 | 22.2 | 14.5 |
| Argel (SW Med) | 37° | 13.6 | 11.2 | 24.7 | 15.7 |
| Greece (NE Med) | 39° | 14.2 | 9.4 | 25.8 | 21.1 |
| Lebanon (SE Med) | 31° | 16.1 | 14.3 | 27 | 25.2 |

Records collected off the western Iberian margin revealed ice-rafting depositions in the area associated with extreme cooling events (summer SST as low as 4.8°C) during the LGM (de Abreu et al. 2003). Pailler and Bard (2002) identified three kinds of regimes for the Iberian margin during the past 160 ky: a fist one with high SST (17-22°C) and low productivity typical from the interglacial periods; a second one with 4-5°C colder SST and high organic matter accumulation; and a last one characterized by extremely low biological productivity and very low SST (6-12°C). Those regimes were clearly related to the Heinrich events and Dansgaard-Oeschger cycles. Using data collected from two cores, one located in a region under the periodic influence of the polar front (43°30'N 30°24'W) and another located further south out of the influence of the polar waters (37°05'N 32°02'W), Calvo et al. (2001) estimated a maximum difference of 6-7°C between the two locations (separated by less than 6° of latitude) during the LGM. The same authors pointed out that differences of such magnitude between close sites in LGM have not been reported anywhere else in the world's ocean and that they were due to the strong cooling in the northern core. Crowley (1981) estimated smaller SST differences (2°-3°C) between 30° and 40°N during the last 150 Ky.

Glacial/interglacial cycles were also felt in the Mediterranean during the Pleistocene. The connection between the Atlantic and the Mediterranean was severely reduced or even closed in the region of the Strait of Gibraltar during the Quaternary due to sea level fluctuations (Thiede 1978; Bianco 1990). Although evidence of transportation of ice-rafted detritus into the Mediterranean was not found (Thiede et al. 1978; Martrat et al. 2004), there is strong evidence of very abrupt temperature changes in the last 250 Ky corresponding to the Heinrich events and Dansgaard-Oeschger cycles (Martrat et al. 2004). During the last glacial, tropical planktonic foramineferal assemblages were restricted to the eastern basin of the Mediterranean (Ionian and Levantine Seas) (Luz and Bernstein 1976). Thiede et al. (1978) described a simple regional distribution pattern of four planktonic foraminiferal assemblages in the glacial Mediterranean. The two cool assemblages were concentrated in the northwest basins, the intermediate one occurred as a belt across its central part and the tropical assemblage was restricted to the southwest region of the Mediterranean. Based on a large fossil data set and using comprehensive calibrations and powerful methodologies, Hayes et al. (2005) reconstructed SSTs of the glacial Mediterranean. These authors described a west-east SST gradient, 4°C greater than that existing today, during LGM summer and winter. During the glacial summer, a west-east gradient of 9°C was described, while during the glacial winter the gradient was of 6°C. SSTs ranged from 14°C in the Alboran Sea to 23°C in the south east of the basin during the summer and from 7°C to 16°C during the winter. The Gulf of Lions was exceptionally cold reaching 10°C in the summer and 7°C in the winter. During the winter, the major changes occurred in the western basin, with SSTs 6°C lower than modern values. Maximal decreases of 5°C were registered in the eastern basin during glacial winter. During the glacial summer, a warm anomaly of 16°C off the east coast of Spain was identified. SSTs

at the Strait of Sicily and along the North African coast as far as the Balearic basin were estimated at 17°C for the summer.

Altogether, studies on the climatic changes experienced by the North Atlantic Ocean and Mediterranean Sea during the Pleistocene, clearly show that distinct areas within these regions were differentially affected. The shores of western Europe endured polar conditions during the glacial maxima, with very cold waters also present along the northwestern African coast and, to some extend, the Canary Islands. At the Azores, temperature drops were moderate, while Madeira, the tropical western coast of Africa and some southern and eastern Mediterranean areas were little affected.

## 2. Oceanography of the central northeastern Atlantic Ocean and the Mediterranean Sea

North Atlantic Ocean circulation

The large scale ocean circulation in the North Atlantic is characterized by two gyres: the cyclonic subpolar gyre and the anticyclonic subtropical north Atlantic gyre. The subpolar gyre is sometimes difficult to define. It includes the northeastward flowing North Atlantic Current, the East and West Greenland Currents and the Labrador Current (Juliano and Alves in press). The north Atlantic subtropical gyre is delimited by the westward flowing North Equatorial Current, the Caribbean Current, the "Loop" Current (inside the Gulf of Mexico), the Florida Current, the Gulf Stream, the eastward flowing North Atlantic Current and the Canary Current, along the northwest African Coast. The eastward flowing Azores Current (south of Azores) is a very prominent zonal current within the gyre (Juliano and Alves in press).

Figure 1 depicts the major surface circulation currents of the central-eastern Atlantic Ocean. In the north Atlantic subtropical gyre, the Gulf Stream (GS), a fast-moving current, transports warm water from the subtropics northwards along the coast of North America. The GS intensity abruptly changes near 54°W, east of which it splits in a southern branch flowing southwestwards and in two main systems (centered near 39° and 41.5°N, Reverdin et al. 2003): the North Atlantic Current (NAC) flowing northeastwards and the Azores Current (AC) moving eastwards until it reaches the Gulf of Cadiz, where some of its water is entrained in the Gibraltar outflow of Mediterranean water (Sy 1988; Johnson and Stevens 2000; Reverdin et al. 2003). Further to the east, but still west of the Mid-Atlantic Ridge, the NAC divides into a branch that crosses the ridge as the permanent subpolar front flowing northwards, and into a regime, composed of a variable number of branches, to the south (Sy 1988). A minimum of two and a maximum of four branches have been observed in variable locations (Sy et al. 1992). The AC is connected to its source region through a cyclonic meander centred at around 35°N 47.5°W. Although this meander is a permanent feature, seasonal variation is identified at the connection between the AC and the GS (Le Traon and De May 1994; Alves and de Verdière 1999). In the winter, the area

of origin of the AC consists of a single flow, but in the summer, its source region flow separates into two bands, one flowing directly towards the AC region and the other forming a southwest loop before merging into the AC (Klein and Siedler 1989). The zonal AC is a quasi-permanent eastward flow that provides an important part of the upper ocean transport to the eastern basin and displays a strong seasonal variability (Gould 1985; Klein and Siedler 1989). The AC is centred around 34ºN, it has a meridional extension of about 5º in latitude and meanders all along its trajectory (Juliano and Alves in press). The surface mean velocity of the AC reaches 30-40 cm $s^{-1}$ (Ollitrault 1995).
In the Azores-Canaries basin the AC splits into three major southward flowing branches: one east of the Middle-Atlantic Ridge, another in the central basin and a third one near the western coast of Africa. The exact location of these branches varies seasonally and interannually (Klein and Siedler 1989). The easternmost branch feeds the Canaries Current (CC), which flows along the coast of Africa and through the Canary archipelago, being associated with coastal upwelling, filaments and eddies (Barton et al. 1998; Johnson and Stevens 2000). The two other branches join the North Equatorial Current (or Cape Verde Current) that flows to the west and closes the gyre when it merges with the Gulf Stream (Maillard and Käse 1989). This multibranch system is even more complex since it changes with the time of the year. Not only the intensity of these flows is much higher during the winter, but particular current systems are also formed resulting from interactions of the AC and the NAC (Santos et al. 1995). During the winter, east of the Azores, the northeastward flowing Southwest European Current (SEC) is formed from the confluence of one branch of the NAC and AC (Santos et al. 1995). According to Klein and Siedler (1989), in the summer, the AC narrows and moves further south increasing the mesoscale variability. However, Tokmakian and Challenor (1993) and Cromwell et al. (1996) showed that mesoscale variability is slightly higher in the winter than in the summer. This contradiction may be due to interannual variability, as pointed out by Cromwell et al. (1996).

According to what has been described above, the circulation at the Azores region is characterized by a complex system of flows characterized by strong seasonal variation. The NAC influences the northern islands, while the AC dominates the southern ones and brings the subtropical thermohaline front (the Azores Front Current) close to the islands (Santos et al. 1995). The complexity of the circulation system of the region is increased by strong meanders and eddies originated by the Azores Front Current (Gould 1985; Alves et al. 2002), which generates considerable mesoscale variability, with consequences for the whole regional ecosystem (Santos et al. 1995). Although the general mean circulation moves eastwards there is evidence of sporadic mean events in the opposite direction (from Africa or Madeira towards Azores) (Santos et al. 1995).

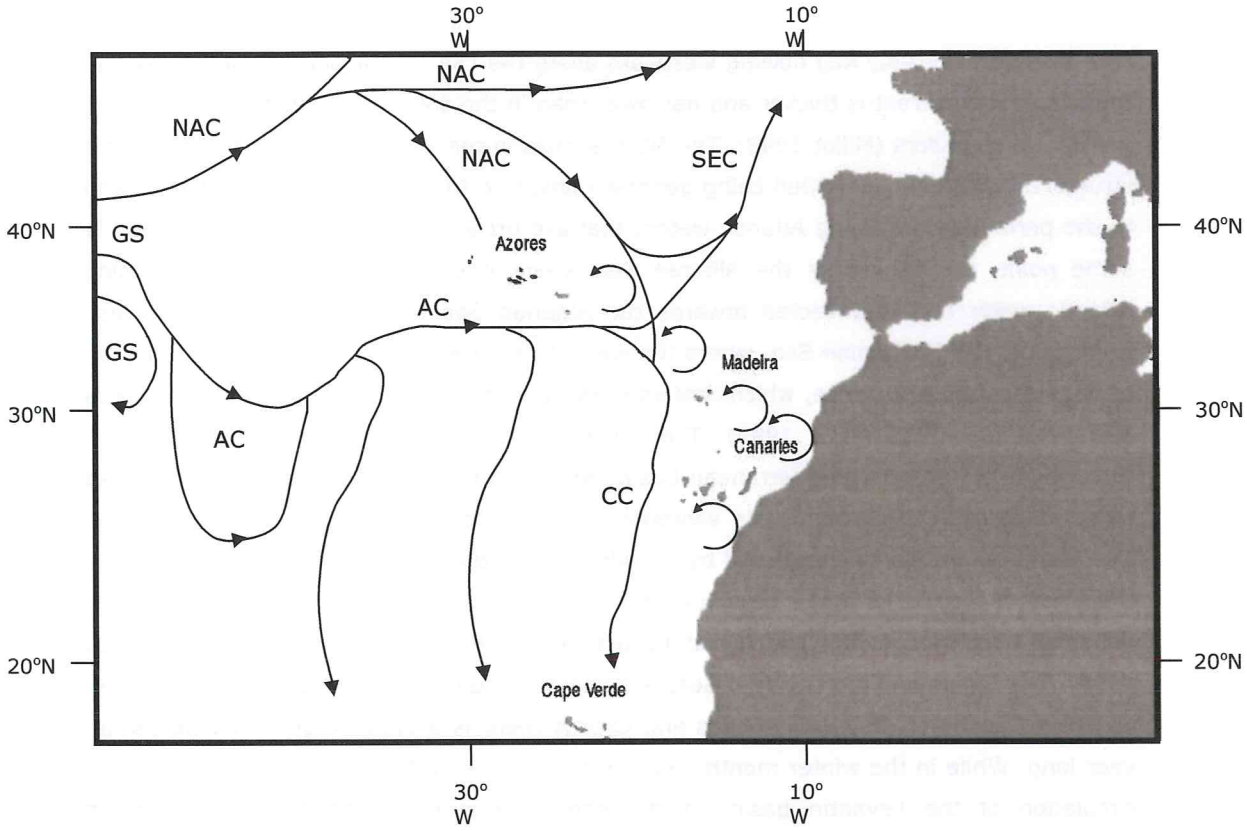


**Figure 1** Surface currents of the central-eastern Atlantic Ocean. Gulf Stream (GS), North Atlantic Current (NAC), Azores Current (AC), Southwest European Current (SEC).

Circulation in the Mediterranean Sea

The Atlantic Ocean and the Mediterranean Sea are connected via the Strait of Gibraltar, which is characterized by a two-layer flow regime (Bryden and Kinder 1991). While the Atlantic water flows into the Mediterranean in the upper layer, the Mediterranean water flows outward in the lower layer. The Mediterranean water then descends the continental shelf in the Gulf of Cadiz and underlies the north Atlantic water, spreading westwards and northwards throughout the northeast Atlantic Ocean reaching the Azores at about 1000m depth (Özgöken et al. 2001). Surface currents of the Mediterranean Sea are shown in Fig. 2. The water in the Alboran Sea describes a quasi-permanent anticyclonic gyre in the west (Garcard and Richez 1985) and a more variable circuit in the east, which is also anticyclonic most of the time (Viúdez et al. 1996). In this regard, the vein that flows from Spain to Algeria is known as the Almeria-Oran jet (AOJ). The AOJ is influenced by mesoscale activity and meanders and eddies have been depicted around the western and eastern gyres (Tintoré et al. 1991; Davies et al. 1993). The Atlantic-Mediterranean water flows further east along the Algerian coast forming the Algerian Current (AlgC), which is associated with mesoscale eddy activity. The AlgC and its associated eddies spread directly to the Balearic Islands, bringing Atlantic water into the region (Millot 1999). The northern part of the western Mediterranean basin is characterized by a seasonally changing current

(the Northern Current, NC) flowing westward along the continental slope (Millot 1999). In the winter this current is thicker and narrower than in the summer and it develops intense mesoscale meanders (Millot 1999). The NC branches in the Balearic Sea, where mesoscale structures have been identified being generally linked to Northern Current instabilities and to the permanent incoming Atlantic waters that are brought by the AlgC (Millot 1999). At some point, the NC enters the Alboran Sea where it encounters the strong incoming Atlantic water and is deflected towards the Algerian Sea (Millot 1999). The AlgC also branches in the Tyrrhenian Sea, where the water flows towards the eastern Mediterranean basin. Mesoscale turbulence, which confers seasonal and annual variability to the area, has also been identified (Millot 1999). The Atlantic surface water continues its spreading throughout the eastern Mediterranean basin, where, in the Levantine Sea, it is subjected to episodes of deep convection in wintertime (Malanotte-Rizzoli and Bergamasco 1989). The Atlantic water carried eastward by the AlgC enters the eastern Mediterranean through the Strait of Sicily. The major branch of the AlgC reaches the Levantine basin, where it is deflected northward at the Israeli and Turkish coasts (Malanotte-Rizzoli and Bergamasco 1989). The Ionian and the Levantin Seas are characterized by a cyclonic circulation both in winter and summer. Between Rhodes and Cyprus there is a cyclonic gyre that persist all year long. While in the winter months the Rhodes gyre in embodied in the larger cyclonic circulation of the Levantin basin, in the spring-summer months this gyre is more pronounced and localized near Rhodes (Malanotte-Rizzoli and Bergamasco 1989). Similarly, another cyclonic feature that persists all year long, the southern Adriatic gyre, remains isolated in the summer, merging with the general cyclonic circulation of the Ionian Sea in winter (Malanotte-Rizzoli and Bergamasco 1989). Southwest of the Greek Peloponnesus an intense anticyclonic quasi-circular spot has also been identified throughout the year, which is more intense and broader during spring and summer (Malanotte-Rizzoli and Bergamasco 1989). Additionally, two seasonal anticyclonic gyres have been identified: one extending from the Egyptian coast into the southeast Levantine interior (absent in December) and another south of Cyprus (from spring through late fall) (Malanotte-Rizzoli and Bergamasco 1989).

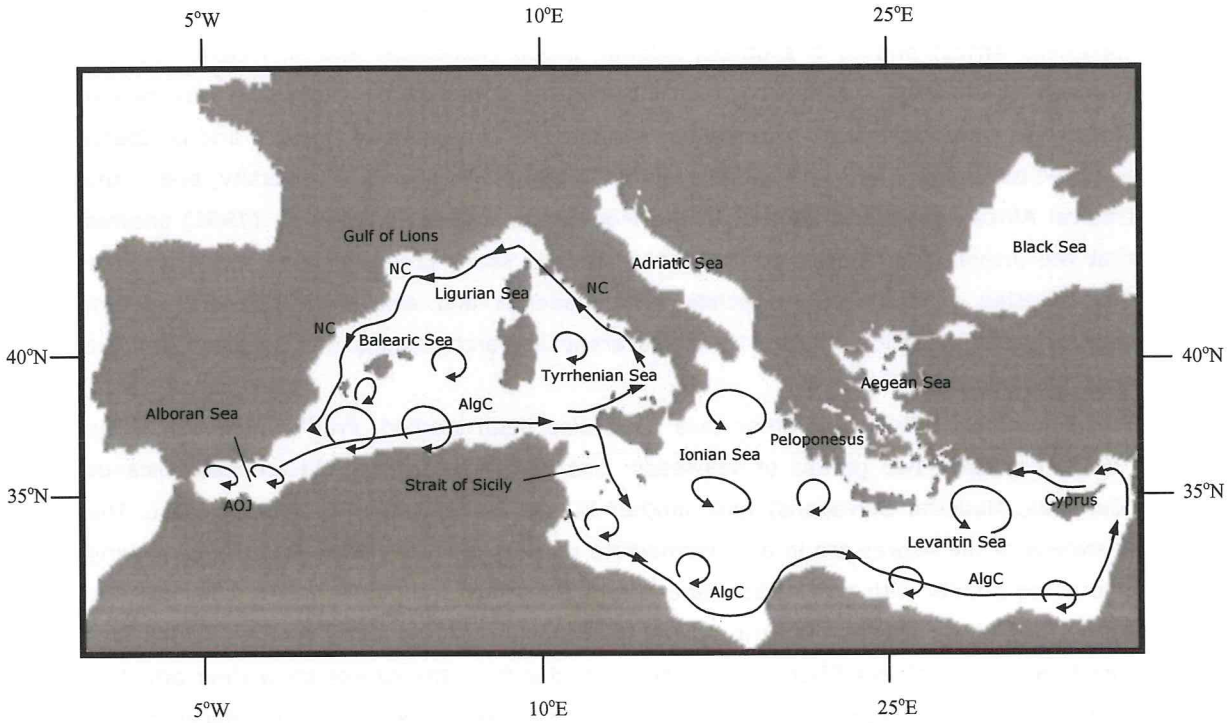


**Figure 2** Surface currents of the Mediterranean Sea. Almeria-Oran jet (AOJ), Algerian Current (AlgC), Northern Current (NC).

## 3. Biogeography of the coastal fauna of the area

The Lusitanian Province

The northeastern Atlantic and the Mediterranean have been described as a single biogeographic unit, the Lusitanian Province, which harbors warm water species mainly. The Lusitanian Province has its northern limit in the western entrance of the English Channel and extends southward until Cape Verde in the western coast of Africa, including the Macaronesian archipelagos of Azores, Madeira and Canaries (Briggs 1974).

Biogeographic affinities of the marine fauna of the Lusitanian Province have been proposed for different taxa. Using cluster analysis based on a database of littoral fishes, Santos et al. (1995) found a great affinity between the Azores, Madeira and Canaries. This group clustered with a second one composed by Morocco, Mauritania and Gulf of Guinea. A third cluster was formed by Portugal and the Mediterranean and a fourth by the Gulf of Biscay and the British Isles. The same authors emphasized the temperate affinities of the Azores littoral fish and pointed out that the Azores is the least species rich of the Macaronesian archipelagos, probably because of its greater distance to the continental coast. The results presented by Santos et al. (1995) were further supported by a cluster analysis of similarity values of blenniids among the eastern Atlantic zoogeographical areas (Almada et al. 2001). Five groups were identified: i) a north temperate group; ii) a tropical group (tropical African coast and Mauritania); iii) Cape Verde Islands; iv) a south temperate group

(southern Africa) and v) a southern Atlantic group (Ascension and S. Helena Islands) (Almada et al. 2001). Within the north temperate group three subgroups with higher similarities were identified: i) Azores and Madeira; ii) Canaries and Morocco and iii) Iberia and Mediterranean. The same authors identified two peaks of species diversity, one in the tropical African coast and another in the Mediterranean Sea. Brito et al. (1991) showed that the inshore fish species of the Canary islands share great affinities with the other Macaronesian archipelagos (especially with Madeira) and also with the northwestern tropical coast of Africa. Of the three Macaronesian archipelagos the Canaries has the strongest tropical affinity.

Biogeographical affinities of other taxa have also been studied. Prud'homme van Reine (1988) described two groups of seaweeds: one in the subtropical Macaronesian islands (Canaries, Madeira, Selvagens) and another in the warm temperate African coast. The seaweeds of the Azores are in an intermediate position between these two groups having also some affinity with the Mediterranean and the warm temperate North America. The Azores has fewer species than the other Macaronesian islands and show less endemism. Like fishes, the molluscs Rissoidae and Anabathridae from the Azores show clear affinities with the Atlantic coasts of Europe and North Africa as well as with the other Macaronesian islands (Gofas 1990). Ávila (2000) and Ávila and Albergaria (2002) showed that the Azores shared a great number of molluscs species with the western Mediterranean, Madeira, mainland Portugal and Canaries and described thirteen endemic species to the archipelago. A strong genetic affinity between bivalves of the genus *Lasaea* from the Azores, Madeira and eastern Atlantic coast was described by Ó Foighil et al. (2001). These authors suggested a stepping-stone like colonization of the islands from eastern mainland sources. A direct colonization of the Azores from the eastern Atlantic coast was also postulated, since some clades included haplotypes found in the Azores and Iberia, but not in Madeira. According to Ó Foighil et al. (2001) this direct colonization might have been possible by larvae transport via countercurrent sporadic phenomena and eddies. The majority of the echinoderms found in the Azores occur in the coasts of Europe, but also in the Mediterranean and northwestern Africa (Britton et al. 2000). Some species are also common in other Macaronesian islands and a few in the western Atlantic coasts. In contrast to a main European affinity, sponges and crustaceans from the Azores show a stronger affinity with the Mediterranean (Boury-Esnault and Lopes 1985; Wirtz and Martin 1993). Carballo et al. 1997 showed a great affinity between sponges from the Mediterranean, the Iberian and northern Africa coasts and the Macaronesian islands. The Azorean hydroids show a major relationship with the Caribbean and northwestern American but also some affinities with the Mediterranean and little endemism (Cornellius 1992).

The Atlantic-Mediterranean transition

Although the general idea is that the Atlantic and the Mediterranean form a single biogeographic unit and that the Strait of Gibraltar does not represent an important

zoogeographical boundary (Ekman 1967) some authors tended to subdivide the area. Briggs (1974) pointed out that there is no firm evidence in either fishes or invertebrates supporting the Atlantic and the Mediterranean coasts as different biogeographic units. The extent of genetic differentiation between Atlantic and Mediterranean populations has been estimated for different coastal organisms, revealing contrasting patterns. While some species show no genetic partition between the Atlantic and the Mediterranean, others reveal genetic differentiation between the two basins. Similarly, some species show genetic homogeneity along the Mediterranean, while others exhibit population structure with genetic breaks occurring at different locations. Fishes like the wrasse *Thalassoma pavo* and the sparid *Diplodus sargus* have no genetic differentiation between Atlantic and Mediterranean populations (Costagliola et al. 2004; Bargelloni et al. 2005). The goby *Pomatoschistus minutus* shows some genetic differentiation between the Atlantic and the western Mediterranean although not very pronounced (Stefanni and Thorley 2003). Several invertebrates like the sponge *Crambe crambe* (Duran et al. 2004a), the Norway lobster *Nephrops norvegicus* (Stamatis et al. 2004) and the limpet *Patella ulyssiponensis* (Sá-Pinto et al. 2005) reveal no signs of genetic partition between the Atlantic and the Mediterranean. The distribution of genetic diversity along the Atlantic and Mediterranean populations of the oyster *Ostrea edulis* fits an isolation-by-distance pattern with the Atlantic being less variable than the Mediterranean (Launey et al. 2002; Diaz-Almela et al. 2004). In contrast, microsatellite analysis of the labrid *Coris julis* from the Atlantic and the Mediterranean, show a high degree of genetic differentiation between the two basins (Aurelle et al. 2003). Two other coastal fishes, the goby *Pomatoschistus microps* (Gysels et al. 2004) and the sparid *Diplodus puntazzo* (Bargelloni et al. 2005) reveal a genetic break between the Atlantic and the Mediterranean at the Almeria-Oran jet. Genetic differentiation between the Atlantic and the Mediterranean basins have also been described for several invertebrate taxa such as the mussel *Mytilus galloprovincialis* (Quesada et al. 1995), the bivalve *Cerastoderma glaucum* (Nikula and Väinölä 2003), the flounders *Platichthys flesus* and *P. stellatus* (Borsa et al. 1997), the sea urchin *Paracentrotus lividus* (Duran et al. 2004b), the sea star *Asterina gibbosa* (Baus et al. 2005), the crabs *Carcinus maenas* (Roman and Palumbi 2004), *Xantho hydrophilus* and *X. poressa* (Reuschel and Schubert 2006), the limpet *Patella rustica* (Sá-Pinto et al. 2005), the scallops *Pecten maximus* and *P. jacobaeus* (Ríos et al. 2002; Saavedra and Peña 2005) and the barnacles *Chthamalus stellatus* and *C. montagui* (Pannacciulli et al. 1997). The Almeria-Oran jet and the Strait of Gibraltar have been suggested as barriers to gene flow for these taxa.

The Macaronesian islands

Few studies attempted to evaluate the extent of differentiation within the Macaronesian islands. The marine gastropod *Littorina striatta* showed no genetic structure between Azores, Madeira, Canaries and also Cape Verde, although the last archipelago yielded higher diversity indices (De Wolf et al. 2000). Similarly, the barnacle *Chthamalus stellatus* showed genetic homogeneity between Azores and Madeira (Pannacciulli et al. 1997). A

phylogenetic survey of several limpets revealed genetic differentiation between Macaronesian and mainland *Patella ulyssiponensis* with low genetic structure within the Macaronesian islands for this limpet, although the Azores was slightly different from the other islands (Sá-Pinto et al. 2005). Some differentiation for *P. candolei- P. lugrubis* complex was also described, with four main clades: Azores, Madeira/Desertas, Selvagens/Canaries and Cape Verde (Sá-Pinto et al. 2005).

The Azores

The Azores archipelago has received particular attention due to its isolated location and young age. The archipelago is composed of nine islands located between 37º and 40ºN and 25º and 31º W. The Azores are in permanent formation since the Miocene (Féraud et al. 1980). The oldest island, Santa Maria, dates from about 8 million years (My), while Pico is as young as 1 My (Azevedo et al. 1991; Serralheiro and Madeira 1993). The majority of the Azorean coastal fauna is very modern and comprises species coming predominantly from the eastern Atlantic coast (southern Europe and northwestern Africa) and the Mediterranean (Morton and Britton 2000). A few species from other Atlantic sources are also found in the islands. From the biogeographic affinities described above it is clear that the marine fauna of the Azores exhibit several biogeographic affinities and that the archipelago has been colonized from different sources. Several means of colonization can be involved depending on the biologic characteristics of the species. Egg and larval transport by ocean currents and eddies coming from Madeira, Canaries, the Atlantic coast of Europe and the African coast has been suggested to be the primary source of colonization for coastal fishes (Santos et al. 1995). Seaweeds and hydroids can also be transported attached to floating objects (rafting) being able to reach the Azores from the American coast (see the case of *Hypleurochilus bananensis* that reached the Azores from the American coast, having been initially identified as *Blennius fucorum*, Nieto and Alberto 1990). Some sponges and crustaceans can survive in deeper waters and be transported by the deep-water current coming from the Mediterranean.

Within the Mediterranean

Within the Mediterranean there is also evidence of population structure for some species. The sponge *Crambe crambe* shows three populations along the Mediterranean: a western one along the coast of Spain including the Balearic Islands, a central one comprehending Marseille and Corsica and a third and more divergent one in Naples (Duran et al. 2004c). Stefanni and Thorley (2003) showed strong restriction to gene flow at the Siculo-Tunysian Strait for the goby *Pomatoschistus minutus* and a very differentiated population in the Adriatic. A strong genetic break at the Greek Peloponnese was reported for the wrasse *Thalassoma pavo* (Costagliola et al. 2004) and the bivalve *Cerastoderma glaucum* (Nikula and Väinölä 2003). Borsa et al. (1997) identified these two regions as effective barriers to gene flow for the flounders *Platichthys flesus* and *P. stellatus*.

The barriers to gene flow occurring between the Atlantic and the Mediterranean and within the Mediterranean have been associated with seawater level changes and cooling effects during the Pleistocene glaciations (eg. Quesada et al. 1995, Stefanni and Thorley 2003, Nikula and Väinölä 2003), current water circulation patterns (Ríos et al. 2002; Bargelonni et al. 2005; Baus et al. 2005), and/or physical, chemical or ecological differences of each basin (Borsa et al. 1997, Pannasciulli et al. 1997; Baus et al. 2005).

Endemism

The warm-temperate Mediterranean-Atlantic has an endemism of about 40-50% (Briggs 1974). While the majority of the shore fishes of Portugal and Morocco also occur in the Mediterranean, this Sea has a great number of species that are not found in the Atlantic coasts. In addition, after the excavation of the Suez Canal in 1869, the Mediterranean was invaded by fishes from the Red Sea. These species, called the Lessepsian migrants, added at least 62 new fishes to the Mediterranean (Golani et al. 2004).
Briggs (1966) described a pattern of marine shore endemism for the Atlantic Ocean, where the endemic rate was very low in the north and middle Atlantic, and much greater in the southern Atlantic. Overall, the endemic rate was 0% in the Azores, 3% in Madeira, 4% in Cape Verde and 27-50% in Santa Helena. According to this author, low sea surface temperatures experienced in the northern areas of the Atlantic during the Pleistocene glaciations could have promoted extinctions in this area, explaining the residual level of endemism in the northern and central Atlantic. McDowall (1968, 1971) criticized Briggs (1966) approach based on the fact that he compared levels of endemism for different phyla. The same author pointed that other factors such as island age, island area, population size, degree of isolation and species characteristics could also have contributed for the low endemism of the shore fauna of the north Atlantic islands. More recent studies revised by Morton and Britton (2000) confirmed the low level of endemism of the north Atlantic islands, especially the Azores, for several taxa. At present, only one shore fish species, the labrid *Centrolabrus caeruleus* (described as *Symphodus caeruleus* by Almada et al. 2002), is recognized as endemic to the Azores (Azevedo 1999). Santos et al. (1995) pointed out the low level of endemism of the Azores islands and suggested that the majority of the marine fauna of the islands must have arrived after the Pleistocene glaciations having thus little time to differentiate.

Speciation centers and colonization routes

According to the biogeographical patterns described above, a few models of speciation of the Atlantic-Mediterranean coastal species have been proposed. Due to its higher species diversity the Mediterranean has been suggested as a center of speciation for many invertebrates. Ávila et al. (2000) suggested that the molluscs of the Azores may have reached the islands from the Mediterranean and mainland Portugal, having Madeira as an intermediate location. The same author speculated about the role of the submarine banks

between Portugal and the islands in a stepping-stone like colonization. The phylogenetic survey of Sá-Pinto et al. (2005) pointed to the importance of the Mediterranean and Macaronesian islands as speciation centers for the limpets of the genus *Patella*. According to Santos et al. (1995) the littoral fishes of the Azores could have colonized the islands from the western Africa and Macaronesian islands, on one side, and from the European Atlantic coast on the other. Eddies that circulate in the region must have had an important role in the transport of eggs and larvae, which might also have profited from the seamounts that occur in the region. Zander (1973) assumed that during the Pleistocene most blennioid species could not have survived in the cold Mediterranean and that the ancestors of the modern forms might have evolved from forms that survived in the warmer refuges in the western African coast. Based on behavioral studies of three species of *Tripterygion* from the Mediterranean, Wirtz (1978) proposed that a common ancestor from the eastern Atlantic invaded the Mediterranean three times. Between each invasion the Atlantic and Mediterranean forms became separated promoting speciation in the Mediterranean. Latter, Zander (1980) described the Macaronesian islands as centers of speciation for some of the eastern Atlantic blennioids, from which the new species have invaded the western European shores. Based on the higher level of diversity and endemism in the Mediterranean and tropical west Africa and on the pattern of species distribution in the Atlantic islands, Almada et al. (2001) proposed a scenario for the migration routes of blenniids in the region, that can be extended to other warm water fish species. These authors suggested that the warm water fauna of the Atlantic-Mediterranean region was severely affected by the drop in sea surface temperatures during the Pleistocene, being able to survive only in warmer refuges located in the tropical western Africa and Mediterranean. Recolonization of the northern areas would have been possible from these refuges after warmer conditions were restored. According to this hypothesis, the tropical West Africa and Mediterranean constitute diversification and speciation centers for the northeastern Atlantic marine fauna.

## 4. Approach and goals

Most inshore fishes have a bipartite live style with pelagic early life stages more prone to dispersal and coastal associated older stages with restricted dispersal ability. Thus, coastal fishes, especially the less vagile ones, are excellent organisms to study the biogeographic affinities and evolutionary relationships of the marine fauna of a given region.

With the development of molecular tools, emerging disciplines like phylogeography and historical demography have proved to be very useful in revealing the evolutionary relationships of populations over time. Phylogeography is a discipline that focuses on the principles and processes that are responsible for the geographic distribution of genealogical lineages within and among closely related species (Avise 2000). It thus combines information from a multitude of disciplines such as phylogenetics, population

genetics, demography, paleontology and ethology. The ultimate goal of phylogeography is to identify the evolutionary processes (selection, dispersal, vicariance) responsible for the geographical distribution of the contemporary lineages. When aiming at understanding the processes that generated the genetic distribution pattern that we see today, it is very useful not only to look at phylogenetic relationships, but also to assess demographic changes undergone by the focal populations in the past. The study of historical demography has improved much with the development of mathematical models based on the coalescent theory. The coalescent process describes the ancestry of a sample of genes. The conceptual basis of the coalescence relies on thinking backwards in time. If we take modern genes and trace their ancestry backwards from ancestor to ancestor, we will encounter common ancestors. Eventually we will reach the gene that is ancestral to all the modern genes in our sample (the most recent common ancestor). Because the time necessary to reach a common ancestor is ultimately related to the size of a population (and also to the mutation rate of the gene in study), models based on coalescence can be developed to estimate valuable parameters such as present and past population sizes (Avise et al. 1988), sudden reductions in population sizes over time (bottlenecks) (Rogers and Harpending 1992) and coalescence time (time elapsed from the present to the most common ancestor). More recently, the coalescent theory has been improved and extended to populations of varying demography and structures (Tajima 1989a,b; Hudson 1998; Withlock and Barton 1997) and also to accommodate recombining genes (Hey and Wakeley 1997). New models that allow the estimation of recombination frequency, population growth rate and migration events have been developed.

This study is centered on the evolutionary relationships of the warm water coastal fishes of the northeastern Atlantic and the Mediterranean. It consists of studies on twelve species from six families (Blenniidae, Labridae, Pomacentridae, Scaridae, Sparidae and Tripterygiidae), with an Atlantic-Mediterranean distribution. In order to shed light on the historical relationships of the different populations, phylogeographic and demographic approaches were applied.

In this study the following questions are addressed:

- Are Atlantic and Mediterranean populations genetically structured?
- Do populations carry genetic signatures of local extinctions and recolonizations triggered by the Pleistocene glaciations? Can Pleistocene refuges be identified and colonization routes recovered?
- Are the colonization routes in agreement with the current system of the area?
- Do species with different thermal tolerances and dispersal capabilities show contrasting patterns?

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# CHAPTERS

# Chapter 1

## Historical colonization and demography of the Mediterranean damselfish, *Chromis chromis*

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# Historical colonization and demography of the Mediterranean damselfish, *Chromis chromis*

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## Abstract

**The desiccation of the Mediterranean Sea during the Messinian Salinity Crisis 6.0–5.3 million years ago (Ma), caused a major extinction of the marine ichthyofauna of the Mediterranean. This was followed by an abrupt replenishment of the Mediterranean from the Atlantic after the opening of the Strait of Gibraltar. In this study, we combined demographic and phylogeographic approaches using mitochondrial and nuclear DNA markers to test the alternative hypotheses of where (Atlantic or Mediterranean) and when (before or after the Messinian Salinity Crisis) speciation occurred in the Mediterranean damselfish, *Chromis chromis*. The closely related geminate transisthmian pair *Chromis multilineata* and *Chromis atrilobata* was used as a way of obtaining an internally calibrated molecular clock. We estimated *C. chromis* speciation timing both by determining the time of divergence between *C. chromis* and its Atlantic sister species *Chromis limbata* (0.93–3.26 Ma depending on the molecular marker used, e.g. 1.23–1.39 Ma for the control region), and by determining the time of coalescence for *C. chromis* based on mitochondrial control region sequences (0.14–0.21 Ma). The time of speciation of *C. chromis* was always posterior to the replenishment of the Mediterranean basin, after the Messinian Salinity Crisis. Within the Mediterranean, *C. chromis* population structure and demographic characteristics revealed a genetic break at the Peloponnese, Greece, with directional and eastbound gene flow between western and eastern groups. The eastern group was found to be more recent and with a faster growing population (coalescent time = 0.09–0.13 Ma, growth = 485.3) than the western group (coalescent time = 0.13–0.20 Ma, growth = 325.6). Our data thus suggested a western origin of *C. chromis*, most likely within the Mediterranean. Low sea water levels during the glacial periods, the hydrographic regime of the Mediterranean and dispersal restriction during the short pelagic larval phase of *C. chromis* (18–19 days) have probably played an important role in *C. chromis* historical colonization.**

*Keywords*: *Chromis*, coalescence, Mediterranean, Messinian Salinity Crisis, phylogeography, speciation

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## Introduction

The Mediterranean Sea underwent at least one episode of desiccation and replenishment, the so-called Messinian Salinity Crisis (MSC) that occurred between 6.0 and 5.3 million years ago (Ma) (Hsü *et al.* 1977; Krijgsman *et al.* 1999; Duggen *et al.* 2003). Except for a small number of species capable of surviving in brackish or hypersaline lagoons, the marine ichthyofauna of the Mediterranean became extinct during this desiccation episode. After that period, the Mediterranean abruptly refilled from the Atlantic following the opening of the Strait of Gibraltar. At present, the Mediterranean Sea is defined as a warm-temperate sea and harbours about 540 species of fish. Briggs (1974) estimated that approximately 52 species (9.6%) are endemics.

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Subsequent studies, however, showed that some of the so-called endemics are also found in the Atlantic areas adjacent to the entrance of the Mediterranean (Almada *et al.* 2001). Few species are shared with the Indian Ocean; these are recent Lessepsian migrants invading from the Red Sea through the Suez Canal (approximately 60 species, Golani 1999). The vast majority of the Mediterranean marine fish species originated from the adjacent Atlantic fish fauna by invading via the Strait of Gibraltar (e.g. Bargelloni *et al.* 2003). Mediterranean endemics are either the result of *in situ* speciation (occurring after the MSC), or experienced an extinction of their Atlantic populations after colonizing the Mediterranean. In addition, since the Mediterranean waters remained warmer that those of the adjacent Atlantic during glacial peeks (Thiede 1978), many species now present in the warm temperate Atlantic, likely survived the cold phases of the glacial cycles within the Mediterranean, recolonizing the Atlantic when more favourable temperatures were re-established during interglacial phases like the present one (Almada *et al.* 2001). Thus, species of Mediterranean fishes provide a unique opportunity to understand the processes of colonization, evolution, and local adaptation.

In this study, we have focused on the Mediterranean damselfish, *Chromis chromis* (Pomacentridae). The species is broadly distributed in the Mediterranean over rocky reefs and seagrass beds, usually in waters shallower than 25 m (Lythgoe & Lythgoe 1971; Riedl 1983). Besides its Mediterranean distribution, some individuals are observed outside the Strait of Gibraltar, along the Atlantic coast of Portugal (Wood 1977; personal observation).

The genus *Chromis* comprises 75 species that are distributed worldwide (Allen 1991; Tang 2001; Quenouille *et al.* 2004). *C. chromis* is found in the Mediterranean and the Atlantic areas adjacent to this sea. *Chromis limbata*, the most likely sister species of *C. chromis* (Wood 1977; L. Rocha *et al.*, unpublished), is restricted to the Macaronesian islands (Azores, Madeira, and Canaries) and the western coast of North Africa (between Senegal and Congo, Wood 1977; L. Rocha *et al.*, unpublished). Eastern and central Atlantic *Chromis* species also include *Chromis lubbocki* (Cape Verde Islands), *Chromis sanctahelena* (Saint Helena Island), *Chromis cadenati* (Senegal to Ghana), and *Chromis multilineata* (from western Africa to the western Atlantic) (Allen 1991). This latter species is considered a transisthmian geminate, which diverged from its eastern Pacific sister species *Chromis atrilobata*, at the rise of the Isthmus of Panama, 3.1–3.5 million years ago (Ma) (L. Rocha *et al.*, unpublished).

Several genetic studies have focused on the phylogeographic relationships of marine organisms both between the Atlantic and the Mediterranean Sea and within the Mediterranean. Some studies have shown a strong genetic divergence between Atlantic and Mediterranean faunas, due to the isolation of both seas during the Pleistocene glaciations and to the present-day hydrographic barriers, while others found very high levels of gene flow between these two regions. For instance, Bargelloni *et al.* (2003) found strong to no differentiation between the Atlantic and the Mediterranean for five teleosts species of the family Sparidae. The wrasse (*Thalassoma pavo*) (Costagliola *et al.* 2004), and the chub mackerel (*Scomber japonicus*, Zardoya *et al.* 2004) were described as having high gene flow levels between the Atlantic and the Mediterranean. Stamatis *et al.* (2004) found no signs of an Atlantic–Mediterranean divide for the Norway lobster (*Nephrops norvegicus*). In contrast, Pannacciulli *et al.* (1997) found marked genetic differentiation between Atlantic and Mediterranean populations in two species of *Chthamalus* barnacles, with the Almeria-Oran front (Fig. 1) preventing extended gene flow between these two regions. Pérez-Losada *et al.* (1999, 2002) described genetic differentiation between Atlantic and Mediterranean populations of the cuttlefish *Sepia officinalis*, and so did Quesada *et al.* (1995) for the mussel *Mytilus galloprovincialis*. Narciri *et al.* (1999) described two groups of populations of the sea bass *Dicentrarchus labrax* and postulated that the divide may correspond to the Almeria-Oran oceanographic front. Comparing nuclear and cytoplasmic markers for the same species, Lemaire *et al.* (2005) suggested the existence of a hybrid zone in the Alborean Sea.

Similarly, within the Mediterranean Sea, studies showed high levels of gene flow, or alternatively strong population differences. Kotoulas *et al.* (1995) and Pujolar *et al.* (2002) found no genetic structure within the Atlantic for the swordfish *Xiphias gladius*. The two populations east of the discontinuity found between the Atlantic and the Mediterranean for the mussel *M. galloprovincialis* analysed by Quesada *et al.* (1995) were homogenous in haplotype frequency. In contrast, some studies evidenced population structure within the Mediterranean with genetic breaks observed at different places. Indeed, some studies evidenced a restriction of gene flow at the 'saddle' between Sicily and Tunisia (Fig. 1) (e.g. the goby *Pomatoschistus minutus*, Stefanni & Thorley 2003, and the mackerel *S. japonicus*, Zardoya *et al.* 2004), while others found a strong break in Greece just south of the Peloponnese (Fig. 1) (e.g. the anchovy *Engraulis encrasicolus*, Magoulas *et al.* 1996; the sea bass *Dicentrarchus labrax*, Bahri-Sfar *et al.* 2000; the bivalve *Cerastoderma glaucum*, Nikula & Väinölä 2003; and the wrasse *T. pavo*, Costagliola *et al.* 2004). Borsa *et al.* (1997) identified three geographically isolated populations of the flounders *Platichthys* in the Mediterranean, separated by the two breaks mentioned above. The Mediterranean damselfish may conform to scenarios described for other species, with the two extreme situations being strong population structure between the Atlantic and the Mediterranean and within the Mediterranean, or high gene flow and no population structure.

Dispersal and vicariance have both probably played an important role in shaping the different phylogeographic

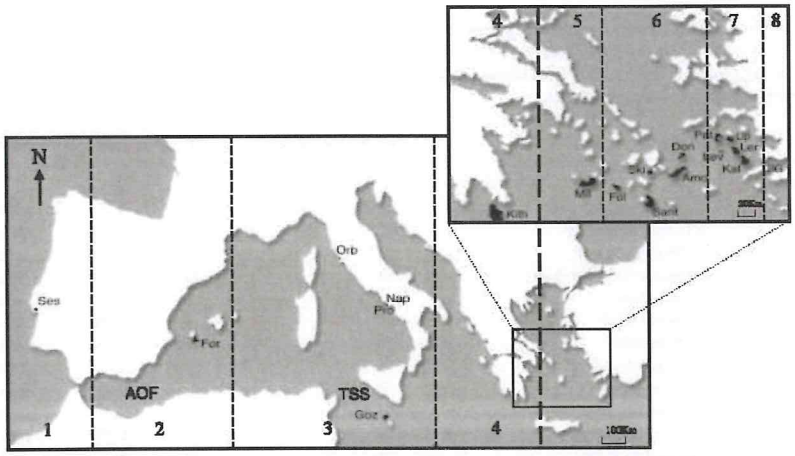


**Fig. 1** *Chromis chromis* sampling locations in the Atlantic and the Mediterranean. Samples were collected in the following localities: Sesimbra (Ses), Portugal; Formentera (For), Spain; Orbetello (Orb), Naples (Nap) and Procida (Pro), Italy; Gozo (Goz), Malta; Kithira (Kit), Milos (Mil), Folegandros (Fol), Santorini (Sant), Skinousa (Ski), Amorgos (Amo), Donoussa (Don), Levitha (Lev), Patmos (Pat), Lipsos (Lip), Leros (Ler), Kalimnos (Kal), Greece; and Bodrum Gulf (BG),Turkey. Dashed lines limit regions (nos 1 8) used for testing degrees of differentiation for multiple groupings. The Tunisia Sicily saddle is indicated by 'TSS', and the Almeria-Oran front is indicated by 'AOF'.

patterns mentioned above. Low sea water levels and changes in ocean circulation patterns during the glacial periods could have played an important role in the segregation of populations in the Mediterranean. Dispersal restriction during the pelagic larval phase has probably contributed to the current situation. In the case of *C. chromis*, after a relatively short pelagic larval duration (18–19 days, Raventós & Macpherson 2001), fishes tend to be sedentary over seagrass or rocky reefs at depths ranging from 3 to 35 m, where they live for a maximum of 9 years (Dulcic & Kraljevic 1995). They reproduce repeatedly throughout the spawning season (June–September) (Picciulin *et al.* 2004). Males establish territories, prepare nests and court females. Females lay demersal eggs that are guarded by males until hatching. As for other pomacentrids, *C. chromis* has been reported to exhibit reproductive parasitism (Picciulin *et al.* 2004). In this study, our goal was to test the alternative hypotheses related to speciation in Mediterranean fishes, and specifically of where and when speciation occurred in the Mediterranean damselfish, *C. chromis*. If it speciated within the Mediterranean, we would predict the divergence from its sister species, *C. limbata*, as well as the coalescence time of *C. chromis*, to have occurred after the MSC. In contrast, if *C. chromis* colonized the Mediterranean from the Atlantic, the split between *C. chromis* and *C. limbata* could have pre-dated the MSC, resulting in a divergence time from *C. limbata*, and coalescence of *C. chromis*, to preceed the formation of the present-day Mediterranean Sea. In addition, we may also find a signature of recent expansion in *C. chromis* populations, possibly showing a migration trend and expansion going eastward inside the Mediterranean. A third possibility is for an Atlantic origin of *C. chromis* and the split between *C. chromis* and *C. limbata* to have occurred after the MSC. This hypothesis, however, requires both a rapid colonization of the Mediterranean and an extinction of Atlantic populations of *C. chromis* over a relatively short time.

In order to determine if speciation occurred recently within the Mediterranean Sea, or if it occurred in the Atlantic, with a subsequent colonization event, we investigated the following questions: (i) Can a phylogeographic approach coupled with historical demographic parameters estimate speciation time in *C. chromis* in relation to the MSC? (ii) Are population structure and migration patterns within the Mediterranean bearing signatures of recent colonization and expansion?

To answer our questions, we decided to combine a phylogeographic approach using mitochondrial and nuclear markers, with a demographic study of the species. We used *C. chromis* as our focal species, *C. limbata* as its sister species, and also the geminate transisthmian pair *C. multilineata* and *C. atrilobata* as a way to calibrate the molecular clock and estimate the mutation rate for this closely related group of species.

## Materials and methods

### *Sampling and DNA extraction*

Sampling localities, dates of collections and number of individuals are given in Table 1 and Fig. 1. Samples of

**Table 1** Collection localities of *Chromis chromis*, *Chromis limbata* and outgroup species, *Chromis multilineata* and *Chromis atrilobata*, used in the present study. Number of individuals, number of haplotypes (Hn) and Haplotype diversity (Hd) (for mitochondrial control region) were calculated using DNASP (Rozas *et al.* 2003). Numbers after *C. chromis* localities represent regions described in Fig. 1. Locality labels from Figs 1 and 2, and Fig. 3 are between parentheses

| Species | Locality | No. of individuals | Hn | Hd | Collection date |
|---|---|---|---|---|---|
| *C. chromis* (CCH) | | | | | |
| Portugal | 1 Sesimbra (Ses) | 14 | 14 | 1 | November 2004 |
| Spain | 2 Formentera (For) | 22 | 20 | 0.991 | October 2003 |
| Italy | 3 Orbetello (Orb) | 10 | 10 | 1 | June 1996 |
| | ° Naples/ Procida (Nap/ Pro) | 13 | 10 | 0.949 | June 2001/July 2003 |
| Malta | ° Gozo (Goz) | 6 | 6 | 1 | April 2003 |
| Greece | 4 Kithira (Kit) | 7 | 6 | 0.952 | May 2003 |
| | 5 Milos (Mil) | 7 | 6 | 0.952 | May 2003 |
| | ° Folegandros (Fol) | 8 | 8 | 1 | June 2003 |
| | ° Santorini (Sant) | 7 | 6 | 0.952 | June 2003 |
| | ° Skinousa (Ski) | 10 | 8 | 0.933 | June 2003 |
| | ° Amorgos (Amo) | 6 | 5 | 0.933 | July 2003 |
| | ° Donoussa (Don) | 10 | 10 | 1 | August 2003 |
| | 6 Levitha (Lev) | 9 | 7 | 0.944 | August 2003 |
| | ° Patmos (Pat) | 9 | 6 | 0.889 | September 2003 |
| | ° Lipsos (Lip) | 8 | 7 | 0.964 | August 2003 |
| | ° Leros (Ler) | 9 | 8 | 0.792 | September 2003 |
| | ° Kalimnos (Kal) | 9 | 9 | 1 | July 2003 |
| Turkey | 7 Bodrum Gulf (BG) | 21 | 17 | 0.976 | October 2003 |
| Total for *C. chromis* | | 185 | 116 | 0.625 | |
| *C. limbata* (CLI) | | | | | |
| Portugal | Azores (Az) | 4 | | | December 2003 |
| | Madeira (Mad) | 4 | | | September 2003 |
| Spain | Canaries (Can) | 4 | | | February 2004 |
| *C. multilineata* (CMU) | | | | | |
| Panama | San Blas (Atlantic coast) (Pan) | 2 | | | March 1997 |
| *C. atrilobata* (CAT) | | | | | |
| Mexico | B. Tortugas (Pacific coast) (Mex) | 1 | | | October 1999 |
| Galapagos | Santa Cruz (SC) | 2 | | | December 1999 |
| | Española (Esp) | 2 | | | January 2000 |
| | Floreana (Flo) | 1 | | | January 2000 |

*Chromis chromis* were obtained from one location in the Atlantic and 18 locations in the Mediterranean corresponding to 17 populations (Naples and Procida locations were treated as a single population due to their proximity). Six of the Mediterranean locations were in the western basin, the remaining 12 were in the eastern basin. *Chromis limbata* (*C. chromis* sister species) was collected from three Atlantic islands (Azores, Madeira, and the Canaries). We used *Chromis multilineata* and *Chromis atrilobata* as outgroups. Samples were collected by spear or hand nets while scuba diving. Fin clips were cut immediately after collection of the individuals and stored at ambient temperature in 95% ethanol. Tissues were digested overnight at 55 °C in 700 µL of extraction buffer (400 mM NaCl, 10 mM Tris, 2mM EDTA, 1% SDS). We purified the DNA by standard chloroform extraction and isopropanol precipitation (Sambrook *et al.* 1989).

### *Polymerase chain reaction and DNA sequencing*

Amplification of the 5′ hypervariable portion of the mitochondrial control region (also called D-loop) was accomplished with universal primers CR-A and CR-E (Lee *et al.* 1995), and used a cycling profile of 45 s at 94 °C, 45 s at 52 °C, 1 min at 72 °C, for 35 cycles. In addition, we amplified and sequenced segments of the mitochondrial 16S rRNA and cytochrome *b* genes as well as the nuclear 1st intron of the alpha-tropomyosin (TROP) for a randomly chosen subset of our samples. Cytochrome *b* and 16S rRNA were amplified for 45 s at 94 °C, 45 s at 48 °C, and 1 min at 72 °C for 35 cycles, with the following primers: GLUDG-L and CB3H, and 16SAR and 16SBR (Kocher *et al.* 1989). Tropomyosin intron was amplified for 30 s at 94 °C, 1 min at 60 °C, and 2 min at72 °C for 35 cycles with the following primers: TR1F and TR1R (Hassan *et al.* 2002). Each 13-µL

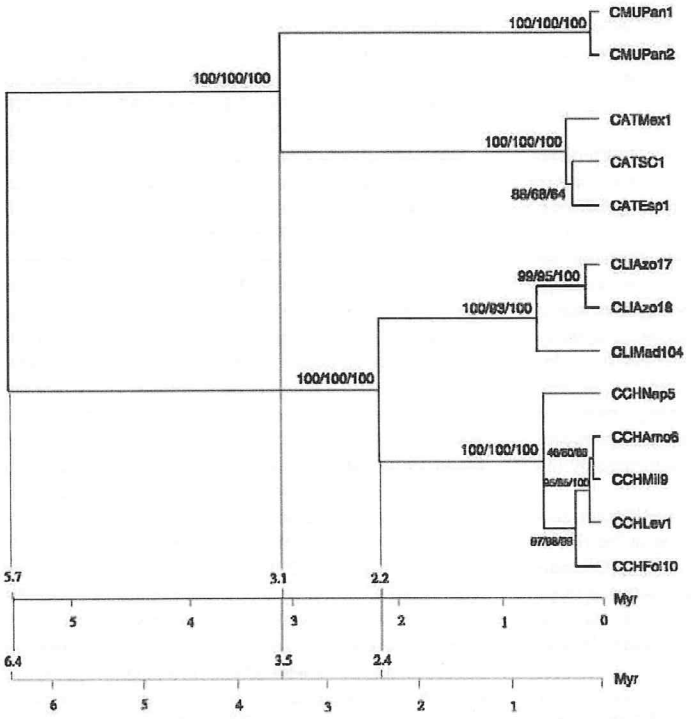


**Fig. 2** Phylogenetic relationship between *Chromis chromis* and *Chromis limbata*, with *Chromis multilineata* and *Chromis atrilobata* used as outgroups. Phylogenetic reconstruction was based on the combined mitochondrial control region, 16S rRNA, cytochrome *b* and the nuclear 1st intron of the tropomyosin gene sequences using a maximum-likelihood method, with HKY + G model and an enforced molecular clock. Alternative reconstruction methods, neighbour-joining, maximum parsimony and maximum likelihood (with or without enforcing a molecular clock) resulted in the same topology. Labels are described in Table 1. The length of each branch is proportional to the number of nucleotide substitutions. Bootstrap values for each node are shown as percentages for neighbour-joining, parsimony and likelihood methods, respectively. Timescales presented at the bottom of the figure are based on the split of *Chromis atrilobata* and *Chromis multilineata* coinciding with the rise of the Isthmus of Panama (3.1–3.5 Ma).

reaction contained 5–50 ng of DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 1.25 U of *Taq* DNA polymerase (PerkinElmer), 150 mM of each dNTP, and 0.3 mM of each primer. After purification following the manufacturer's protocol (Applied Biosystems), direct sequencing was performed with an ABI 3100 automated sequencer (Applied Biosystems). Sequencing was performed in one direction only for the mitochondrial control region, cyt *b* and 16S. The tropomyosin intron was sequenced in both directions. None of the sequences contained ambiguous heterozygous positions, but for two that contained a single heterozygous (A and G) position that was encoded as R (for purines). When removing that position from the analysis, results remained unchanged, thus the position was kept for the final analysis.

### *Data analysis*

*DNA sequences and phylogenetic analyses.* We used the computer program CLUSTAL V implemented by Sequence Navigator (Applied Biosystems) to align the sequences. Number of haplotypes and haplotype diversity were calculated using the software package DNASP (Rozas *et al.* 2003). Phylogenetic relationships based on mitochondrial control region, 16S, cyt *b* and TROP sequences were assessed using representative individuals from each species (described in Fig. 2). Character congruence between the four fragments was tested using the incongruence-length difference test (ILD) (Farris *et al.* 1995) available in PAUP (version 4.0; Swofford 1998). We used three methods of phylogenetic inference: maximum parsimony (using heuristic search, TBR branch swapping, random addition of taxa and no weighting), neighbour joining and maximum likelihood, with the substitution model established using MODELTEST 3.06 (Posada & Crandall 1998) under hLRT (HKY + G, ti/tv ratio = 2.5511, gamma value = 0.1573, base frequencies A = 0.2786, C = 0. 1957, T = 0.2564 and G = 0.2693). All methods were implemented by the software package PAUP (Swofford 1998). Additionally, we inferred phylogenetic relationships of all *C. chromis* specimens based on mitochondrial control region sequences using maximum-parsimony and neighbour-joining methods (with the substitution model obtained using MODELTEST under hLRT: HKY + G, ti/tv ratio = 2.1676, gamma value = 0.4752, base frequencies A = 0.3676, C = 0. 1871, T = 0.1405 and G = 0.3048). Topological confidence was evaluated for all phylogenetic analyses, with 1000 bootstrap replicates (Felsenstein 1985). For maximum parsimony, bootstrap was performed using the fast-step

method (only one tree kept at each replicate). Alternative topologies were tested using the Shimodaira–Hasegawa test (Shimodaira & Hasegawa 1999) implemented in PAUP (Swofford 1998).

*Genetic divergence and molecular clock calibration.* Genetic divergence between sister species (*C. chromis*/*C. limbata* and *C. multilineata*/*C. atrilobata*) was estimated for mitochondrial control region, cyt *b*, 16S and TROP, using substitution models obtained with MODELTEST. In order to account for polymorphism in each species, divergence was estimated as the average pairwise distance between species minus the average pairwise distance within species. In order to test for homogeneity of rates of molecular evolution for D-loop, cyt *b*, 16S and TROP, we compared maximum-likelihood topologies with or without enforcing a molecular clock, using a likelihood-ratio test (Shimodaira–Hasegawa test; Shimodaira & Hasegawa 1999), implemented by the software package PAUP (Swofford 1998).

The rate of divergence between *C. multilineata* and *C. atrilobata* was calibrated using the timing of the rise of the Isthmus of Panama (3.1–3.5 Ma; Coates & Obando 1996) as the minimum time of divergence between these two species. This molecular clock was used to estimate the minimum divergence time between *C. chromis* and *C. limbata*.

*Population structure.* Gene flow ($F_{ST}$ and *Nm*) was estimated using ARLEQUIN (version 2.000; Schneider *et al.* 2000). Population structure was estimated by an analysis of molecular variance (AMOVA; Excoffier *et al.* 1997) using ARLEQUIN. Populations were grouped in different regions and alternative groupings were tested with an AMOVA to find the best fit for our data, which defined western and eastern regions of the Mediterranean. Corrections for simultaneous multiple comparisons were applied using sequential Bonferroni correction (Rice 1989).

To test for isolation by distance (IBD) we applied the Mantel test (Mantel 1967) to two matrices, $F_{ST}$ values and log geographical distances in kilometres between localities. We used IBD 1.4 (Bohonak 2002) to perform the Mantel test, using 1000 replicates to test significance.

### *Historical demography*

*Chromis chromis* sample locations were divided in western and eastern regions according to the results of the AMOVA. The historical demography of the western and eastern groups was first examined using mismatch distributions analysis and Tajima's *D* test of neutrality (Tajima 1989), in order to evaluate possible events of population expansion and decline. Theoretical studies have shown that populations in long stable demographic equilibrium show a chaotic mismatch distribution, while recent rapid population expansions or bottlenecks are reflected in a unimodal mismatch distribution (Rogers & Harpending 1992; Rogers 1995). Tajima's *D* test is classically used to test neutrality, but it can also be used to test population growth as a population that has been experiencing expansion may result in a reject of the null hypothesis of neutrality (significant negative *D* values).

Estimates of $\Theta$ (= 2 $N\mu$, where $\mu$ is the mutation rate for mitochondrial control region), were made for each region as well as *C. chromis* as a whole. The parameter $\Theta$ was estimated under two conditions: an unconstrained exponential growth parameter, and an assumption of constant $N$ ($g = 0$). We used FLUCTUATE (Kuhner *et al.* 1998) to estimate the maximum likelihood of the parameters $\Theta$ and g (the exponential growth parameter in units $\mu^{-1}$). Seeds for all analyses were generated randomly and the default transition to transversion ratio was used. Analyses were repeated 10 times per region to ensure stability of parameters estimates. Final analyses of each data set employed 10 short Monte Carlo chains of 200 steps each and 5 long chains of length 20 000, with a sample increment of 20. Exchanges and range expansions (immigration) between western and eastern regions were estimated using MIGRATE version 2.0 (Beerli & Felsenstein 2001; Beerli 2004). Again, analyses were repeated 10 times, to ensure stability of parameter estimates. Final analyses of each data set employed 10 short Monte Carlo chains with 500 recorded genealogies and five long chains with 5000 recorded genealogies, and a sample increment of 20. A chi-squared goodness-of-fit test was performed to test the null hypothesis that migration in both directions had equal rates. The time of coalescence was estimated by assuming that coalescence was reached when the population size was reduced to 1% of its present-day value, following Wares & Cunningham (2001). In order to estimate coalescence time, we estimated the mutation rate ($\mu$) as $\mu$ = substitutions per site per generation. Generation time, a value necessary to estimate coalescence time, was estimated at 3 years, the approximate time for sexual maturity for *C. chromis* and *C. limbata* (Mapstone & Wood 1975; Dulcic & Kraljevic 1995).

## Results

### *DNA sequences and phylogenetic analyses*

The 16S rRNA and cytochrome *b* sequences were obtained for 13 samples, 5 *Chromis chromis*, 3 *Chromis limbata*, 3 *Chromis atrilobata*, and 2 *Chromis multilineata*. Sequences for these two genes were 539 and 616 bp long, respectively. Nuclear sequences for the 1st intron of the alpha-tropomyosin gene (347 bp) were obtained for 30 individuals, 15 *C. chromis*, 7 *C. limbata*, 6 *C. atrilobata*, and 2 *C. multilineata*. No indels were found in this intron. The null hypothesis of congruence between all four loci (D-loop, cyt *b*, 16S and TROP) was not rejected ($P = 1$), thus data for all loci were combined. All methods of phylogenetic inference resulted

**Table 2** Divergence of sister species *Chromis atrilobata* and *Chromis multilineata* based on mitochondrial and nuclear genes (column 1). Model of substitution for each marker obtained using MODELTEST 3.06 (Posada & Crandall 1998) (column 2). Sequence divergence (using substitution model obtained in MODELTEST) and rate of sequence divergence [per million years (Myr)] for *Chromis atrilobata* and *Chromis multilineata* are given in columns 3 and 4. Rate of divergence between *C. multilineata* and *C. atrilobata* was calibrated by the rise of the Isthmus of Panama [3.1–3.5 million years ago (Ma)]. This molecular clock was used to estimate divergence time between *Chromis chromis* and *Chromis limbata* (column 6), based on their sequence divergence (column 5)

| | | *C. atrilobata*/*C. multilineata* | | *C. chromis*/*C. limbata* | |
|---|---|---|---|---|---|
| | Model | % divergence | % divergence/Myr | % divergence | Divergence time (Ma) |
| D-loop | HKY + G | 48.53 | 6.93–7.83 | 19.2 | 1.23–1.39 |
| cyt *b* | HKY + G | 16.53 | 2.36–2.67 | 5.79 | 1.09–1.22 |
| 16S | K80 | 1.29 | 0.18–0.21 | 1.20 | 2.89–3.26 |
| Tropomyosin | JC | 4.66 | 0.67–0.75 | 1.40 | 0.93–1.05 |

in the same topology. A maximum-likelihood phylogeny, with an enforced molecular clock, is presented in Fig. 2. As expected, individuals from nominal species (*C. chromis*, *C. limbata*, *C. multilineata* and *C. atrilobata*) grouped in well-supported clades, with *C. chromis* being the sister species of *C. limbata*, and *C. multilineata* grouping with *C. atrilobata* (Fig. 2).

Mitochondrial control region sequences were obtained from 205 individuals including 185 *C. chromis*, 12 *C. limbata*, 6 *C. atrilobata*, and 2 *C. multilineata*. Number of haplotypes and haplotype diversity are shown in Table 1. Phylogenetic relationships of *C. chromis* individuals based on the mitochondrial control region resulted in two major clades that were recovered both by the neighbour-joining and maximum-parsimony methods, but with low bootstrap support (less than 50%) (Fig. 3). In addition, samples did not partition according to geographical regions (i.e. there were no fixed differences between regions). Enforced geographically partitioned topologies were found to be significantly worse than the topology presented in Fig. 3 (Shimodaira–Hasegawa test, $P < 0.001$).

### *Genetic divergence and molecular clock calibration*

Genetic divergences between species are given in Table 2 for each separate locus and can be visualized in the combined phylogram presented in Fig. 2. As expected mutation rates for the mitochondrial loci were highest for the control region, intermediate for cyt *b*, and slowest for 16S rRNA (e.g. McMillan & Palumbi 1997; Bernardi *et al.* 2001). The divergence between the pair of geminate species, *C. multilineata* and *C. atrilobata*, was higher than the divergence between the target species, *C. chromis* and *C. limbata*, for all molecular markers used. The ratio between the divergence across the Isthmus of Panama (*C. atrilobata*/*C. multilineata*) and Atlantic islands/Mediterranean (*C. limbata*/*C. chromis*) was 2.52 for the mitochondrial control region, 2.85 for cyt *b*, 1.07 for 16S rRNA and 3.33 for the nuclear tropomyosin intron. We compared maximum-likelihood topologies with or without enforcing a molecular clock using a Shimodaira–Hasegawa test (Shimodaira & Hasegawa 1999) for D-loop, cyt *b*, 16S and tropomyosin. Topologies were not significantly different (difference in Log $L = 0$; $P = 1$ for all markers). Therefore we assumed homogeneity of rates of molecular evolution for these markers.

The rise of the Isthmus of Panama, which is assumed to be responsible for the split of *C. multilineata* and *C. atrilobata*, occurred between 3.1 and 3.5 Ma (Coates & Obando 1996). This vicariant event has been used in other studies to calibrate mutation rates and estimate mutation rates ($\mu$) in closely related lineages (Bermingham & Lessios 1993; Knowlton *et al.* 1993; Bermingham *et al.* 1997; Lessios 1998; Donaldson & Wilson 1999; McCartney *et al.* 2000).

Using this calibrated clock, the time of divergence between *C. chromis* and *C. limbata* ranged from 0.93 to 3.26 Ma depending on the molecular marker used (Table 2).

### *Population structure*

As mentioned above, mitochondrial control region sequences of *C. chromis* individuals clustered in two major clades. $F_{ST}$ and AMOVA tests were performed with all *C. chromis* individuals. In order to detect if the implicit phylogenetic signal was artificially producing significant results for these tests, we performed the same analysis on a subset of individuals from only the larger of the two clades. Both procedures gave similar results, indicating that the presence of the clades was not responsible for the significance of the tests. We therefore performed the remainder of the population analysis using all samples.

Comparing to other damselfishes (Fauvelot *et al.* 2003), all *C. chromis* populations analysed showed high haplotype diversity values (ranging from 0.792 to 1, Table 1). Population structure of *C. chromis* was first assessed by looking at gene flow between the 18 populations in our study (Table 3). Gene flow between populations was found

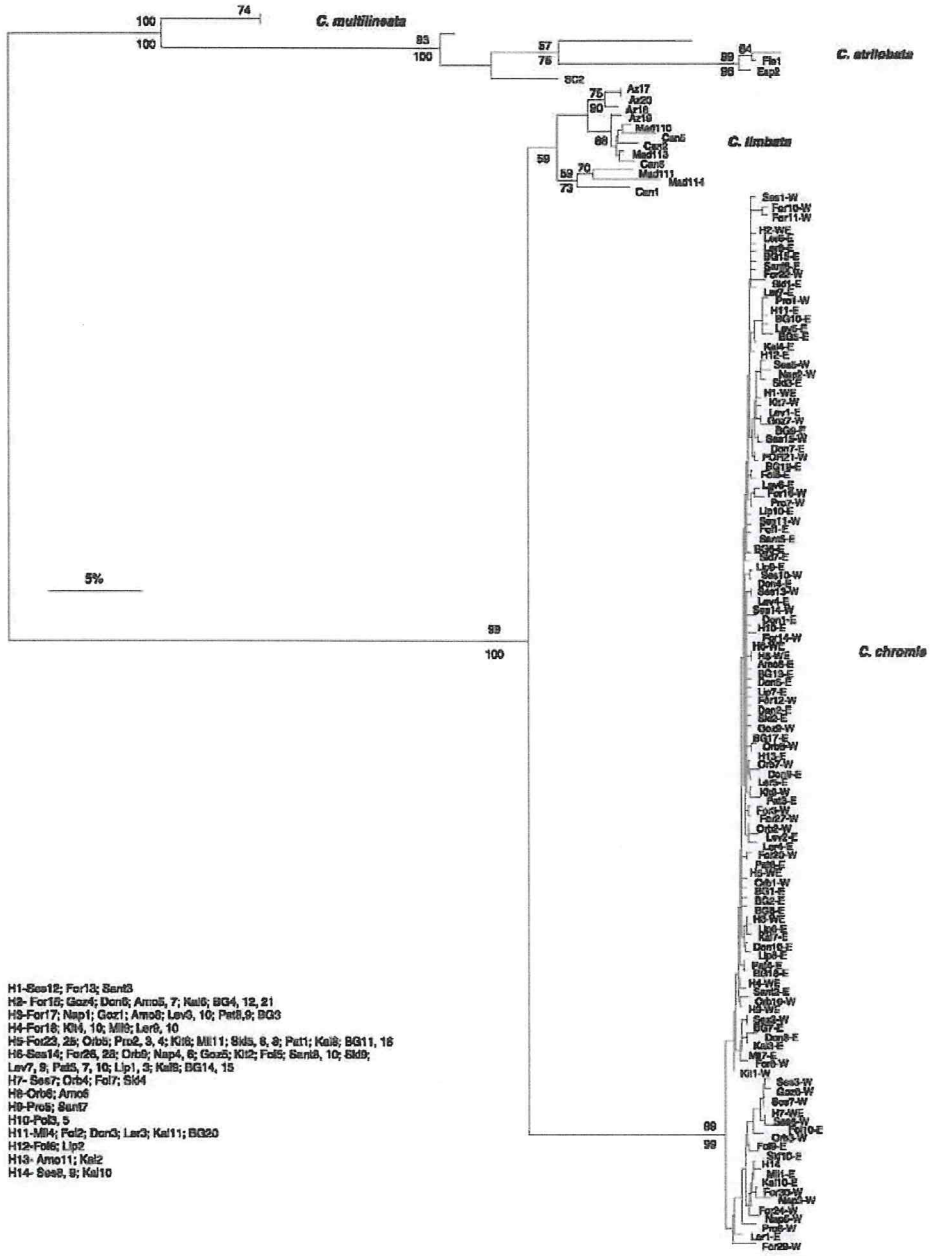


**Fig. 3** Phylogenetic relationship within *Chromis chromis* and its sister species *Chromis limbata* with *Chromis multilineata* and *Chromis atrilobata* used as outgroups. The phylogenetic tree, based on mitochondrial control region (D-loop), was obtained using the neighbour-joining method (with HKY + G model), implemented by the software package PAUP (version 4.0, Swofford 1998). Maximum parsimony resulted in the same topology. Labels are described in Table 1. Additionally individuals belonging to the western or eastern groups are indicated by a W or E, respectively, after each label (see text). The length of each branch is proportional to the number of nucleotide substitutions. Bootstrap values above 50% for each node are shown as percentages for neighbour joining and parsimony, above and below the nodes, respectively.

 

**Table 3** Gene flow among *Chromis chromis* populations represented by *Nm* (below the diagonal) and $F_{ST}$ (above the diagonal), calculated from mitochondrial control region sequences using ARLEQUIN version 2.000 (Schneider *et al.* 2000). Significant *P* values ($P < 0.05$) are indicated by an asterisk (after Bonferroni correction). Nm values below one migrant per generation are in bold

| | Ses | For | Orb | Nap/Pro | Goz | Kith | Mil | Fol | Sant | Ski | Don | Amo | Lev | Pat | Lip | Ler | Kal | B.G. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Ses | | 0.07 | 0.08 | 0.08 | 0.00 | 0.12 | 0.51* | 0.53* | 0.12 | 0.54* | 0.64* | 0.59* | 0.54* | 0.67* | 0.55* | 0.63* | 0.62* | 0.35* |
| For | 6.64 | | 0.00 | 0.00 | 0.04 | 0.00 | 0.45* | 0.49* | 0.00 | 0.52* | 0.60* | 0.58* | 0.47* | 0.63* | 0.48* | 0.60* | 0.59* | 0.25* |
| Orb | **5.75** | inf | | 0.00 | 0.07 | 0.06 | 0.39* | 0.43* | 0.00 | 0.48* | 0.60* | 0.56* | 0.43* | 0.64* | 0.44* | 0.61* | 0.59* | 0.20 |
| Nap/Pro | 5.75 | 303 | inf | | 0.09 | 0.37 | 0.48* | 0.51* | 0.00 | 0.53* | 0.64* | 0.62* | 0.51* | 0.69* | 0.52* | 0.65* | 0.63* | 0.28* |
| Goz | inf | 10.91 | 7.08 | 4.87 | | 0.11 | 0.56 | 0.59* | 0.13 | 0.60* | 0.74* | 0.71 | 0.59* | 0.82* | 0.63* | 0.75 | 0.73 | 0.36* |
| Kith | 3.67 | 94.20 | 7.87 | 13.04 | 4.02 | | 0.57 | 0.62* | 0.00 | 0.61* | 0.75* | 0.75 | 0.60* | 0.84* | 0.64* | 0.78* | 0.75* | 0.33* |
| Mil | **0.48*** | **0.61*** | **0.78*** | **0.55*** | **0.39** | **0.37** | | 0.01 | 0.53 | 0.15 | 0.30 | 0.26 | 0.00 | 0.37 | 0.00 | 0.32 | 0.27 | 0.05 |
| Fol | **0.44*** | **0.51*** | **0.66*** | **0.46*** | **0.34*** | **0.31*** | 46.32 | | **0.57*** | **0.28** | **0.47*** | 0.41 | 0.08 | **0.56*** | 0.05 | **0.48*** | **0.44*** | 0.10 |
| Sant | 3.67 | inf | 305 | inf | 3.36 | inf | **0.44** | **0.38*** | | **0.58*** | **0.72*** | **0.72*** | **0.55*** | **0.81*** | **0.59*** | **0.75*** | **0.72*** | 0.28 |
| Ski | **0.43*** | **0.47*** | **0.54*** | **0.44*** | **0.33*** | **0.31*** | 2.82 | 1.31 | **0.36*** | | 0.01 | 0.03 | 0.16 | 0.15 | 0.22 | 0.10 | 0.00 | **0.29*** |
| Don | **0.28*** | **0.34*** | **0.34*** | **0.28*** | **0.17*** | **0.16*** | 1.19 | **0.57*** | **0.19*** | 61.43 | | 0.06 | 0.28 | 0.08 | 0.38 | 0.07 | 0.00 | **0.38*** |
| Amo | **0.35*** | **0.36*** | **0.39*** | **0.31*** | **0.20** | 0.16 | 1.40 | **0.72** | **0.20*** | 17.18 | 7.99 | | 0.27 | 0.11 | 0.36 | 0.00 | 0.00 | 0.37 |
| Lev | **0.43*** | **0.56*** | **0.67*** | **0.48*** | **0.34*** | **0.33*** | inf | 5.42 | **0.40*** | 2.55 | 1.27 | 1.34 | | 0.36 | 0.00 | 0.32 | 0.28 | 0.09 |
| Pat | **0.25*** | **0.30*** | **0.28*** | **0.23*** | **0.11*** | **0.10*** | **0.85** | **0.40*** | **0.12*** | 2.75 | 5.85 | 3.84 | **0.88** | | 0.45 | 0.15 | 0.10 | 0.42 |
| Lip | **0.41*** | **0.53*** | **0.64*** | **0.45*** | **0.30*** | **0.28*** | inf | 8.55 | **0.35*** | 1.78 | **0.83** | **0.89** | inf | **0.60** | | 0.42 | 0.36 | 0.06 |
| Ler | **0.29*** | **0.33*** | **0.32*** | **0.27*** | **0.16** | **0.14*** | 1.07 | **0.53*** | **0.17*** | 4.61 | 6.90 | inf | 1.07 | 2.87 | **0.69** | | 0.03 | 0.40 |
| Kal | **0.31*** | **0.35*** | **0.35*** | **0.30*** | **0.18** | **0.16*** | 1.37 | **0.64*** | **0.19*** | inf | inf | inf | 1.31 | 4.25 | **0.87** | 17.25 | | 0.38 |
| B.G. | **0.93*** | 1.50* | 1.94* | 1.29* | 1.90* | 1.01* | 10.08 | 4.27 | 1.28 | 1.23* | **0.80*** | **0.85** | 5.22 | **0.70** | 7.52 | **0.75** | **0.23** | |

to be remarkably low, with more than half of the *Nm* values below the threshold value of one migrant per generation, 89 out of 153 pairwise comparisons (58.2%), and 117 (76.5%) below five migrants per generation (Table 3). We detected no significant correlation between $F_{ST}$ and log-geographical distance in *C. chromis* populations ($r^2 = 0.016$, $P > 0.05$).

A series of AMOVAs with alternative groupings showed that the highest degree of differentiation was found when partitioning populations into two groups (western and eastern), with the boundary between these groups falling between the Greek islands of Kithira and Milos (Table 4, Fig. 1). Gene flow between the Kithira and Milos populations was indeed very low ($F_{ST} = 0.57$, $Nm = 0.37$), but not the lowest observed. Average number of migrants per generation (*Nm*) within western and eastern groups was 9.63 and 1.28, respectively, while average *Nm* between these two regions was only 0.45 migrant per generation.

**Table 4** Results of hierarchical analysis of molecular variance (AMOVA) of mtDNA control region haplotypes performed in ARLEQUIN (version 2.000; Schneider *et al.* 2000). Significance ($p$) is defined as the probability of finding a higher among-group variance component and $\phi_{CT}$ than the observed value. Significant *P* values ($P < 0.05$) are indicated by an asterisk (after Bonferroni correction). Region labels are described in Table 1 and Fig. 1

| Region groupings | $\phi_{SC}$ | $\phi_{ST}$ | $\phi_{CT}$ | % variance among groups | *P* |
|---|---|---|---|---|---|
| (1) (2–8) | 0.417 | 0.459 | 0.071 | 7.14 | 0.11347 |
| (1, 2) (3–8) | 0.388 | 0.483 | 0.156 | 15.58 | 0.07491 |
| (1–3) (4–8) | 0.284 | 0.521 | 0.331 | 33.11 | 0.00040* |
| (1–4) (5–8) | **0.240** | **0.533** | **0.385** | **38.48** | **0.00000*** |
| (1–5) (6–8) | 0.284 | 0.510 | 0.316 | 31.55 | 0.00000* |
| (1–6) (7–8) | 0.379 | 0.464 | 0.138 | 13.76 | 0.03822 |
| (1–7) (8) | 0.435 | 0.377 | −0.103 | −10.33 | 0.94614 |
| (1) (2–4) (5–8) | 0.250 | 0.512 | 0.349 | 34.94 | 0.00000* |

### *Historical demography*

Historical demography was assessed by determining historical population size and growth using the control region sequences of all the *C. chromis* and also partitioning the data in western and eastern groups (Table 5). In all cases, populations were growing at a relatively slow rate (Table 5). Migrations between western and eastern groups were also determined. Migration between these regions was reduced, with a small eastward trend that was statistically significant (49.478 eastward migrants vs. 0.476 westward migrants). A chi-squared goodness-of-fit test assuming equal rates of migration in both directions rejected this null hypothesis ($\chi^2 = 6.4$, d.f. = 1, $P < 0.05$). In addition, in both western and eastern groups, bimodal mismatch distributions (not shown) and Tajima's *D* test (west $D = -1.29$, $P = 0.096$; east $D = -1.11$, $P = 0.13$) show no significant bottleneck or recent demographic expansion for these groups.

Relative historical population size was determined, allowing us to estimate the coalescence time for *C. chromis* and its eastern and western groups. Considering a generation time of 3 years (Mapstone & Wood 1975; Dulcic &

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# Chapter 2

## Molecular validation of the specific status of *Parablennius sanguinolentus* and *Parablennius parvicornis* (Pisces: Blenniidae)

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# Molecular validation of the specific status of *Parablennius sanguinolentus* and *Parablennius parvicornis* (Pisces: Blenniidae)*

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SUMMARY: *Parablennius sanguinolentus* and *P. parvicornis* have been classified as either two distinct species or as two sub-species depending on the different criteria used to classify them. An analysis of fragments of mitochondrial 12S and 16S rDNA showed that the genetic distance between samples of *P. sanguinolentus* and *P. parvicornis* is similar or higher than those found for other blenniids that are widely recognized as distinct species. These results, together with the distinct geographical distributions and meristic differences, support the conclusion that *P. sanguinolentus* and *P. parvicornis* should be considered as two different species.

*Keywords*: speciation, 12S mitochondrial rDNA, 16S mitochondrial rDNA, glaciations, NE Atlantic, Mediterranean.

RESUMEN: CONFIRMACIÓN CON DATOS MOLECULARES DEL ESTATUS ESPECÍFICO DE *PARABLENNIUS SANGUINOLENTUS* Y *PARABLENNIUS PARVICORNIS* (PISCES: BLENNIIDAE). – *Parablennius sanguinolentus* y *P. parvicornis* han sido clasificados por diferentes autores como especies distintas o bien como subespecies. El análisis de fragmentos de las subunidades 12S y 16S del ADN ribosómico de la mitocondria muestra que la distancia genética entre los dos taxones es comparable o bien superior a las distancias entre especies de blénidos claramente reconocidas como válidas. Este resultado, sumado a las ligeras diferencias merísticas y a distribuciones geográficas bien distintas, soporta el reconocimiento de estos taxa como especies válidas.

*Palabras clave*: especiación, 12S ADNr, 16S ADNr, glaciaciones, Atlántico Nororiental, Mediterráneo.

## INTRODUCTION

There has been considerable controversy over the taxonomic status of the blenniids *Parablennius sanguinolentus* (Pallas, 1811) and *Parablennius parvicornis* (Valenciennes, 1836). Several authors have used different criteria to classify *P. sanguinolentus* and *P. parvicornis* as two different species (see Bath, 1977; Bath, 1990; Santos *et al.*, 1997), or as two sub-species of *P. sanguinolentus* (see Arruda, 1979; Zander, 1980; Almeida and Harmelin-Vivien, 1983).

The only notable characters that distinguish these taxa from each other are: an additional dorsal spine in *P. sanguinolentus*, small teeth anteriorly to the canines in the upper jaw in *P. parvicornis*, which

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TABLE 1. – List of Blenniidae species and outgroup taxa included in the phylogenetic analysis, sample localities, number of specimens and corresponding Genbank accession numbers.

| | Sampling localities | Genbank accession number | | | |
|---|---|---|---|---|---|
| | | 12S rDNA | N | 16S rDNA | N |
| *P. sanguinolentus* | Lebanon | AF414697, AF414698 | 2 | AF428242 | 1 |
| | Greece | AF414700 | 1 | AY345188 | 1 |
| | Croatia | AF414699 | 1 | AY345187 | 1 |
| | Italy | AF414701, AF414702 | 2 | AY345189 | 1 |
| | Mainland Portugal | AF414703, AF414704, AF414705 | 3 | AF428241, AY098837 | 2 |
| *P. parvicornis* | Cape Verde | AF414712, AY345216 | 2 | AY345190, AY345191 | 2 |
| | Canaries | AY345210, AY345211, AY345212, AY345213 AY345214, AY345215 | 6 | AY345200, AY345201, AY345202, AY345203, 345204, AY345205 | 6 |
| | Madeira | AF414706, AF414707, AF414708, AY345206 AY345207 | 5 | AYAF428239, AF428240, AY345192, AY345193, AY345194 | 5 |
| | Azores | AF414709, AF414710, AF414711, AY345208, AY345209 | 5 | AF428238, AY345196, AY345197, AY345198, AY345199 | 5 |
| *P. gattorugine* | Greece | AF414715 | 1 | AY098835 | 1 |
| *P. ruber* | Azores | AF414716 | 1 | AY098834 | 1 |
| *P. incognitus* | Azores | AY098788 | 1 | AY098829 | 1 |
| *P. pilicornis* | Mainland Portugal | AY098795 | 1 | AY098831 | 1 |
| *T. delaisi* | Madeira | AY098812 | 1 | AY098850 | 1 |
| *L. nuchipinnis* | Cape Verde | AY098807 | 1 | AY098847 | 1 |

are absent in *P. sanguinolentus* (Bauchot, 1966), and slight differences in pigmentation (Zander, 1979).

According to Zander (1986), Bath (1990), Oliveira *et al.* (1992), Gonçalves *et al.* (1993), and Santos *et al.* (1997), the distribution area of *P. sanguinolentus* includes the Mediterranean and the Atlantic coast between France (Gulf of Biscay) and Morocco, although Bath (1990), did not list *P. sanguinolentus* as inhabiting African shorelines. The distribution of *P. parvicornis* includes the West African coast, from Morocco or Mauritania to the Congo River including the NE Atlantic archipelagos of Azores, Madeira and the Canaries and Cape Verde islands.

In this paper we discuss the taxonomic status of *P. sanguinolentus* and *P. parvicornis* using mitochondrial rDNA data.

## MATERIALS AND METHODS

The species and outgroup taxa included in the phylogenetic analysis, their sample localities, number of specimens and corresponding Genbank accession numbers are listed in Table 1.

The blenniids *Parablennius gattorugine* (Brünnich, 1768), *Parablennius ruber* (Valenciennes, 1836), *Parablennius pilicornis* (Cuvier, 1829) and *Parablennius incognitus* (Bath, 1968), were also analysed to provide a wider frame of reference to clarify the relationships between the populations that are morphologically classified as *Parablennius sanguinolentus* and *Parablennius parvicornis*. *Tripterygion delaisi* (Cadenat and Blanche, 1971) (Tripterygiidae) and *Labrisomus nuchipinnis* (Quoy and Gaimard, 1824) (Labrisomidae), which represent families that are closely related to the blenniids (Stepien *et al.*, 1997), were used as outgroups.

Samples were collected underwater and in tide pools and fixed in 96% ethanol. Total genomic DNA was extracted either from muscle tissue or from fin-rays using a proteinase K/SDS based extraction buffer and phenol/chloroform with ethanol precipitation (Maniatis *et al.*, 1982).

The following primer sequences were used to amplify two fragments of the mitochondrial DNA: for 12S rDNA, 12SFor 5'-AAC TGG GAT TAG ATA CCC CAC-3' and 12SRev 5'-GGG AGA GTG ACG GGC GGT GTG-3'; for 16S rDNA, ' 16SFor 5'-AAG CCT CGC CTG TTT ACC AA-3' and 16SRev 5'-CTG AAC TCA GAT CAC GTA GG-3'. These primers were designed in our laboratory and have already been used in previous studies (e.g. Henriques, *et al.*, 2002).

For both fragments, PCR mixtures were prepared with a total volume of 20 ml, with: 1.5 mM $MgCl_2$, 200 mM of each dNTP, 0.5 mM of each primer, 0.5 units of *Taq* polymerase (Gibco BRL, Life Technologies Inc., Gaithersburg, MD), 1x buffer supplied by the manufacturer and approximately 20 ng of genomic DNA. The amplification process was performed in a Biorad Gene-Cycler™ as follows: 4 minutes at 94°C and 30 cycles of 1 minute at 94°C, 1 minute at 55°C and 1 minute at 72°C. After this sequence, these products where kept at 72°C for 10 minutes. PCR products were purified with a GFX

PCR DNA purification kit (Amersham-Pharmacia), following the manufacturer's recommendations. Automatic sequencing of PCR purified products was performed with a CEQ 2000 XL, Beckman Coulter, with the same primers.

Alignments were made using Clustal X 1.81 (Thompson *et al.*, 1997), with default settings. Character congruence between the two fragments was tested using the incongruence-length difference test (ILD) (Farris *et al.*, 1995), available in PAUP 4.0b10 Win (Swofford, 2002). The null hypothesis of congruence between the two data sets was not rejected ($P$=1). Therefore, we analysed the 12S and 16S rDNA sequences combined in one single fragment (but see Dolphin *et al.*, 2000). Regions where the alignment was ambiguous were removed from the analysis, which resulted in a fragment with 847 bp. The degree of saturation was assessed by plotting transitions and transversions against uncorrected *p*-distances.

Sequences were analysed with three methods of phylogenetic inference: maximum-parsimony (MP), maximum-likelihood (ML) and minimum evolution (neighbour-joining - NJ) (Saitou and Nei, 1987). The phylogenetic analysis was performed with PAUP 4.0b10 Win (Swofford, 2002). Bootstrapping (Felsenstein, 1985), was used to assess robustness of the nodes in the trees with 1000 replicates for MP and NJ and 100 replicates for ML. The heuristic search option "random addition of taxa" and tree bisection reconnection (TBR), with the MULPARS option in effect, was used with the three inference methods. MP analysis was conducted with the ACCTRAN option.

In order to choose the model of molecular evolution that best fitted our data we used the program Modeltest 3.06 (Posada and Crandall, 1998). For the combined 12S-16S rDNA dataset the ML settings selected, according to the results of the Modeltest, corresponded to the general time reversible model (GTR+G) with rate heterogeneity. The distribution of rates at the variable sites was assumed to follow a gamma distribution with a shape parameter equal to 0.2668. NJ was based on the distance estimator derived from the ML settings selected for the combined fragment.

## RESULTS AND DISCUSSION

We analysed a total of 367 bp of the mitochondrial 12S rDNA and 480 bp of the mitochondrial 16S rDNA, which makes a combined sequence of 847 bp. Of these, 267 sites were variable and 137 sites were parsimony informative. The TS/TV ratio was 1.53. The base frequencies were: A=0,2950; C=0,2501; G=0,2289 and T=0,2260. There was no evidence of saturation either for transitions or transversions.

The three methods of phylogenetic inference converged into the same topology represented in Figure 1.

All samples of *P. sanguinolentus* and *P. parvicornis* formed a well supported monophyletic group that was well differentiated from the remaining species of *Parablennius*. Within this group, all the samples of *P. sanguinolentus* formed a well supported clade as did those of *P. parvicornis*. It is interesting to note that all samples of *P. sanguinolentus* from Mainland Portugal to Lebanon are represented by the same haplotype. The genetic distance (*p*-distance) between *P. sanguinolentus* and *P. parvicornis* was 2.43% (SD = 0.07; Min = 2.40; Max = 2.64). The *p*-distance in relation to the variation within the *P. parvicornis* clade was 0.17% (SD = 0.14; Min = 0; Max = 0.60). The highest values were found between fish from Cape Verde and some Azorean samples, which are the more peripheral and distant localities within our study area.

The results of this study unambiguously confirm the distinctiveness of *P. sanguinolentus* and *P. parvicornis* which has been suggested by previous morphological studies (e.g. Bauchot, 1966). Furthermore, the genetic divergence between *P. sanguinolentus* and *P. parvicornis* (2.4-2.6%) is markedly higher than the values found for other pairs of blenniids widely recognized as distinct species (e.g. *Parablennius gattorugine* / *Parablennius ruber*: *p*-distance = 1.6%, present study; *Lipophrys canevai* / *Lipophrys nigriceps*: *p*-distance = 1.3%, Almanda *et al.*, 2005).

One of the authors (A. Brito) conducted three expeditions along the African coast (in 1986, 1988 and 1998), in order to examine the distribution of *P. sanguinolentus* and *P. parvicornis* by sampling the intertidal fish fauna. *P. sanguinolentus* was not found south of the Casablanca area (34°N), a conclusion supported by Brownell (1978). *P. parvicornis* was never collected north of Cape Blanc (21° N), a finding also supported by Bath and Wirtz (1992). South of this point, on the coast of Senegal, it is a common fish. These results show that *P. sanguinolentus* and *P. parvicornis* are separated by a gap of at least 13° of latitude.

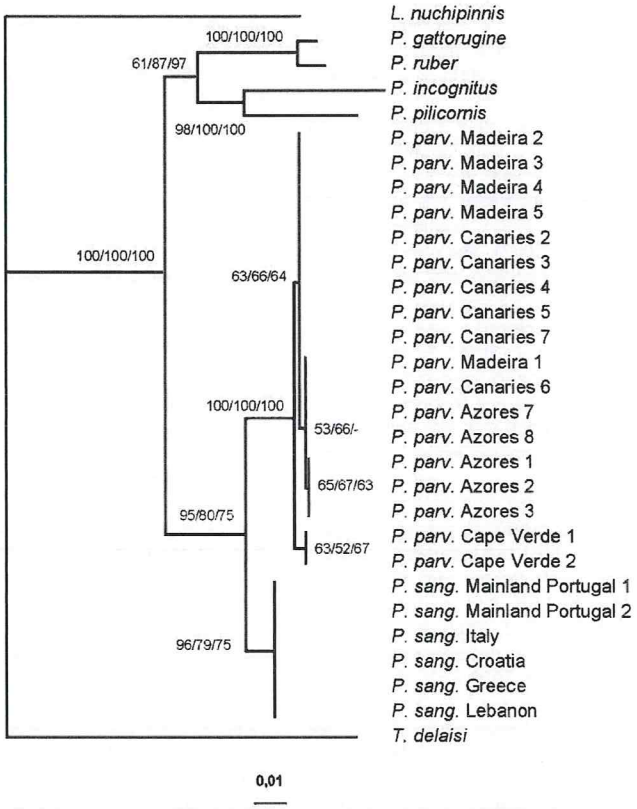


FIG. 1. – Phylogenetic tree obtained for the combined 12S-16S rDNA sequences. *L. nuchipinnis* and *T. delaisi* have been used as outgroups. Bootstrap values for each node are shown as percentages of maximum-parsimony (tree length = 421; consistency index (CI) = 0.82; homoplasy index (HI) = 0.18; retention index (RI) = 0.82), of maximum-likelihood (GTR+G model) and of neighbour-joining (distance based on ML settings) respectively (MP/ML/NJ). The topology of the trees is the same for all inference methods. Branch lengths are proportional to the genetic divergence between haplotypes.

From a biogeographical point of view it is interesting to note that the northern limit of *P. parvicornis* is much more to the south on the West African coast than it is near the Atlantic islands. Indeed, it reaches its northern limit at the Azores well to the north of the entrance to the Mediterranean. This distributional pattern compares well with the results of previous studies (e.g. Santos *et al.*, 1995 and the references therein), that have demonstrated that there are significant affinities between the ichthyofauna of the Azores and those of Madeira and the Canaries. These authors discussed the possible oceanographic processes fish of tropical African origin may use to reach the Azores via the Canaries and Madeira.

Further genetic studies using larger samples for each geographical location and rapidly evolving markers, such as the control region of the mitochondrial DNA, could help to test this biogeographical hypothesis.

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# Chapter 3

## Phylogeny of the shanny, *Lipophrys pholis*, from the NE Atlantic using mitochondrial DNA markers

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Short communication

# Phylogeny of the shanny, *Lipophrys pholis*, from the NE Atlantic using mitochondrial DNA markers

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## 1. Introduction

The distribution of the shanny includes the NE Atlantic from Norway to Mauritania and the Atlantic islands, with some records in the Western Mediterranean. Although several studies have been carried out on the morphology (Arruda, 1979; Bath, 1976; Lowe, 1843), ecology (Faria and Almada, 2001; Almada and Faria, 2000), ontogeny (Faria et al., 2002), and its diet composition (Maze et al., 1999) not much has been done on the genetics (Guillemaud et al., 2000).

Some authors have drawn attention to morphological differences between *Lipophrys pholis* of the European continental coasts and the Atlantic islands. One of the first studies to propose subspeciation in *L. pholis* is Arruda (1979) who identifies unique features for the Azorean shanny.

This observation led Zander (1980) to propose a model of re-colonisation of blennies for the Mediterranean after the Messinian crisis (Hsü et al., 1973). Zander suggests that as the temperature and sea level decreased, those Mediterranean blennies moved towards Atlantic islands. One of these blennies could have been an ancestor of *L. trigloides* which probably gave rise to an ancestor similar to *L. pholis*. At the end of the glaciation, this "new" species invaded the African and then the European coasts. Finally, Zander justifies the disjunct distribution of the shanny by differences in competition pressures that lead to the giant race in Madeira (*Blennius bufo*) and a northern European shanny (*B. pholis*).

In the present study, we use molecular tools along with meristic counts with the aim of resolving the phylogeny of the shanny. Phylogenetic inferences are based on the analysis of 12S, 16S, and partial control region (CR) sequences of the mtDNA from three localities along NE Atlantic coast and two Atlantic islands.

## 2. Materials and methods

### 2.1. Morphology

A total of 283 specimens were analysed from the collections listed in Appendix A.

The specimens from "LiphAz1" to "LiphAz5" as well as the larger individual from MMF 24241 were preserved in ethanol therefore they have also been used for the molecular studies.

In addition to meristic counts, for samples from Azores, Portugal mainland, and France, the number of cephalic pores in the nasal area has also been investigated, since their absence was characterising the Azorean population (Arruda, 1979).

### 2.2. Molecular analysis

A total of 28 shannies were included in the comparison of the control region (CR), 12S, and 16S rDNA. Sequences were analysed separately including 20 individuals for the CR and nine for the 12S and 16S (one

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individual was shared in all genes studied). Samples were collected from the United Kingdom (Oban and Plymouth), mainland Portugal (Gelfa, Avencas, Amoreira, and Lagos), and two Atlantic islands: Azores (Salão in Fayal) and Madeira (Ponta Delgada). As the projects were carried out at different time, amplifications of CR, 12S, and 16S were not all done on the same fishes.

In addition, the sequences of *Lipophrys trigloides* were included as outgroups.

All fish were fixed in 95% ethanol before being stored at −20 °C. mtDNA was extracted from white muscle following the procedure of Sambrook et al. (1989), with slight modifications (Stefanni, 2000). Fragments of the mtDNA including partial CR were amplified using the L-Pro1 and H-DL1 primers (Ostellari et al., 1996), while for 12S and 16S the primers used were 12SF, 12SR, 16SF, and 16SR (Henriques et al., 2002), respectively. The same primers were also used for sequencing, as described in Stefanni (2000).

### 2.3. Sequence alignment and phylogenetic analyses

All sequences were aligned using SEAVIEW (Galtier et al., 1996) and CLUSTAL X (Thompson et al., 1997). Character congruence between 12S and 16S was tested using the incongruence-length difference test (ILD; Farris et al., 1995) available in PAUP 4.0 (Swofford, 1999). The null hypothesis of congruence between the two data sets was not rejected ($p = 1$) which led us to analyse the 12S and 16S rDNA sequences combined in one single fragment.

Hierarchical series of likelihood ratio tests (Huelsenbeck and Rannala, 1997), implemented using MODELTEST 3.06 (Posada and Crandall, 1998) were used to identify the appropriate nucleotide substitution models. Likelihood ratio tests of the model's fit with and without the assumption of a molecular clock were conducted.

Phylogenetic trees of the haplotypes were constructed using PAUP. The trees were rooted with the *L. trigloides* haplotypes as outgroup. The neighbor-joining (NJ) method (Saitou and Nei, 1987) was used to construct a phylogenetic tree from the maximum likelihood (ML) distances estimated under the selected models. The support for internal branches within the NJ tree was assessed using the bootstrap (Felsenstein, 1985) with 1000 replicates. A maximum parsimony (MP) analysis was performed using the default options for heuristic search to find the best MP tree(s). The length ($L$), consistency index (CI), and retention index (RI) of the MP tree(s) were calculated with parsimony-uninformative sites excluded.

The degree of correlation between geographical distances and genetic differentiation was estimated by regression of a matrix of pairwise linearised $\Phi_{ST}$ values (Slatkin, 1993), calculated using the program ARLEQUIN 2.000 (Schneider et al., 2000), against a matrix of spatial distribution of the sampling localities. Geographical distances were calculated following the coastal line and for the islands as the shortest distance between one and the other according to the isolation by distance (IBD) model.

## 3. Results

### 3.1. Morphology

#### 3.1.1. Meristics

For the mainland samples, frequency distributions of ray counts in the second dorsal (D2) and anal (A) fins show a shift towards higher numbers at higher latitudes (Table 1). Counts of the rays of the first dorsal fins (D1) are stable at 12 with rare exceptions of 11 (Table 1). Counts for rays of the second dorsal and anal fins show high frequencies of 18 and 19. Towards the north the frequency of fishes with 19 fin rays increases, with some individuals in the north having 20

Table 1
Meristics of *L. pholis*

| Location | D1 | | D2 | | | | A | | | | N |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | XI | XII | 17 | 18 | 19 | 20 | II + 17 | II + 18 | II + 19 | II + 20 | |
| *Mainland* | | | | | | | | | | | |
| UK | 2 | 17 | 0 | 46 | 69 | 4 | 1 | 27 | 83 | 8 | 119 |
| France | 0 | 15 | 1 | 6 | 8 | 0 | 0 | 4 | 10 | 1 | 15 |
| Portugal | 3 | 11 | 0 | 8 | 6 | 0 | 0 | 4 | 10 | 0 | 14 |
| Morocco | 1 | 69 | 0 | 48 | 22 | 0 | 0 | 32 | 38 | 0 | 70 |
| *Islands* | | | | | | | | | | | |
| Azores[a] | 3 | 26 | 0 | 1 | 26 | 27 | 0 | 0 | 19 | 35 | 54 |
| Madeira | 0 | 5 | 0 | 2 | 3 | 0 | 0 | 2 | 3 | 0 | 5 |
| Savages | 0 | 2 | 0 | 0 | 2 | 0 | 0 | 0 | 2 | 0 | 2 |
| Canaries | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 1 |

D1, first dorsal fin; D2, second dorsal fin; A, anal fin; N, total number of specimens.
[a] Ray counts for D1 do not include the specimens from the islands of Santa Maria.

rays. In contrast to the mainland, the Azores population is mainly characterised by 19 and 20 fin rays in both the second part of the dorsal fin and the anal fin (Table 1). At the other oceanic islands (Madeira, Savages, and Canaries) we did not find individuals with 20 fin rays in the second dorsal or anal fin, but samples were too small for statistical comparisons (Table 1). Frequency distributions of fin rays among the other five populations (four mainland and Azores) differ significantly (Kruskall–Wallis test, $p < 0.001$ for both D2 and A). The method of multiple comparison (Siegel and Castellan, 1988) revealed that the Azores were different from all other populations ($p < 0.05$ for both D2 and A). Furthermore, Morocco differed from the UK in one comparison (D2: $p < 0.05$), which we interpret as a result of the shift towards higher fin ray counts mentioned above.

#### 3.1.2. Sensory pores

The number of sensory pores in the inter-orbital nasal area was identical in all fishes examined. Thus, the extra pore that Arruda (1979) described as a unique feature for the Azorean population was found in mainland populations as well.

### 3.2. Genetic analysis

#### 3.2.1. CR

All shannies were successfully sequenced and the 20 sequences define 17 haplotypes (GenBank Accession Nos. AY966017–33). All fishes sharing the same haplotype are from the same sampling locality. All sequences correspond to the first hypervariable and part of the central domains of the control region with a length ranging from 450 bp (Oban, UK) to 454 bp (Azores). Five indels are required to align the sequences, giving a total length of 455 bp for all the sequences. Two indels partition the Azorean shanny haplotypes from the others, two partition the Azorean with two and three mainland Portugal haplotypes, respectively, and one is affecting the two Scottish and three Portuguese shannies haplotypes.

The appropriate model of nucleotide substitution was the HKY (Hasegawa et al., 1985) model with invariable sites ($I$), rate heterogeneity ($G$), and no clock. The transition/transversion (ti/tv) ratio, proportion of invariable sites (i), and $\gamma$ shape parameter ($\alpha$) were estimated to be ti/tv = 2.9294, $i = 0$, and $\alpha = 0.3877$, respectively. The base frequencies were estimated to be A = 0.3601, C = 0.1874, T = 0.3099, and G = 0.1426.

#### 3.2.2. 12S + 16S

A total of eight fish were successfully sequenced from which seven haplotypes were defined (GenBank Accession Nos. for 12S and 16S rDNA: AY097015, AY098765–7, AY098825, AY098770, AY0987012–4, AY0987017, and AY0987019–24). The combined fragment corresponded to a total of 863 and 369 bp for the 12S and 494 bp for the 16S. No indels were required for the alignment and the appropriate model of nucleotide substitution was K80 with ti/tv = 2.

### 3.3. Phylogenetics

Phylogenetic analysis from CR sequences described by the neighbor-joining (NJ) tree contains 13 internal branches with bootstrap (BS) values greater than 50% (Fig. 1). The branch separating the Azorean shanny is extremely well supported (BS = 100) while the one including samples from Madeira, mainland Portugal, and Scotland has a much lower value (BS = 72). Inside the latter clade, branches with BS values >50% define small ($n = 2$ or 3) monophyletic groupings of haplotypes from different sampling localities.

Of the 455 sites considered only 79 are parsimony-informative. The maximum parsimony (MP) search retained six best MP trees ($L = 163$, CI = 0.859, and RI = 0.832). The strict component consensus tree of the MP trees, which displays all and only those groups found in all the MP trees, resembles the NJ tree. In this tree, the first split separates the Azorean shanny from the monophyletic clade composed by two other groups. One clade which contains the same three haplotypes from mainland Portugal, Avencas (Av10, Av13, and Av17) as presented in the NJ tree, and the second contains haplotypes from all the localities, except for the Azores. The patristic distance between the Azorean

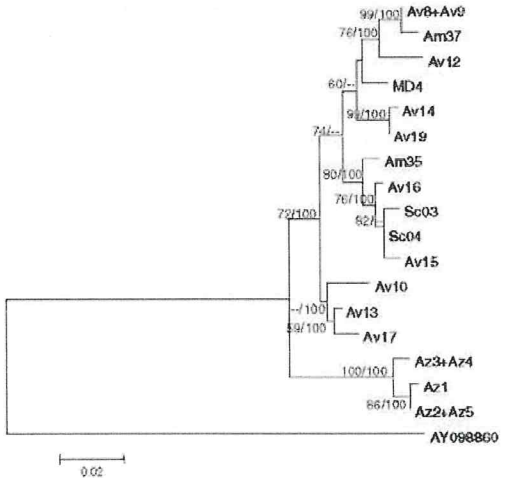


Fig. 1. Neighbor-joining tree constructed from HKY+I+G distances with ti/tv = 2.9294, $i = 0$, and $\alpha = 0.3877$. Numbers above internal branches indicate bootstrap values out of 1000 replicates (only if >50%) for NJ and MP strict consensus trees. Az, Azores; Am, Amoreira; Av, Avencas; MD, Madeira; Sc, Oban.

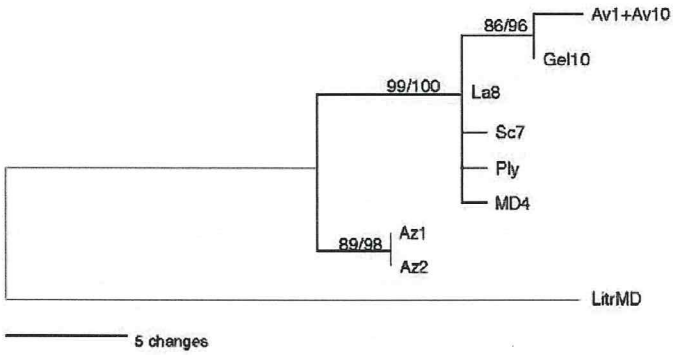


Fig. 2. Maximum parsimony tree for 12S + 16S with numbers above internal branches indicating bootstrap values out of 1000 replicates (only if >50%) for MP and NJ trees. Az, Azores; Av, Avencas; Gel, Gelfa; La, Lagos; MD, Madeira; Ply, Plymouth; Sc, Oban; Litr, *L. trigloides*.

clade and the clade containing the remaining samples is between 1.55 and 1.69%.

Not taking into account the Azorean samples, pairwise $\Phi_{ST}$ values between sites varied between 0.093 (Amoreira—Oban) and 0.935 (Oban—Madeira). The latter is of the same order of magnitude when comparing the Azorean haplotypes with the ones from the other localities. However, $p$ values showed very high significance only when any of the sites is compared with the Azores.

The corrected sequence divergence between the Azores sequences and all the others (Madeira, mainland Portugal, and Scotland) is 4.42% while the sequence divergence within the two groups is 0.34 and 2.53%, respectively.

The estimates of the number of migrants ($N_m$) calculated from the $\Phi_{ST}$ values (Slatkin, 1993) are much reduced between the two most remote sites ($N_m = 0.0345$) as well as between the islands of Madeira and Azores ($N_m = 0.0338$). The reduction in gene flow between localities is not supported by the IBD model and the correlation between geographical distances and genetic differentiation is not significant ($p = 0.076$).

For the phylogenetic analysis of the combined 12S + 16S fragment, all inference methods converged on the same tree topology (Fig. 2). MP yielded a single most parsimonious tree of 54 steps. All methods resulted in the same samples of shannies as a monophyletic group clearly divided in two clades with very high support values. One clade contains the Azorean fish while in the other clade all the remaining localities are grouped together (mainland Portugal, Madeira, and UK). The patristic distance between the Azorean clade and the one containing the remaining samples is 1.24%.

## 4. Discussion

The phylogenetic analyses and the population genetics strongly support the presence of two groupings of shanny, one for the Azores and one for the mainland Europe which includes the island of Madeira. The clade constituted by the Azorean sample is highly supported by NJ and MP trees using CR and 12S + 16S. Genetics show unique haplotypes for the Azorean shanny. Further support to this finding is the substantial divergence between Azorean sample and the others. Patristic distances between these two groups are 0.77 and 1.58% for the two fragments at slower rate of evolution (12S and 16S, respectively) and 1.55–1.67% for the CR. While in the 12S the two Azorean fishes share the same haplotype, in the 16S they differ from each other, and the distances between them and the group containing the remaining shannies have an average of 0.61%.

It is important to note that the patristic distance between the Azorean shanny and those from mainland Portugal, UK, and Madeira are of the same order of magnitude (or even higher) than the values between some of recognised distinct species of blennies. For example, the $p$ distance between *Parablennius gattorugine* and *P. ruber* is 1.6% and between *Lipophrys canevai* and *L. nigriceps* is 1.3% (Almada et al., 2005).

As for 12S and 16S, the CR also describes unique haplotypes for the Azorean shanny. They are defined by 12 substitutions and four insertions, and are mainly encountered (more than 80% of them) in the first domain. Within the two clades, the level of nucleotide diversity is very different (Azores: $\pi = 0.008$; continental Europe + Madeira: $\pi = 0.056$) differences which are also reflected in the number of shared haplotypes (Azores: 40%; continental Europe + Madeira: 7%). These rough values also suggest that the population of shanny in the Azores may be very limited in size when compared to the continental one.

When all sampling localities are compared one to another, pairwise values of $\Phi_{ST}$ result statistically significant only when Azores is included. This is further supported by the lack of correlation between genetic differentiation and geographical distance.

When comparing $p$ distances from partial CR sequences of several *Lipophrys* species (available from GenBank), the lowest value, after the 0.09 between "Azorean" *L. pholis* and "mainland Portugal" *L. pholis*, was 0.27 between *L. adriaticus* and *L. caboverdensis*, followed by a 0.32 between *L. adriaticus* and *L. dalmatinus*, and 0.41 between *L. canevae* and *L. caboverdensis*.

Interruption of gene flow in *L. pholis* between Azores and continental Europe (most probably through Madeira and Canaries islands) might have happened more recently than the Messinian crisis (5.9 Mya) as proposed by Zander (1980). Applying mutational rates compatible with universal molecular clocks, 1% for region at slow-paced rate (as 12S and 16S), places the split between continental and Azorean shannies at 1.23 Mya, during the early Pleistocene. It is well known that CR yields a mutation rate which is several times faster compared to the rest of the mitochondrial genome (Brown et al., 1993). Taking into account that early Pleistocene is a plausible period for this separation to occur, the mutation rate affecting the CR of *L. pholis* would range between 2.9 and 5.5%, values also encountered in other fish species with similar transtion/transversion rate (McMillian and Palumbi, 1997). A reliable calibration of the CR clock for teleosts is provided by the comparison between two amphi-Panamic geminate species of snook (Donaldson and Wilson, 1999), and using this estimate, separation between continental and Azorean shannies occurred about 1 Mya.

Early Pleistocene was characterised by an inter-glacial period with relatively high global temperature and little ice locked up in polar caps (Lambeck et al., 2002). With such warm conditions in the northern hemisphere we might expect to find the anticyclone known as the "Azores High" located further north than today. In such a scenario, we would expect that the trade winds would also be blowing at a higher latitude, maybe from the coasts of southern Europe westward. In this situation, the Azores would receive a constant supply of eggs and larvae from marine organisms breeding in Europe. Such a regular gene flow would possibly have been affected by interruptions, especially during the glaciations that were becoming progressively intense.

During the last glacial maximum, probably several times during the Pleistocene, the temperatures in the European shores would have been so low that *L. pholis* would have become extinct, re-colonizing from more southern refugia during inter-glacials. On the contrary, at the Azores, the drop in sea surface temperature was apparently much smaller, about 3 °C (Climap, 1981; Crowley, 1981). As a result, the present population of Azores would have survived there, eventually adapting to long cold periods. This difference in population histories may help to explain the genetic differentiation that we found and the contrast in patterns of meristic characters.

The strong differentiation of the Azorean shanny which is indicated by both, genetic and meristics data and the fact that this fish is extremely rare in the Azores, suggests that the taxonomic and conservation status of this species should be carefully revised.

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## Appendix A. Appendix

List of collections of *L. pholis* used for morphological study: BMNH—British Museum of Natural History, London, UK; SMNS—Senckenberg Natural History Museum, Frankfurt, Germany; MMF—Museum Municipal do Funchal, Madeira, Portugal; DOP—Department of Oceanography and Fisheries, Fayal, Azores, Portugal; RMNH—State Natural History Museum, Leiden, The Netherlands. The specimens were originally collected from the following locations:

1. United Kingdom, 119 specimens: BMNH 47015; $n=11$, Devon; $n=2$, Loch Kishorn; $n=3$, Isle of Lewis—Hebrides; $n=28$, Pembrokeshire; $n=5$, Bude—Cornwall; $n=15$, Eastborne—Sussex; $n=1$, Mullet Peninsula; $n=2$, Pembrokeshire; $n=3$, Orkney; $n=4$, Isle of Mann; $n=3$, Brighton; $n=13$, Rosscarbery Bay; $n=13$, Lannacombe Bay; $n=5$, Guernsey; BMNH 47020, $n=11$, Cornwall.
2. France, 15 specimens: BMNH 47020; $n=10$, Roscoff—Brittany; DOP LIP PHO Fr 1–3, $n=3$, Beg-Meil—Brittany; RMNH 23844, $n=1$, Roscoff—Britanny; RMNH 23864, $n=1$, Roscoff—Brittany.
3. Morocco, 70 specimens: SMNS 13541 $n=24$, Tan Tan; SMNS 13532, $n=1$, Agdu; SMNS 13540, $n=8$, 15 km south of Tan Tan; SMNS 13506, $n=9$, Cap Rhin; SMNS 13538, $n=10$, Tarfaya; SMNS 13536, $n=5$, Sidi Ifni; 13544, $n=13$, Flayounne.
4. Portugal mainland, 14 specimens: DOP LIP PHO Parede 1–14; $n=14$, Parede.
5. Madeira Island, five specimens: BMNH 1863.9.10.12 $n=3$, Lowe 1863; MMF 22421 $n=2$.
6. Porto Santo Island, 1 specimen: RMNH 29592, south west coast.

7. Savages Iles, two specimens DOP LIP PHO Selvagem 1, $n = 1$, Selvagem Grande; MMF 201 $n = 1$, Selvagem Islands.
8. Azores islands (Central group: Fayal and Pico islands), 29 specimens: BMNH 19870, $n = 1$, Porto de Salão, Fayal; DOP LIP PHO Az 1-2, Porto de Salão, Fayal, Clipe-project; DOP LIP PHO Az 3–5, Porto de Salão, Fayal; DOP LIP PHO Az 6–25, north coast Fayal; DOP 161–395-LIP PHO-(6;1) $n = 3$, São Roque, Pico, Expedition Azores 89; (Oriental group: São Miguel and Santa Maria islands), 27 specimens: DOP LIP PHO Santa Maria 1–25, $n = 25$ (lost during transfer of collection); RMNH 35033, $n = 1$, Baia São Lourenço, Santa Maria; RMNH 35032, $n = 1$, south coast São Miguel.
9. Canary Islands, one specimen: DOP 132–395-LIP PHO-(6;1), Tenerife Norte.

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# Chapter 4

## Historical population dynamics and demography of the eastern Atlantic pomacentrid *Chromis limbata* (Valenciennes, 1833)

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# Historical population dynamics and demography of the eastern Atlantic pomacentrid *Chromis limbata* (Valenciennes, 1833)

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**Abstract**

Recent studies have focused on the relationship between the marine fauna of the eastern Atlantic and the Mediterranean Sea, but within the Atlantic, little is known about genetic relationships between populations of the Macaronesian islands. In this study, we tested whether the paleo-climatology and paleo-oceanography of the region could predict the genetic relationships among three eastern Atlantic populations (Azores, Madeira, and Canaries) of a damselfish, *Chromis limbata*, and compared our results with its Mediterranean and adjacent Atlantic sister species, *Chromis chromis*. We combined phylogeographic and coalescent approaches using the fast evolving mitochondrial control region gene. No population structure was found for the three archipelagos. The coalescence time estimated for *C. limbata* (0.857–1.17 Mya) was much greater than that estimated for *C. chromis*. We propose that this difference reflects differences in glaciating extents in the Northeastern Atlantic and the Mediterranean. Diversity indexes (Hd and genetic distances) together with historical demographic parameters of *C. limbata* ($\Theta$ and $g$) revealed a more stable population history when compared to *C. chromis*. Our results suggest that the Macaronesian populations of *C. limbata* have probably been less affected by the last glaciation than the Mediterranean populations of *C. chromis*. Migration across the three archipelagos was estimated and a prevailing northwest trend was detected. This result supports the idea of a colonization of the Azores by warm water fish from Madeira or the westernmost Canary islands which acted as major glacial refugia for the tropical and subtropical marine fauna during the glaciations.


*Keywords:* *Chromis limbata*; Northeastern Atlantic islands; Phylogeography; Glaciations; D-loop

## 1. Introduction

To understand the relationships between speciation and dispersal/population dynamics in marine species, genetic connectivity between populations are usually studied across geographic boundaries. Recently, studies have focused on the relationship between the eastern Atlantic and Mediterranean (Alvarado Bremer et al., 2005; Bargelloni et al., 2003, 2005; Costagliola et al., 2004; Domingues et al., 2005; Lemaire et al., 2005; Naciri et al., 1999; Stamatis et al., 2004; Zardoya et al., 2004). In contrast, little is known about genetic relationships between populations of the Macaronesian islands (Azores, Madeira, Canaries, and Cape Verde; Almada et al., 2005; Guillemaud et al., 2000). These isolated oceanic islands constitute interesting model systems for the study of colonization processes, as several climatic and oceanographic phenomena have played a major role in the history of the ichthyofauna of these islands (Almada et al., 2001; Miller, 1984; Santos et al., 1995; Zander, 1980).

During the Pliocene, the eastern Atlantic experienced a progressive cooling that reached its extreme with the Pleistocene glaciations (Adams et al., 1999; Briggs, 1996). The western coast of Portugal was particularly affected by a

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very pronounced southward migration of the polar front, which caused a significant dropping of sea water temperatures in this region (Crowley, 1981; Dias et al., 1997). The Madeira islands, located further south, were less affected, while the Canaries were severely affected due to its proximity to the continent, although probably less in the western islands, which nowadays present the higher temperatures (Barton et al., 1998) and warmer fauna of all the temperate Macaronesia (Brito et al., 2001). The Cape Verde islands, although remaining considerably warm, were clearly out of the Tropical bio-region (Briggs, 1996). The sea surface temperatures in the Azores region experienced a small cooling (2–3 °C) (Crowley, 1981). Several authors (Briggs, 1974; Miller, 1984; Santos et al., 1995) suggested that this drop in sea surface temperatures has probably resulted in mass extinctions of littoral fish at the Azores, and that most of the organisms now present would have recolonized the islands after this glaciating event from some southern regions such as Madeira.

In marine organisms, oceanographic conditions play an important role in colonization processes, particularly in those whose dispersal is restricted to their planktonic larval phase. The Northeastern Atlantic current system is dominated by the Gulf Stream, which splits into two main branches, the North Atlantic Current (flowing north) and the Azores Current (flowing east). Close to the Azores islands, each of these currents divides into two branches, one of which flows south, feeding the Madeira and Canaries currents (Santos et al., 1995; Stramma, 1984). This multibranch system is even more complex due to seasonal variations of the mean current directions, and as it is a source of meanders and eddies (Santos et al., 1995; Stramma, 1984). Although dominant average ocean current circulation reaches the Northeastern Atlantic islands from the west, the marine littoral fauna and flora of the temperate Macaronesia (Azores, Madeira, and Canaries) share affinities with the eastern coasts of the Atlantic and the Mediterranean (Boury-Esnault and Lopes, 1985; Brito et al., 2001; Brito and Ocaña, 2004; Gofas, 1990; Lloris et al., 1991; Prud'homme van Reine, 1988; Weerdt, 1989; Wirtz and Martins, 1993). This is probably the result of episodic anomalies of the water movements described above (Santos et al., 1995), and of the temperate conditions that the northwest African upwelling confers to these islands.

*Chromis limbata* (Valenciennes, 1833) is a species restricted to the Macaronesian islands (Azores, Madeira, and Canaries) and the western coast of Africa (between Senegal and Congo, Edwards, 1986; Wood, 1977; L. Rocha et al., unpublished). The Cape Verde islands bear an endemic and a tropical amphiatlantic *Chromis* species (Edwards, 1986). *Chromis chromis* (Linnaeus, 1758), the most likely sister species of *C. limbata* (Edwards, 1986; Wood, 1977; L. Rocha et al., unpublished) is found in the Mediterranean and adjacent Atlantic. *Chromis limbata* inhabits rocky areas from 3 to 50 m, where it forms aggregations in midwater (Brito et al., 2002). During the summer, nesting males defend territories and take care of the eggs that are attached to the substratum (Mapstone and Wood, 1975). In the case of *C. chromis*, after a pelagic larval phase of 18–19 days (Raventós and Macpherson, 2001) fish settle to adult grounds. It is likely that *C. limbata* have similar life history parameters.

The goal of this study was to test whether the paleo-climatology and paleo-oceanography of the region could predict the genetic relationships among the three eastern Atlantic populations (Azores, Madeira, and Canaries) of *Chromis limbata*. Our working hypothesis was that relict populations of *C. limbata* from the Madeira refugium would have been the source of re-colonization of the other two populations. To address this question, we combined a phylogeographic and coalescent approach using the fast evolving mitochondrial control region gene.

## 2. Materials and methods

### 2.1. Sampling and DNA extraction

Samples of *C. limbata* were obtained from one island of the archipelagos of the Azores, Madeira, and the Canaries (Fig. 1). *C. chromis* (*C. limbata* sister species) was used as outgroup. Samples were collected by spear fishing or hand nets while scuba diving. Fin clips were cut immediately after collection of the individuals and stored at ambient temperature in 95% ethanol. Tissues were digested overnight at 55 °C in 700 µl of extraction buffer (400 mM NaCl, 10 mM Tris, 2 mM EDTA, 1% SDS). We purified the DNA by standard chloroform extraction and isopropanol precipitation (Sambrook et al., 1989).

### 2.2. DNA amplification and sequencing

Amplification of the 5′ hypervariable portion of the mitochondrial control region (also called D-loop) was accomplished with universal primers CR-A and CR-E (Lee et al., 1995), and used a cycling profile of 45 s at 94 °C, 45 s at 52 °C, 1 min at 72 °C, for 35 cycles. Each 13 µl reaction contained 5–50 ng of DNA, 10 mM Tris–HCL (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 1.25 u of *Taq* DNA polymerase (Perkin-Elmer, Norwalk, Conn.), 150 mM of each dNTP, and 0.3 mM of each primer. After purification following the manufacturer's protocol (Applied Biosystems, Forter City, CA), direct sequencing was performed with an ABI 3100 automated sequencer (Applied Biosystems).

### 2.3. Data analysis

We used the computer program Clustal V implemented by Sequence Navigator (Applied Biosystems) to align the sequences. Number of haplotypes and haplotype diversity were calculated using the software package DNAsp (Rozas et al., 2003).

Phylogenetic relationships of *C. limbata* individuals were assessed using the neighbor-joining and maximum parsimony methods implemented by the software package PAUP

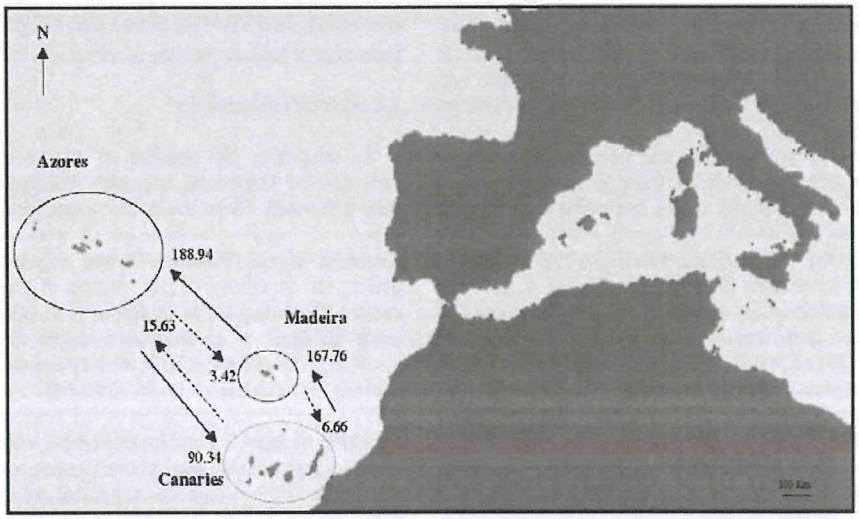


Fig. 1. *Chromis limbata* sampling locations. *C. limbata* samples were collected in one island of the archipelagos of the Azores, Madeira, and the Canaries. Arrows indicate the direction of migration between the islands and numbers next to the arrows are the number of immigrants per generation, estimated using Migrate version 2.0 (Beerli, 2004).

(Version 4.0; Swofford, 1998). Since, we were assessing an intraspecific phylogeny, we adopted a model of evolution with as few assumptions as possible and selected Kimura-2-distance (Kimura, 1980). Topological confidence was evaluated for both methods with 1000 bootstrap replicates (Felsenstein, 1985). Additionally, a network of haplotypes was constructed using the statistical parsimony method (Templeton et al., 1992) implemented in TCS (Version 1.21, Clement et al., 2000).

Gene flow (Fst and Nm) was estimated using ARLEQUIN (Version 2.000; Schneider et al., 2000). Population structure was estimated by an analysis of molecular variance (AMOVA; Excoffier et al., 1997) using ARLEQUIN 2.0.

The historical demography of the islands was first examined using mismatch distributions analysis and Tajima's test of neutrality (Tajima, 1989), to evaluate possible events of population expansion and decline. These analyses were performed in ARLEQUIN 2.0. Theoretical studies have shown that populations in long stable demographic equilibrium show a chaotic mismatch distribution, while recent rapid population expansions or bottlenecks are reflected in a unimodal (approximately Poisson) profile (Rogers, 1995; Rogers and Harpending, 1992). Mismatch distributions were established and their fit to Poisson distributions was assessed by Monte Carlo simulations of 1000 random samples. The sum of squared deviations (SSD) between observed and expected mismatch distributions was used as a test statistics, its $p$ value representing the probability of obtaining a simulated SSD larger or equal to the observed one (Schneider and Excoffier, 1999). Tajima's $D$ test are classically used to test neutrality, but they can also be used to test population growth as a population that has been experiencing expansion may result in a rejection of the null hypothesis of neutrality (significant negative $D$ value).

Estimates of $\Theta$ ($=2\,\mathrm{N}\,\mu$, where $\mu$ is the mutation rate for mitochondrial control region), were made for the entire *C. limbata* sample using FLUCTUATE (Kuhner et al., 1998). The parameter $\Theta$ was estimated under an assumption of unconstrained exponential growth. Seeds for all analyses were generated randomly. Analyses were repeated 10 times per region to ensure stability of parameters estimates. Final analyses of each dataset employed 10 short Monte Carlo chains of 200 steps each and 5 long chains of length 20,000, with a sample increment of 20.

The time of coalescence of the islands was estimated by assuming that coalescence was reached when the population size was reduced to 1% of its present day value, following Wares and Cunningham (2001). In order to estimate coalescence time, we used the mutation rate ($\mu$) for mitochondrial control region as $8.24\times10^{-8}$–$9.30\times10^{-8}$ (Domingues et al., 2005).

Exchanges and range expansions (immigration) between each island were estimated using MIGRATE 2.0 (Beerli and Felsenstein, 2001; Beerli, 2004). Again, analyses were repeated 10 times, to ensure stability of parameter estimates. Final analyses of each dataset employed 10 short Monte Carlo chains with 5000 recorded genealogies and 5 long chains with 50,000 recorded genealogies, and a sample increment of 20. We applied an exhaustive search using 4 heated chains {1, 4, 7, 10} and an interval between swapping trees of 1.

## 3. Results

### 3.1. Population diversity and phylogenetic analysis

A total of 62 mitochondrial control region sequences were obtained for *Chromis limbata*. Three *C. chromis*

sequences were used as outgroup. Number of haplotypes, diversity indexes and uncorrected p-distances are shown in Table 1. The two most southern islands (Madeira and Canaries) showed higher diversity indexes and p-distances than the Azores.

Both methods of phylogenetic inference gave similar topologies (Fig. 2). Individuals from the three islands did not partition into distinct clades, indicating some level of gene flow between islands. Phylogenetic relationships, however, partitioned the samples in two major clades, one containing 10 individuals (6 from Madeira and 4 from the Canaries) and the other containing the remaining samples. The smaller clade was only weakly supported (boostrap replicates, 59 and 51% for Maximum-Likelihood and Maximum Parsimony, respectively). In the other clade, *C. limbata* sequences grouped in small and low supported clades, which include individuals from the three islands. Evolutionary relationships among haplotype sequences were also represented in the form of statistical parsimony networks (Fig. 3). *C. limbata* revealed a complex pattern with three networks and 13 haplotypes that could not be connected under the confidence limit of 95% (Templeton et al., 1992). The two larger networks included individuals from the three islands, which supports a lack of geographical structure. The entire network is also characterized by several closed loops instead of linear relationships connecting haplotypes, suggesting the presence of homoplasy (Templeton et al., 1992). Two haplotypes present exclusively in the Azores were inferred to be ancestral, as they yielded the highest outgroup weights in each of the networks (0.467 and 0.162) (Castelloe and Templeton, 1994).

### 3.2. Population structure

Population structure was first assessed by looking at gene flow between the Azores, Madeira, and Canary islands (Table 2). Consistent with the phylogenetic description above, gene flow was high between the three archipelagos. Number of migrants was higher between Madeira and the Canary islands than between either of these islands and the Azores. The Azores population showed similar Fst values for the two southern island groups. The analysis of molecular variance showed no population structure for the Azores, Madeira, and the Canaries. There were a high percentage of within population variation for the three archipelagos (89.5%), and a low percentage of variation between populations (10.5%).

### 3.3. Historical demography

To investigate the presence of a past demographic expansion or bottleneck, mismatch distribution analyses were performed. Given the lack of population differentiation for *C. limbata*, the three islands were analyzed in a combined sample. Methods showed contrasting results, making the occurrence of past changes in population size unclear. According to the goodness of fit test, *C. limbata* could be fitted to an expansion model (SSD = 0.0046, $p = 0.356$). This method is however very conservative, rarely rejecting the expansion model (Schneider and Excoffier, 1999). Indeed, visual inspection of the mismatch distributions (Fig. 4) showed a multimodal profile, which is typical of demographic stable populations (Rogers and Harpending, 1992). This outcome was supported by a non-significant Tajima's $D$ value ($D = -0.999$, $p = 0.158$).

Theta ($\Theta$) and growth ($g$) values were estimated for *C. limbata* (Table 3).When compared to populations of *C. chromis* (Domingues et al., 2005), *C. limbata* was growing at a slow rate (Table 3).

Migration between the Azores, Madeira, and Canary islands was determined. The prevailing migration direction was towards the northwest (Fig. 1). Number of migrants was higher from the Canaries into Madeira and from Madeira into the Azores. Migration in the other directions could not be excluded, although it occurs in a much smaller extend (6.66 from Madeira into the Canaries and 3.42 from the Azores into Madeira; see Fig. 1).

## 4. Discussion

In a previous paper, Domingues et al. (2005) estimated the timing of speciation of *C. chromis* based on the mitochondrial control region, using two approaches: (i) determining the time of divergence between *C. chromis* and its sister species *C. limbata* (using a molecular clock based on the divergence between the transisthmian geminate species *C. multilineata* and *C. atrilobata*); ii) estimating the age of the most recent common ancestor using the time of coalescence for the two sister species. The divergence time of *C. chromis*

Table 1
Collection localities of *Chromis limbata* and outgroup species, *C. chromis*, used in the present study and diversity indexes for mitochondrial control region

| Locality | Number individuals | Hn | Hd | $\pi$ | p-distance (average) | Collection date |
|---|---|---|---|---|---|---|
| *Chromis limbata* | | | | | | |
| Azores (Azo) | 25 | 12 | 0.48 | 0.036 | 0.033 | Feb. 2004 |
| Madeira (Mad) | 18 | 17 | 0.94 | 0.058 | 0.057 | Sep. 2003 |
| Canaries (Can) | 19 | 19 | 1 | 0.050 | 0.046 | Apr. 2004 |
| Total | 62 | | | | | |
| *Chromis chromis* | | | | | | |
| Sesimbra (Ses) (Portuguese Atlantic coast) | 3 | | | | | Mar. 1997 |

Number of individuals, number of haplotypes (Hn), Haplotype diversity (Hd), Nucleotide diversity ($\pi$), and average uncorrected p-distance for each population are shown.

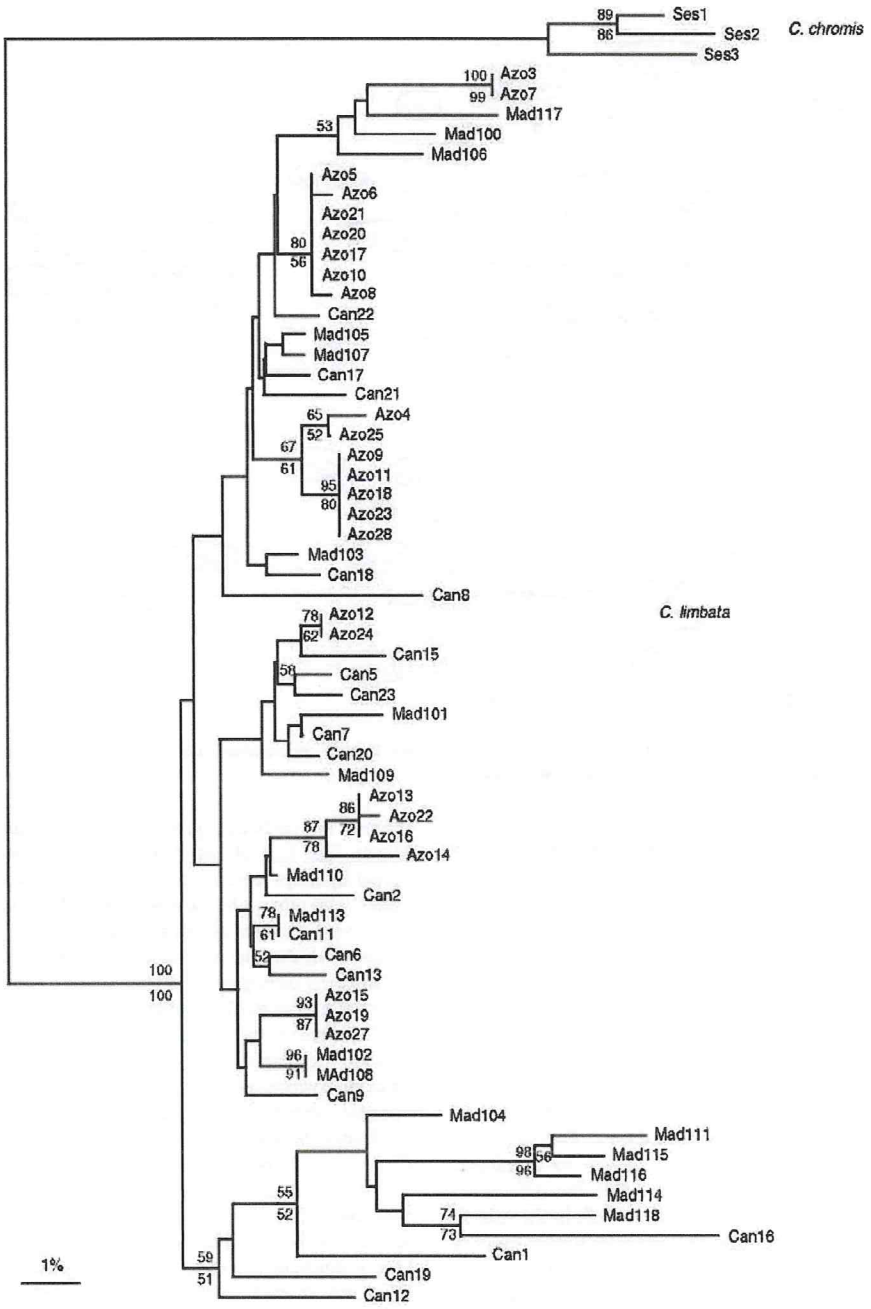


Fig. 2. Phylogenetic relationship within *Chromis limbata* using *C. chromis* as outgroup. A neighbor-joining tree is shown with neighbor-joining (above the nodes) and maximum parsimony (below the nodes) bootstrap support above 50%. Labels are described in Table 1. The length of each branch is proportional to the number of nucleotide substitutions. *Scale bar*: 1% Kimura-2 genetic distance.

from its sister species *C. limbata* was estimated at 0.93–3.26 Mya, while its time of coalescence was reached 0.14–0.21 Mya (Table 3). In the case of *C. limbata*, coalescence time was estimated at 0.857–1.17 Mya. These results are in agreement with the general idea that the divergence time of two sister species is most likely an overestimate of their

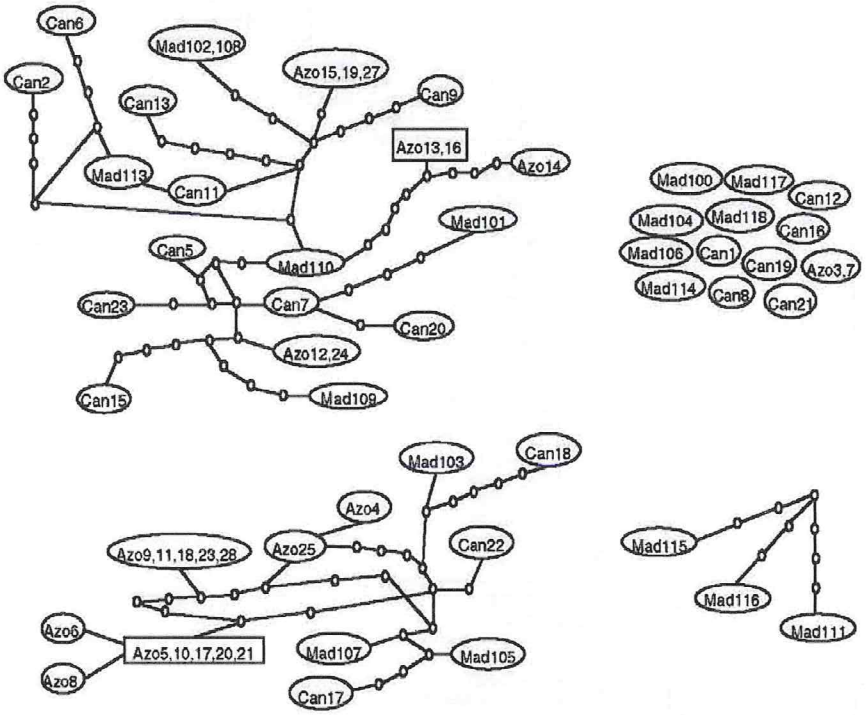


Fig. 3. Statistical parsimony networks of D-loop sequences for *C. limbata* samples. Empty circles represent missing haplotypes. Ancestral haplotypes for each network (Castelloe and Templeton, 1994) are displayed in a square.

Table 2
Gene flow among *Chromis limbata* populations represented by Fst (below the diagonal) and Nm (above the diagonal), calculated from mitochondrial control region sequences using ARLEQUIN version 2.000 (Schneider et al., 2000)

| | Azores | Madeira | Canaries |
|---|---|---|---|
| Azores | | 2.623* | 4.189* |
| Madeira | 0.160* | | 13.744 |
| Canaries | 0.107* | 0.035 | |

Significant $p$ values ($p < 0.05$) are indicated by an asterisk.

actual coalescence time (Edwards and Beerli, 2000). As stated above, the coalescence time estimated for *C. limbata* is much greater than that estimated for *C. chromis* by Domingues et al. (2005). This difference may reflect the differentiate impact of glaciations in the two areas. While in the Atlantic islands, specially in Madeira, the drop in sea surface temperature was negligible (Crowley, 1981), in the Mediterranean warm water fish were reduced to small pockets in the southern Mediterranean (Thiede, 1978), which must have implied a major population collapse of *C. chromis*.

No population structure was found among *C. limbata* from the Azores, Madeira, and Canary islands. None of the phylogenetic inference methods yielded highly supported clades encompassing individuals from one of the islands exclusively. Similarly, the statistical parsimony network showed no particular groupings of individuals across islands. The lack of population differentiation was supported by a low percentage of variation between populations (10.5%) shown by the AMOVA analyses. Indeed, gene flow among the three islands was high (Table 2), with all the comparisons showing Nm values greater than 1.

Historical demographic parameters of *C. limbata* (Table 3) reveal a more stable population history when compared to *C. chromis* of the Western Mediterranean and adjacent Atlantic (Domingues et al., 2005). In addition to the differences of coalescence times discussed above, two lines of evidence support this conclusion. Western Mediterranean *C. chromis* showed a smaller $\Theta$ (and thus smaller Ne) than *C. limbata*. Although the difference is not very large ($\Theta = 0.541$ and 0.572 for the Western Mediterranean *C. chromis* and *C. limbata*, respectively), it is important to remember that the Western Mediterranean and adjacent Atlantic have a much higher area. As for the growth parameter, *C. chromis* showed much higher values than *C. limbata*. Large $g$ values are typical of populations expanding their geographical range following regressions due to glaciating events. These observations, which suggest a relatively stable population of *C. limbata* with moderate growth during the Pleistocene, would explain the apparent contradiction between the results of the mismatch analysis and the non-significant Tajima's $D$ value.

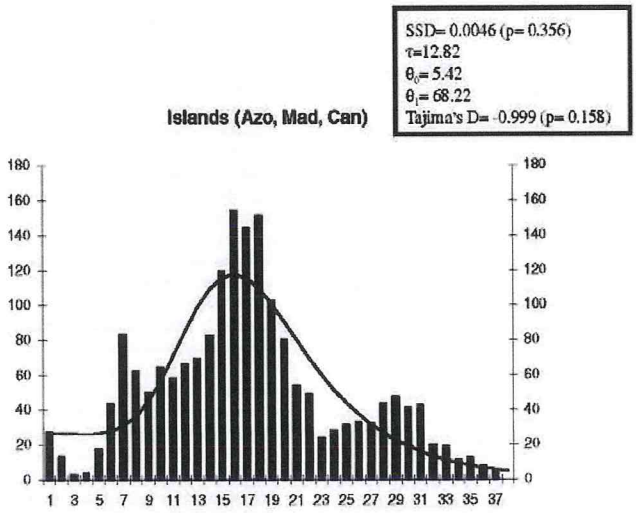


Fig. 4. Mismatch distribution established for *Chromis limbata* mitochondrial control region. The bars represent the observed frequency of the pairwise differences among haplotypes, while the line shows the expected curve predicted for a population that has undergone a demographic expansion in the past. The parameters of the model of sudden expansion (Rogers and Harpending, 1992) are presented as well as goodness of fit test to the model: SSD sum of squared deviations; $\theta_0$ pre-expansion and $\theta_1$ post-expansion population sizes; $\tau$ time in number of generations, elapsed since the sudden expansion episode. Tajima's (1989) $D$ test value and its statistical significance are also given.

Table 3
Demographic parameters of *Chromis limbata* based on mtDNA control region

| | $\Theta$ | $g$ | Coalescence time (Mya) |
|---|---|---|---|
| *C. limbata* | | | |
| Azo, Mad, Can | 0.572 (±0.037) | 52.678 (±5.072) | 0.857–1.17 |
| *C. chromis* | | | |
| Mediterranean | 0.897 (±0.178) | 308.026 (±45.979) | 0.14–0.21 |

Estimates of $\Theta$ (compound parameter representing the effective population size and mutation rate), $g$ (growth parameter), and coalescence time. Parameters were estimated using FLUCTUATE (Kuhner et al., 1998). The standard deviation is presented between parentheses after $\Theta$ and $g$.

In the Azores region during the glacial peaks the drops in sea surface temperatures were small (2–3 °C), due to a complex and stabilizing system of interactions between the Gulf Stream and North Atlantic Current (Crowley, 1981). However, as noted by the same author, the planktonic foraminiferal record in this region has experienced large variations during the last 150,000 years. Indeed, Santos et al. (1995) suggested that this temperature drop, although not very strong, has probably resulted is mass extinctions of littoral fish from the Azores. Following this idea, most of the organisms now present would have recolonized the islands after the last glaciating event from some southern, less affected regions like the Northwestern coast of Africa south of Cape Blanco, the westernmost Canary islands and Madeira. Indeed, both the haplotypic and nucleotidic diversity indexes as well as the uncorrected p-distances were lower for the more affected population of Azores than for the southern Madeira and Canary islands. When looking at migration across the three archipelagos, a prevailing northwest trend is evident. This estimation agrees with the colonization process proposed by Santos et al. (1995) for the Azores. According to these authors, the western Africa and Macaronesian islands have been the main source of eggs and larvae transported by several eddies and having small islets and shallow seamounts as "stepping-stones" for the dispersal of warm water organisms to the Azores. Also the proximity of the Canary islands to the continental African coast combined with the above mentioned eddies may have played an important role in the colonization process of Madeira and Azores.

In summary, our study shows that the Macaronesian populations of *C. limbata* have probably been less affected by the last glaciation than the Mediterranean populations of *C. chromis* studied by Domingues et al. (2005). It also supports the colonization model proposed by Santos et al. (1995) for the warm water fish of the Azores and the possible role of Madeira and the westernmost Canary islands as a major glacial refugia for the tropical and subtropical marine fauna.

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# Chapter 5

## Phylogeography and evolution of the triplefin *Tripterygion delaisi* (Pisces, Blennioidei)

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RESEARCH ARTICLE

# Phylogeography and evolution of the triplefin *Tripterygion delaisi* (Pisces, Blennioidei)

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**Abstract** The genus *Tripterygion* (Risso 1826) is restricted to the eastern Atlantic and the Mediterranean, and comprises only three species. *T. melanuros* and *T. tripteronotus* are essentially endemic to the Mediterranean, while *Tripterygion delaisi* occurs in the Atlantic and in the Mediterranean. Two subspecies of *T. delaisi* have been described (*T. d. xanthosoma* in the Mediterranean and *T. d. delaisi* in the Atlantic). Several scenarios have been proposed for the evolution of *T. delaisi* subspecies, but so far its subspeciation process is not clear. In this study we present a population survey of *T. delaisi* including specimens from the two recognized subspecies. We combined a phylogeographic approach with estimates of the direction of migration (between the Atlantic and the Mediterranean) and of the coalescence time of the two subspecies, using polymorphic mitochondrial and nuclear genes. The results of this study clearly support the existence of two *Tripterygion delaisi* clades, one in the eastern Atlantic islands and another in the Atlantic coasts of Europe and in the Mediterranean. Historical migration between the islands and Western Europe plus Mediterranean was restricted, and showed a westbound trend, with a higher number of migrants going from the Western Europe plus Mediterranean into the islands. We estimated the time of coalescence of both groups of *T. delaisi* to be more recent than the onset of Pleistocene glaciations (1.7 Mya). Our results are consistent with previous hypothesis that consider successive dispersal events of a *Tripterygion* ancestor from the western African coast colonizing the Atlantic islands and the Mediterranean, promoting the evolutionary divergence between these areas.

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## Introduction

Despite the fact that the Mediterranean and adjacent Atlantic have been considered the same biogeographical province (Briggs 1974), several recent studies using molecular markers addressed levels of differentiation between these regions. Studies on different marine organisms have shown contrasting results. Some species exhibit little gene flow and strong genetic divergence between Atlantic and Mediterranean populations (Kotoulas et al. 1995; Borsa et al. 1997; Chikhi et al. 1997; Naciri et al. 1999; Aurelle et al.

2003; Bargelloni et al. 2003, 2005), while others show very high levels of gene flow (Bargelloni et al. 2003, 2005; Costagliola et al. 2004; Duran et al. 2004; Zardoya et al. 2004). An emerging pattern is shown in cases of closely related species. In such cases one of two sister species is present in the Mediterranean and adjacent Atlantic while the other is found in tropical west Africa and Macaronesian islands (eg. *Chromis chromis* and *C. limbata*, Domingues et al. 2005; *Parablennius sanguinolentus* and *P. parvicornis*, Almada et al. 2005a). Thus the study of the boundary between these regions may shed light on the role of biogeographic breaks in the formation and maintenance of speciation events.

Several scenarios have been proposed for the evolution and phylogeography of the ichthyofauna of the northeastern Atlantic and the Mediterranean. Based on small cryptic fishes (blennioids), Zander (1980) described the Macaronesian islands as a center of speciation for the Atlantic blennioids from which the new species have colonized the western European shores. Almada et al. (2001) proposed the tropical coast of Africa and the Mediterranean as possible speciation centers of the northeastern Atlantic blenniids, serving also as refugia during the glacial periods.

Triplefin blennioids (family Tripterygiidae) comprise a number of small demersal fish living in rocky habitats. The genus *Tripterygion* (Risso 1826) is restricted to the eastern Atlantic and the Mediterranean (Zander 1986; Wirtz 1990). In recent years only three species of this genus have been recognized in the entire area. *Tripterygion melanurus* (Guichenot 1850), and *T. tripteronotus* (Risso 1810) are essentially endemic to the Mediterranean (but there are indications pointing to the existence of an Atlantic population on the coasts of northwest Morocco; Brownell 1978). *T. delaisi* (Cadenat and Blache 1970) is distributed in two disjunct areas: a southern area comprising western tropical Africa north to Senegal and the Macaronesian islands, and a second area comprising the Mediterranean sea and adjacent northeastern Atlantic waters south to Casablanca and Morocco, and north to the British Isles. Indeed, although Zander (1986) mentioned the species as ranging from southern England to Senegal, there are no records of triplefins between Casablanca and Senegal. Moreover a recent survey did not detect it in Western Sahara (Falcón et al. 2002). The name *T. xanthosoma* (Zander and Heymer 1970) was recognized for the Mediterranean, but Wirtz (1980) placed *T. xanthosoma* in synonymy with the eastern Atlantic *T. delaisi*. In spite of this, Wirtz (1980) recognized that there were small differences among populations of different locations that could be statistically detected if sufficiently large samples were analyzed. Zander (1986), based on the courtship behavior and color of the males, considered that the differences between the Mediterranean and Atlantic populations of *T. delaisi* were sufficient to ascribe them to two different subspecies (*T. d. xanthosoma* in the Mediterranean and *T. d. delaisi* in the Atlantic). Thus, the three species are sympatric in the Mediterranean where they differ in microhabitats preferences, both in terms of depths and level of illumination (Wirtz 1978). *T. delaisi* lives at a depth of 3–40 m, *T. tripteronotus* between 0 and 6 m and *T. melanurus* between 0 and 18 m (Wirtz 1978). *T. delaisi* prefers darker places while *T. tripteronotus* and *T. melanurus* occurs in small caves (Wirtz 1978). Interestingly, the western African populations of *T. delaisi* from Senegal do not show these habitat restrictions occupying all depth levels and light zones that were described for the three species in the Mediterranean (Wirtz 1980). All three species exhibit territorial breeding and male parental care of benthic eggs. Dispersal is restricted to their planktonic larval phase, which is very similar for the three species (17–18 days in the case of *T. delaisi* and *T. tripteronotus*, and 15–18 days for *T. melanurus*; Raventós and Macpherson 2001).

The presence of a single species widely distributed in the eastern Atlantic, and in the Mediterranean and two other species ecologically very distinct in the Mediterranean, has raised the interest of many biologists to understand the biology and evolutionary history of the genus. Different hypotheses have been proposed for the speciation of *Tripterygion*. Zander (1973) suggested that during the last glaciations *Tripterygion* evolved into two different populations along the western African coast: a cold-resistant northern population and a southern population better adapted to warmer waters. After the glaciations, each of these populations might have migrated into the Mediterranean evolving there into two different species: *T. xanthosoma* (now *T. delaisi)* and *T. tripteronotus*. Wirtz (1978, 1980) proposed several invasions of the Mediterranean by a primary west african *Trypterygion*. This author suggests that the fluctuation of the sea level during the last glaciations caused the isolation of the Mediterranean from the Atlantic, promoting speciation within the Mediterranean. According to this model, the Mediterranean population of *T. delaisi* resulted from the last group of invaders. Jonge and Videler (1989) suggested that *T. delaisi* evolved into *T. tripteronotus* within the Mediterranean after the last glaciations, either sympatrically or in allopatry. Geertjes et al. (2001) hypothesized that the divergence between the three species started before the Pleistocene and that *T. melanurus* and *T.*

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*tripteronotus* survived the Pleistocene glaciations in refugia within the Mediterranean. According to the same authors, *T. tripteronotus* and *T. delaisi* might have diverged sympatrically in the Mediterranean. Recently, Carreras- Carbonell et al. (2005) attempted to clarify the phylogenetic relationships and taxonomic status of *Tripterygion* using four mitochondrial markers and one nuclear gene, which unfortunately showed a single haplotype for the three species. According to the same authors the speciation process of *Tripterygion* may have resulted from a rapid radiation event after the Messinian Salitiny Crisis (5.2 Mya) leading to a trichotomy. The same authors showed that the two subspecies of *T. delaisi* have originated much more recently, probably during the climatic Quaternary fluctuations (1.10–1.23 Mya). Interestingly, they found that of the five specimens of *T. delaisi* from the Atlantic they analyzed, one, from northwestern Spain, clearly clustered with the Mediterranean samples, while the remaining four (two from Azores and two from the Canaries) formed a very distinct cluster. Thus, Carreras-Carbonell et al. (2005) stated that further sampling of *T. delaisi* in the Atlantic is necessary to assess the direction of migration between the Atlantic and the Mediterranean in order to determine the subspeciation process.

In this study we present a population survey of *Tripterygion delaisi* including specimens from the two recognized subspecies. Our goals were to evaluate the extent of the genetic divergence between the northeastern Atlantic islands versus the European western coast and the Mediterranean populations, and to clarify the process of subspeciation of *T. delaisi*. We analyze polymorphic mitochondrial and nuclear genes to compare populations of *T. delaisi* from the Atlantic (Azores, Madeira, Canaries, and the continental coast of Portugal) and the Mediterranean. To shed some light on the process of evolution of *T. delaisi* subspecies, a phylogeographic approach was combined with estimates of the direction of migration (between the Atlantic and the Mediterranean) and of the coalescence time of the two subspecies.

## Materials and methods

### Sampling and DNA extraction

Samples of *Tripterygion delaisi* were obtained from three eastern Atlantic islands (Azores, Madeira, and the Canaries), from the Portuguese Atlantic coast (Arrábida) and from the Mediterranean (Vivara and Capri in Italy, Croatia and Cyprus). The other two species of the genus *Tripterygion*, *T. tripteronotus* and *T. melanurus*, were also included in the analysis and *T. melanuros* was used as outgroup, following the phylogenetic relationships recovered by Carreras-Carbonell et al. (2005). Samples were collected by hand nets while scuba diving. Fin clips were cut immediately after collection of the individuals and stored at ambient temperature in 95% ethanol. Tissues were digested overnight at 55°C in 700 µl of extraction buffer (400 mM NaCl, 10 mM Tris, 2mM EDTA, 1% SDS). We purified the DNA by standard chloroform extraction and isopropanol precipitation (Sambrook et al. 1989).

### DNA amplification and sequencing

Amplification of the 5′ hypervariable portion of the mitochondrial control region (also called D-loop) was accomplished with universal primers CR-A and CR-E (Lee et al. 1995), and used a cycling profile of 45 s at 94°C, 45 s at 52°C, 1 min at 72°C, for 35 cycles. Each 13 µl reaction contained 5–50 ng of DNA, 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 1.25 u of *Taq* DNA polymerase (Perkin-Elmer, Norwalk, CT), 150 mM of each dNTP, and 0.3 mM of each primer. In addition, we amplified and sequenced the second intron of the S7 ribosomal protein gene, using the primers S7RPEX2F and S7RPEX3R (Chow and Hazama 1998), and an annealing temperature of 56°C. After purification following the manufacturer's protocol (Applied Biosystems, Forter City, CA), direct sequencing was performed with an ABI 3100 automated sequencer (Applied Biosystems).

### Data analysis

We used the computer program Clustal V implemented by Sequence Navigator (Applied Biosystems) to align the sequences. Number of haplotypes and haplotype diversity were calculated using the software package ARLEQUIN (vers. 2.000; Schneider et al. 2000). For the nuclear marker, we could not ascribe the two alleles of heterozygous positions to one of the sequences of each individual. Sample size was not enough for a maximum likelihood estimation of the allelic constitution of each sequence. Thus, we adopted a reliable criterion, using one sequence per individual scoring the variable sites with alternative nomenclature.

Phylogenetic relationships were assessed using Maximum Parsimony and Neighbor-Joining. As we were dealing with very closely related species, with small genetic distances, we adopted the p distance following Nei and Kumar (2000). Both methods were implemented by the software package PAUP (vers. 4.0; Swofford 1998). Topological confidence was evaluated for

Maximum Parsimony and Neighbor-joining with 1,000 bootstrap replicates (Felsenstein 1985).

Gene flow (Fst) was estimated using ARLEQUIN (vers. 2.000; Schneider et al. 2000). Corrections for simultaneous multiple comparisons were applied using sequential Bonferroni correction (Rice 1989). Population structure was evaluated by an analysis of molecular variance (AMOVA; Excoffier et al. 1997) grouping the populations into two groups: eastern Atlantic islands versus Arrábida and Mediterranean. The AMOVA was implemented in ARLEQUIN (vers. 2.000; Schneider et al. 2000).

### Historical demography

Estimates of $\Theta = 2N\mu$, where $\mu$ is the mutation rate for mitochondrial control region, were made for the two groups described above. The parameter $\Theta$ was estimated under an unconstrained exponential growth parameter. We used FLUCTUATE (Kuhner et al. 1998) to estimate the maximum likelihood of the parameters $\Theta$ and g (the exponential growth parameter in units $\mu^{-1}$). Seeds for all analyses were generated randomly. Analyses were repeated 10 times per region to ensure stability of parameters estimates. Final analyses of each dataset employed ten short Monte Carlo chains of 200 steps each and five long chains of length 20,000, with a sample increment of 20. The time of coalescence was estimated by assuming that coalescence was reached when the population size was reduced to 1% of its present day value, following Wares and Cunningham (2001). In order to estimate coalescence time, we used the mutation rate ($\mu$) for mitochondrial control region as $8.24 \times 10^{-8} - 9.30 \times 10^{-8}$ that was estimated using an internally calibrated molecular clock (Domingues et al. 2005). Exchanges and range expansions (immigration) between the island and Arrábida plus Mediterranean were estimated using MIGRATE 2.0 (Beerli and Felsenstein 2001; Beerli 2004). This software gives the value $M$ ($M = m/\mu$), which is the number of migrants scaled by the mutation rate. Again, analyses were repeated 10×, to ensure stability of parameter estimates. Final analyses of each dataset employed ten short Monte Carlo chains with 5,000-recorded genealogies and five long chains with 50,000 recorded genealogies, and a sample increment of 20.

## Results

### DNA sequences and phylogenetic analysis

Mitochondrial control region sequences were obtained from 72 individuals including 66 *Tripterygion delaisi*, three *T. tripteronotus*, and three *T. melanurus*. In addition, second intron of the S7 ribosomal protein gene sequences was obtained from 56 samples, 50 *T. delaisi*, three *T. tripteronotus*, and three *T. melanurus*. Sequences of the mitochondrial control region and S7 intron were 352 and 450 bp long, respectively. Number of haplotypes and haplotype/genotypic diversities are shown in Table 1.

Both methods of phylogenetic inference resulted in a similar topology. Neighbor-joining phylogenies based on the mitochondrial control region and S7 intron sequences are presented in Figs. 2a and b. For both genes, *T. delaisi* were found to partition in two major sister clades, one including samples from the eastern Atlantic islands and the other including samples from mainland Portugal (Arrábida) and the Mediterranean. The two clades were defined by 16 fixed differences in the control region. For the nuclear marker S7, heterozygous individuals make the analysis of fixed differences more complex during direct sequencing. We found that seven nucleotides are uniquely found on the Atlantic islands and two nucleotides are uniquely found on the mainland and the Mediterranean, resulting in the phylogenetic separation of those two regions into two different clades as described above. These clades were well supported for the mitochondrial control region but showed lower bootstrap values for the S7 intron (less than 50% for maximum parsimony).

### Population structure

Population structure was first assessed by looking at gene flow between the seven populations in our study (Table 2). As expected, gene flow estimates were higher in the nuclear marker than in the mitochondrial marker (Table 2). As described above, no gene flow was detected between the mainland/Mediterranean and the Atlantic Islands. Within these regions, markers showed a higher level of gene flow within the island group (Fst ranging from 0.085 to 0.523 for Dloop and from 0.000 to 0.053 for S7), than within the mainland Portugal and Mediterranean populations (Fst ranging from 0.260 to 0.981 for Dloop and from 0.000 to 1.000 for S7). While these numbers were mostly driven by small sample sizes in the Mainland/Mediterranean group, results still held when only considering the two largest populations (Italy and Arrábida).

The analysis of molecular variance showed a high degree of differentiation between the two groups of populations (86.56% and 64.58% for Dloop and S7, respectively). Uncorrected p distances between these two groups were high (average = 0.068, SD = 0.009, min = 0.047, max = 0.083 for Dloop; and average = 0.024,

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**Table 1** Collection localities, diversity indexes and date of collection of *Tripterygion delaisi*, *T. tripteronotus* and *T. melanurus* used in the present study

| Locality | Number of individuals | | *k* | | Hd | Gd | Collection date |
|---|---|---|---|---|---|---|---|
| | *Dloop* | *S7* | *Dloop* | *S7* | *Dloop* | *S7* | |
| *Tripterygion delaisi* | | | | | | | |
| Azores | 13 | 10 | 2 | 6 | 0.15 | 0.60 | March 2004 |
| Madeira | 4 | 3 | 1 | 2 | 0.25 | 0.67 | ? |
| Canaries | 16 | 13 | 8 | 6 | 0.50 | 0.46 | February 2005 |
| Arrábida, Portugal | 16 | 15 | 1 | 11 | 0.06 | 0.73 | February 2004 |
| Italy (Capri, Vivara) | 12 | 7 | 5 | 5 | 0.42 | 0.71 | June–July 2003 |
| Croatia | 2 | 1 | 2 | 1 | 1 | 1 | ? |
| Cyprus | 3 | 1 | 3 | 1 | 1 | 1 | May 2002 |
| Total | 66 | 50 | | | | | |
| *Tripterygion melanurus* | | | | | | | |
| Italy (Vivara) | 3 | 3 | | | | | June 2003 |
| *Tripterygion tripteronotus* | | | | | | | |
| Spain (Cabo Gata) | 1 | 1 | | | | | July 2004 |
| Italy (Vivara) | 1 | 2 | | | | | June 2003 |
| Greece (Limnos) | 1 | 0 | | | | | July 2003 |

Number of individuals, number of haplotypes (*k*), haplotype diversity (Hd) and genotypic diversity (Gd) for mitochondrial control region and S7 intron are given

**Table 2** Fst values for *Tripterygion delaisi* populations calculated from mitochondrial control region sequences (above the diagonal) and S7 intron (below the diagonal), using ARLEQUIN version 2.000 (Schneider et al. 2000)

| | Azores | Madeira | Canaries | |
|---|---|---|---|---|
| Azores | | 0.085$^{+}$ | 0.523$^{+*}$ | |
| Madeira | 0.002 | | 0.488$^{+}$ | |
| Canaries | 0.053 | 0.000 | | |
| | Arrábida | Italy | Croatia | Cyprus |
| Arrábida | | 0.606$^{+*}$ | 0.981$^{+}$ | 0.845$^{+}$ |
| Italy | 0.003 | | 0.488$^{+}$ | 0.402$^{+}$ |
| Croatia | 0.300 | 0.000 | | 0.260 |
| Cyprus | 0.076 | 0.162 | 1.000 | |

Significant *P* values ($P < 0.05$) before Bonferroni correction are indicated by +, and after Bonferroni correction are indicated by an asterisk

SD = 0.008, min = 0.004, max = 0.043 for S7) when compared to the distances within each group (average = 0.008, SD = 0.007, min = 0.000, max = 0.036 for Dloop and average = 0.004, SD = 0.004, min = 0.000 max = 0.018 for S7 for the eastern Atlantic islands and average = 0.006, SD = 0.005, min = 0.000, max = 0.017 for Dloop and average = 0.009, SD = 0.006, min = 0.027 max = 0.000 for S7, for Arrábida plus the Mediterranean).

### Historical demography

Historical demography was assessed by determining historical population size and growth using the control region and S7 intron sequences, for the two groups of populations of *Tripterygion delaisi*: islands and Arrábida plus Mediterranean (Table 3). The islands group showed a much higher growth rate than the Arrábida plus Mediterranean group for the mitochondrial control region. However, for the S7 intron this relation was the opposite, although the difference between the growth values of the two populations was not as high (Table 3).

Relative historical population size was determined, allowing us to estimate the coalescence time of the two groups of *T. delaisi*. Using the mutation rate ($\mu$) for mitochondrial control region as $8.24 \times 10^{-8}$ – $9.30 \times 10^{8}$ (Domingues et al. 2005), the islands populations of *T. delaisi* was 1% of its present size approximately 5,000–6,000 ya (Table 3). The coalescence time for Arrábida plus Mediterranean populations was 175,000–210,000 ya. Historical migration between the Atlantic islands and Arrábida plus Mediterranean was restricted, with a trend of migration from the Mediterranean into the Atlantic islands [22.04 ($\pm$ 1.10)/$\mu$ Islands immigrants vs. 0.09 ($\pm$ 0.27)/$\mu$ Mediterranean immigrants; Fig. 1].

## Discussion

The results of this study support the existence of two *Tripterygion delaisi* clades, one in the eastern Atlantic islands and another in the Atlantic coasts of Europe and in the Mediterranean. All phylogenetic reconstruc-

**Table 3** Demographic parameters of *Tripterygion delaisi* based on mtDNA control region and S7 intron

| | Θ (growth) | g | Coalescence time (ya) |
|---|---|---|---|
| Dloop | | | |
| Islands | 0.005 (± 0.005) | 10,000 (± 0.000) | 5,000–6,000 |
| Arrábida and Mediterranean | 0.016 (± 0.003) | 275.12 (± 8.19) | 175,000–210,000 |
| S7 | | | |
| Islands | 0.033 (± 0.001) | 59.90 (± 6.474) | |
| Arrábida and Mediterranean | 0.052 (± 0.002) | 369 (± 20.253) | |

Estimates of Θ (compound parameter representing the effective population size and mutation rate) and g (growth parameter) based on the mitochondrial control region and S7 intron for both groups of *T. delaisi* (islands and Atlantic plus Mediterranean populations). Coalescence time of *T. delaisi* populations based on mitochondrial control region data is shown in the column 4. Parameters were estimated using FLUCTUATE (Kuhner et al. 1998). The standard deviation is presented between parentheses after each estimator

tion methods partition *T. delaisi* into two major clades (eastern Atlantic islands vs. Arrábida and Mediterranean). High-uncorrected p distances between these two groups for the mitochondrial control region sequences (0.07) and the S7 intron (0.02), also supports the existence of two highly divergent groups of *T. delaisi*. Indeed, this value is only slightly lower than the distance between each group of *T. delaisi* and the other *Tripterygion* species: *T. tripteronotus* (0.10–0.19 for Dloop and 0.01–0.04 for S7) and *T. melanurus* (0.08–0.12 for Dloop and 0.02–0.06 for S7). These values are similar to the distance between mitochondrial control region sequences of other blennioid sister species available in the GenBank (*Parablennius parvicornis*/*P. sanguinolentus* = 0.13; *P. pilicornis*/*P. salensis* = 0.11; *Lipophrys pholis*/*L. trigloides* = 0.17; *L. adriaticus*/*L. dalmatinus* = 0.21).

In addition to the phylogenetic data, gene flow between the eastern Atlantic islands and Arrábida plus Mediterranean was very low (average Fst = 0.94 and 0.70 for Dloop and S7 intron, respectively). This was also confirmed by the AMOVA analyses showing that the total genetic variance was strongly explained by the existence of two groups of populations of *T. delaisi* (eastern Atlantic islands versus Arrábida and Mediterranean). Moreover, migration between these regions was remarkably restricted.

Several studies have shown restricted gene flow between the Atlantic and the Mediterranean for different marine organisms. Some studies described a strong genetic divergence between Atlantic and Mediterranean faunas, ascribed to the relative isolation of both seas during the Pleistocene glaciations and to present day barriers such as the hydrology in the strait of Gibraltar. Genetic discontinuities between the Atlantic and the Mediterranean were found for four sparids (Bargelloni et al. 2003, 2005), for the cuttlefish *Sepia officinalis* (Pérez-Losada et al. 1999, 2002) and the mussel *Mytilus galloprovincialis* (Quesada et al. 1995). Other species such as the ornate wrasse (*Thalassoma pavo*,

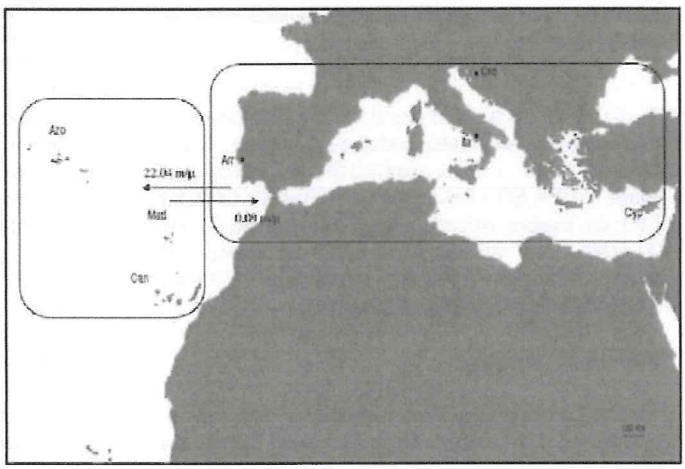


**Fig. 1** *Tripterygion delaisi* sampling locations. *T. delaisi* samples were collected in the northeastern Atlantic islands, Portuguese Atlantic coast, and in the Mediterranean. Labels are Azores (*Azo*), Madeira (*Mad*), Canaries (*Can*), Arrábida (*Arr*), Italy (*Ita*), Croatia (*Cro*), and Cyprus (*Cyp*). The extend of migration between the two clades of *T. delaisi* are indicated in the figure. Number of migrants scaled by the mutation rate were estimated using MIGRATE 2.0 (Beerli 2004 )

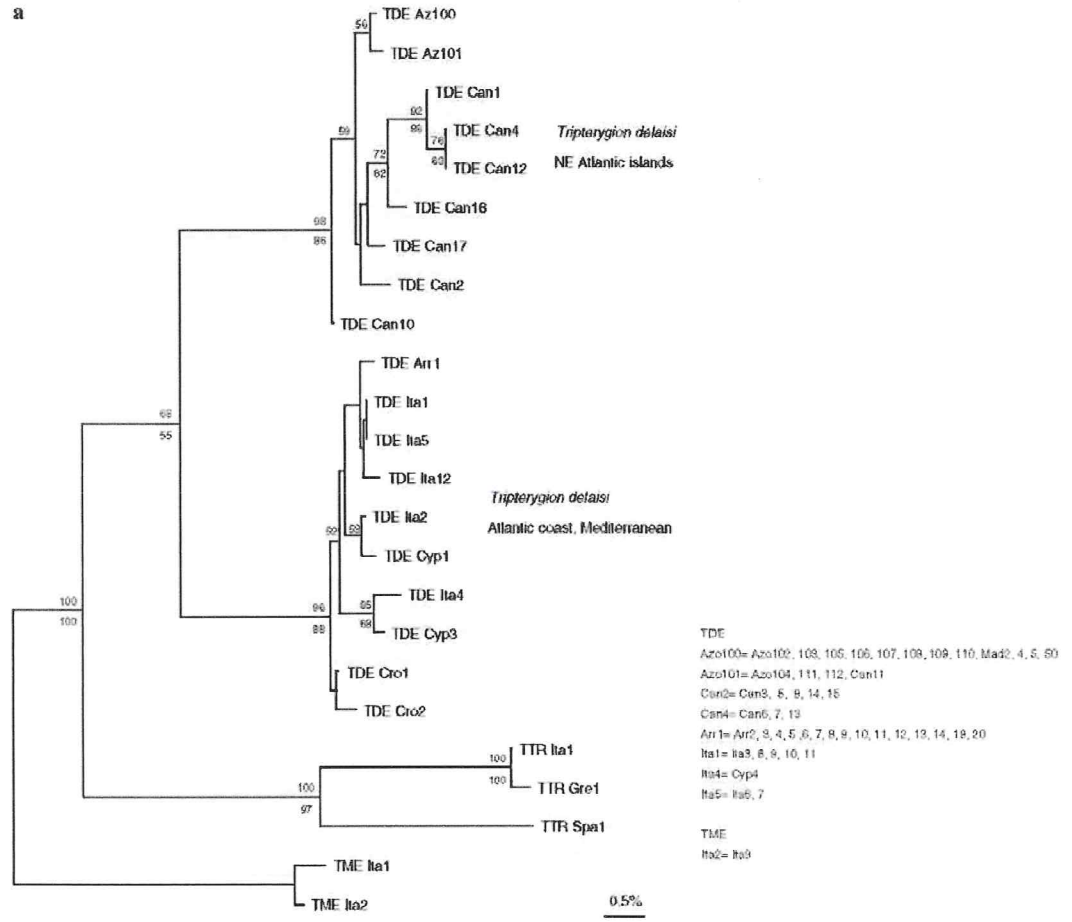


**Fig. 2** Phylogenetic relationship within *Tripterygion delaisi* for the mitochondrial control region sequences (**a**) and S7 intron (**b**). *T. tripteronotus* and *T. melanurus* were also included and *T. melanurus* was used as outgroup., Neighbor-joining trees are shown with neighbor-joining (above the nodes) and maximum parsimony (below the nodes) bootstrap support above 50% indicated at the nodes. Labels are *T. delaisi* (TDE), *T. tripteronotus* (TTR), *T. melanuros* (TME), Azores (Azo), Madeira (Mad), Canaries (*Can*), Arrábida (Arr), Spain (Spa), Italy (Ita), Croatia (Cro), Greece (Gre), and Cyprus (Cyp). The length of each branch is proportional to the number of nucleotide substitutions. Scale bar: 0.5% Uncorrected *P* distance

Costagliola et al. 2004), the chub mackerel (*Scomber japonicus*, Zardoya et al. 2004), the withe sea-bream (*Diplodus sargus*, Bargelloni et al. 2005) and the Norway lobster (*Nephrops norvegicus*, Stamatis et al. 2004) were described as having high gene flow levels between the Atlantic and the Mediterranean and no signs of an Atlantic-Mediterranean divide. These contrasting patterns may stem from different biological characteristics of the species. Larval ecology in particular, is well known to affect the extent of gene flow (eg. Riginos and Victor 2001). Results for *T. delaisi* are contradictory. Fst values between Arrábida and Italy (the most western location of the Mediterranean in our study) are high for the mitochondrial control region but very low for the S7 intron (0.606 and 0.003, respectively, Table 2). All individuals from Arrábida are represented by a single mitochondrial control region haplotype, which is not present in any of the other populations. However, the uncorrected p distances between Arrábida and Italy are very small (0.005), indicating that the haplotype of Arrábida is similar to Italy's.

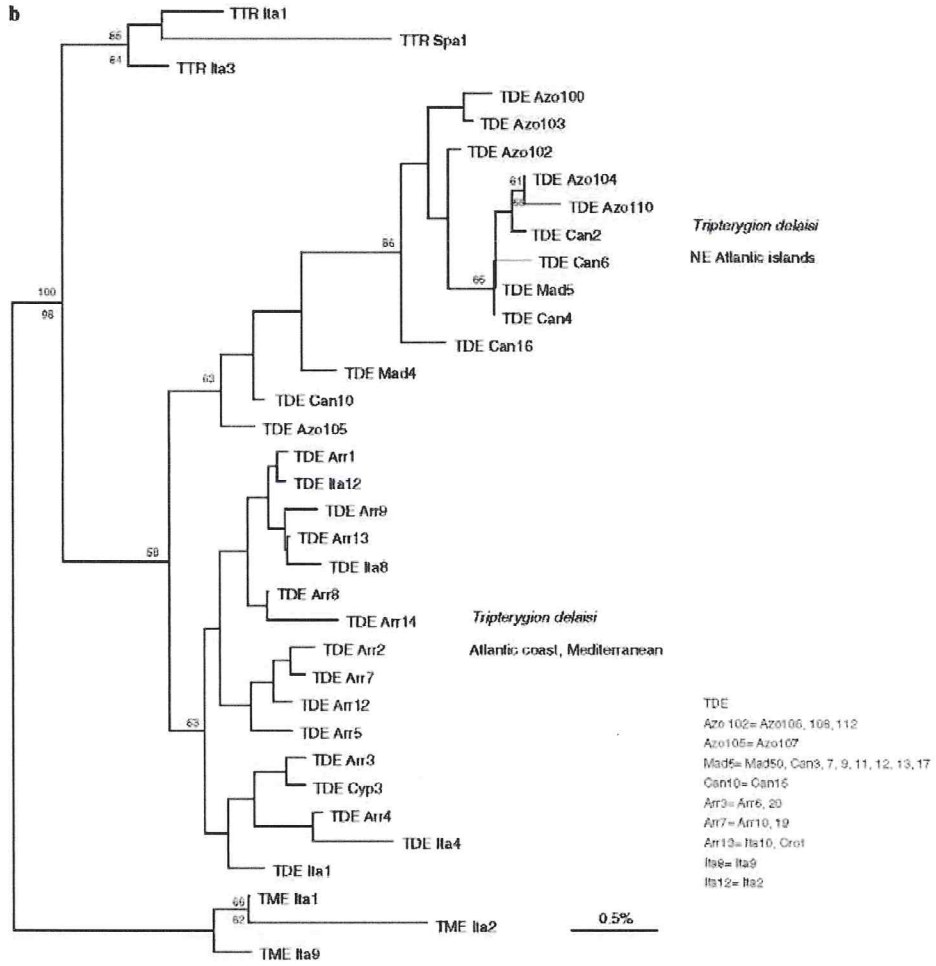


**Fig. 2** continued

Zander (1986) described Atlantic and Mediterranean subspecies of *T. delaisi*. However, both phylogenetic and population analysis in this study showed that the individuals from the Atlantic coast of Portugal, like the one from norwestern Spain included in Carreral-Carbonell et al. (2005), are more closely related to the Mediterranean fish than to those from the Atlantic islands. This biogeographic pattern is also seen in other sister species such as the blennids *Parablennius parvicornis*/*P. sanguinolentus* (Almada et al. 2005a), and the pomacentrids *Chromis limbata*/*C. chromis* (Domingues et al. 2005). In these cases, one of the species is restricted to the Macaronesian islands and the Atlantic tropical coast of Africa, while the other is found in the Mediterranean and Atlantic coast of southwest Europe. In the blenniids, a family closely related to the tripterygids, Almada et al. (2005b) showed that at least two clades (*Macrolipophrys* and *Parablennius*) have species in Tropical Africa that have counterparts in the Mediterranean and adjacent Atlantic waters. The examples cited above and the distribution of the two *T. delaisi* clades point to the conclusion that, in many instances, the main split is not between coastal Atlantic and Mediterranean populations. Instead, many clades split in two groups: one comprising populations from western Africa, and often the Macaronesian islands,

and another including the Mediterranean plus a more or less extensive range of the northeastern Iberian Atlantic.

The times of coalescence of the eastern Atlantic islands population and the Mediterranean plus Arrábida population of *T. delaisi* were more recent than the onset of Pleistocene glaciations, which occurred, 1.7 Mya (Briggs 1996) (Table 3). This result shows a more recent separation of the two *T. delaisi* clades than what was suggested by Carreras-Carbonell et al. (2005). These authors estimated the evolutionary rate for 12SrDNA and 16SrDNA mitochondrial genes assuming that the speciation process of *Tripterygion* started when the Strait of Gibraltar reopened. Using this calibration they found that the two clades of *T. delaisi* were separated 1.23 ± 0.45 and 1.10 ± 0.49 Mya for 12S and 16S, respectively. Using the 12S genetic distances from Carreras-Carbonell et al. (2005), and applying an internally calibrated molecular clock based on the closely related blenniid geminate transisthmian pair *Ophioblennius atlanticus* and *O. steindachneri* (sequences available at GenBank), we estimated the time of separation of the two *T. delaisi* clades at 0.64 ± 0.23 Mya. This result is more in agreement with our coalescence time estimation, specially taking into account that the divergence time of two sister species has been shown to most likely be an overestimate of their actual coalescence time (Edwards and Beerli 2000).

The biogeographical hypothesis proposed by Zander (1980), considered the eastern Atlantic islands, especially the Azores, as speciation centers that exported colonizers to the Mediterranean and adjacent Atlantic coast. This hypothesis predicts an older coalescence time of the islands population in relation to the Mediterranean and adjacent Atlantic coasts. The present results, however, do not support this prediction as the islands population showed a more recent origin than the Arrábida plus Mediterranean populations for the mitochondrial control region sequences (Table 3). Moreover, although migration between the islands and Arrábida plus Mediterranean was restricted, it showed a westbound trend, with a higher number of migrants going from the Arrábida plus Mediterranean into the islands [22.04 (± 1.10)/μ] than the opposite [0.09 (± 0.27)/μ; Fig. 1].

Our results, are consistent with the hypotheses of Wirtz (1978, 1980) and Almada et al. (2001), that consider successive dispersal events from the western African coast which could follow two routes: (a) one that would successively colonize the Atlantic islands; (b) another directed northwards, along the continental coast of Africa, that would colonize the Mediterranean. The southern Mediterranean would act as a refugium during glacial periods and as a secondary speciation center for warm water species, which would colonize the Atlantic shores of the Iberian Peninsula (and in some cases the western European shores more to the north), and northwestern Africa, during interglacial periods like the present one (Almada et al. 2001). Indeed, during some glacial maxima (e.g. 18,000 years ago) the polar front, with water temperatures of 4°C, moved as far south as 37°N (Dias et al. 1997). These extremes of low temperature must have eliminated all tropical, subtropical, and warm temperate fauna along the shores of southwestern Europe and northwestern Africa. Thus, *T. delaisi* Arrábida population might have been the result of a post-glacial colonization from the Mediterranean. The low genetic diversity of this population shown for the mitochondrial control region supports this hypothesis.

This scenario is also in agreement with the speciation processes proposed by Jonge and Videler (1989) and Geertjes et al. (2001) for the other Mediterranean triplefins. These authors consider different processes of speciation, all occurring within the Mediterranean, which has acted as a refugium during the Pleistocene glaciations.

Our hypothesis is reinforced by the lack of records of *Tripterygion* in the African Atlantic coast from Casablanca south to Senegal (Brownell 1978). Recent studies of coastal fishes in the Sahara coast have not recorded specimens of this triplefin blennioid (Falcón et al. 2002). It is likely that the situation is similar to that of the sister species *Parablenius parvicornis* and *P. sanguinolentus*. *P. parvicornis* occurs in tropical Africa and the Macaronesian islands while *P. sanguinolentus* are restricted to the Mediterranean and adjacent north Atlantic waters (Almada et al. 2005a). This species pair is also absent from the western African coast north of Cape Blanco (Mauritania). It seems that the African upwelling has played an important role as a biogeographical barrier for littoral species, promoting the divergence of the tropical populations and those of the Mediterranean and adjacent Atlantic.

The presence of *T. delaisi* in the Macaronesian islands could be related to the larval transport from the African coast by water filaments that reach the Canaries (Barton et al. 1998; Rodríguez et al. 1999), or to the maintenance of the populations in the warmer zones of the islands (Madeira or the westernmost Canary Islands) during the last glacial period, as it seems to have occurred in other cases (Miller 1984). The complex eddies system of the Canary Current may have allowed a northwards dispersal to the Azores (Stramma 1984; Santos et al. 1995).

In this study, we show the existence of two highly divergent clades of *Tripterygion delaisi*, that correspond to the two clades identified by Carrera-Carbonell et al. (2005). Are these two clades equivalent to the two subspecies *Tripterygion delaisi xanthosoma* and *T. d. delaisi* defined by Zander and Heymer (1970)? With molecular data only, this question cannot be adequately answered. Detailed morphological, behavioral, biogeographical and genetic work encompassing the entire distribution area of what is now called *T. delaisi* is urgently needed to address this issue. The study of specimens from Senegal is particularly important in this respect.

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# Chapter 6

## Mitochondrial and nuclear markers reveal isolation by distance and effects of Pleistocene glaciations in the northeastern Atlantic and Mediterranean populations of the white seabream (*Diplodus sargus*, L.)

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# Mitochondrial and nuclear markers reveal isolation by distance and effects of Pleistocene glaciations in the northeastern Atlantic and Mediterranean populations of the white seabream (*Diplodus sargus*, L.)

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**Abstract**

Phylogeography of *Diplodus sargus* from the northeastern Atlantic and the Mediterranean was assessed using sequences from the mitochondrial control region and the first intron of the S7 ribosomal protein gene. The relationship between genetic and geographic distances supported an isolation by distance model, with the Azores having a peripheral position. The geographic distribution of the genetic diversity, together with the historical demography of the populations studied can be explained by the effect of the Pleistocene glaciations in the northeastern Atlantic warm water fauna. *D. sargus* might have disappeared from western Europe during glacial peaks and suffered considerable demographic reductions in the Canaries and Mauritania, surviving in less affected areas such as Madeira, Azores and the Mediterranean. The mismatch analysis and the Fu's *Fs* values provide clear evidence of expansion in western Iberia (S. Pedro), Canaries, Mauritania and also in the eastern Mediterranean. Atlantic and Mediterranean populations of *D. sargus* showed no signs of genetic differentiation. *D. sargus* are active swimmers that can undergo extensive movements along the shores. This and the presence of planktonic eggs and larvae would allow rapid mixing between Mediterranean and Atlantic fish, erasing signs of population differentiation.


*Keywords:* *Diplodus sargus*; Isolation by distance; Northeastern Atlantic; Mediterranean; Pleistocene glaciations

## 1. Introduction

*Diplodus sargus* is an Atlantic–Mediterranean species complex belonging to the family Sparidae, with only one subspecies living out of this area. The complex includes six subspecies: *D. s. sargus* in the Mediterranean

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and Black Sea; *D. s. cadenati* in the eastern Atlantic (from the Bay of Biscay to Senegal including the islands of the Azores, Madeira and Canaries); *D. s lineatus* which is endemic to Cape Verde islands; *D. s. helenae* in St. Helena island; *D. s ascensionis* in Ascension island; and *D. s. kotschyi* from the Persian Gulf and northern Indian Ocean. Another subspecies of the complex, *Diplodus sargus capensis*, which occurs from Angola to Mozambique and southern Madagascar, has been recently considered a species (*Diplodus capensis*) by Heemstra and Heemstra (2004).

The diversification of *D. sargus* seems to have resulted from a rapid series of colonization events originated in the eastern Atlantic, which is supported by morphological (De la Paz et al., 1973) and molecular (Summerer et al., 2001) phylogenies.

Like other Sparids, *D. sargus* has commercial value and is of great interest for aquaculture. Young are euryhaline, entering brackish waters and lagoons in the spring. The adults can be found in a diverse range of habitats, including coastal rocky reefs, sandy bottoms, and seagrass beds (*Posidonia oceanica* in the Mediterranean). They congregate in schools of 5–50 individuals, feeding on polychaetes, mollusks and sea urchins (Bauchot and Hureau, 1986; pers observ.). *D. sargus* are non-guarders and have pelagic eggs and larvae.

Bargelloni et al. (2005) assessed the extent of genetic differentiation of *D. sargus* in the Mediterranean including a sample from the Atlantic immediately outside of the Gibraltar Strait. Results, based on the analysis of allozymes and a fragment of the mitochondrial control region, showed lack of population structure and no appreciable genetic differences between the Mediterranean and Atlantic samples. A molecular phylogeny by Summerer et al. (2001) yielded similar results.

Although the Mediterranean populations of *D. sargus* have been studied, little is known about the distribution of genetic variability within the Atlantic. The northeastern Atlantic has experienced severe climatic and sea level fluctuations during the Pleistocene glaciations (Briggs, 1996; Adams et al., 1999). Current research suggests that these glaciation events were very influential in shaping patterns of genetic variability and the geographic distribution of marine fauna from this region (Almada et al., 2001; Domingues et al., 2006, 2007; Stefanni et al., 2006). The warm-water fauna of the most affected regions like the western coast of Europe and, to some extent, the Azores and Canaries islands (Crowley, 1981; Dias et al., 1997) must have not survived in those regions. Most of the organisms now present would have recolonized these areas after the glaciating events from some southern regions such as Madeira, the western African tropical coast, or the Mediterranean (Briggs, 1974; Miller, 1984; Santos et al., 1995).

Previous phylogeographic and phylogenetic studies of warm water benthic fish of the northeastern Atlantic (*Chromis limbata*, Domingues et al., 2006; *Tripterygion delaisi*, Domingues et al., 2007) showed that the postglacial recolonization 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 refugia. On the contrary, a study on *Liphophris pholis*, a benthic fish more tolerant to cooler waters, showed a high level of differentiation for the Azorean population, strongly suggesting that it survived the modest sea cooling of the Azores during the glaciations (Stefanni et al., 2006).

In this paper we tested the hypothesis that the relationships among the populations of *D. sargus* follow a pattern characterized by isolation by distance combined with substantial gene flow among populations. This hypothesis stems from the following features of *D. sargus*: 1) the species is benthopelagic and not benthic, which means that, dispersal is achieved not only by eggs and larvae but also by adults that can move to depths much greater (deeper than 50 m in the Atlantic, Bauchot and Hureau, 1986) than those tolerated by the species included in the studies mentioned above; 2) judging from the present day distribution of the species, it must have survived the drops in sea surface temperatures estimated for Mauritania, Madeira and the Azores (Crowley, 1981) and the Mediterranean (Thiede, 1978) having became extinct in the Atlantic shores of western Europe (Dias et al., 1997) and perhaps in the further eastern islands of the Canaries (Crowley, 1981).

In this study we focus on the phylogeography of *D. sargus* covering eastern and western Mediterranean, and the northeastern Atlantic including the Azores, Madeira and Canaries archipelagos and also the Portuguese and Mauritanian coasts. Sequences from a fragment of the mitochondrial control region and the 1st intron of S7 ribossomal protein gene were obtained and genetic diversities, gene flow levels and historical demography of the populations were determined. Results were interpreted in light of palaeoclimatic events, contemporary oceanic current patterns and the ecology of the species.

## 2. Materials and methods

### 2.1. Sample collection and laboratorial procedures

Individuals of *D. sargus* were obtained from five locations in the Atlantic and six locations in the

Mediterranean (Fig. 1 and Table 1). Additionally, one individual of *Diplodus vulgaris* was collected from São Pedro and used as outgroup. Fish were collected by spear fishing while scuba diving, by line fishing or with hand nets in tide pools. Sampled individuals were non-spawning adults. Fin clips were cut immediately after collection of the individuals and stored at ambient temperature in 95% ethanol. Total genomic DNA was extracted by SDS proteinase K procedure and purified by standard chloroform and isopropanol precipitation (Sambrook et al., 1989). Amplification of the 5′ hypervariable portion of the mitochondrial control region (also called D-loop) was accomplished with universal primers L-Pro1 and H-DL1 (Ostellari et al., 1996), and used a cycling profile of 1 min at 92 °C, 1 min at 50 °C, 1 min at 72 °C, for 30 cycles. Each 13 µl reaction contained 5–50 ng of DNA, 10 mM Tris HCL (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 1.25 u of *Taq* DNA polymerase (Perkin-Elmer, Norwalk, Conn.), 150 mM of each dNTP, and 0.3 mM of each primer.

In addition, we amplified and sequenced the first intron of the S7 ribosomal protein gene, using the primers S7RPEX1F and S7RPEX2R (Chow and Hazama, 1998) and an annealing temperature of 56 °C. PCR amplification was performed as described for the mitochondrial control region. After purification following the manufacturer's protocol (Applied Biosystems, Forter City, CA), direct sequencing was performed with an ABI 3100 automated sequencer (Applied Biosystems).

### 2.2. Data analysis

#### 2.2.1. Mitochondrial control region

Sample size was increased with two mitochondrial control region sequences of *Diplodus sargus sargus* from Calvi (France) and two sequences of *D. s. cadenati* from Agadir (Morocco) available in GenBank (Accession numbers: AF365348 AF365349 AF365350 AF365351).

Sequences were aligned using the CLUSTAL V (Higgins et al., 1991) implemented by Sequence Navigator (Applied Biosystems). Gaps were not included in the analyses. Population diversity indexes (number of haplotypes, haplotype and nucleotide diversities, p-distances and % of private haplotypes) were calculated using the software package ARLEQUIN (vers. 2.000; Schneider et al., 2000).

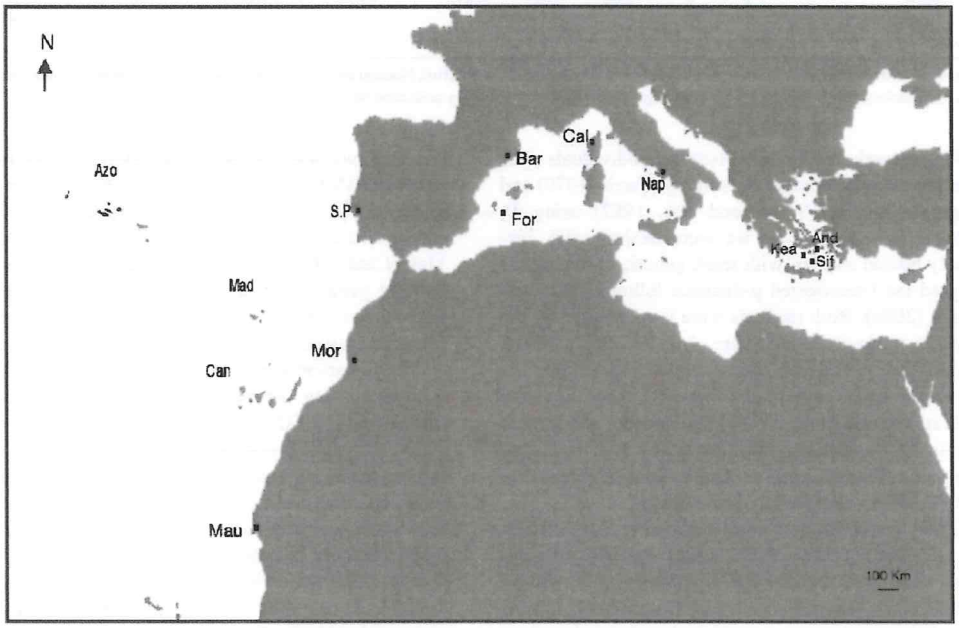


Fig. 1. *Diplodus sargus* sampling locations. Individuals included in this study were collected from the Mediterranean (western and eastern basins) and from the Atlantic in the European and African coasts as well as in the astern Atlantic islands of Azores, Madeira and Canaries. Labels are shown in Table 1.

Table 1
Collection localities of *Diplodus sargus* used in the present study and diversity indexes for mitochondrial control region

| Localities | N | Hn | Hd | $\pi$ | p-distance (average) | p-distance (max) | p-distance (min) | % private H | Collection date |
|---|---|---|---|---|---|---|---|---|---|
| *Atlantic* | | | | | | | | | |
| Portugal | | | | | | | | | |
| Azo—Azores (Faial) | 18 | 8 | 0.850 | 0.027 | 0.024 | 0.042 | 0.000 | 87.5 | Nov. 2003 |
| Mad—Madeira (Funchal) | 20 | 15 | 0.968 | 0.035 | 0.034 | 0.054 | 0.000 | 73.3 | Sep. 2003 |
| S.P—S. Pedro | 21 | 21 | 1.000 | 0.036 | 0.033 | 0.055 | 0.000 | 95.2 | Nov. 2003 |
| Spain | | | | | | | | | |
| Can—Canaries (Tenerife) | 15 | 14 | 0.990 | 0.034 | 0.032 | 0.047 | 0.000 | 85.7 | Nov. 2005 |
| Morocco | | | | | | | | | |
| Mor—(Agadir) | 2 | 2 | | | | | | | |
| Mauritania | | | | | | | | | |
| Mau—(Nouakchott) | 17 | 17 | 1.000 | 0.033 | 0.032 | 0.055 | 0.000 | 70.6 | Nov. 2005 |
| *Western Mediterranean* | | | | | | | | | |
| Spain | | | | | | | | | |
| For—Formentera | 1 | | | | | | | | Oct. 2003 |
| Bar—Barcelona | 27 | 23 | 0.971 | 0.024 | 0.022 | 0.04 | 0.000 | 78.3 | Dec. 2006 |
| France | | | | | | | | | |
| Cal—Calvi | 2 | 2 | | | | | | | |
| Italy | | | | | | | | | |
| Nap—Naples | 6 | 6 | 1.000 | 0.026 | 0.050 | 0.037 | 0.005 | 83.3 | Oct. 2003 |
| *Eastern Mediterranean* | | | | | | | | | |
| Greece | | | | | | | | | |
| Kea—Kea | 9 | 9 | 1.000 | 0.028 | 0.027 | 0.045 | 0.005 | 55.6 | Jun. 2006 |
| Sif—Sifnos | 8 | 8 | 1.000 | 0.033 | 0.032 | 0.050 | 0.003 | 100 | Jun. 2006 |
| And—Andros | 1 | | | | | | | | Aug. 2006 |
| Total | 147 | | | | | | | | |

Number of individuals (N), number of haplotypes (Hn), Haplotype diversity (Hd), Nucleotide diversity ($\pi$), Uncorrected p–distances and percentage of private haplotypes (% private H) for each population are shown. Samples collection date is shown for each location in the last column.

Phylogenetic relationships between individuals were assessed using Maximum Parsimony (Farris, 1970) and Neighbor–Joining (Saitou and Nei, 1987) using *D. vulgaris* as outgroup. As we were dealing with very closely related species, with small genetic distances, we adopted the Uncorrected p-distance following Nei and Kumar (2000). Both methods were implemented by the software package PAUP (vers. 4.0; Swofford, 1998). Topological confidence was evaluated for Maximum Parsimony and Neighbor–Joining with 1000 bootstrap replicates (Felsenstein, 1985). Uncorrected p-distances for all the populations were estimated and visualized using a multidimensional scaling analysis performed in STATISTICA (version 7.1; Statsoft Inc.).

Gene flow (Fst) was estimated using ARLEQUIN (vers. 2.000; Schneider et al., 2000). Pairwise comparisons were estimated for the populations with similar sample sizes. Samples from the three Greek islands (Kea, Sifnos and Andros) were analyzed together since they are geographically very close. Corrections for simultaneous multiple comparisons were applied using sequential Bonferroni correction (Rice, 1989). Population structure was assessed by an analysis of molecular variance (AMOVA; Excoffier et al., 1997) implemented in ARLEQUIN (vers. 2.000; Schneider et al., 2000). To test for isolation by distance (IBD) we applied the Mantel test (Mantel, 1967) to two matrices, Fst values and log geographical distances in Km between localities. We used IBD 1.4 (Bohonak, 2002) to perform the Mantel test, using 1000 replicates to test significance.

The historical demography of each population was examined using mismatch distributions analysis performed in ARLEQUIN (vers. 2.000; Schneider et al., 2000). Theoretical studies have shown that populations in long stable demographic equilibrium show a chaotic mismatch distribution, while recent rapid population expansions or bottlenecks are reflected in a unimodal (approximately Poisson) profile (Rogers, 1995; Rogers and Harpending, 1992). Mismatch distributions were established and their fit to Poisson distributions was assessed by Monte Carlo simulations of 1000 random samples. The sum of square deviations (SSD) between observed and expected mismatch distributions was used as a test statistics, its *P* value representing the probability

of obtaining a simulated SSD larger or equal to the observed one (Schneider and Excoffier, 1999). In addition Fu's *F*s neutrality test (Fu, 1997) was used to detect possible population expansions.

*2.2.2. First intron of the S7 ribosomal protein gene*

Sequences were aligned using the CLUSTAL V implemented by Sequence Navigator (Applied Biosystems). Heterozygous positions could not be ascribed to each sequence of each individual. Thus, we scored and converted those positions into a codominant genetic marker dataset. Linkage disequilibrium was tested using the program GENETIX 4.04 (Belkhir et al., 1996–2004) and only unlinked loci were used in subsequent analyses. Samples from the Mediterranean were analyzed in two populations: western Mediterranean (Barcelona and Naples) and eastern Mediterranean (Greek islands). Gene diversity, allelic frequencies, observed and expected heterozygosity and exact probability tests for deviations from Hardy–Weinberg equilibrium (HWE) were performed using the same program. All tests were conducted using 1000 permutations. The proportion of shared alleles (Ps) for pairs of populations was calculated as the number of shared alleles summed over loci divided by twice the number of loci, as in Bowcock et al. (1994). A genetic distance matrix between pairs of populations was obtained by-ln (Ps). UPGMA cluster analysis was conducted using the PHYLIP software package (Felsenstein, 1989).

Fst, population structure and isolation by distance were estimated using the same procedures as for the mitochondrial control region fragment.

## 3. Results

### *3.1. Genetic diversity and phylogenetic analysis*

*3.1.1. Mitochondrial control region*

A total of 143 *D. sargus* mitochondrial control region sequences were obtained (GenBank Accession numbers: EF468518–EF468623). Fragments were 385 bp long. All populations showed high genetic diversity (Table 1). Phylogenetic trees recovered using Maximum Parsimony and Neighbor–Joining yielded similar topologies (Fig. 2). The phylogenetic trees showed no partition between Atlantic and Mediterranean populations of *D. sargus*. Four haplotypes (H5, H9, H10 and H11; Fig. 2) were shared between the Atlantic and Mediterranean and samples from both regions grouped together. Clades containing haplotypes from only one region showed very low bootstraps values.

*3.1.2. First intron of the S7 ribosomal protein gene*

The first intron of the S7 ribosomal protein gene was amplified for a total of 127 individuals (GenBank Accession numbers: EF467669–EF467796). A total of 308 bp were successively sequenced. Individuals differed only in base frequencies in heterozygous positions. A total of 47 polymorphic positions were found. As we could not ascribe the two alleles of heterozygous positions to one of the sequences of each individual, we used those positions as a dataset of codominant genetic markers. After eliminating loci that were shown to be linked, our dataset was represented by 13 loci. Allelic frequencies of these 13 loci are shown in Table 2. None of the loci showed deviations from Hardy–Weinberg equilibrium. Gene diversities estimated based on the 13 loci are also shown in Table 2. The Azores appears as the least genetically diverse population.

### *3.2. Population structure*

The relationship across populations based on the genetic distances calculated for the mitochondrial control region can be visualized in the form of a multidimensional scaling plot (Fig. 3). This plot shows the Azores as the most differentiated population. The UPGMA tree built using the distance based on the proportion of S7 intron shared alleles (Fig. 4) also shows the Azores as a more differentiated population.

Gene flow was high between all the populations (Table 3). Fst estimates, based on the mitochondrial control region sequences, between the Azores and all the other population were higher than all other pairwise comparisons, suggesting the Azores as the most isolated population. Fst values between Atlantic and Mediterranean populations also yielded significant values. The isolation of the Azores and Mediterranean is not shown by the Fst values estimated from the S7 data. Indeed, this marker yielded very low and not significant Fst values for all pairwise comparisons except for Azores versus the Greek islands. To assess the existence of population structure within *D. sargus* we applied an AMOVA analysis. Additionally, to evaluate the differentiation between Atlantic and Mediterranean we applied an AMOVA grouping population into Atlantic and Mediterranean groups. Results showed lack of population structure for *D. sargus*. In both cases, a high percentage of the variance in the data derived from within-population variance (Table 4). Only a very small percentage of the data variance was attributable to the separation of Atlantic and Mediterranean populations and, in the case of D-loop, this value was very similar to the variance among populations within each group

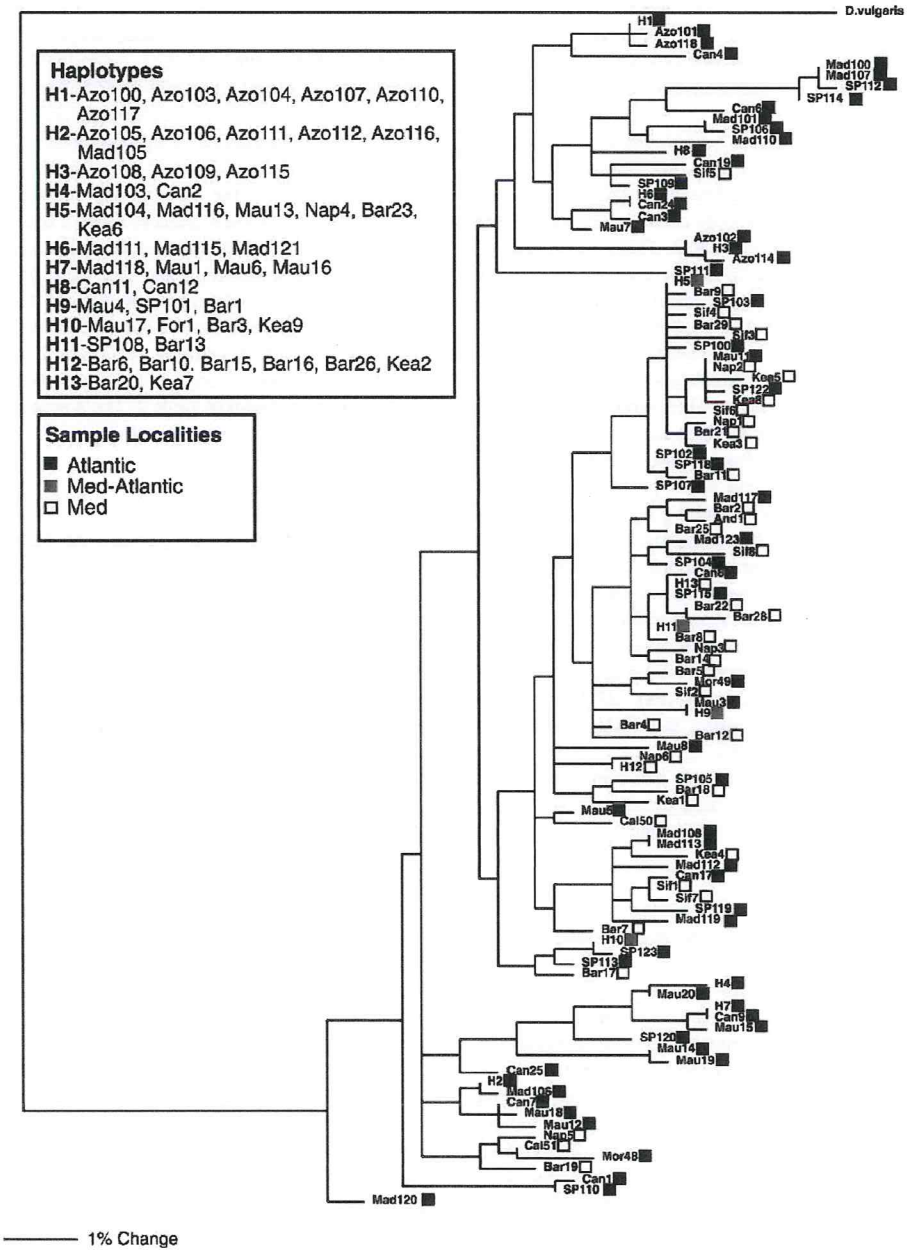


Fig. 2. Phylogenetic relationships of Atlantic and Mediterranean *D. sargus* using *D. vulgaris* as outgroup. None of the major branches showed bootstrap supports above 50%. Labels are described in Table 1. The length of each branch is proportional to the number of nucleotide substitutions. Scale bar: 1% uncorrected p genetic distance.

Table 2
Sample size (N), Gene diversity and allelic frequencies obtained from the heterozygous positions of the first intron of the S7 ribosomal gene sequences

| | Azo | Mad | Can | Mau | S.P | W. Med | E. Med |
|---|---|---|---|---|---|---|---|
| N | 17 | 17 | 11 | 11 | 21 | 33 | 18 |
| Gene diversity | 0.567 | 0.741 | 0.758 | 0.684 | 0.785 | 0.754 | 0.787 |
| *Locus 1* | | | | | | | |
| Freq. Allele 1 | 0.000 | 0.029 | 0.000 | 0.045 | 0.048 | 0.045 | 0.056 |
| Freq. Allele 2 | 1.000 | 0.971 | 1.000 | 0.955 | 0.952 | 0.955 | 0.944 |
| *Locus 2* | | | | | | | |
| Freq. Allele 1 | 0.000 | 0.088 | 0.000 | 0.091 | 0.071 | 0.000 | 0.000 |
| Freq. Allele 2 | 1.000 | 0.912 | 1.000 | 0.909 | 0.929 | 1.000 | 1.000 |
| *Locus 3* | | | | | | | |
| Freq. Allele 1 | 0.000 | 0.000 | 0.000 | 0.000 | 0.024 | 0.000 | 0.056 |
| Freq. Allele 2 | 1.000 | 1.000 | 1.000 | 1.000 | 0.976 | 1.000 | 0.944 |
| *Locus 4* | | | | | | | |
| Freq. Allele 1 | 0.000 | 0.059 | 0.091 | 0.045 | 0.024 | 0.030 | 0.083 |
| Freq. Allele 2 | 1.000 | 0.041 | 0.909 | 0.955 | 0.976 | 0.970 | 0.917 |
| *Locus 5* | | | | | | | |
| Freq. Allele 1 | 0.000 | 0.000 | 0.045 | 0.000 | 0.024 | 0.030 | 0.000 |
| Freq. Allele 2 | 1.000 | 1.000 | 0.955 | 1.000 | 0.976 | 0.970 | 1.000 |
| *Locus 6* | | | | | | | |
| Freq. Allele 1 | 0.147 | 0.176 | 0.182 | 0.045 | 0.071 | 0.106 | 0.083 |
| Freq. Allele 2 | 0.853 | 0.824 | 0.818 | 0.955 | 0.929 | 0.894 | 0.917 |
| *Locus 7* | | | | | | | |
| Freq. Allele1 | 0.118 | 0.000 | 0.000 | 0.045 | 0.071 | 0.015 | 0.028 |
| Freq. Allele 2 | 0.882 | 1.000 | 1.000 | 0.955 | 0.929 | 0.985 | 0.972 |
| *Locus 8* | | | | | | | |
| Freq. Allele 1 | 0.235 | 0.324 | 0.318 | 0.273 | 0.333 | 0.545 | 0.611 |
| Freq. Allele 2 | 0.765 | 0.676 | 0.682 | 0.727 | 0.667 | 0.455 | 0.389 |
| *Locus 9* | | | | | | | |
| Freq. Allele 1 | 0.000 | 0.000 | 0.045 | 0.000 | 0.000 | 0.000 | 0.028 |
| Freq. Allele 2 | 1.000 | 1.000 | 0.955 | 1.000 | 1.000 | 1.000 | 0.972 |
| *Locus 10* | | | | | | | |
| Freq. Allele 1 | 0.000 | 0.000 | 0.045 | 0.000 | 0.024 | 0.000 | 0.000 |
| Freq. Allele 2 | 1.000 | 1.000 | 0.955 | 1.000 | 0.976 | 1.000 | 0.000 |
| *Locus 11* | | | | | | | |
| Freq. Allele 1 | 0.000 | 0.029 | 0.000 | 0.000 | 0.024 | 0.000 | 0.000 |
| Freq. Allele 2 | 1.000 | 0.971 | 1.000 | 1.000 | 0.976 | 1.000 | 1.000 |
| *Locus 12* | | | | | | | |
| Freq. Allele 1 | 0.000 | 0.029 | 0.091 | 0.045 | 0.024 | 0.076 | 0.083 |
| Freq. Allele 2 | 1.000 | 0.971 | 0.909 | 0.976 | 0.924 | 0.917 | 0.949 |
| *Locus 13* | | | | | | | |
| Freq. Allele 1 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.015 | 0.000 |
| Freq. Allele 2 | 0.000 | 0.000 | .0000 | .0000 | 0.000 | 0.015 | 0.000 |
| Freq. Allele 3 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 0.970 | 1.000 |

Frequencies are shown for the 13 unliked loci used in the study. Labels are described in Table 1.

(Table 4). The IBD test showed a significant correlation between Fst and log geographical distances ($r^2=0.542$ and 0.688; $P=0.018$ and 0.011 for D-loop and S7 respectively), pointing to the existence of genetic isolation by geographic distance.

### 3.3. Historical demography

Mismatch distributions based on the mitochondrial control region sequences were estimated (Fig. 5) and SSD tests were performed (Table 5). The model of sudden expansion was only rejected for the Azores and Barcelona, although visual inspection of the mismatch distribution show a unimodal profile for the later population. $P$ values for Madeira were close to the limit of rejection. Fu's $Fs$ values were significantly negative for all the populations except for the Azores and Madeira.

Estimates of expansion parameter $\tau$ were similar for all the populations. This parameter is equal to $2t\mu$,

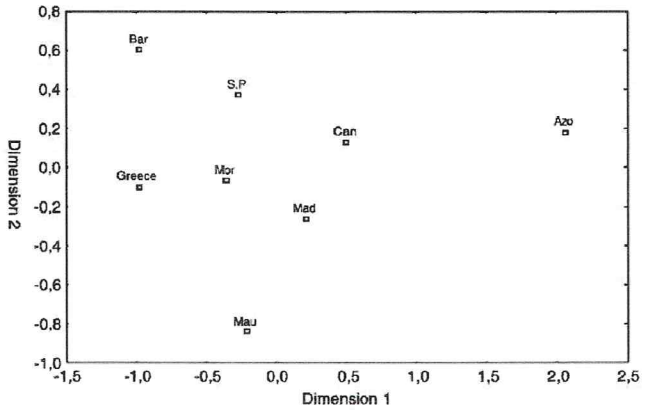


Fig. 3. Multidimensional scaling based on *Diplodus sargus* mitochondrial control region Uncorrected p-distances. See Table 1 for labels.

where $t$ is the time of the expansion and $\mu$ is the mutation rate. Thus, population expansion occurred at approximately the same time in all samples. Although estimates of $\theta$ are less accurate than $\tau$ (Schneider and Excoffier, 1999), values of $\theta_0$ are similar and very low, suggesting that *D. sargus* has undergone a bottleneck before the population expansion.

## 4. Discussion

Our data confirm the results of other molecular phylogenies (Summerer et al., 2001; Bargelloni et al., 2005) which found no appreciable genetic differences between Atlantic and Mediterranean *D. sargus* and no evidence supporting the distinction between *D. s. cadenati* and *D. s. sargus*. Bargelloni et al. (2005) considered two hypotheses to explain this lack of differentiation. Either *D. sargus* is a recent immigrant in the Mediterranean or historical bottlenecks and recolonization processes prevented strong differentiation of Atlantic and Mediterranean basins. Our data showed high genetic diversities for the Mediterranean, even in the eastern basin, and lack of clear evidence of population expansion in the western basin. These findings make a recent invasion of the Mediterranean unlikely.

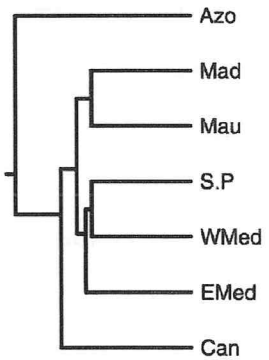


Fig. 4. Phenogram based on the genetic distance obtained from the proportion of S7 intron shared alleles between *Diplodus sargus* populations. The phenogram was estimated using the UPGMA cluster analysis. See Table 1 for labels.

The relationship between genetic and geographic distances supported an isolation by distance model as predicted by the hypothesis outlined in the introduction. If we consider the Fst values and the Uncorrected p-distances for the mitochondrial control region (Table 3 and Fig. 3), together with the pattern of S7 ribosomal protein gene shared alleles (Fig. 4) a number of features emerge: 1) the peripheral position and low genetic diversity of the Azores; 2) the substantial connection among the Azores, Madeira and Canary islands; and 3) the proximity of western Portugal and the Mediterranean. The same pattern was also described for other species such as *Ophioblennius atlanticus* (Muss et al., 2001), *Chromis limbata* (Domingues et al., 2006) and *Tripterygion delaisi* (Domingues et al., 2007).

The Fsts computed from S7 were generally lower than those for the D-loop. This is not surprising since, because mtDNA is haploid and only maternally inherited, mitochondrial genes have a fourfold lower effective population size than the nuclear ones, which makes fixation of mutations much slower in the nuclear genes. The only significant difference was found when comparing the Azores with the Greek islands, which are the most geographically distant sites. Thus, overall our

Table 3
Fst values for *Diplodus sargus* populations calculated from mitochondrial control region sequences (below the diagonal) and S7 intron (above the diagonal) using ARLEQUIN (version 2.000 Schneider et al., 2000)

| | Azores | Madeira | Canaries | Mauritania | S. Pedro | Barcelona | Greek islands |
|---|---|---|---|---|---|---|---|
| Azores | | 0.006 | 0.005 | 0.000 | 0.000 | 0.077 | 0.118* |
| Madeira | 0.173* | | 0.000 | 0.000 | 0.000 | 0.030 | 0.055 |
| Canaries | 0.142* | 0.000 | | 0.000 | 0.000 | 0.019 | 0.042 |
| Mauritania | 0.216* | 0.047 | 0.060 | | 0.000 | 0.040 | 0.068 |
| S. Pedro | 0.190* | 0.018 | 0.045 | 0.049* | | 0.025 | 0.047 |
| Barcelona | 0.328* | 0.150* | 0.196* | 0.139* | 0.048* | | 0.000 |
| Greek islands | 0.300* | 0.096* | 0.147* | 0.100* | 0.017 | 0.027 | |

Significant $P$ values ($P< 0.05$) after Bonferroni correction are indicated by an asterisk.

results provide evidence supporting our hypothesis, specifically suggesting that the Azorean population persisted through the glacial cycles, and that gene flow is probably substantial among populations conforming to a pattern of isolation by distance.

The geographic distribution of the genetic variability shown by our data is easily explained by the paleo–biogeographic history of the eastern Atlantic. Briggs (1974) proposed that the severe Pleistocene climatic fluctuations that occurred in the northeastern Atlantic (Adams et al., 1999) had a strong impact in the coastal fauna of the region, leading to local extinctions and latitudinal shifts of many taxa. The coasts of Biscay and western Iberia were particularly affected by a very pronounced southern migration of the polar front (Crowley, 1981; Dias et al., 1997) reaching temperatures too low for *D. sargus* to survive. The sea surface temperatures in the Azores region decreased about 2–3 °C (Crowley, 1981). Such a decline would bring the sea surface temperatures at the Azores to values similar to those prevailing nowadays in western Iberia where *D. sargus* breeds and grows successfully. Thus, it is unlikely that the Azorean population was eliminated by the glaciations. The archipelago of Madeira, located further south, was even less affected, while the Canaries (at least the eastern islands) were severely affected due to its proximity to the continent and to the influence of upwelling effects (Barton et al., 1998). The Mauritanian population, being geographically close to the thermal stable tropical coast of Africa must have quickly received fish from the southern refugia after the Pleistocene glaciations and acted later as a source for the northern colonization. Taking this into consideration, recent research suggests that Saharan upwelling filaments are capable of transporting larvae from the African neritic zone into oceanic areas and towards the Canary archipelago (Rodríguez et al., 1999, 2004). Indeed, *D. sargus* from Madeira and Mauritania showed high genetic diversities and low percentages of private haplotypes (Table 1). Another region where warm water pockets persisted during the Pleistocene, although in areas much smaller than today, is the Mediterranean (Thiede, 1978). The mismatch analysis and the Fu's *Fs* values showed a clear population expansion for the eastern Mediterranean basin (Greek islands), but not for the western basin (Barcelona). The historical demography of *D. sargus* based on the mitochondrial control region supports the hypothesis outlined above, which assumes that *D. sargus* disappeared from the Atlantic shores of Europe during glacial peaks and suffered considerable demographic reductions in the Canaries and Mauritania, surviving in Madeira, Azores and in some regions of the Mediterranean. Interestingly, the values of Fu's *Fs* and SSD only provide clear evidence of expansion in western Iberia (S. Pedro), Canaries, Mauritania and the Greek islands.

Table 4
Results of hierarchical analysis of molecular variance (AMOVA)

| | Among groups | Among populations within groups | Within populations |
|---|---|---|---|
| All populations | – | 12.20*/2.76* | 87.80/97.24 |
| Mediterranean vs Atlantic | 7.41*/6.16 | 7.13*/−0.84 | 84.86*/94.69* |

Percentage of the data variance are shown for the mtDNA control region and the first intron of S7 ribossomal gene respectively. Significant values ($P<0.05$) are indicated by an asterisk.

The dual pattern of refugia in the western tropical African coast and Madeira and in the Mediterranean, probably explains splits between sister taxa like those between *Parablennius parvicornis* and *P. sanguinolentus* (Almada et al., 2005); *Chromis limbata* and *C. chromis* (Domingues et al., 2006); or the forms of *Tripterygion delaisi* from the oceanic islands and the Mediterranean populations (Domingues et al., 2007). Why then does *D. sargus* lack the patterns of population differentiation that seem to be common in other warm water species previously examined? It is important to

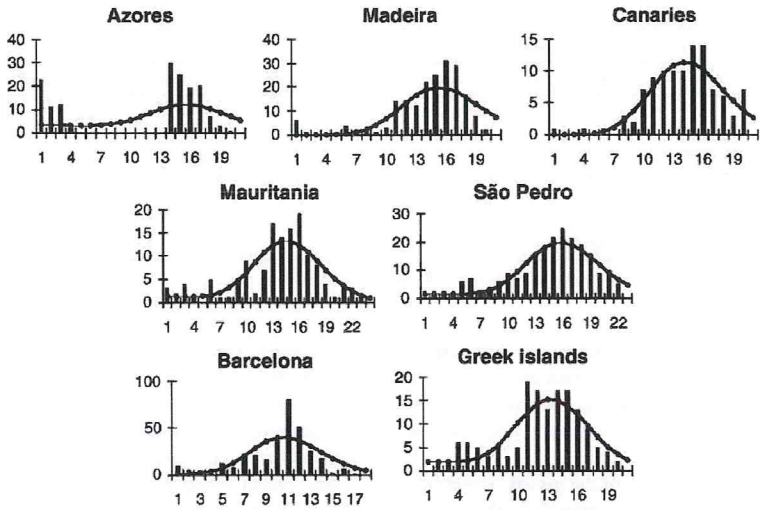


Fig. 5. Mismatch distribution for *Diplodus sargus* populations based on the mitochondrial control region. The bars represent the observed frequency of the pairwise differences among haplotypes, while the line shows the expected curve predicted for a population that has undergone a demographic expansion in the past.

note that several of the species studied so far were rocky littoral or sub-littoral species that as adults show very restricted movements and are confined to the upper meters of the water column (*P. sanguinolentus* 1 m, Zander, 1986; *T. delaisi* 3–40 m, Wirtz, 1978; *C. limbata* 5–45 m, Allen, 1991). In addition, blennids, tripterygiids and pomacentrids all show male parental care of demersal eggs, meaning that the planktonic phase is restricted to the larval stage. *D. sargus* spawns planktonic eggs, attains a size that is much larger than the species mentioned above and both juveniles and adults are active swimmers. Although precise data on the extent of their movements could not be found, it is likely that they can undergo extensive movements along the shores, a possibility that is absent for the adults of blenniids and tripterygiids and even probably small pomacentrids like *Chromis*. This mobility of the adults would allow rapid mixing between Mediterranean and Atlantic fish. On the other hand, not being a strictly benthic fish, *D. sargus* may have profited, at least occasionally, from the numerous submarine banks and seamounts that have been mapped between the European mainland coast and the Azores archipelago (Kitchingman and Lai, 2004; Kitchingman et al., 2007). It is known that *D. sargus* can be found below 50 m in the Atlantic (Bauchot and Hureau, 1986). Several of the seamounts mentioned above reach such depths (eg. Gorringe-40 m, Lagabrielle and Auzende, 1982; Àmpere-18 m, Josephine-50 m, D. João de Castro-13 m, Cardigos et al., 2005). With sea level drops of

Table 5
Estimated values for the expansion model for each population: SSD (sum of square deviations) and its probability *P*; $\theta_0$ and $\theta_1$ (compound parameter representing the mutation rate and the female efective population size before and after expansion respectively); and $\tau$ (time in generations, upper and lower bounds of 95% CI in parenthesis) Fu's *Fs* neutrality test and its probability P

| | SSD | *P* | $\theta_0$ | $\theta_1$ | $\tau$ | Fu's *Fs* | *P* |
|---|---|---|---|---|---|---|---|
| Azores | 0.066 | 0.00 | – | – | – | 2.492 | 0.888 |
| Madeira | 0.014 | 0.08 | 0.000 | 5010 | 14.75 (10.43–18.00) | −1.822 | 0.228 |
| Canaries | 0.005 | 0.75 | 0.011 | 4730 | 13.66 (8.29–16.73) | −4.145 | 0.046 |
| Mauritania | 0.011 | 0.41 | 0.006 | 114.65 | 14.064 (7.13–17.31) | −8.145 | 0.000 |
| S.Pedro | 0.003 | 0.83 | 0.015 | 139.24 | 15.37 (9.29–18.88) | −11.670 | 0.000 |
| Barcelona | 0.027 | 0.00 | – | – | – | −11,067 | 0.001 |
| Greek islands | 0.007 | 0.55 | 0.001 | 78.61 | 12.93 (9.47–16.09) | −9.690 | 0.000 |

120–140 m in the glacial maxima (Lambeck et al., 2002) many of these seamounts were above sea water level, making the number of available stepping stones higher than today. If this interpretation is correct, we expect many other benthopelagic species, especially those with planktonic eggs, to show a less differentiated population structure than the benthic ones, when the Azores and Madeira are compared to southwestern Europe and the Mediterranean.

All phylogeographic studies published so far emphasize the strong affinities of the Azorean populations with those of Madeira, Canaries and western Africa. In general, the migratory flow tends to prevail towards the Azores. Santos et al. (1995) and references therein showed that eddies capable of transporting fish, eggs and larvae from Madeira towards the Azores are frequent in the area. This, associated with the probable local extinctions of warmer water species during the glaciations at this archipelago, likely combine to explain the low level of endemism of these islands which have puzzled marine biogeographers for many years (Briggs, 1974). More studies with different types of organisms with distinct ecology and life histories will help to improve our understanding of the present and past biogeography of the area.

## 5. Conclusions

The present work reveals no signs of differentiation between the Atlantic and Mediterranean populations of *D. sargus*, confirming previous results. Gene flow patterns of the northeastern Atlantic and Mediterranean populations of *D. sargus* follow an isolation by distance model, with the Azorean being more isolated and less diverse than the other populations. Gene flow seems, however, to be higher than in benthic fishes with demersal eggs studied in the same geographical area. The geographic distribution of the genetic diversity, together with the historical demography of the populations studied can be explained by the effect of the Pleistocene glaciations in the northeastern Atlantic warm water fauna. *D. sargus* might have disappeared from the Atlantic coast of Europe during glacial peaks and suffered population bottlenecks in the Canaries and Mauritania, surviving in Madeira, Azores and in the Mediterranean.

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# Chapter 7

## Molecular data confirm the validity of the Portuguese blenny (*Parablennius ruber*, Valenciennes, 1836) and its presence inWestern Europe

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# BRIEF COMMUNICATIONS

## Molecular data confirm the validity of the Portuguese blenny (*Parablennius ruber*, Valenciennes, 1836) and its presence in Western Europe

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DNA sequence analysis confirms the distinction between *Parablennius ruber* and *Parablennius gattorugine*, simultaneously validating the presence of the former species in Western Europe where it has been reported for >150 years. A possible scenario involving speciation of *P. ruber* at the Azores and subsequent transport of larvae to Europe, a process that may be still occurring nowadays, could explain this pattern of occurrence. 

Key words: Northeastern Atlantic; biogeography; Blenniidae; mitochondrial ribosomal DNA; speciation.

The Portuguese blenny (*Parablennius ruber*, Valenciennes, 1836) is a blenniid fish, which constitutes a major element of the rocky subtidal ichthyofauna of the Azores (Santos, 1987; Azevedo & Homem, 2002). Almada *et al.* (2005) showed, based on molecular data, that *P. ruber* is a sister species of *Parablennius gattorugine* (Linnaeus, 1758), with which it forms a very differentiated clade within the genus *Parablennius*. The similarity between the two species is so strong that, although described in the 19 century (Valenciennes, 1836), for many years, the validity of *P. ruber* was questioned and it was often not distinguished from *P. gattorugine*. The species was revalidated independently by Bath (1982) and by Almeida (1982). Both the authors showed that *P. ruber* differs from *P. gattorugine* in a number of morphological traits that include the morphology

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of the lateral line system and the shape of the orbital tentacles (Fig. 1). Surprisingly, although the species was commonly viewed as a fish from the Macaronesian Islands, both the original description of Valenciennes (1836) and the revalidation by Almeida (1982) were based on material collected in the shores of mainland Europe, from the western coast of France to the southern coast of Portugal. This is remarkable because *P. gattorugine* is quite abundant in both the Atlantic and the Mediterranean shores of Europe. The finding of these specimens of *P. ruber* indicates that, at least from the time of Valenciennes (1836), *P. ruber* is sympatric with *P. gattorugine* although surveys of European littoral fishes very rarely report its presence. In recent years, the presence of this species in the shores of western Britain and Ireland became clearly noted (pictures and records available at http://www.habitas.org.uk/marinelife/species.asp?item=ZG6370; B. Picton, pers. comm.). One of these pictures was also examined by P. Wirtz (pers. comm.) who also confirmed the identification.

Two distinct issues are addressed within this note: first, using the mitochondrial 12S and 16S rDNA, Azorean *P. ruber* samples are compared with those of *P. gattorugine* from the Mediterranean and from the Atlantic shores of Europe, in order to test, using molecular data, the consistency of the distinction between the two species as suggested by their morphologies. Additionally, the DNA of fish morphologically classified as *P. ruber* and caught in Northwest Portugal was compared both with material from *P. gattorugine* and with Azorean samples of *P. ruber*. This comparison was made to test the hypothesis that the morphological similarities between continental and insular fish classified as *P. ruber* reflect true genetic affinity.

Samples of *P. ruber* were collected from the Azores (Faial 38°40′ N; 27°10′ W, eight specimens, PRAZ1–PRAZ8) and from the northwest coast of mainland Portugal (Moledo do Minho, Caminha 41°50′ N; 8°50′ W, three specimens, PRMM1–PRMM3). Samples of *P. gattorugine* were collected from mainland Portugal (S. Pedro do Estoril, Cascais 38°41′ N; 09°25′ W, eight specimens, PGSP1–PGSP8), England (Plymouth 50°25′ N; 04°05′ W, one specimen, PGEN1), the Mediterranean from Eastern Italy (Chioggia 45°13′ N; 12°17′ E, two specimens, PGIT1 and PGIT2) and Eastern Greece (Lavrio

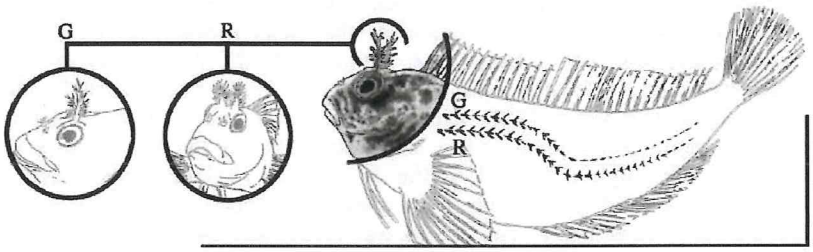


FIG. 1. Diagrammatic representation outlining the more salient differences between *Parablennius gattorugine* (G) and *Parablennius ruber* (R). The differences at the level of the orbital tentacles and the lateral line are especially conspicuous. In *P. ruber* the supraorbital tentacles tend to be bilobed, the main stem is short with many branches arising almost at the same level. In *P. gattorugine* there is a central stem along which thinner branches arise at different levels. The shape of the supraorbital tentacles is, however, quite variable in *P. gattorugine*.

 

37°42′ N; 24°04′ E, one specimen, PGGR1). Sequences are available at GenBank under accession numbers AY098778, AY098779, AY098834, AY098835 and DQ160198–DQ160205. The voucher specimens are deposited in the fish collection of the Eco-Ethology Research Unit at the Instituto Superior de Psicologia Aplicada, Lisbon. *Parablennius pilicornis* (Cuvier, 1829) and *Parablennius sanguinolentus* (Pallas 1814) were used as out-groups (GenBank accession numbers AY098796, AY098831, AF414700 and AY345187). Fin clips were cut immediately after collection of the individuals and stored at ambient temperature in 96% ethanol. Total genomic DNA was extracted from fin rays using a proteinase K/sodium dodecyl sulphate (SDS) based extraction buffer following the Sambrook *et al.* (1989) protocol.

Fragments of the 12S and 16S rDNA were amplified using primers described in Henriques *et al.* (2002), and polymerase chain reaction and sequencing conditions described in Almada *et al.* (2005). Sequence alignments were made using ClustalX 1.81 (Thompson *et al.*, 1997) with default settings. Character congruence between the two fragments was tested using the incongruence-length difference test (Farris *et al.*, 1995) available in PAUP (version 4.0; Swofford, 1998). The null hypothesis of congruence between the two data sets was not rejected ($P = 1$), which led us to analyse the 12S and 16S rDNA sequences combined in one single fragment.

Phylogenetic relationships were assessed using maximum parsimony (MP) and neighbour-joining (NJ) methods, implemented by the software package PAUP. Bootstrapping (Felsenstein, 1985) was used to assess robustness of the nodes in the trees with 1000 replicates. As the authors were dealing with very closely related species with very small genetic distances, patristic distances were adopted (following Nei & Kumar, 2000). An analysis of molecular variance (AMOVA; Excoffier *et al.*, 1997) was performed using ARLEQUIN 2.0 (Schneider *et al.*, 2000), to test the genetic differentiation between the samples of *P. ruber* and *P. gattorugine*.

A total of 367 bp of the mitochondrial 12S rDNA and 480 bp of the mitochondrial 16S rDNA were analysed, resulting in a combined sequence of 847 bp. In Fig. 2, the results of the phylogenetic analysis are summarized. A single phylogenetic MP tree of 139 steps was recovered and had the same topology as the NJ tree. *Parablennius ruber* and *P. gattorugine* form a monophyletic clade clearly separated from the other species of the genus *Parablennius* used as out-groups. All samples of *P. gattorugine* form a well-supported clade, which is sister to another equally well-supported clade that contains all *P. ruber* samples. The samples from mainland Portugal, group unambiguously with the Azorean samples of *P. ruber*. The mean interspecific uncorrected *p* distances are 1·67% (S.D. = 0·12%) and 1·71% (S.D. = 0·14%) for 12S and 16S, respectively. The intraspecific distances are 0·91% (S.D. = 0·17%) and 0·11% (S.D. = 0·17%) for *P. ruber* (12S and 16S, respectively), and 0% (S.D. = 0%) and 0·096% (S.D. = 0·11%) for *P. gattorugine* (12S and 16S, respectively).

The AMOVA analysis showed that 84·99% of the total genetic variation was due to interspecific differences, while the intraspecific variation accounted for 15·01%. The fixation index was highly significant ($P = 0$) for 1023 permutations.

The results presented in this study provide molecular support for the distinction between *P. ruber* and *P. gattorugine*. They also demonstrate that *P. ruber* is present both in the Azores and in the northwestern shore of mainland Portugal. This conclusion could be criticized due to the fact that the molecular markers

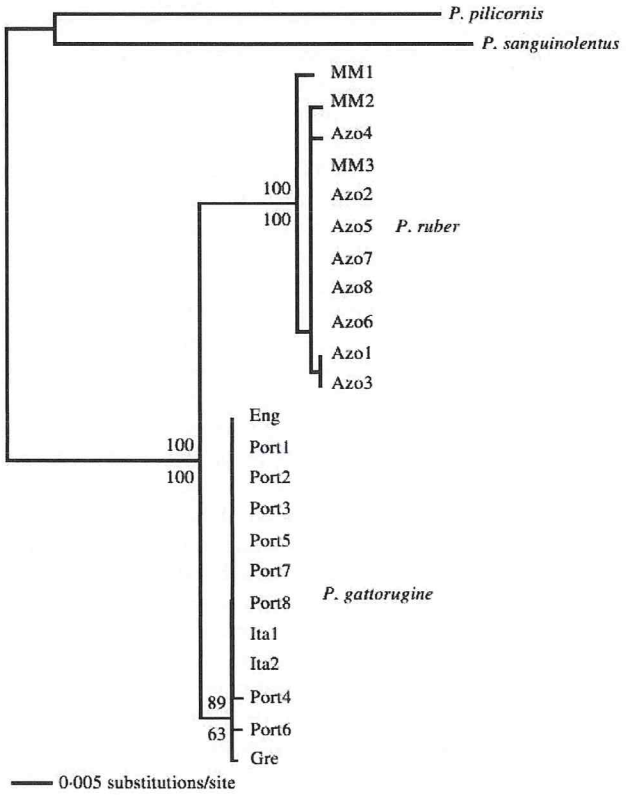


FIG. 2. Phylogenetic relationship of *Parablennius ruber* and *Parablennius gattorugine* using *Parablennius pilicornis* and *Parablennius sanguinolentus* as out-groups. A neighbour-joining tree is shown with neighbour-joining (above the nodes) and maximum parsimony (below the nodes) bootstrap support at the major nodes. Labels are: MM (Moledo do Minho, Northern Portugal), Azo (Azores), Port (Southern Portugal), Eng (Plymouth, England), Ita (Italy), Gre (Greece). The length of each branch is proportional to the number of nucleotide substitutions.

used are maternally inherited, and the presence of hybrids could not be excluded. This possibility seems unlikely as all the specimens, from both mainland Europe and Azores, all genetically classified as *P. ruber*, were unequivocally assigned to the same species based on all available morphological diagnostic criteria.

In recent years, many warm water organisms have been recorded in European waters for the first time, while the distribution of others have shifted, tracking changes in sea surface temperatures (Southward *et al.*, 1995; Kröncke *et al.*, 1998; O'Brien *et al.*, 2000). In the case of *P. ruber*, it is interesting that the original description by Valenciennes (1836) was based on specimens from the western coast of France, which means that this species already occurred in Europe more than one and a half century ago. The material included by Almeida (1982), in his revalidation of the species, was from northwest Portugal and from Algarve, in the south of the country. Bath (1982) used material from Azores and from


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Madeira, implying that the species was also found at these archipelagos. Recent surveys (N. Monteiro, unpubl. data), on the shore of Madeira, could not detect *P. ruber*, suggesting that the species may now be uncommon at this archipelago.

Concerning the presence of *P. ruber* in the Atlantic shores of Western Europe, the issue that requires clarification is the apparent contradiction between the relatively old presence of this species in Europe and its rarity or absence in most surveys of fishes of the European rocky shores. One has to admit the possibility that the species was not recorded because of insufficient sampling or inadequate identification. This seems, however, unlikely for areas like the British Isles where thorough surveys of the inshore ichthyofauna have been conducted for many years. Thus, the possibility that *P. ruber* is regularly present on the west European shores, although at low numbers should not be ruled out. If it occurs in very small numbers, it is possible that *P. ruber* may not be able to establish viable populations in Europe. The authors suggest that these populations may be, to a considerable extent, replenished by occasional larval transport from the Azores. The idea that blenniid larvae are able to undergo long distance transport was already proposed by Muss *et al.* (2001) to explain the distribution of *Ophioblennius atlanticus* (Valenciennes, 1836) across the Atlantic Ocean. Rafting on algae is another form of long distance transport that was demonstrated for blenniids. Specimens of *Hypleurochilus fissicornis* (Quoy & Gaimard, 1824), a species living in the southwestern Atlantic, were collected from a raft, off Azores (Santos *et al.*, 1997), a transport that involves a much greater distance that the one between Azores and Western Europe.

Azevedo & Homem (2002) showed that, at the Azores, *P. ruber* breeds in winter, the time when winds blowing from southwest are stronger, maximizing the likelihood of larval transport from the Azores to Western Europe. Thus, *P. ruber* may be a species conforming to the model of blenniid speciation proposed by Zander (1973, 1980). According to this model, blenniids transported from the European shores, could sometimes reach the Atlantic Islands where they could survive the glacial periods undergoing some degree of independent evolution to become incipient new species. These new species would then be transported back to Europe by the predominant currents and, if reproductive isolation was sufficiently strong, could become a new component of the European ichthyofauna, when temperatures become more favourable. The idea that the Macaronesian Islands like the Madeira and the Azores were colonized from eastern Atlantic sources is supported by the fact that from the eight blenniid species found at the Azores, six were 'Lusitanian'. They are shared with the warm temperate eastern Atlantic shores of Europe and Africa extending in most cases into the Mediterranean. One species (*O. atlanticus*) is shared with the tropical Africa and the tropical western Atlantic, and another (*Parablennius parvicornis*, Valenciennes, 1836) occurs in the Macaronesia and in the tropical Africa. The situation of the blenniid fauna of Madeira is very similar and many Azorean fish probably derived from a Madeiran source (Santos *et al.*, 1995). Thus, at least in the past, it is certain that blenniids dispersed from the eastern Atlantic shores to the Macaronesian Islands (Almada *et al.*, 2001). Such a scenario may well apply to *P. ruber*. It very likely derived from a *P. gattorugine*-like ancestor that colonized the Azores. It may have evolved there, surviving for one or more glacial periods. During glacial peeks, the Atlantic shores of

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# Chapter 8

## Phylogeography and demography of the Blenniid *Parablennius parvicornis* and its sister species *P. sanguinolentus* from the northeastern Atlantic Ocean and the western Mediterranean Sea

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Short communication

# Phylogeography and demography of the Blenniid *Parablennius parvicornis* and its sister species *P. sanguinolentus* from the northeastern Atlantic Ocean and the western Mediterranean Sea

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## 1. Introduction

Studies on the phylogeography and historic demography of the marine fish fauna of the northeastern Atlantic Ocean and the Mediterranean Sea have demonstrated the impact of the Pleistocene glaciations on their populations (Domingues et al., 2006, 2007a,b; Stefanni et al., 2006). Estimates of sea surface temperatures (SST) of the northeastern Atlantic over the last 280 Kyr showed a clear glacial/interglacial evolution and a steep north–south SST gradient between 37 and 45°N during the last glacial period (Calvo et al., 2001). SST at the Azores region were estimated to be 2–3°C lower than present day values (CLIMAP, 1976; Crowley, 1981). The archipelago of Madeira, located further south, experienced negligible variations in SST, while the eastern islands of the Canaries were more affected due to their proximity to the continent (Fig. 1, Calvo et al., 2001). Santos et al. (1995a) mentioned that the drop in SST at the Azores, might have been enough to promote the local disappearance of the warm water marine fish in the region. The same authors propose the Madeira Islands and the western coast of Africa as glacial refugia and source of fish for post-glacial colonization of the Azores. Almada et al. (2001) argued that the warmer water fish of the Atlanto-Mediterranean area survived in two distinct glacial refugia which acted as sources of post-glacial colonization: one in the west coast of Tropical/Subtropical Africa and Madeira, from which fish reached the Azores, and another inside the Mediterranean, from which the northeastern Atlantic waters adjacent to the Mediterranean entrance were colonized during the interglacials. These ideas have been supported by molecular studies on species like the pomacentrid *Chromis limbata* (Domingues et al., 2006) and the blenniid *Tripterygion delaisi* (Domingues et al., 2007a).

The Mediterranean has also been impacted by the Plesitocene glaciations experiencing considerable reduction in SST (Hayes et al., 2005), except for some preserved warm water pockets in the southern regions of this Sea (Thiede, 1978). Effects of the cooling events of the Mediterranean have also been identified by the molecular analysis of *C. chromis* (Domingues et al., 2005), *T. delaisi* (Domingues et al., 2007a) and *Diplodus sargus* (Bargelloni et al., 2005; Domingues et al., 2007b).

The species pair *Parablennius parvicornis* (Valenciennes, 1836) and *P. sanguinolentus* (Pallas, 1814) (Piscies: Blenniidae) constitutes a promising system to test the biogeographical hypothesis presented above. These blenniids have been described as two distinct species (Almada et al., 2005a) and their sister status has been demonstrated in a molecular phylogeny of the northeastern Atlantic and Mediterranean blenniids (Almada et al., 2005b). Almada et al. (2005a) commented on the pattern of geographic distribution of the species pair, highlighting the interest of a population survey to address biogeographical issues. *Parablennius parvicornis* occurs in the western African coast, south of Cape Blanc to the Congo river, including the

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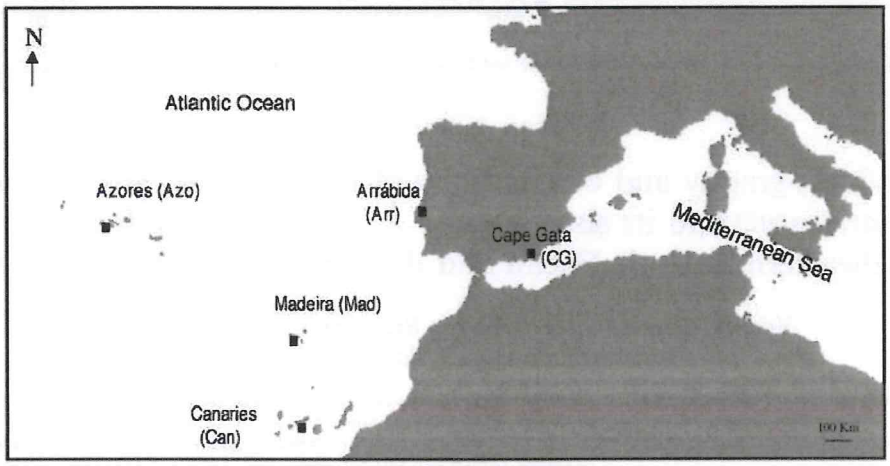


Fig. 1. *Parablennius parvicornis* and *P. sanguinolentus* sampling locations. Individuals of *P. parvicornis* were collected in the islands of Fayal (Azores), Madeira, and Tenerife (Canaries). Samples of *P. sanguinolentus* were collected in Cape of Gata.

archipelagos of Azores, Madeira, Canaries and Cape Verde. The distribution of *P. sanguinolentus* includes the Mediterranean and the Atlantic coast between the Gulf of Biscay and Morocco (north of Casablanca, Almada et al., 2005a and references therein). The two species are allopatric and are separated by a gap of at least 13° of latitude. These fishes are intertidal and are found in well-illuminated sites in sheltered areas with algae-covered rocks (Bath, 1990; Zander, 1986). Like other blenniids, *P. parvicornis* and *P. sanguinolentus* have demersal adhesive eggs guarded by the male (Santos, 1989) and planktonic larvae that remain in the water column for over a month (Santos et al., 1995b; Raventós and Macpherson, 2001).

In this note we analyze mitochondrial control region sequences of populations of *P. parvicornis* from the Atlantic archipelagos of Azores, Madeira and Canaries and *P. sanguinolentus* from a western Mediterranean location. We aim at determining whether the phylogeography and historical demography of these species fit the biogeographical pattern proposed by Almada et al. (2001) and Santos et al. (1995a).

## 2. Materials and methods

Samples of *P. parvicornis* were collected from the Azores (Fayal), Madeira (Funchal) and Canaries (Tenerife). Individuals of *P. sanguinolentus* were collected from Spain (Cape of Gata). A sequence of *P. sanguinolentus* from Arrábida (western coast of Portugal) available from GenBank database was also included in the analyses. Collection localities and date of collection are shown in Fig. 1 and Table 1. Fin clips were cut immediately after collection of the individuals and stored at ambient temperature in 95% ethanol. Total genomic DNA was extracted by SDS proteinase K procedure and purified by standard chloroform and isopropanol precipitation (Sambrook et al., 1989). Amplification of the 5′ hypervariable portion of the mitochondrial control region (also called D-loop) was accomplished with primers Lpro1 (Ostellari et al., 1996) and 12S (Nesbö et al., 1998), using an annealing temperature of 50 °C. Direct sequencing was performed with both primers with an ABI 3100 automated sequencer (Applied Biosystems) yielding a final fragment of 556 bp.

Sequences were aligned using CLUSTAL V (Higgins et al., 1991) implemented by Sequence Navigator (Applied Biosystems). Genetic diversity indexes (number of haplotypes, haplotype diversity and nucleotide diversity) were calculated. Population structure was determined by an analysis of molecular variance (AMOVA; Excoffier et al., 1997) and gene flow (Fst) between populations was estimated. These analyses were performed in the program ARLEQUIN (version 2.000; Schneider et al., 2000).

A network of haplotypes was constructed using the statistical parsimony method (Templeton et al., 1992) implemented in TCS (version 1.21, Clement et al., 2000).

The historical demography of each population was examined using mismatch distributions analysis (Rogers and Harpending, 1992; Rogers, 1995) performed in ARLEQUIN (version 2.000; Schneider et al., 2000). The parameters of the expansion $\theta_0$, $\theta_1$ and $\tau$ were computed and the time of the expansion ($t$) was estimated using the formula $\tau = 2t\mu$, where $\mu$ is the mutation rate. In the absence of an estimate of $\mu$ for the mitochondrial control region of blenniids, we used $\mu = 8.24 \times 10^{-8}$ that was estimated using an internally calibrated molecular clock for two pomacentrid sister species separated by the closure of the isthmus of Panama (Domingues et al., 2005). This value is very similar to the one applied by Bowen et al. (2006) after a revision of

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Table 1
Collection localities of *Parablennius parvicornis* and *P. sanguinolentus* used in the present study and diversity indexes for the mitochondrial control region sequences

| | *N* | nH | Hd | $\pi$ | Date of collection | GenBank Accession Nos. |
|---|---|---|---|---|---|---|
| *Parablennius parvicornis* | | | | | | |
| Azores (Azo) | 22 | 8 | 0.736 | 0.002 | November 2002/June 2006 | EF554601 EF554622 |
| Madeira (Mad) | 29 | 23 | 0.980 | 0.005 | September 2003 | EF554623 EF554651 |
| Canaries (Can) | 18 | 16 | 0.987 | 0.005 | November 2005 | EF554652 EF554669 |
| *Parablennius sanguinolentus* | | | | | | |
| Arrábida (Arr) | 1 | | | | | AY090789 |
| Cape of Gata (CG) | 14 | 6 | 0.604 | 0.001 | July 2004 | EF554670 EF554683 |

Number of individuals (*N*); number of haplotypes (nH); haplotype diversity (Hd); and nucleotide diversity ($\pi$) for each population are shown. Date of collection and GenBank Accession Nos. are shown in the two last columns.

D-loop molecular clock calibrations for several tropical Atlantic fish species. In addition Fu's Fs neutrality test (Fu, 1997) was used to detect possible population expansions.

## 3. Results

A total of 83 D-loop sequences were obtained and deposited in GenBank database (Table 1). Madeira and Canaries showed similar haplotype and nucleotide diversities, which were higher than the values for Azores and Cape of Gata (Table 1). The AMOVA analysis showed that 24.36% ($P < 0.001$) of the data variance was explained by differences among populations. Gene flow was shown to be higher between Madeira and Canaries (Fst = 0.016, $P = 0.261$) than between Azores and the other archipelagos (Fst = 0.385, $P = 0.000$ for Azores and Madeira and Fst = 0.280, $P = 0.000$ for Azores and Canaries).

The haplotype networks of the two species are not connected at the confidence level of 95% (Fig. 2). Ancestral haplotypes in each network were inferred as the ones that yielded the highest outgroup weights (Castelloe and Templeton, 1994). Both networks showed very simple patterns with few mutational steps separating the most divergent haplotypes from the ancestror (two steps in the case of *P. sanguinolentus* and 6 steps in the case of *P. parvicornis*). *Parablennius parvicornis* show two common haplotypes, differing by four mutations, from which the remaining haplotypes derive by one or two mutations. Haplotypes were shared between Azores, Madeira and Canaries populations and genetic partition between the three populations was not evident. Few network reticulations are observed suggesting the existence of moderate homoplasy.

The model of sudden expansion was not rejected for any of the populations (Table 2) and mismatch distributions were unimodal (figures not shown). While the values of $\theta$ (the compound parameter representing the mutation rate and the female effective population size) were similar before the expansion ($\theta_0$) in the four populations, the values corresponding to the same parameter after the expansion ($\theta_1$) were much higher in the Azores and Cape of Gata. Population expansions seemed to have occurred more recently in the Azores and Cape of Gata than in Madeira and Canary islands.

## 4. Discussion

Our results showed that *P. parvicornis* from the Atlantic archipelagos of Azores, Madeira and Canaries are genetically connected with a particular strong connection of Madeira and Canaries. In this study we wanted to test whether populations of *P. parvicornis* were differentially affected by the Pleistocene glaciations. Evolutionary relationships among *P. parvicornis* haplotypes resulted in a star-like network (Fig. 2), which is consistent with a recent demographic expansion following a considerable reduction in population size. In the parsimony network Azorean haplotypes tend to be closer to the ancestral one while in Madeira and Canaries there are haplotypes linked by more mutational steps to the most common one. This suggests that if bottlenecks took place in these islands the loss of genetic diversity was less accentuated for Madeira and Canaries than for the Azores. The historical demography analysis revealed the existence of a past population expansion for all the populations (Table 2). Comparison of $\theta_0$ and $\theta_1$ values (the compound parameter representing the mutation rate and the female effective population size before and after the expansion, respectively), showed that the demographic expansion of *P. parvicornis* was more accentuated in the Azores than in Madeira and Canary islands. According to CLIMAP (1976) and Crowley (1981) SST during the Pleistocene glaciations decreased about 2–3 °C in the Azores region. Santos et al. (1995a) suggested that such a decrease would have been enough to promote the local disappearance of warm water species such as *P. parvicornis* from the Azores. The less affected tropical western coast of Africa, the archipelago of Madeira, the westernmost Canary islands and some regions of the Mediterranean have been shown to act as refugia for species that were not able to survive the cold phases in the northern Atlantic locations (Domingues et al., 2006, 2007a,b). Our findings support this hypothesis. Indeed, *P. parvicornis* from the Azores show a strong and recent population expansion that

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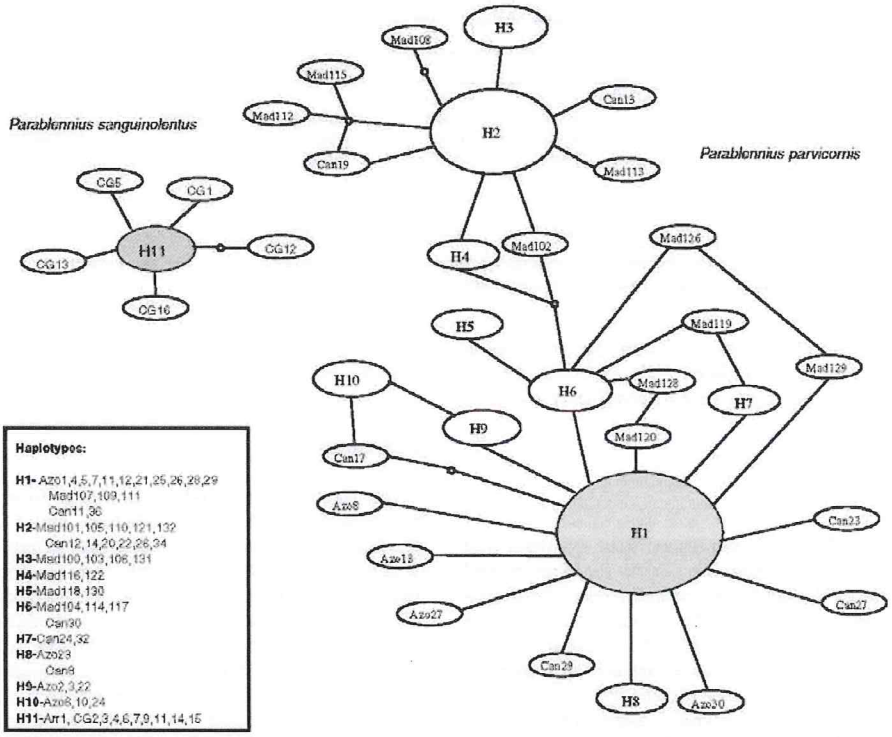


Fig. 2. Statistical parsimony network of *Parablennius parvicornis* D-loop haplotypes. Empty circles represent missing haplotypes. Ancestral haplotypes for each network (Castelloe and Templeton, 1994) are in grey. The size of the circles is proportional to the haplotype frequency. Shared haplotypes are defined in the table. See Fig. 1 for labels.

Table 2
Estimated values for the expansion model for each population of *Parablennius parvicornis* and *P. sanguinolentus* obtained from the D-loop sequences

| | SSD | *P* | $\theta_0$ | $\theta_1$ | τ | *t* (Kyr ago) | Fu's Fs | *P* |
|---|---|---|---|---|---|---|---|---|
| *Parablennius parvicornis* | | | | | | | | |
| Azores | 0.006 | 0.380 | 0.000 | 1693 | 0.228 2.189 | 1.61 15.5 | −4.247 | $P < 0.05$ |
| Madeira | 0.004 | 0.460 | 1.184 | 40.78 | 1.132 5.177 | 8.01 36.1 | −23.149 | $P < 0.001$ |
| Canaries | 0.018 | 0.360 | 0.005 | 8.198 | 2.051 8.389 | 14.5 59.3 | −13.419 | $P < 0.001$ |
| *Parablennius sanguinolentus* | | | | | | | | |
| Cape of Gata | 0.001 | 0.840 | 0.000 | 1252 | 0.000 1.911 | 0.00 13.5 | −5.997 | $P < 0.001$ |

SSD (sum of square deviations) and its probability *P*; $\theta_0$ and $\theta_1$(compound parameter representing the mutation rate and the female effective population size before and after expansion, respectively); and τ (time in generations). The time of the expansion (*t*) is also presented. Fu's Fs neutrality test and its probability *P* are shown in the last two columns.

might have occurred after the Younger Dryas at about 12 Kyr (Table 2), when, although already after the Last Glacial Maximum, a large-scale cooling occurred (Lambeck et al., 2002). According to our data, demographic expansions of *P. parvicornis* in Madeira and Canaries were less pronounced and occurred earlier than in the Azores. Interestingly, Azorean fish show lower genetic diversity than Madeira and Canaries populations (Table 1). Lower levels of genetic diversity are typical of recent populations or of populations that have experienced a recent bottleneck, like the one we propose to have occurred in *P. parvicornis* from the Azores. According to this scenario, *P. parvicornis* from the Azores resulted from a post-glacial colonization having its origin in southern, and thus less affected, regions like Madeira. Eddies moving from Madeira towards the Azores, which persist for many weeks and retain the characteristic of the water mass that originated them, have been documented (Santos et al., 1995a and references therein). These eddies can easily transport the pelagic larvae of *P. parvicornis*, which

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remain in the water current for over a month (Santos et al., 1995b).

In this study, we were also interested in assessing the effects of this climatic event on one population of *P. sanguinolentus* (the sister species of *P. parvicornis*) from the western Mediterranean. Considerably reductions in SST during the Pleistocene have also been described for the Mediterranean Sea (Hayes et al., 2005), with warm water fish species being confined to southern warmer pockets (Thiede, 1978). The genetic diversity indices and demographic parameters obtained for *P. sanguinolentus* from Cape of Gata yielded a pattern similar to the one obtained for the Azorean *P. parvicornis*. It is thus likely that this population of *P. sanguinolentus* has been drastically reduced during the Pleistocene glaciations having its origin in a post-glacial colonization from the preserved regions of the Mediterranean.

Results on *P. parvicornis* and its sister species *P. sanguinolentus* presented in this study add to the growing evidence of a biogeographical scenario for the Atlanto-Mediterranean warm water benthic species. Previous work on other warm water benthic fish species such as *Tripterygion delaisi* (Domingues et al., 2007a) and *C. limbata/C. chromis* (Domingues et al., 2006) pointed to the existence of two groups of populations: one including the Mediterranean and the Atlantic coast of western Europe and another encompassing the western tropical coast of Africa and the Atlantic archipelagos of the Macaronesia. This pattern may reflect the different effects of the Pleistocene glaciations on warm water fishes that must have become extinct or suffered considerable reductions, in some regions where sea surface temperatures were seriously reduced (western coast of Europe, Azores, eastern Canaries and northern Mediterranean), surviving in less affected regions that acted as refugia. Recolonization of the affected locations may have been possible in the last 10 Kyr, from the western tropical coast of Africa, the western Canaries and Madeira islands, in the case of the Azores, and the southwestern Mediterranean in the case of the Atlantic shores of Iberia (Almada et al., 2001). As suggested by Almada et al. (2001) the operation of this double system of refugia through the entire series of glaciations may have also promoted speciation with the formation of multiple sister species pairs involving one Afro-Macaronesian species and one in the Mediterranean and adjacent Atlantic waters.

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# Chapter 9

## Genetic divergence in the Atlantic-Mediterranean Montagu's blenny *Coryphoblennius galerita* (Linnaeus 1758) revealed by molecular and morphological characters

# Genetic divergence in the Atlantic-Mediterranean Montagu's blenny *Coryphoblennius galerita* (Linnaeus 1758) revealed by molecular and morphological characters

*Article in press in Molecular Ecology*

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Key words- *Coryphoblennius galerita*, northeastern Atlantic, Mediterranean, population structure, mt/n DNA, morphology

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Running title: Genetic divergence of *Coryphoblennius galerita*

**Abstract**

*Coryphoblennius galerita* is a small intertidal fish with a wide distribution and limited dispersal ability, occurring in the northeastern Atlantic and Mediterranean. In this study we examined Atlantic and Mediterranean populations of *C. galerita* to assess levels of genetic divergence across populations and to elucidate historical and contemporary factors underlying the distribution of the genetic variability. We analyze three mitochondrial and one nuclear marker and 18 morphological measurements. The combined dataset clearly supports the existence of two groups of *C. galerita*: one in the Mediterranean and another in the northeastern Atlantic. The latter group is subdivided in two subgroups: Azores and the remaining northeastern Atlantic locations. Divergence between the Atlantic and the Mediterranean can be the result of historical isolation between the populations of the two basins during the Pleistocene glaciations. Present day barriers such as the Gibraltar Strait or the "Almeria-Oran jet" are also suggested as responsible for this isolation. Our results show no signs of local extinctions during the Pleistocene glaciations, namely at the Azores, and contrast with the biogeographical pattern that has been observed for Atlantic-Mediterranean warm water species, in which two groups of populations exist, one including the Mediterranean and the Atlantic coast of western Europe, and another encompassing the western tropical coast of Africa and the Atlantic islands of the Azores, Madeira and Canaries. Species like *C. galerita* that tolerate cooler waters, may have persisted during the Pleistocene glaciations in moderately afected locations, thus being able to accumulate genetic differences in the more isolated locations such as the Azores and the Mediterranean. This study is one of the first to combine morphological and molecular markers (mitochondrial and nuclear) with variable rates of molecular evolution to the study of the relationships of the Atlantic and Mediterranean populations of a cool water species.

## Introduction

The study of genetic divergence and speciation in the marine environment represents a great challenge. Marine populations tend to be large and marine species often have high fecundity and larvae that can disperse over long distances. Thus, marine species are usually expected to show high levels of gene flow and low geographical differentiation. However, increasing evidence indicates that at least partially isolated populations may occur quite commonly in marine systems (eg. Doherty *et al.* 1995; Shulman & Bermingham 1995; Bernardi 2000; Riginos & Nachman 2001; Stefanni & Thorley 2003; Taylor & Hellberg 2003; Baus *et al.* 2005). Several mechanisms have been proposed by which marine species with high dispersal potential can diverge genetically. These include vicariance processes caused by past barriers; oceanographic currents; habitat discontinuities; local adaptation; larval behavior; isolation by distance and limited dispersal to new areas promoting genetic differentiation (Palumbi 1994; Riginos & Nachman 2001 and references therein).

In this regard, the northeastern Atlantic, including the Macaronesian islands, together with the Mediterranean constitute very interesting study cases. Different areas within these regions were differentially affected by drops in sea surface temperature associated with the Pleistocene glaciations. The shores of west Europe endured polar conditions during the glacial maxima, with very cold waters also present along the northwestern African coast (Crowley 1981; Dias 1997) and, to some extend, the Canary Islands (Lamb 1977, Crowley 1981). At the Azores, temperature drops were moderate (about 2-3°C; Crowley 1981), while Madeira, the tropical western coast of Africa and some southern Mediterranean areas were little affected (Thiede 1978). These fluctuations led to local extinctions and latitudinal shifts of many taxa, namely those that are only capable of living in warmer waters (Almada *et al.* 2001; Domingues *et al.* 2006). After warmer conditions were restored, recolonization probably occurred from some less affected regions that may have acted as refugia. Thus, vicariance and dispersal have very likely played an important role in the evolutionary history of the marine fauna of the northeastern Atlantic and the Mediterranean.

Oceanic currents should also be taken in consideration when identifying the factors that influence the distribution of the genetic variability in a particular region, especially when studying coastal organisms whose dispersal capabilities are restricted to their planktonic larval phases. The northeastern Atlantic current system is dominated by a multibranch complex system that has its origin in the Gulf Stream (Stramma 1984). The circulation flows predominantly to the east and northeast, bringing warm water to Europe. However, one branch of the system turns south originating the cold water Canaries current which runs parallel to the shores of southwest Europe and Northwest Africa, reaching Madeira and the Canaries (Santos *et al.* 1995; Stramma 1984) (Fig. 1). However, meanders and eddies also cause sporadic transport of water and plankton from Madeira to Azores (Santos *et al.* 1995). The impact of currents in the Canary Islands also generates a complex system of eddies that promotes transport towards the north in the western islands (Molina *et al.* 1996). These eddies make the connection between the Canaries and Madeira possible, with the Salvage islands possibly acting as a stepping-stone. Between the West African shore and the Canary Islands, upwelling filaments can also cause sporadic transport towards the islands (Barton *et*

*al.* 1998; Rodríguez *et al.* 1999; Bécognée *et al.* 2006). The Atlantic and the Mediterranean communicate by the Gibraltar strait, which is characterized by a two-layer flow regime. Atlantic waters inflow in the upper layer and Mediterranean waters outflow in the lower layer (Malanotte-Rizzoli & Bergamasco 1989; Özgökmen *et al.* 2001). Close to the Gibraltar strait, the water in the Alboran Sea describes a quasi-permanent anticyclonic gyre that generates another oceanographic barrier known as the "Almerian-Oran jet" (Millot 1999) (Fig. 1).

The effects of the Pleistocene glaciations together with the circulation patterns described above for the Atlantic and the Mediterranean have been suggested to be responsible for a major biogeographic break between the two regions. Results, however, are not conclusive since the extent of the differentiation varies across species. Indeed, some species such as the seabream *Diplodus puntazzo* (Bargelloni *et al.* 2005), the sea bass *Dicentrarchus labrax* (Lemaire *et al.* 2005), the cuttlefish *Sepia officinalis* (Pérez-Losada *et al.* 2002) and the sponge *Crambe crambe* (Duran *et al.* 2004) show high levels of genetic differentiation between Atlantic and Mediterranean populations, while others like the damselfish *Chromis chromis* (Domingues *et al.* 2005), the seabream *Diplodus sargus* (Bargelloni *et al.* 2005, Domingues *et al.* 2007a), the wrasse *Thalassoma pavo* (Costagliola *et al.* 2004) and the Norway lobster *Nephrops norvergicus* (Stamatis *et al.* 2004) show no genetic partition between the Atlantic and the Mediterranean.

The phylogeographic patterns of Atlantic-Mediterranean fish described in recent studies have revealed genetic signatures of the effects of Pleistocene glaciations on the different populations. Incidentally, these patterns are consistent with transport of eggs and larvae that are possible with the current regime described for these water masses. Warm water species such as *Tripterygion delaisi* (Domingues *et al.* 2007b), *Chromis chromis/ C. limbata* (Domingues *et al.* 2006) and *Parablennius sanguinolentus /P. parvicornis* (Almada *et al.* 2005) show two groups of populations: one including the Mediterranean and adjacent European Atlantic coast and another that comprises the western coast of Africa and the Macaronesian islands. According to the studies mentioned above, this dual pattern can be explained by local extinctions at the time of the glacial peaks in the more affected regions (Azores, Canaries and European Atlantic coast) followed by post-glacial colonization from warmer refugia such as Madeira and the Tropical coast of Africa, in the case of the Azores, and the southern regions of the Mediterranean, in the case of the Atlantic Europe. The differentiation between these two groups is not only supported by molecular divergences but also by differences in morphologic characters. *Parablennius parvicornis* / *P. sanguinolentus* differ in the number of spines in the dorsal fin, presence or absence of extra teeth in the upper jaw and pigmentation (Almada *et al.* 2005 and references therein). Although the two groups of *Tripterygion delaisi* have not been described as distinct species, differences in the number of rays of the second dorsal, and in the number of scales in the lateral line have been described, as well as color and behavioral variation between the two forms (Domingues *et al.* 2007b and references therein). Interestingly, cool water species such as *Liphophrys pholis* (Stefanni *et al.* 2006) and *Parablennius ruber* (Almada *et al.* 2007) seem to have been able to survive the cooler periods in the Azores. In the case of *L. pholis*, the Azorean population shows a strong genetic differentiation that is accompanied by meristic differences in the number of rays of the dorsal and anal fins (Stefanni *et al.* 2006).

*Coryphoblennius galerita* (Linnaeus 1758), the single member of its genus, is a small benthic fish living in exposed rocky shores in the littoral zone (Zander 1986). The species is present in the eastern Atlantic (from the southwestern coast of Britain to Cape Juby in the Saharian coast, including the Azores, Madeira and Canary Islands) and in the Mediterranean including the Black Sea (Zander 1986; Quéro *et al.* 1990; Falcón *et al.* 2002). This species breeds from spring to summer in the Atlantic, although the extent of the breeding season varies with latitude (Almada *et al.* 1996). In the Mediterranean breding can also take place in winter (Richtarski & Patzner 2000). Females spawn demersal eggs that are cared for by the males until hatching (Almada *et al.* 1983; Milton 1983). Dispersal is restricted to the planktonic larval phase of about 26-27 days (Raventós & Macpherson 2001). The large distribution range of *C. galerita,* together with its limited dispersal ability, suggests the existence of population structure for this species. Bath (1978), based on morphological data (fin rays and coloration), found differences in the Atlantic and Mediterranean populations, and also between fishes from Madeira and Canaries. Indeed, Almada *et al.* (2005), in a study of the phylogeny of the northeastern Atlantic and Mediterranean blenniids, found substantial divergence between conserved mtDNA sequences of *C. galerita* from the Mediterranean and the Atlantic. The same authors also found that in the Atlantic there was a clear genetic divergence between mainland shores of Europe and Madeira on one hand and the Azores on the other. However, sample sizes were small and no definitive conclusions were attempted on this issue.

Several factors make the study of *Coryphoblennius galerita* very promising. It inhabits areas that experienced very distinct glacial effects and differ in present day ecological and oceanographic conditions. In this study we examined Atlantic and Mediterranean populations of *C. galerita*, including the eastern Atlantic archipelagos of the Azores, Madeira and Canaries, to assess the levels of genetic divergence of this widely distributed blenniid. Since it is well known that the combined analysis of patterns seen in different loci is very informative when studying spatial and temporal genetic structure (Slatkin & Maddison 1989), we employed three mitochondrial and one nuclear markers with different rates of molecular evolution. Additionally, we combined the molecular survey with an analysis of morphological characters. Judging from the geographical distribution of *C. galerita*, which has its northern limit in the southwestern coast of Britain, this species must have been able to survive the cold temperatures of the Pleistocene glaciations in moderately affected regions such as the Azores Islands. We thus expect the distribution of *C. galerita* genetic diversity to conform to the scenario described for the cooler water species, where strong genetic differentiation between populations is found. This may also be accompanied by some degree of morphological differentiation. This is one of the first studies to apply a comparative analysis of molecular and morphological traits to a cool water species. We believe that this approach will shed light on the evolutionary history of the species and will also contribute to the elucidation of the biogeographical scenarios that are now emerging for the northeastern Atlantic and the Mediterranean.

## 6. Materials and methods

*Sampling*

A total of 132 *Coryphoblennius galerita* were collected from 7 locations in the Atlantic and 4 locations in the Mediterranean (Fig. 1). Samples were collected in tide-pools using hand nets. Fin clips were cut immediately after collection of the individuals and stored at ambient temperature in 95% ethanol.

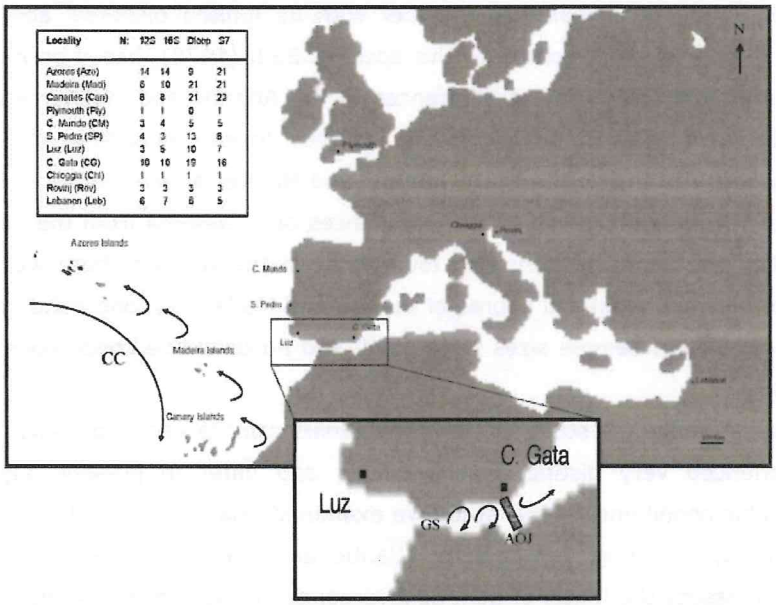


**Figure 1** *Coryphoblennius galerita*'s sampling locations. Samples were collected in the Atlantic archipelagos of Azores (Fayal, Azo); Madeira (Madeira, Mad) and Canaries (Tenerife, Can); in Plymouth (England, Ply); and in the Atlantic coast of Portugal in C. Mundo (CM), S. Pedro (SP) and Luz. Samples from the Mediterranean were collected from Cape of Gata (CG), Spain; Chioggia (Chi), Italy; Rovinj (Rov), Croacia; and one location in Lebanon (Leb). Numbers of individuals sequenced for each of the markers are shown in the table. Arrows indicate major current flow patterns. The Canaries Current is indicated by 'CC', the Gibraltar Strait is indicated by "GS" and the Almeria-Oran jet' is indicated by 'AOJ'.

*Molecular analysis*

*DNA extraction, amplification and sequencing.* Total genomic DNA was extracted by SDS proteinase K procedure and purified by standard chloroform and isopropanol precipitation (Sambrook *et al.* 1989). Fragments of the conserved 12S and 16S rDNA mitochondrial genes were amplified for a subset of our samples using primers and PCR conditions described in Almada *et al.* (2005). Amplification of the 5′ hypervariable portion of the mitochondrial control region (also called D-loop) was performed for a larger number of individuals, using primers and PCR conditions described in Ostellari *et al.* (1996). In addition, we amplified the first intron of the S7 ribosomal protein gene, using primers S7RPEX1F and S7RPEX2R (Chow and Hazama 1998), and an annealing temperature of 56°C. After purification following the manufacturer's protocol (Applied Biosystems, Forter City, CA), direct sequencing was performed with an ABI 3100 automated sequencer (Applied Biosystems).
For the first intron of the S7 ribosomal protein gene, the two strands of each individual were recovered using one of two methods. (1) For the individuals that showed heterozygous indels, we used double peaks in chromatograms generated as artifacts in the vicinity of the heterozygous indels to identify the specific sequences present in individual strands, following the approach described by Sousa-Santos *et al.* (2005). (2) Both strands of the individuals that did not possess heterozygous indels, but that showed heterozygous positions, were recovered using a set of specific amplifications. Heterozygous positions were identified as double peaks in the chromatograms obtained with S7RPEX1F and S7RPEX2R primers, and specific primers were designed for the amplification of only one of the sequences of each individual. The last base of each of these primers consisted of one of the bases in the first or last heterozygous positions of the individual. Amplifications were then made using the specific primer in combination with S7RPEX1F or S7RPEX2R under high annealing temperatures in order to assure specificity. A total of 14 primers were required to obtain the two strains of all the individuals (primer sequences and PCR conditions available from authors upon request). Direct sequencing was then performed using S7RPEX1F or S7RPEX2R primers.

*DNA sequences and phylogenetic analyses.* Sequences were aligned using the program CLUSTAL V (Higgins *et al.* 1991) implemented by Sequence Navigator (Applied Biosystems). Diversity indexes were calculated using the software package ARLEQUIN (version 2.000; Schneider *et al.* 2000). The computer program MODELTEST ver. 3.7 (Posada & Crandall 1998) was used to determine a model of sequence evolution that best fitted each gene. The Akaike Information Criterion (AIC) was chosen since it yields more reliable results (Posada & Buckley 2004). Phylogenetic relationships within *Coryphoblennius galerita* were assessed using Bayesian inference, performed in MR BAYES 3.0b4 (Huelsenbeck & Ronquist 2001). The blenniid *Lipophrys trigloides* was used as outgroup. The dataset was divided into four gene partitions (12S, 16S, D-loop and S7), which were assigned separate (unlinked) parameters to accommodate gene-specific differences in evolutionary rate. The general model selected by MODELTEST was used for the analysis, allowing MR BAYES to estimate the parameters in that model. The GTR model with equal rates was chosen for12S and 16S and the GTR model with

gamma rates was used for D-loop and S7. Monte Carlo Markov chains were run for 5,000,000 generations saving a tree every 100 generations. To help ensure that stationarity was reached, we discarded the first 15,000 generations (150 sampled trees) as burn-in and used the remaining 4,985,000 generations (49,850 sampled trees) in all subsequent analysis. A majority rule consensus tree calculated from the 49,850 remaining trees was constructed and used to determine the posterior probabilities of clades. Phylogenetic trees were also obtained using the mitochondrial and the nuclear sequences separately.

*Gene flow and population structure.* Individuals were grouped forming 5 samples (see Fig. 1): Azores, Madeira, Canaries, Portugal (including samples from C. Mundo, S. Pedro, Luz and also the sample from Plymouth) and Western Mediterranean (C. Gata). Samples from the remaining locations of the Mediterranean belonged to the eastern basin and were only included in the phylogenetic analysis. Gene flow (Fst) between the 5 locations was estimated. Corrections for simultaneous multiple comparisons were applied using sequential Bonferroni correction (Rice 1989). Population structure was determined by an analysis of molecular variance (AMOVA; Excoffier *et al.* 1997) using the program ARLEQUIN (version 2.000; Schneider *et al.* 2000). Population average pairwise differences were estimated and results were visualized using a multidimensional scaling analysis implemented by STATISTICA (version 7.0; Statsoft Inc.). These analyses were performed for a combined dataset including the two slow evolving mitochondrial genes (12S and 16S rDNA) and for the D-loop and S7 intron separately.

*Historical demography.* Sequences were pooled forming three groups of populations (Group 1= Azores, Group 2= Madeira, Canaries and Portugal, Group 3= Mediterranean) according to the phylogeny results. The historical demography of the three populations was examined using mismatch distributions analysis performed in ARLEQUIN 2.0. Theoretical studies have shown that populations in long stable demographic equilibrium show a chaotic mismatch distribution, while rapid population expansions or bottlenecks are reflected in a unimodal (approximately Poisson) profile (Rogers 1995; Rogers & Harpending 1992). Mismatch distributions were established and their fit to Poisson distributions was assessed by Monte Carlo simulations of 1000 random samples. The sum of square deviations (SSD) between observed and expected mismatch distributions was used as a test statistics, its P value representing the probability of obtaining a simulated SSD larger or equal to the observed one (Schneider & Excoffier 1999). The parameters of the expansion $\theta_0$, $\theta_1$, and $\tau$ were estimated and the time of the expansion (t) was estimated using the formula $\tau = 2\ t\ \mu$, where $\mu$ is the mutation rate. In the absence of an estimate of $\mu$ for the mitochondrial control region of blenniids, we used $\mu = 8.24 \times 10^{-8}$ that was estimated using an internally calibrated molecular clock for two pomacentrid sister species separated by the closure of the isthmus of Panama (Domingues *et al.* 2005). We did not attempt to estimate the time of the expansion using the S7 intron data, since there is not any estimation of the mutation rate of this intron. In addition Tajima's D neutrality test (Tajima 1989) was used to detect possible population expansions.

*Morphological analysis*

*Measurements* A total of 84 fish were measured: 25 from Azores; 11 from Madeira; 21 from Canaries; 11 from C. Mundo (Portugal) and 16 from the Mediterranean. A total of 18 measurements were taken for each fish, using callipers to a precision of 0.1 millimetres: total length (TL); standard length (SL); head length (HL: from the tip of the snout to the rear edge of the opercular bone); snout length (SnL: pre-orbital); pre-anal length (PreAL: from the tip of the snout to the base of the first ray of the anal fin); pre-dorsal length (PreDL): ibidem to the base of the first ray of the dorsal fin); length of the ventral fin (LV: from the base to the end of the longest ray); length of the pectoral fin (LP: from the base to the end of the longest ray); head height (HH: measured at the level of the opercular bone); pre-orbital width (PreOW); eye diameter (YD); body width at the insertion of the pectoral fin (WIP); body width at the level of the anus (WA); body height at the insertion of the pectoral fin (HIP); mouth perimeter (MP); mouth width (MW); crest width (CW: measured at the base of the crest); crest height (CH). The number of rays of the different fins was also counted. Sex was determined by direct observation of the genital papilla.

*Statistical analysis* To compare the populations Discriminant Analysis were performed on the residuals obtained from log-log Regressions of each continuous measure over the standard length. UPGMA cluster analysis was used to build a phenogram based on the Squared Mahalanobis Distances obtained in the Discriminant Analysis. UPGMA cluster analysis was conducted using the PHYLIP software package (Felsenstein 1989). As crest width and height residuals were the features with the highest contribution to the separation of populations, they were separately analyzed using ANOVA with the Tukey HDS test for post-hoc comparisons among populations. Differences on meristic data were tested using ACTUS (Estabrook & Estabrook 1989), a simulation statistic procedure designed to analyse contingency tables that is not limited by the assumptions of conventional $\chi^2$ Tests and that apart from assessing the overall significance of the table, allows assessment of the significance of deviations between observed and expected frequencies for each individual cell.

Results

*Molecular analysis*

*DNA sequences and phylogenetics analyses* A total of 61 12S rDNA sequences, 66 16S rDNA sequences, 108 D-loop sequences and 218 S7 first intron sequences (corresponding to 109 individuals) were obtained (Fig. 1). Some of the 12S and 16S sequences were available in GenBank (accession numbers: AY098816- AY098816 and AY098749- AY098755). The remaining sequences were registered in GenBank (accession numbers: EF520774-EF520790, EF521602-EF521813 and EF527585-EF527802). The 12S and 16S rDNA sequences were 395 bp and 568 bp long respectively and no gaps were required for their alignment. 12S rDNA

sequences showed a total of 8 polymorphic sites, while 32 polymorphic sites were found for 16S rDNA sequences. Sequences of the mitochondrial control region showed a complex pattern composed by sequence motifs of variable length, which were repeated several times. The motif TATATGTACTA was found in every sequence. However, variations of this motif were found to be characteristic of each geographic region. Sequences from the Mediterranean were composed by a variable number of TATATGTACTAGG repeats, while the motif TATATGTACTATACAC was only found in the Azores and the motif TATATGTACTATACAGTATATGTATGGGTACA was characteristic of Portugal, Canaries and Madeira. These motifs occurred in the central region of the sequences and, due to the variable times that they are repeated, the alignment was very difficult. Thus, we opted to exclude the central region of the sequences and decided to base our analysis on the remaining 312 bp. For the first intron of the S7 ribosomal protein gene a fragment of 636 bp was obtained. There was one fixed difference between Mediterranean and Atlantic individuals and 76 heterozygous positions. Of these, 73 positions were only heterozygous in individuals from the Atlantic or the Mediterranean and the remaining 3 showed heterozygosity in the 2 locations.

Diversity indexes based on the four genes sequences are shown in Table 1. The bayesian phylogeny built using the combined dataset of the four genes shows the existence of two strongly supported monophyletic groups, one including the Mediterranean samples and another comprehending the Atlantic haplotypes (Fig. 2). Within the Atlantic group, Azores haplotypes cluster together in a strongly supported monophyletic clade, while another very well supported clade groups fish from mainland Portugal, England, Madeira and Canaries. Phylogenetic reconstructions based on the mitochondrial genes only showed the same pattern (Fig. S1 in the Supplementary material). The tree based on the nuclear intron groups the Atlantic haplotypes in a monophyletic clade, which includes the Azorean haplotypes. The haplotypes from the Mediterranean did not form a monophyletic clade (Fig. S2 in the Supplementary material).

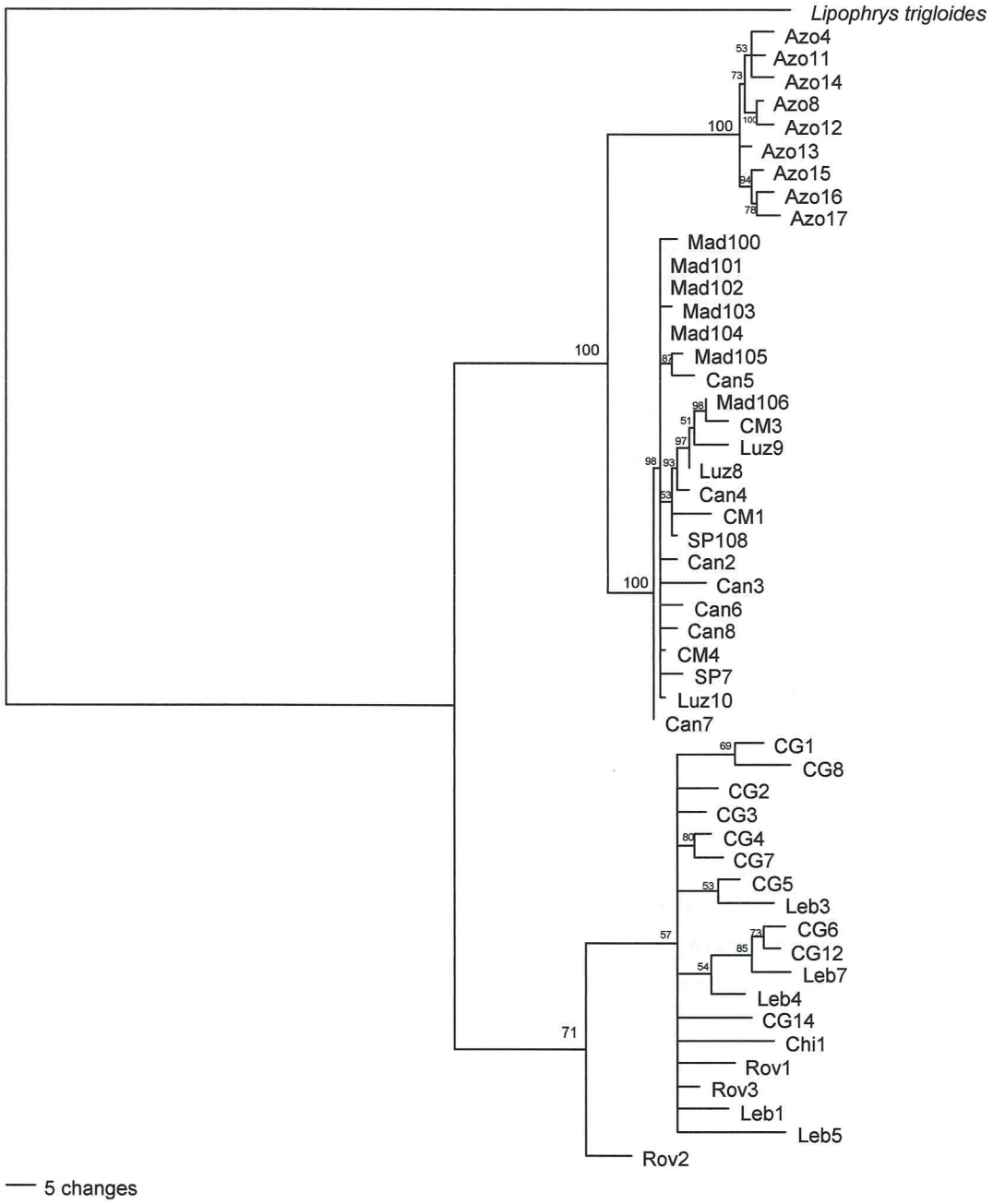


**Figure 2** Bayesian phylogeny of *Coryphoblennius galerita* populations based on sequences from the four genes fragments (12SrDNA, 16SrDNA, D-loop and S7 intron). The dataset was divided into four gene partitions to accommodate gene-specific differences in evolutionary rate. Numbers on branches are posterior probabilities from a consensus tree of all post bur-in topologies visited by the Markov chain. Labels are described in Figure 1.

**Table 1** Collection localities of *Coryphoblennius galerita* used in the present study and diversity indexes for 12srDNA, 16SrDNA, D-loop and S7 intro sequences. Number of haplotypes (Hn), Haplotype/ Gene diversity (Hd/Gd), and Nucleotide diversity ($\pi$) for each population are shown.

| | 12SrDNA | | | 16SrDNA | | | D-loop | | | S7 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Hn | Hd | $\pi$ | Hn | Hd | $\pi$ | Hn | Hd | $\pi$ | Hn | Gd | $\pi$ |
| Azores | 2 | 0.143 | 0.000 | 2 | 0.143 | 0.000 | 5 | 0.556 | 0.005 | 26 | 0.959 | 0.005 |
| Madeira | 1 | 0.000 | 0.000 | 1 | 0.000 | 0.000 | 8 | 0.381 | 0.055 | 21 | 0.928 | 0.002 |
| Canaries | 3 | 0.464 | 0.001 | 1 | 0.000 | 0.000 | 7 | 0.333 | 0.003 | 28 | 0.936 | 0.004 |
| Portugal | 1 | 0.000 | 0.000 | 4 | 0.421 | 0.000 | 15 | 0.536 | 0.010 | 21 | 0.944 | 0.001 |
| Total Atlantic | 5 | 0.539 | 0.005 | 6 | 0.539 | 0.014 | 35 | 0.443 | 0.009 | 73 | 0.949 | 0.003 |
| Cape of Gata, Spain | 2 | 0.556 | 0.001 | 4 | 0.644 | 0.001 | 19 | 1 | 0.016 | 29 | 0.994 | 0.010 |
| Chioggia, Italy | 1 | 0.000 | 0.000 | 1 | - | - | 1 | - | - | 2 | - | - |
| Rovinj, Croacia | 1 | 0.000 | 0.000 | 2 | 0.556 | 0.001 | 3 | 1 | 0.013 | 5 | 9.933 | 0.006 |
| Lebanon | 1 | 0.000 | 0.000 | 5 | 0.857 | 0.004 | 6 | 1 | 0.027 | 9 | 0.978 | 0.011 |
| Total Mediterranean | 2 | 0.395 | 0.001 | 8 | 0.676 | 0.002 | 18 | 0.621 | 0.036 | 45 | 0.996 | 0.010 |

*Gene flow and population structure.* Gene flow (Fst) between *Coryphoblennius galerita* locations was determined (Table 2). Gene flow between the Mediterranean and all the other locations was very restricted for all the molecular markers. Fst values between the Azores and the other locations were high for the mitochondrial markers, but not for the S7 intron.

The AMOVA based on the mitochondrial markers showed that a high percentage of the total genetic variance was explained by the variance among populations (96.67% P=0.000 and 85.06%, P=0.000 for 12S+16SrDNA and D-loop respectively) pointing to the existence of population structure for *Coryphoblennius galerita*. However, for the S7 intron 56.40% (P=0.000) of the total variation was due to variation within populations. Population average pairwise differences were estimated and results were visualized using a multidimensional scaling diagram (Fig. 3). According to the 12S +16S rDNA and D-loop the Mediterranean and the Azores appear as very differentiated populations. The Madeira and Canary Islands together with mainland Portugal appear as closely related populations. The picture is slightly different for the S7 intron, as only the Mediterranean appears as a very differentiated population.

**Table 2** Fst values for *Coryphoblennius galerita* populations calculated from the 12S and 16S rDNA (top table below the diagonal); mitochondrial control region sequences (top table, above the diagonal) and S7 intron (bottom table). Significant P values ($P < 0.05$) after Bonferroni correction are indicated by an asterisk. Fst values above 0.5 are bolded.

| | Azores | Madeira | Canaries | Portugal | W. Med |
|---|---|---|---|---|---|
| Azores | x | **0.544*** | **0.930*** | **0.842*** | **0.856*** |
| Madeira | **0.984*** | x | 0.261* | 0.281* | **0.896*** |
| Canaries | **0.970*** | 0.071 | x | 0.023 | **0.893*** |
| Portugal | **0.972*** | 0.002 | 0.000 | x | **0.879*** |
| W. Med | **0.981*** | **0.977*** | **0.955*** | **0.959*** | x |

| | Azores | Madeira | Canaries | Portugal | W. Med |
|---|---|---|---|---|---|
| Azores | x | | | | |
| Madeira | 0.007 | x | | | |
| Canaries | 0.028 | 0.000 | x | | |
| Portugal | 0.014 | 0.013 | 0.026 | x | |
| W. Med | **0.596*** | **0.639*** | **0.618*** | **0.633*** | x |

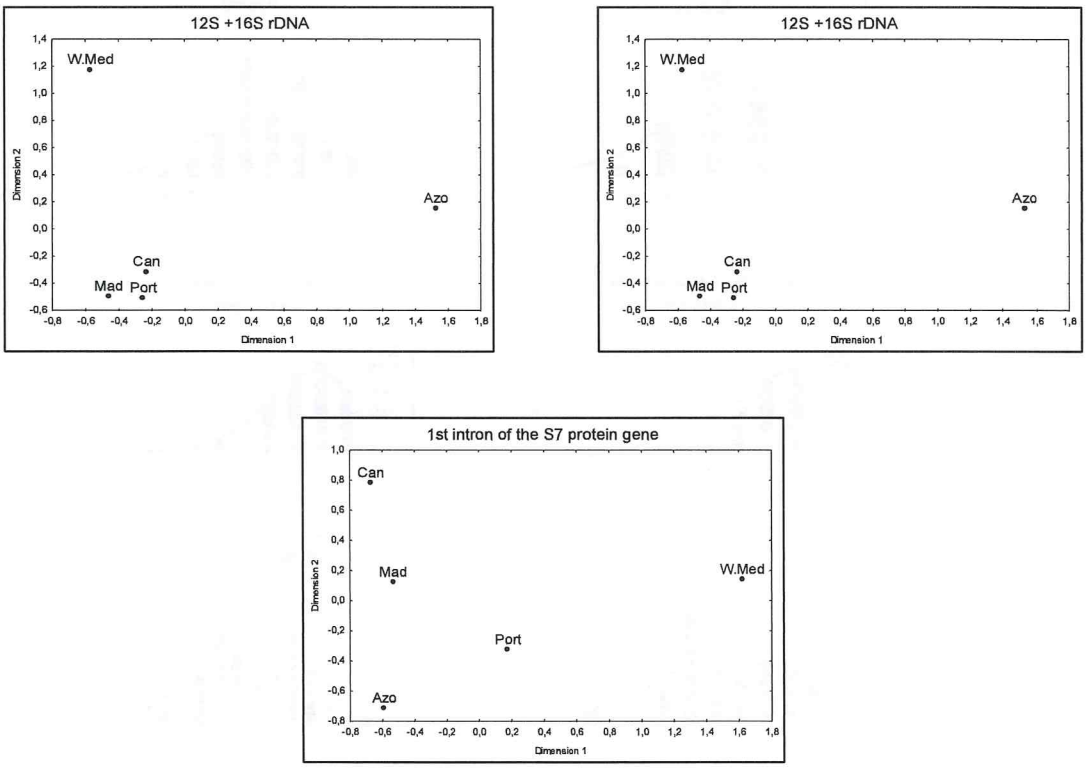


**Figure 3** Multidimensional scaling based on *Choryphoblennius galeritaI's* population average pairwise differences for 12S rDNA + 16S rDNA (A); D-loop (B) and first intron of the S7 protein gene (C). In the D-loop graph Madeira and Portugal plots overlap.

*Historical demography.* Mismatch distributions based on D-loop and S7 sequences were estimated for 3 populations (Azores; Madeira + Canaries + Portugal; and Western Mediterranean) according to the results obtained in the phylogenetic reconstruction. SSD tests were performed for each population. In the case of the mitochondrial control region the Azores population is represented by nine sequences only. Thus, the mismatch analysis concerning mitochondrial sequences from the Azores must be viewed with caution and only as an exploratory tool to inspect the data. Despite the mismatch distributions of the Mediterranean population were not unimodal (Fig. 4), the model of sudden expansion was not rejected for any of the populations (Table 3). Tajima's D values were significantly negative for the group including Madeira, Canaries and Portugal. Mismatch distributions based on the nuclear sequences were not unimodal for the Azores (Fig. 4) and SSD test rejected the model of sudden expansion for this population only (Table 3). However, Tajima's D values were significantly negative for Azores and the group including Madeira, Canaries and Portugal. The times of the expansion of the Azores and the remaining locations of the Atlantic are more recent than the Mediterranean estimates.

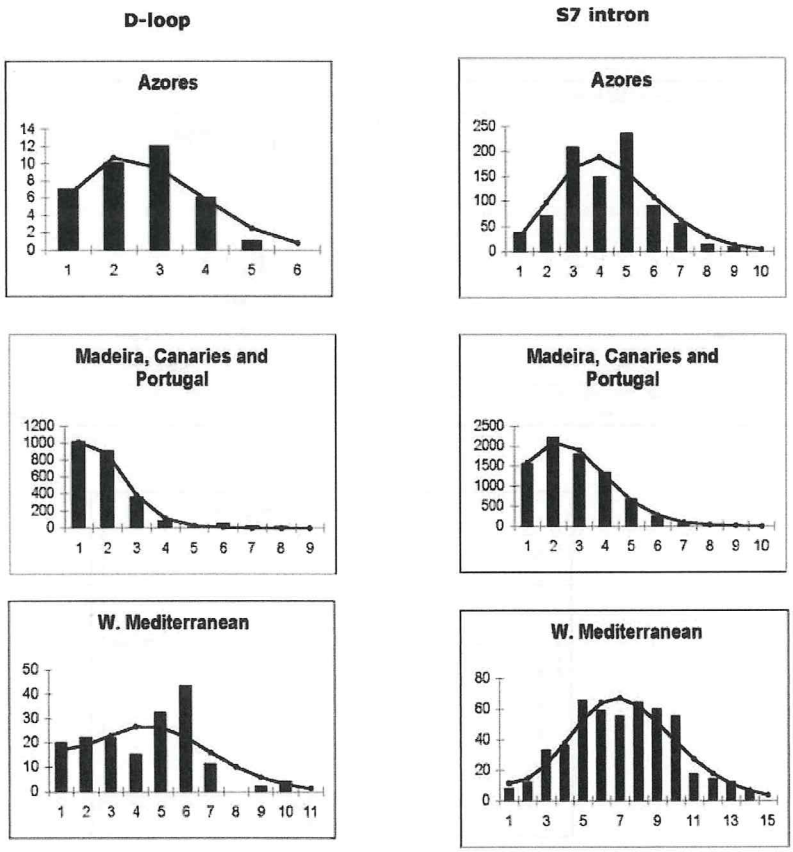


**Figure 4** Mismatch distributions of *Coryphoblennius galerita* D-loop and S7 intron haplotypes. The bars represent the observed frequency of the pairwise differences among haplotypes, while the line shows the expected curve predicted for a population that has undergone a demographic expansion in the past.

**Table 3** Estimated values for the expansion model for each population: SSD (sum of square deviations) and its probability P; $\theta_0$ and $\theta_1$ (compound parameter representing the mutation rate and the female effective population size before and after expansion respectively); and $\tau$ (time in generations). The time of the expansion (t, in million years) is also presented. Tajima's D neutrality test and its probability P. Significant P values ($P < 0.05$) are indicated by an asterisk.

| D-loop | SSD | P | $\theta_0$ | $\theta_1$ | $\tau$ | t (Kyr) | D | P |
|---|---|---|---|---|---|---|---|---|
| Azores | 0.007 | 0.758 | 0.000 | 821.2 | 1.759 | 0-22.5 | -0.843 | 0.232 |
| Madeira Canaries Portugal | 0.001 | 0.620 | 0.000 | 467.6 | 0.872 | 1.18-8.64 | -2.150 | 0.000* |
| W. Mediterranean | 0.026 | 0.260 | 0.000 | 9.709 | 4.560 | 13.4-52.5 | -0.317 | 0.404 |
| **S7** | | | | | | | | |
| Azores | 0.014 | 0.020* | - | - | - | - | -1.486 | 0.038* |
| Madeira Canaries Portugal | 0.001 | 0.920 | 0.246 | 7.676 | 1.841 | - | -1.958 | 0.002* |
| W. Mediterranean | 0.004 | 0.500 | 1.168 | 45.922 | 5.607 | - | 0.099 | 0.500 |

*Morphological analysis*

A discriminant function analysis using 17 continuous measurements generated a significant model (Wilk's $\lambda$ = 0.062, Approx. $F_{68.249}$ = 3.776, $P < 0.001$). The first two discriminant functions explained 86% of the data variability. The measurements which yielded a significant P value were PreAL, PreDL, PreOW, MW, CH and CW. Except for crest height (CH) and width (CW), measurements did not show a consistent pattern of variation. The ANOVA applied to CH and CW showed significant differences among populations for both morphological characteristics (HC: $F_{(1,2)}$ = 16.947; $P < 0.001$ and WC: $F_{(1,2)}$ = 18.835; $p < 0.001$). Tukey HDS test for post-hoc comparisons based on CW yielded significant results for comparisons of the Mediterranean with all other populations. The same test based on CH yielded significant results for comparisons of Azores against all the populations and also for comparisons of Madeira against all the populations. Crest width differentiates the Mediterranean individuals from all the others, while crest height separates the Azorean and Madeiran individuals from the ones in the remaining locations. Fish from the Azores show a high and wide crest, while Mediterranean representatives possess a low and narrow crest (Fig. 5). In addition, the Mediterranean fish present filaments only on the upper edge of the crest, while in the Atlantic the filaments occur around the entire crest (Fig. 5). Overall classification success was approximately 86%, with the Azores and the Mediterranean showing values above 90%. Figure 6 depicts the placement of the 5 populations in the two dimensional space defined by the first two discriminant functions. The Mediterranean is separated from the other populations. The Azores is somehow differentiated, being closer to Madeira than to the other locations. The Canary Islands and Portugal show a close connection. Figure 7 shows the phenogram based on the Squared Mahalanobis Distances resulting from the discriminant analysis. According to these distances the Mediterranean is well differentiated from the other populations. Azores/ Madeira and Canaries/ Portugal are morphologically close to each other. The results of the analysis of contingency tables of the meristic data are shown in Table 4. Although some values differed significantly from a random distribution, the distribution of the number of rays does not follow a geographic pattern.

**Table 4** Results of the analysis of contingency tables of the meristic data from *Coryphoblennius galerita*. The first value is the number of times that the simulated values did not exceed the observed ones. The second value is the number of times that the observed values did not exceed the simulated ones. Significant P values (< 0.05) are indicated by an asterisk.

| | 2nd dorsal fin ($\chi^2$ = 167.507, df = 8, P < 0.001) | | | Anal fin ($\chi^2$ = 46.664, df = 12, P < 0.001) | | | |
|---|---|---|---|---|---|---|---|
| | Number of rays | | | Number of rays | | | |
| | 15 | 16 | 17 | 16 | 17 | 18 | 19 |
| Azores | 1000/0* | 985/24* | 0/1000* | 524/716 | 809/271 | 156/928 | 823/539 |
| Madeira | 1000/1* | 994/16* | 0/1000* | 385/803 | 757/333 | 533/639 | 551/1000 |
| Canaries | 860/477 | 983/32* | 16/995* | 272/878 | 347/742 | 975/46* | 541/1000 |
| Portugal | 0/1000* | 5/995* | 1000/0* | 874/155 | 761/266 | 47/965 * | 664/488 |
| Mediterranean | 485/1000 | 969/54 | 96/936 | 10/1000* | 15/997* | 1000/0* | 823/539 |

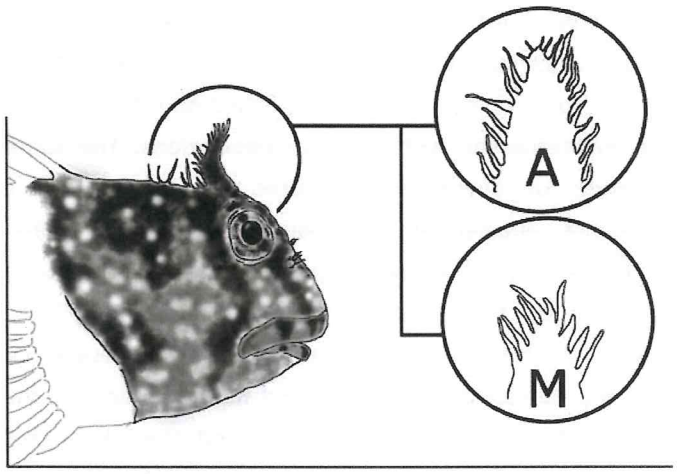


**Figure 5** Diagramatic representation of the crest of *Coryphoblennius galerita* from the Azores (A) and the Mediterranean (M).

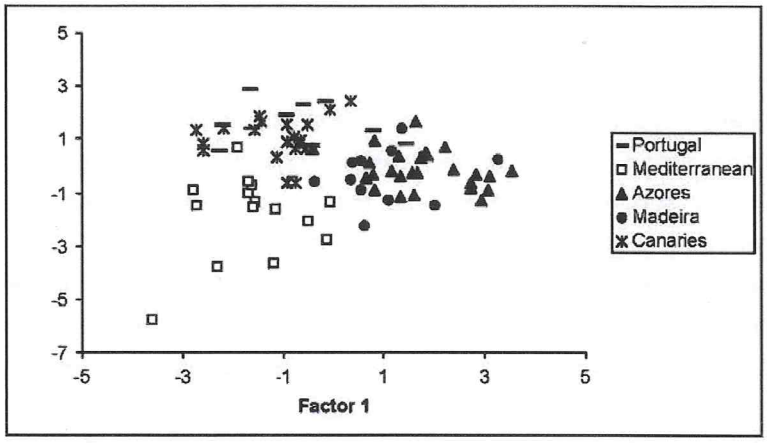


**Figure 6** Representation of the five *Coryphoblennius galerita* populations in the two dimensional space defined by the first two discriminant functions of a discriminant analysis including 17 continuous morphological measurements.

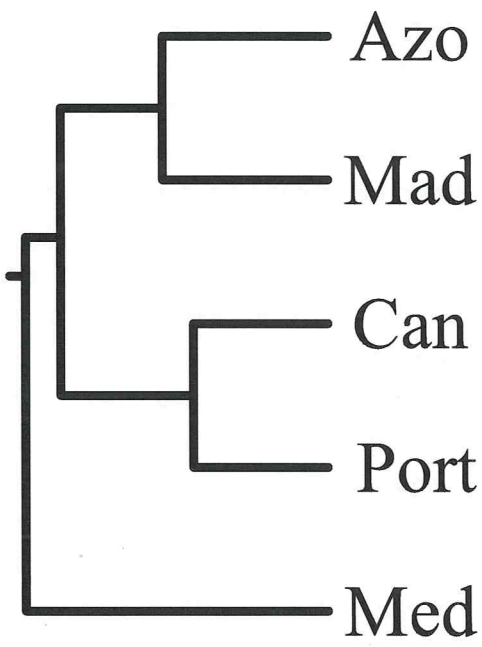


**Figure 7** Phenogram based on the Squared Mahalanobis Distances resulting from the discriminant analysis using 17 continuous morphological measurements of *Coryphoblennius galerita.* The phenogram was estimated using the UPGMA cluster analysis.

Discussion

This study clearly supports the existence of two clades of *Coryphoblennius galerita*: one in the Mediterranean and another in the eastern Atlantic. The eastern Atlantic group is subdivided in two subgroups: Azores; and the group formed by Madeira, Canaries and mainland Portugal (including also the sample from England). The Mediterranean differentiation is strongly supported by all molecular markers. Even the samples from the eastern basin clearly fell in the same clade. According to the mitochondrial markers, the Azores is also clearly differentiated from the other Atlantic locations. Interestingly, 12S and 16S rDNA uncorrected p distances between Atlantic and Mediterranean populations of *C. galerita* (1.3% and 3.3% respectively) and between the Azores and the remaining locations of the Atlantic (1.0% and 3.0% respectively) are very similar, and in some cases even higher, than the distances between several closely related blenniid species pairs (Almada *et al.* 2005; Carreras- Carbonell *et al.* 2005; Stefanni *et al.* 2006). Although the genetic differentiation of the Azores population was very clear according to the three mitochondrial markers, this differentiation was not detected with the nuclear intron. This is not surprising since lack of recombination in the mitochondrial DNA makes the whole genome a single genetic entity. Moreover, since mtDNA is haploid and only maternally inherited, mitochondrial genes have a fourfold lower effective population size than the nuclear ones, which makes fixation of mutations much slower in the nuclear genes.

The AMOVA analysis also reveals a major barrier between Mediterranean and Atlantic populations of *Coryphoblennius galerita*. Indeed, individuals from Cape of Gata, in the western Mediterranean, and those from the western coast of Portugal, despite being geographically close, show distinct mitochondrial and nuclear genotypes and a very restricted gene flow. When the patterns derived from the nuclear and mitochondrial markers are combined, the picture that emerges is one that involves a major split between Mediterranean and Atlantic populations, with some separation although less marked, between the Azores and the group formed by mainland Portugal, Madeira and Canaries. Interestingly this is also supported by the analysis of morphological characters (crest height and width). While crest width distinguishes two groups (Mediterranean from all the Atlantic fish) with a minimum in the Mediterranean and a maximum at Azores, crest height separates fish from Azores and Madeira from all the others, showing a minimum in the Mediterranean and a maximum at Azores. Bath (1978) using meristic characters and color patterns had already noted a clinal variation in *C. galerita* from the eastern end of the Mediterranean to the oceanic islands.

Taken together the results presented above suggest the existence of an effective barrier that prevents gene flow between Mediterranean and Atlantic populations of *Coryphoblennius galerita*. Several studies have proposed the Gibraltar strait or/and the "Almerian-Oran jet" as barriers to gene flow in different marine organisms (*e.g.* Pérez-Losada *et al.* 2002; Duran *et al.* 2004; Bargelloni *et al.* 2005; Lemaire *et al.* 2005). The connection between the Atlantic and the Mediterranean is known to has been severely reduced or even closed in the region of the Gibraltar strait during the Quaternary due to sea level fluctuations (Bianco 1990). This could have promoted the isolation of the two basins with subsequent genetic divergence. On a shorter time scale, the complex pattern of gyres and eddies of the Alboran sea can

constitute an effective physical barrier to small coastal fish like *C. galerita*, whose dispersal is restricted to the planktonic larval phase. On the other extreme, high levels of gene flow were found between the western coast of Portugal and the archipelagos of Madeira and Canaries. The Canaries current can assure the transport of larvae between these locations. It is interesting to note that the breeding season of *C. galerita* along the Portuguese coast lasts from March to September (Almada *et al.* 1996), which coincides with upwelling events in the Atlantic coast. This phenomenon is due to strong northwestern winds that, once reaching the coast, create offshore currents flowing to southwest. Larvae from the Atlantic coast of Portugal can then be easily pushed offshore joining the Canaries current that might promote their mixing with larvae from the islands. Thus, the population of Madeira is likely to have contributions from sources more to the north, namely southwestern Europe, together with fish persisting locally. It is well known that there are transport mechanisms capable of carrying fish larvae from Madeira to the Azores (Santos *et al.* 1995). This transport, even if sporadic, would prevent a complete isolation of the Azorean populations, explaining why, despite the great geographical isolation of the archipelago, the differentiation of the Azorean fish from those of the remaining Atlantic sites is less marked than that between the Cape of Gata and Portuguese fish, which are separated by a much shorter distance. Another explanation for this discrepant levels of genetic differentiation can be that gene flow between Atlantic and the Mediterranean populations has been reduced for a longer period than gene flow between Azores and the remaining locations in the Atlantic.

The Pleistocene glaciations have been shown to have a great effect in the geographical distribution of the genetic diversity of the Atlantic warm water species. Cooling during glacial peaks has been thought to have caused local extinctions and latitudinal shifts of the marine fauna of the region (Almada *et al.* 2001; Schiebel *et al.* 2002; Domingues *et al.* 2006, 2007b). Our data reveal no signs of regional extinctions, since genetic diversity is high in all populations studied. The Azorean population in particular shows a strong degree of genetic differentiation, at least for the mitochondrial markers, showing the long-term persistence of the population. Although not drastic, the effects of Pleistocene glaciations on the demographic history of *C. galerita* populations were felt, at least in some regions. The historical demography of the species reveals that the Azores and the remaining populations of the Atlantic experienced an expansion at about the same time (between 0 and 22.5 Kyr in the case of the Azorean population and 1.18- 8.64 Kyr in the case of Madeira, Canaries and Portugal). This time frame roughly coincides with the Younger Dryas (YD) at about 12 Kyr, when, although already after the Last Glacial Maximum (LGM), a large-scale cooling occurred (Lambeck *et al.* 2002).

It is interesting to note the emergence of two biogeographical patterns when considering Atlantic/Mediterranean marine fauna. Warm water species, such as *Tripterygion delaisi* (Domingues *et al.* 2007b), *Chromis limbata/ C. chromis* (Domingues *et al.* 2006) and *Parablennius parvicornis/P. sanguinolentus* (Almada *et al.* 2005) show two groups of populations: one including the Mediterranean and the Atlantic coast of western Europe and another encompassing the western tropical coast of Africa and the Atlantic islands of the Azores, Madeira and Canaries. On the other hand, cold resistant species such as *Coryphoblennius galerita* and *Lipophrys pholis* (Stefanni *et al.* 2006) show an accentuated

differentiation of the Azores population, and, in the case of *C. galerita,* a clear divergence between Mediterranean and western European individuals (*L. pholis* does not form stable populations in the Mediterranean; Zander 1986). Similarly, the clade *Symphodus trutta* and *S. caeruleus,* which belongs to a temperate genus, shows that the Azorean population acquired enough distinctive characters to be placed in a different species (*S. caeruleus*) when compared to fish from Madeira and Canaries (*S. trutta*; Almada *et al.* 2002). It is very likely that *S. caeruleus* ancestors persisted in the Azores during the glaciations, being able to diverge and accumulate substantial differences. This pattern may reflect the different effects of the Pleistocene glaciations on fishes with different thermal tolerances. Less tolerant species must have become extinct in some regions where sea surface temperatures were seriously reduced. These regions include the western coast of Portugal, the eastern islands of the Canary archipelago, and to a lesser extent the Azores. Recolonization of these locations may have been possible in the last 10 Kyr, from less affected regions, namely the western tropical coast of Africa and Madeira islands in the case of the Azores and the western Canaries and the southwestern Mediterranean in the case of the Atlantic shores of Iberia (Almada *et al.* 2001; Domingues *et al.* 2006; Domingues *et al.* 2007b). This different postglacial colonization routes would explain the lack of differentiation between Portuguese and western Mediterranean populations on one hand and also the homogeneity among Azorean, Canarian and Madeiran populations and their affinities with western Africa, on the other. The scenario outlined above fits well all the available data for warm water species but is unlikely to hold for species that tolerate cooler waters, like *C. galerita*. Populations of these species might have persisted during the Pleistocene cooling episodes at the less afected areas, including the Azores. Thus, it is not surprising that these species show clear signs of population differentiation in the more isolated locations such as the Azores archipelago and the Mediterranean Sea. Although the shores of southwest Europe were seriously affected at the glacial maxima, causing a likely local extinction of *C. galerita*, the tolerance of the species to relatively cold waters may have allowed its survival in the shores of northwest Africa, from which they could have easily re-invaded Europe.

Our study is one of the first to combine morphological and molecular markers, and to apply molecular markers of different natures (mitochondrial and nuclear) and with variable rates of molecular evolution to the study of the relationships of the Atlantic and Mediterranean populations of a cool water species. It is well known that an accurate investigation of spatial and temporal genetic structure should consider the variety of patterns seen in different loci, because of the stochastic effect of genetic drift in gene frequencies of each locus (Slatkin & Maddison 1989). Moreover, because the phylogenetic tree derived from a single locus may not accurately reflect the history of a species or population (Ball *et al.* 1990), population structure can only be accurately viewed by the concordance of phylogenetic patterns across several loci (Avise 2000).

More studies on these cool water species are needed to test the hypothesis that fishes of tropical and temperate affinities differ in their response to the glacial changes that affected the Atlantic-Mediterranean area.

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## Supplementary material

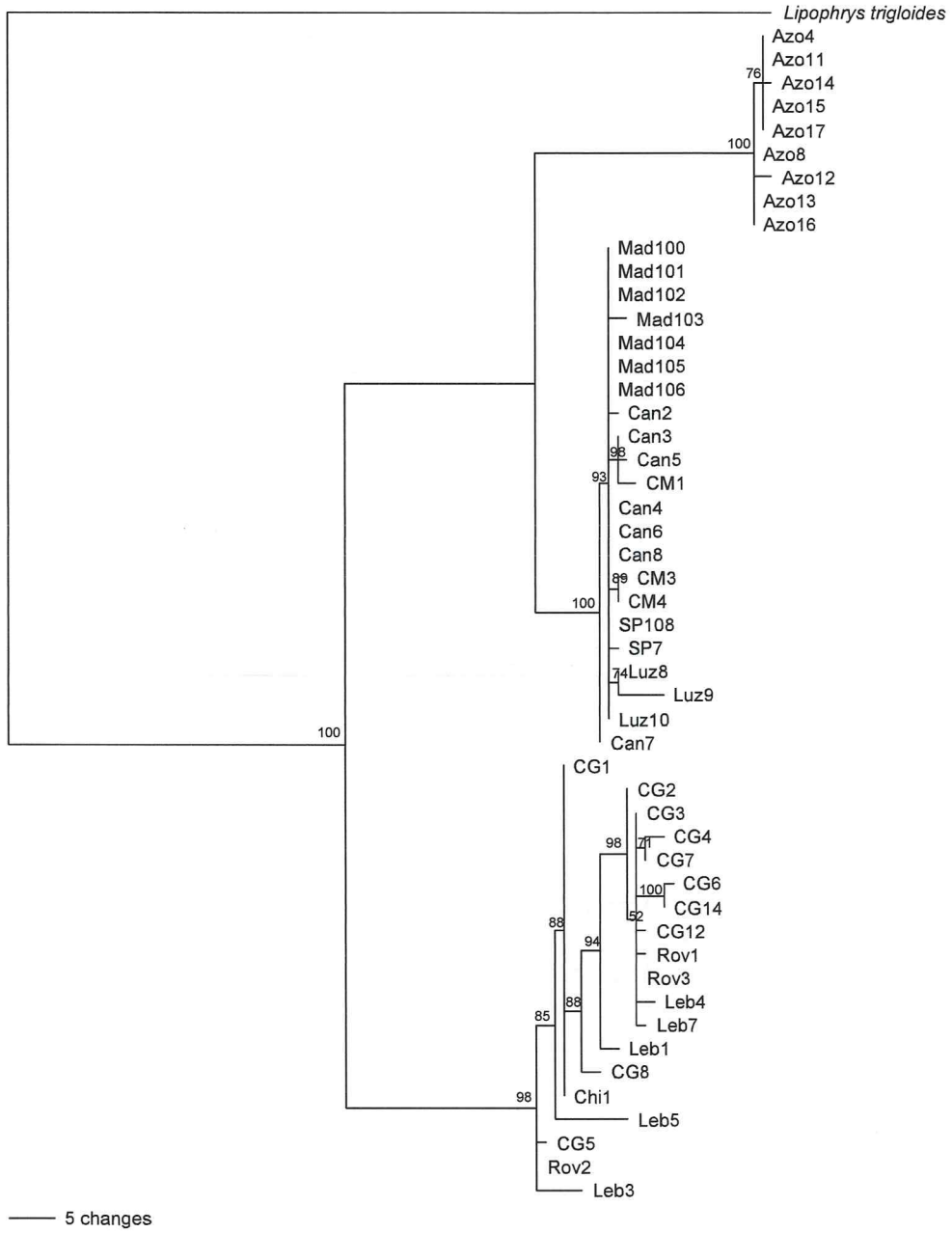


**Figure S1** Bayesian phylogeny of *Coryphoblennius galerita* populations based on sequences from the mitochondrial fragments (12SrDNA, 16SrDNA and D-loop). The dataset was divided into four gene partitions to accommodate gene-specific differences in evolutionary rate. Numbers on branches are posterior probabilities from a consensus tree of all post burn-in topologies visited by the Markov chain. Labels are described in Figure 1.

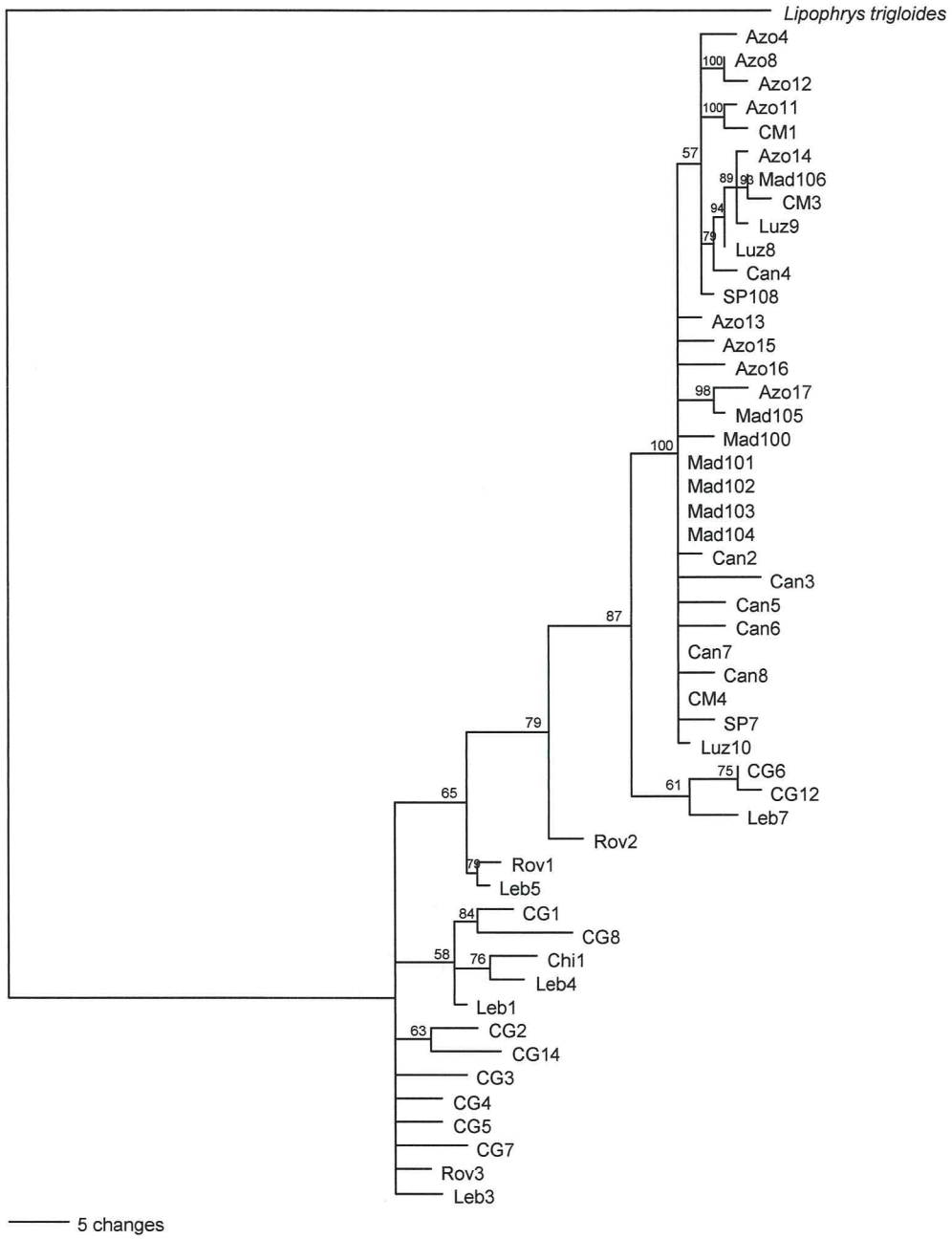


**Figure S2** Bayesian phylogeny of *Coryphoblennius galerita* populations based on sequences from the first intron of the S7 ribosomal protein gene. Numbers on branches are posterior probabilities from a consensus tree of all post burn-in topologies visited by the Markov chain. Labels are described in Figure 1.

# Chapter 10

## Tropical fishes in a temperate sea: evolution of the wrasse *Thalassoma pavo* and the parrotfish *Sparisoma cretense* in the Mediterranean and the northeastern Atlantic islands

# Tropical fishes in a temperate sea: evolution of the wrasse *Thalassoma pavo* and the parrotfish *Sparisoma cretense* in the Mediterranean and the northeastern Atlantic islands

*Submitted to Marine Biology*

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**Abstract**

The northeastern Atlantic and the Mediterranean Sea are historically closely related and share great faunal affinities. The majority of the Mediterranean species have Atlantic origin, with a few species with tropical affinities. These include the parrotfish *Sparisoma cretense* and the wrasse *Thalassoma pavo* that are restricted to the subtropical North-eastern Atlantic, the Macaronesian archipelagos and the southern Mediterranean. The Pleistocene glaciations have been described as having different effects on the fauna of the two regions. During glacial peaks, Mediterranean waters remained warmer than those of the adjacent Atlantic. Within the eastern Atlantic, the effects of Pleistocene glaciations were differentiated. Here, we perform a comparative analysis focusing on *T. pavo* and *S. cretense* populations from the northeastern Atlantic and the Mediterranean to assess the effects of Pleistocene glaciations in these two species. Sequences from the mitochondrial control region were obtained and analyzed combining phylogeographic and demographic approaches. Gene flow between Atlantic and Mediterranean populations was shown to be very high. The Mediterranean populations of *T. pavo* and *S. cretense* showed high levels of genetic diversity, even in the eastern basin, pointing to an ancient colonization of this sea. This suggests that both species must have been able to persist in the Mediterranean during the cold Pleistocene periods. Historical migration estimates revealed a Mediterranean towards Atlantic trend in the case of *Thalassoma pavo*, which may reflect the re-colonization of areas in the Atlantic by fish that survived the cold phases in relatively warmer Mediterranean refugia. Our data also showed that migration in the Macaronesian Archipelago occurred from Madeira towards the Azores, for both *T. pavo* and *S. cretense*, thus supporting a post-glacial colonization of the Azores by fish that persisted in the warmer region of Madeira. Similar geographic distributions, thermal affinities, and means of dispersion for *T. pavo* and *S. cretense* resulted in a similar response to the effects of Pleistocene glaciations, as evidenced by identical phylogeographic patterns.

**Introduction**

Paleogeography and oceanography have played an important role in shaping the present day phylogeographic patterns in Northeastern Atlantic and Mediterranean marine faunas. Approximately 5-6 million years ago (Mya), the Mediterranean Sea experienced a desiccation event, the Messinian Salitiny Crisis (MSC), that lasted for some hundred thousands years and resulted in a major extinction of its marine fauna (Hsü et al. 1977; Krijgsman et al. 1999; Duggen et al. 2003). This event was followed by replenishment from the adjacent Atlantic Ocean, at the base of the Pliocene, suddenly restoring the open marine conditions. More recently, Pleistocene glaciations were accompanied by lowered seawater temperatures and changes in oceanographic characteristics (Briggs 1996; Adams et al. 1999; Lambeck et al. 2002). During these glacial peaks, Mediterranean waters remained warmer than those of the adjacent Atlantic (Thiede 1978). Within the eastern

Atlantic, the effects of Pleistocene glaciations were differentiated. The western coast of Europe and the northwest coast of Africa were particularly affected by a very pronounced southward migration of the polar front, which caused a significant drop in the seawater temperatures of these regions (Crowley 1981, Dias 1997). The Macaronesian archipelagos (Azores, Madeira and Canaries) and Cape Verde islands were also differentially affected. While the Azores region experienced moderate cooling (2-3°C; Crowley 1981), the Madeira archipelago, located further south, was not affected. The Canaries were affected to some extent, and the Cape Verde islands, although remaining considerably warm, were clearly out of the Tropical bio-region (Briggs 1996). These climatic events promoted the extinction of the warm temperate species in the cooler regions of the Atlantic. Although the lowering of sea surface temperatures of the Azores were not very pronounced, they may have still triggered migrations and even local extinction of several littoral fish species, particularly for populations that are not distributed further north than this region. Many species now present in the warm temperate Atlantic are likely to have survived the cold phases of the glacial cycles in less affected regions such as Madeira, the western tropical coast of Africa, and southern Mediterranean, recolonizing the affected regions when more favorable temperatures were reestablished during interglacial phases (Almada et al. 2001). Santos et al. (1995) suggested that a relevant set of the organisms (mainly fish) now present in the Azores would have recolonized the islands from some of the southern regions mentioned above, namely Madeira, after the end of the last glaciation. These geological and climatic events shaped the biota of the Mediterranean, which is currently considered a subtropical temperate sea, with most species being temperate. Most Mediterranean species have Atlantic affinities, which is supported by genetic evidence (Pannacciuli et al. 1997; Pérez Losada et al. 1999; Aurelle et al. 2003; Bargelloni et al. 2003; Costagliola et al. 2004; Duran et al. 2004). A few species show clear tropical affinities. These include the parrotfish *Sparisoma cretense* and the wrasse *Thalassoma pavo*, which belong to the closely related families Scaridae and Labridae respectively. The distributions of both species reflect their preference for warmer water. Indeed, these species are restricted to the subtropical North-eastern Atlantic, the Macaronesian archipelagos and the southern Mediterranean (but are slowly migrating North, due to recent warming trends; Guidetti et al. 2002). In the Atlantic, *T. pavo* occurs from Portugal (Algarve) southwards to Cape Lopez (Gabon) (Quignard and Pras 1986a), and *S. cretense* has its southern limit in Senegal (Quignard and Pras 1986b; González 1993).

Life history characteristics

The genus *Thalassoma* comprises 30 species that are mostly found on tropical coral reefs (Bernardi et al. 2004). Six closely related species are found in the Atlantic Ocean: *T. bifasciatum* and *T. norhonanum* are found in the western Atlantic, and *T. newtoni*, *T. pavo*, *T. sanctaehelenae* and *T. ascensionis* in the eastern Atlantic (Costagliola et al. 2004). *Thalassoma newtoni*, is found in São Tomé and Principe, just off shore from Gabon, the recognized southern distribution limit of *T. pavo* (Quignard and Pras 1986a). It is possible

that this southern limit is due to a misidentification of *T. newtoni*. Nevertheless, individuals from the Cape Verde islands were shown to be *T. pavo* (Costagliola et al. 2004) indicating that the southern distribution of *T. pavo* reaches at least these islands and could possibly go beyond them. In addition, *T. pavo* is found in the warmer regions of the Mediterranean. Like many other wrasses *T. pavo* is a partially protogynous hermaphrodite, with external fertilization and broadcast spawning (Gomon and Forsyth 1990). Pelagic eggs hatch after a few days and active swimming larvae remain in the water column for 38-49 days (Raventós and Macpherson 2001). Juvenile fishes then recruit to rocky reefs and seagrass beds (*Posidonia oceanica*). Costagliola et al. (2004) studied Atlantic and Mediterranean populations of *T. pavo* and found no genetic discontinuities between the two regions. Within the Mediterranean, populations of *T. pavo* showed a genetic restriction of gene flow between eastern and western regions.

The genus *Sparisoma* comprises 13 species that are restricted to the Atlantic Ocean (Robertson and Warner 1978; Bernardi et al. 2000; Robertson et al. 2006) with most species found in the western Atlantic. One species, *S. aff. rubripinne*, occurs in the tropical eastern Atlantic from Mauritania to Gulf of Guinea including Cape Verde and São Tomé and Principe islands (L. Rocha, personal communication). An isolated species, *S. strigatum,* is distributed in the mid Atlantic islands of Saint Helena and Ascension and was shown to be the sister species of *S. cretense* (Bernardi et al. 2000; Robertson et al. 2006). The distribution of *S. cretense* is very similar to the distribution of *Thalassoma pavo*, as they are both found in the Macaronesian archipelagos, Cape Verde islands and in the warmer waters of the Mediterranean. *Sparisoma cretense* is a benthic fish mainly inhabiting rocky bottoms and sea grass beds. This species is essentially gonochoristic, although the potential for sex change is not excluded (González et al. 1994; Girolamo et al. 1999). The reproductive season of *S. cretense* lasts mainly throughout the months of June to October, with variations according the climatic regional conditions (González 1993). Fertilization occurs in the water column and larvae remain pelagic until recruitment to the adult habitat. Unlike most wrasses and parrotfishes, *S. cretense* male coloration is drab while the female is brilliantly colored with yellow patches on an orange background (González et al. 1994).

The evolutionary history of *Sparisoma cretense* and *Thalassoma pavo* presents a number of interesting challenges: 1) How were populations affected when seawater temperatures dropped during the Pleistocene glaciations events? 2) What are the historical dynamics between Atlantic and Mediterranean populations? 3) Do *S. cretense* and *T. pavo* populations show similar phylogeographic and demographic patterns and to what extent may this similarity be attributed to the effects of Atlantic and Mediterranean paleoclimatic history?

To address these questions we used a comparative phylogeographic method based on the fast evolving mitochondrial control region gene. We focus on two fish species with tropical affinities that are restricted to the Mediterranean and Northeastern Atlantic. Sequences from *Thalassoma pavo* available from Costagliola et al. (2004) were integrated in our study and a comparative analysis including *Sparisoma cretense* was performed. New data

analysis tools based on the coalescent theory, allowed the determination of the direction of migration between populations of *T. pavo* and *S. cretense*. Combined with traditional approaches, these methods shed light on the evolutionary history and population dynamics of the two species in these closely related regions.

## Materials and methods

### Sampling and laboratory procedures

A total of 84 *Sparisoma cretense* and 134 *Thalassoma pavo* from the Mediterranean and the islands of the Azores, Madeira, Canaries and Cape Verde were used in this study. We used *S. strigatum* and *T. ascensionis* and *T. sanctahelenae* from Ascension Island and Saint Helena as outgroups. Sampling localities and number of individuals are given in Table 1 and Figure 1. Samples of *S. cretense* were collected by spear or hand nets while scuba diving or free diving. Fin clips were cut immediately after collection of the individuals and stored at ambient temperature in 95% ethanol. Total genomic DNA was extracted by SDS proteinase K procedure and purified by standard chloroform and isopropanol precipitation (Sambrook et al. 1989). Amplification of the 5′ hypervariable portion of the mitochondrial control region (611bp) was accomplished with universal primers L15725 (revised from Sorenson et al. 1999) and CR-E (Lee et al. 1995), and used a cycling profile of 45 sec at 94°C, 45 sec at 52°C, 1 min at 72°C, for 35 cycles. Each 13 μl reaction contained 5-50 ng of DNA, 10 mM Tris HCL (pH 8.3), 50 mM KCl, 1.5mM $MgCl_2$, 1.25 u of *Taq* DNA polymerase (Perkin-Elmer, Norwalk, Conn.), 150 mM of each dNTP, and 0.3 mM of each primer. After purification following the manufacturer's protocol (Applied Biosystems, Forter City, CA), direct sequencing was performed with an ABI 3100 automated sequencer (Applied Biosystems). Partial mitochondrial control region sequences (321 bp) of *Thalassoma pavo* obtained in Costagliola et al. (2004) (GenBank accession numbers AY329698-AY329798) were used in this study. In addition, 3 different locations in the Mediterranean (Kea, Sifnos and Santorini) were included in the analysis, and sample size of Cape Verde was increased. PCR and sequencing procedures followed Costagliola et al. (2004).

**Table 1** Collection localities and diversity indexes of *Thalassoma pavo* and *Sparisoma cretense* used in the present study. Number of individuals (n), number of haplotypes (nH), Haplotype diversity (Hd) and nucleotide diversity ($\pi$) for mitochondrial control region were calculated using DNAsp (Rozas *et al.* 2003). Locality labels are presented between parentheses.

| | *Thalassoma pavo* | | | | *Sparisoma cretense* | | | |
|---|---|---|---|---|---|---|---|---|
| Species Locality | n | nH | Hd | $\pi$ | n | nH | Hd | $\pi$ |
| **Atlantic** | 49 | 30 | 0.915 | 0.011 | 51 | 31 | 0.943 | 0.005 |
| Azores, Portugal (Azo) | 12 | 6 | 0.818 | 0.004 | 10 | 9 | 0.978 | 0.005 |
| Canaries, Spain (Can) | 11 | 6 | 0.800 | 0.005 | 19 | 12 | 0.871 | 0.003 |
| Cape Verde (CV) | 15 | 15 | 1.000 | 0.016 | 12 | 9 | 0.955 | 0.005 |
| **Mediterranean** | 85 | 34 | 0.829 | 0.006 | 33 | 23 | 0.964 | 0.007 |
| Western Mediterranean | | | | | | | | |
| Al-Hoceima, Morocco (Mor) | 3 | 3 | 1.000 | 0.008 | - | - | - | - |
| Genoa, Italy (Gen) | 10 | 6 | 0.778 | 0.007 | - | - | - | - |
| Calvi, Corsica, France (Cal) | 3 | 3 | 1.000 | 0.006 | - | - | - | - |
| Olbia, Sardinia, Italy (Olb) | 10 | 4 | 0.533 | 0.002 | - | - | - | - |
| Pozzuoli, Italy (Poz) | 11 | 5 | 0.618 | 0.004 | - | - | - | - |
| Naples, Italy (Nap) | 3 | 3 | 1.000 | 0.008 | - | - | - | - |
| Ustica, Italy (Ust) | 2 | 2 | 1.000 | 0.003 | - | - | - | - |
| Lipari, Italy (Lip) | 7 | 3 | 0.524 | 0.004 | - | - | - | - |
| Linosa, Italy (Lin) | 3 | 3 | 1.000 | 0.004 | - | - | - | - |
| Lampedusa, Italy (Lamp) | - | - | - | - | 3 | 3 | 1.000 | 0.004 |
| Eastern Mediterranean | | | | | | | | |
| Otranto, Italy (Otr) | 9 | 6 | 0.833 | 0.007 | - | - | - | - |
| Kea, Greece (Kea) | 3 | 2 | 0.667 | 0.002 | 12 | 11 | 0.985 | 0.007 |
| Sifnos, Greece (Sif) | 6 | 5 | 0.933 | 0.007 | 10 | 9 | 0.978 | 0.005 |
| Santorini, Greece (Sant) | - | - | - | - | 8 | 7 | 0.964 | 0.011 |
| Rhodes, Greece (Rho) | 15 | 8 | 0.733 | 0.005 | - | - | - | - |

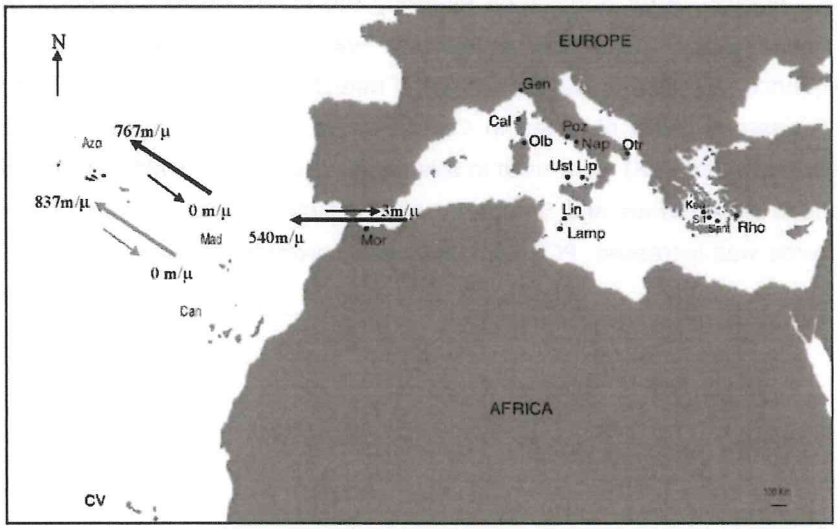


**Figure 1** *T. pavo* and *S. cretense* sampling locations. Both species were collected from the Macaronesian and Cape Verde islands, in the Atlantic, and the Mediterranean (see Table 1 for detailed sampling locations and labels). Extent of migration of *T. pavo* (between Atlantic and Mediterranean populations and between Azores and Madeira) are shown with black arrows. Extent of migration of *S. cretense* (between the Azores and Madeira) are shown with grey arrows. Numbers of migrants in each direction are given close to the arrows.

Genetic diversity and phylogenetic relationships

Sequences were aligned using the computer program Clustal V implemented by Sequence Navigator (Applied Biosystems). Diversity indexes (number of haplotypes, haplotype diversity and nucleotide diversity) of *Sparisoma cretense* and *Thalassoma pavo* were calculated using DNAsp (Rozas et al. 2003). Phylogenetic relationships were inferred using Maximum Parsimony (MP) and Neighbor-Joining (NJ) methods implemented by the software package PAUP (version 4.0; Swofford 1998). As we were dealing with very closely related species, with small genetic distances, we adopted the uncorrected p distance following Nei and Kumar (2000). Topological confidence was evaluated for MP and NJ, with 1000 bootstrap replicates (Felsenstein 1985).

Gene flow and Historical demography

Gene flow (Fst) and corrected average pairwise differences between populations were estimated for both species using ARLEQUIN (vers. 2.000; Schneider et al. 2000). p distances were estimated for each pair of populations. Population structure was estimated by an analysis of molecular variance (AMOVA, Excoffier et al. 1997) by partitioning the data into two groups (Atlantic and Mediterranean).

Exchanges and range expansions (immigration) between Atlantic and Mediterranean and also between Azores and Madeira were estimated for *Sparisoma cretense* and *Thalassoma pavo* using MIGRATE 2.0.3 (Beerli and Felsenstein 2001; Beerli 2004). In the case of *T. pavo*, only samples from the western basin of the Mediterranean were included in the analysis generating similar sampling sizes for the two locations. Analyses were repeated 10 times, to ensure stability of parameter estimates. Final search strategy varied according to the dataset. For *S. cretense* final analyses employed 10 short Monte Carlo chains with 1,000,000 sampled genealogies and 3 long chains with 10,000,000 sampled genealogies. We applied an exhaustive search using four heated chains {1, 4, 7, 10} and an interval between swapping trees of 1. For the *T. pavo* dataset the same heating scheme and number or Monte Carlo chains were used, but 100,000 sampled genealogies for the short chains and 1000,000 sampled genealogies for the long chains were enough to ensure stability. Finally, two runs were performed using the obtained parameters estimated in the previous runs to ensure stability was reached.

## Results

Genetic diversity and phylogenetic relationships

A total of 83 *Sparisoma cretense* and 13 *S. strigatum* partial sequences of the mitochondrial control region were obtained (GenBank accession numbers: EF484419-EF484515). New sequences of *Thalassoma pavo* were also deposited in GenBank (accession numbers: EF484516-EF484537). Number of haplotypes, haplotype diversity and nucleotide diversity for the mitochondrial control region are shown in Table 1. Both species revealed high diversity values for all locations.

Both methods of phylogenetic inference resulted in similar topologies. Figures 2 and 3 show *Thalassoma pavo* and *Sparisoma cretense* Neighbor-Joining phylogenies based on the mitochondrial control region sequences. Haplotypes were shared between populations and none of the species showed clear signs of geographic partitioning. The majority of *T. pavo* haplotypes from Cape Verde grouped together in a clade containing also 2 haplotypes from Italy. However, this clade was weakly supported (bootstrap below 50%). Similarly, all haplotypes from the island of Rhodes, Greece grouped together in a clade with a bootstrap below 50%. For *Sparisoma cretense* all clades included haplotypes from different locations, thus resulting in a tree with no obvious geographic structure.

Gene flow

Fst values and p distances estimated for *Thalassoma pavo* and *Sparioma cretense* populations are shown in Tables 2 and 3 respectively. Gene flow between *S. cretense* and *T. pavo* populations was found to be high. *Sparisoma cretense* in particular, revealed remarkably high levels of gene flow, with all pairwise comparisons showing Fst values below 0.1. Cape Verde samples appeared as a more isolated population for both species. *Thalassoma pavo* showed gene flow restriction between the Canary islands and the other locations but also between the eastern locations of the Mediterranean (Kea, Sifnos and Rhodes) and the remaining locations.

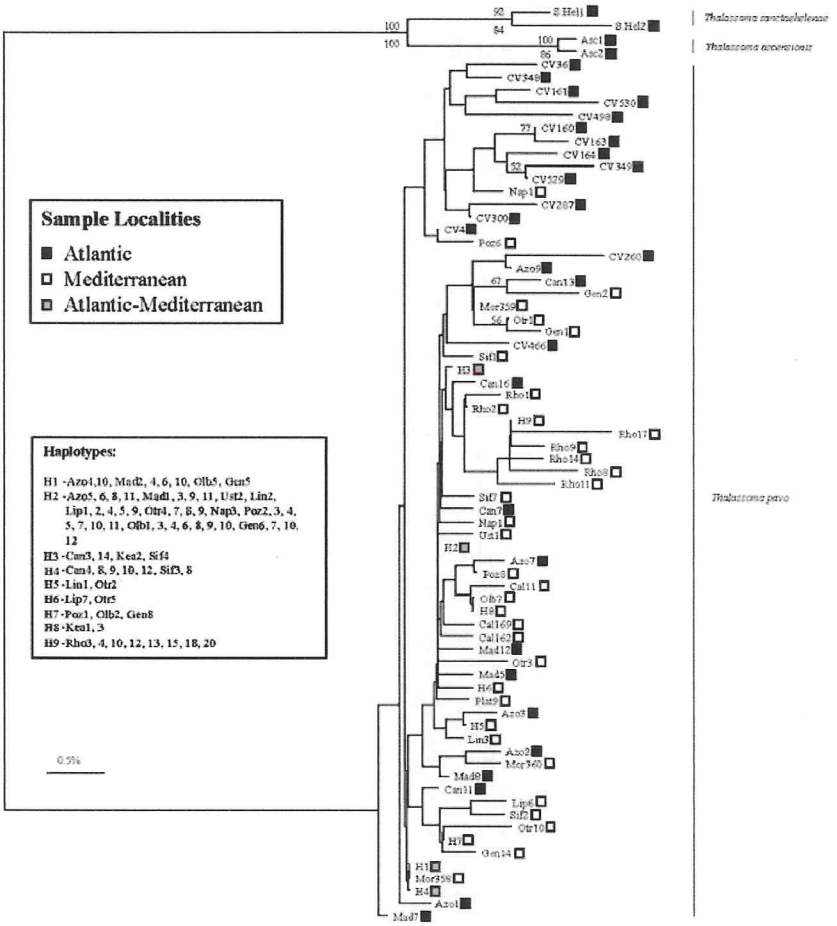


**Figure 2** Phylogenetic relationship within *Thalassoma pavo* for the mitochondrial control region sequences. *T. sanctaehelenae* and *T. ascensionis* were used as outgroup., Neighbor-Joining trees are shown with Neighbor-Joining (above the nodes) and Maximum Parsimony (below the nodes) bootstrap support above 50% indicated at the nodes. Labels are described in Table 1. The length of each branch is proportional to the number of nucleotide substitutions.

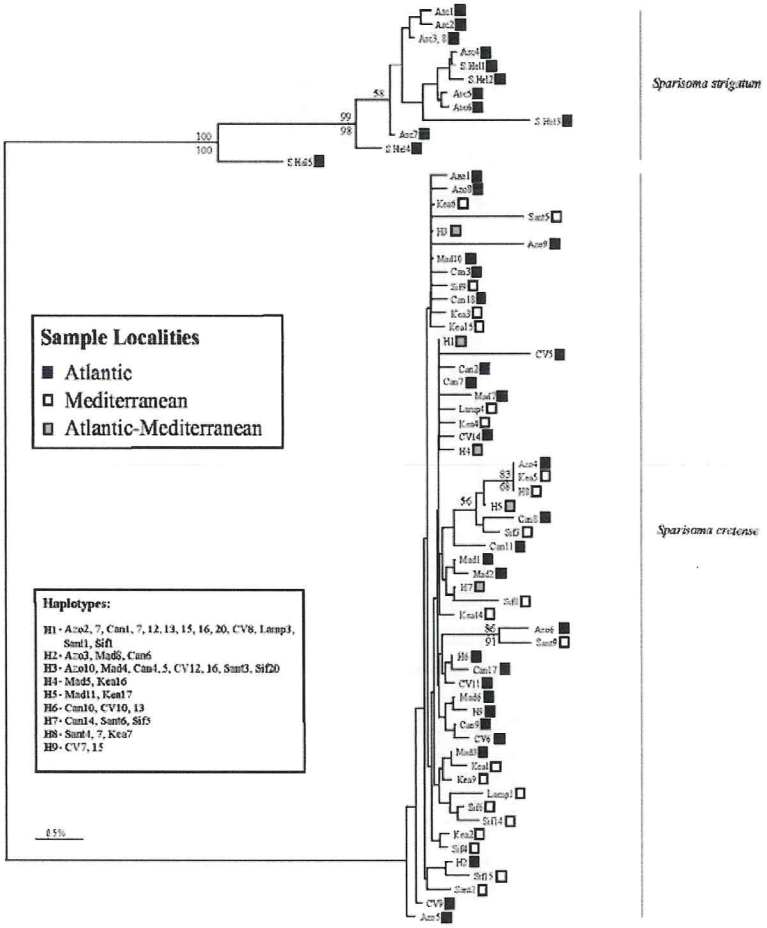


**Figure 3** Phylogenetic relationship within *Sparisoma cretense* for the mitochondrial control region sequences. *S. strigatum* was used as outgroup., Neighbor-Joining trees are shown with Neighbor-Joining (above the nodes) and Maximum Parsimony (below the nodes) bootstrap support above 50% indicated at the nodes. Labels are described in Table 1. The length of each branch is proportional to the number of nucleotide substitution.

**Table 2** Uncorrected p distances and Fst values (above and below the diagonal respectively) among *Thalassoma pavo* populations calculated from mitochondrial control region sequences using ARLEQUIN version 2.000 (Schneider et al. 2000). Fst significant p values (p<0.05) after Bonferroni correction are bolded

| | Azo | Mad | Can | CV | Mor | Gen | Cal | Olb | Poz | Nap | Ust | Lip | Lin | Otr | Kea | Sif | Rho |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Azo | | 0.000 | 0.297 | 0.541 | 0.053 | 0.000 | 0.222 | 0.000 | 0.000 | 0.044 | 0.034 | 0.000 | 0.000 | 0.000 | 0.413 | 0.284 | 0.563 |
| Mad | 0.000 | | 0.303 | 0.544 | 0.066 | 0.003 | 0.256 | 0.002 | 0.011 | 0.047 | 0.047 | 0.003 | 0.047 | 0.013 | 0.464 | 0.292 | 0.575 |
| Can | **0.335** | **0.409** | | 0.780 | 0.360 | 0.308 | 0.606 | 0.337 | 0.347 | 0.388 | 0.398 | 0.341 | 0.379 | 0.353 | 0.189 | 0.000 | 0.607 |
| CV | **0.304** | **0.336** | **0.401** | | 0.473 | 0.575 | 0.953 | 0.643 | 0.617 | 0.328 | 0.744 | 0.655 | 0.730 | 0.649 | 1.119 | 0.793 | 1.225 |
| Mor | 0.122 | **0.245** | **0.417** | **0.205** | | 0.076 | 0.069 | 0.132 | 0.144 | 0.104 | 0.208 | 0.149 | 0.208 | 0.139 | 0.625 | 0.375 | 0.711 |
| Gen | 0.000 | 0.013 | **0.332** | **0.307** | 0.125 | | 0.243 | 0.000 | 0.000 | 0.076 | 0.035 | 0.000 | 0.035 | 0.000 | 0.451 | 0.295 | 0.558 |
| Cal | **0.260** | 0.417 | 0.534 | **0.360** | 0.069 | 0.251 | | 0.194 | 0.220 | 0.313 | 0.208 | 0.208 | 0.208 | 0.208 | 0.486 | 0.583 | 0.753 |
| Olb | 0.000 | 0.003 | **0.468** | **0.368** | 0.380 | 0.000 | **0.440** | | 0.000 | 0.069 | 0.007 | 0.000 | 0.007 | 0.000 | 0.382 | 0.309 | 0.543 |
| Poz | 0.000 | 0.029 | **0.439** | **0.356** | **0.323** | 0.000 | **0.383** | 0.000 | | 0.059 | 0.011 | 0.000 | 0.011 | 0.000 | 0.428 | 0.311 | 0.545 |
| Nap | 0.085 | 0.197 | **0.432** | **0.128** | 0.100 | 0.104 | 0.273 | 0.297 | 0.208 | | 0.104 | 0.074 | 0.104 | 0.081 | 0.521 | 0.375 | 0.621 |
| Ust | 0.000 | 0.083 | 0.417 | 0.229 | 0.137 | 0.000 | 0.186 | 0.089 | 0.000 | 0.066 | | 0.000 | 0.000 | 0.000 | 0.417 | 0.375 | 0.545 |
| Lip | 0.000 | 0.012 | **0.411** | **0.327** | 0.263 | 0.000 | **0.315** | 0.000 | 0.000 | 0.176 | 0.000 | | 0.000 | 0.000 | 0.417 | 0.301 | 0.533 |
| Lin | 0.000 | 0.122 | 0.425 | **0.280** | 0.222 | 0.000 | 0.250 | 0.100 | 0.042 | 0.143 | 0.000 | 0.000 | | 0.000 | 0.417 | 0.340 | 0.545 |
| Otr | 0.000 | 0.033 | **0.375** | **0.331** | 0.192 | 0.000 | 0.238 | 0.000 | 0.000 | 0.119 | 0.000 | 0.000 | 0.000 | | 0.417 | 0.328 | 0.535 |
| Kea | 0.372 | **0.559** | 0.245 | **0.393** | 0.500 | 0.372 | 0.483 | 0.621 | 0.539 | 0.500 | 0.638 | **0.503** | 0.571 | **0.374** | | 0.167 | 0.649 |
| Sif | **0.296** | **0.391** | 0.000 | **0.354** | 0.336 | **0.287** | **0.444** | **0.446** | **0.400** | 0.342 | 0.317 | **0.350** | 0.333 | **0.320** | 0.174 | | 0.587 |
| Rho | **0.499** | **0.565** | **0.548** | **0.540** | **0.584** | **0.488** | 0.599 | **0.578** | **0.500** | **0.555** | **0.510** | **0.525** | **0.524** | **0.488** | **0.570** | **0.520** | |

**Table 3** Uncorrected p distances and Fst values (above and below the diagonal respectively) among *Sparisoma cretense* populations calculated from mitochondrial control region sequences using ARLEQUIN version 2.000 (Schneider et al. 2000). Fst significant p values (p<0.05) after Bonferroni correction are bolded.

| | **Azo** | **Mad** | **Can** | **CV** | **Lamp** | **Kea** | **Sif** | **Sant** |
|---|---|---|---|---|---|---|---|---|
| **Azo** | | 0.000 | 0.004 | 0.023 | 0.056 | 0.000 | 0.011 | 0.000 |
| **Mad** | 0.000 | | 0.000 | 0.011 | 0.018 | 0.000 | 0.000 | 0.000 |
| **Can** | 0.032 | 0.000 | | 0.016 | 0.014 | 0.013 | 0.010 | 0.006 |
| **CV** | 0.037 | 0.021 | 0.043 | | 0.048 | 0.060 | 0.052 | 0.058 |
| **Lamp** | 0.000 | 0.024 | 0.067 | 0.071 | | 0.053 | 0.093 | 0.035 |
| **Kea** | 0.000 | 0.000 | 0.037 | 0.090 | 0.030 | | 0.005 | 0.000 |
| **Sif** | 0.015 | 0.000 | 0.037 | 0.084 | 0.000 | 0.007 | | 0.000 |
| **Sant** | 0.000 | 0.000 | 0.058 | **0.089** | 0.000 | 0.000 | 0.000 | |

The AMOVA analyses showed a significant but weak differentiation between Atlantic and Mediterranean populations. Only a very small percentage of the data variance (9.96%, p=0.00 in the case of *Thalassoma pavo* and 2.24%, p=0.03 in the case of *Sparisoma cretense*) was attributable to the separation between Atlantic and Mediterranean populations.

## Historical demography

Migration between Atlantic and Mediterranean was determined for *Thalassoma pavo*. Migration between the two basins showed a higher value of Atlantic immigrants revealing a westward trend. We were not able to determine migration between Atlantic and Mediterranean populations of *Sparisoma cretense.* Despite several attempted search strategies, stationarity of parameters was not achieved. Both species showed similar levels of migration from Madeira into the Azores and no migration in the opposite direction.

**Discussion**

*Thalassoma pavo* and *Sparisoma cretense* showed no evidence for genetic partition between the Atlantic and the Mediterranean. Gene flow between localities in the two basins was very high for both species. Similarly, AMOVA analyses revealed that only a very small percentage of the data variance was attributable to Atlantic/Mediterranean differentiation, although *T. pavo* showed a slightly higher variance between Atlantic and Mediterranean than *S. cretense*. Even though Cape Verde is not genetically isolated from the other locations, Fst values indicate lower levels of gene flow between this archipelago and the other location for both *T. pavo* and *S. cretense*. Within the Mediterranean, *T. pavo* showed gene flow restriction between Kea, Sifnos and Rhodes and the locations to the west. This analysis confirms the lack of genetic discontinuities between Atlantic and Mediterranean populations of both *T. pavo* and *S. cretense,* and the existence of a genetic discontinuity at the Peloponnesus for *T. pavo*. Thus, despite of increasing the number of available samples, our analysis is still in agreement with the results presented by Costagliola et al. (2004).

The Northeastern Atlantic Ocean and the Mediterranean Sea are known for sharing great fauna affinities shaped by an intimately related paleogeography. Pleistocene glaciations had a differential effect in the two regions, shaping the structure of marine populations, which disappeared as the ice endured and managed to recolonize regions after temperatures stabilized. Distribution ranges of *Thalassoma pavo* and *Sparisoma cretense* reflect a clear preference for warm waters. Some warm water species such as *Chromis chromis/ C. limbata* and *Tripterygion delaisi* (Domingues et al. 2006, 2007), have been suggested to have survived the cold periods in warmer regions like Madeira, the tropical African coast and the southern Mediterranean, where the climatic conditions did not change significantly during the Pleistocene (Thiede 1978; Crowley 1981). Indeed, Mediterranean populations of *T. pavo* and *S. cretense* show high levels of genetic diversity even in the astern basin, pointing to an ancient colonization of this sea. This suggests that both species must have been able to persist in the Mediterranean during the cold periods, possibly in some southern warmer water pockets (Thiede, 1978). Although the direction of historical migration between the Atlantic and the Mediterranean could not be estimated for *S. cretense*, a Mediterranean-Atlantic trend is evident for *T. pavo* (Fig 1). This trend may reflect the re-colonization of the cooler areas of the Atlantic by fish that survived the cold phases in the Mediterranean. Colonization of the Atlantic from the Mediterranean may have been vacillated by the numerous submarine banks and seamounts that occur between Europe and the islands of Madeira and Azores (Kitchingman and Lai 2004; Kitchingman et al. 2007). *Thalassoma pavo* and *Sparisoma cretense* are active demersal (or benthic) fish, with pelagic eggs and larvae, that occur at depths of 50 m or below (Quignard and Pras 1986 a,b), thus being able of reaching these seamounts. The interpretation of the Mediterranean-Atlantic migration trend must, however, be viewed with some caution, since the Atlantic samples come from island locations, which in the case of Cape Verde and the Azores, are very far away from shore, meaning that many haplotypes may not have been

able to reach them. Sampling on the western African shore is urgently needed to improve our understanding of the phylogeography of these fishes.

According to what has been discussed, persistence of *Thalassoma pavo* and *Sparisoma cretense* in the Azores archipelago during the Pleistocene glaciations is difficult to admit. Planktonic foraminifera record of the last 150 ky in the region, revealed variations in Sea Surface Temperatures of 2-3 °C (Crowley 1981). These small fluctuations might have been enough to promote local disappearance of sub-tropical species like *T. pavo* and *S. cretense*, whose northern limit of distribution is in this archipelago. Indeed, Santos et al. (1995) suggested the recolonization of the Azores by sub-tropical species following the end of the glaciations from some southern regions such as Madeira. Bearing in mind that *Thalassoma pavo* has active swimming larvae that remain in the water column for 38-49 days (Raventós and Macpherson 2001), this colonization might have been possible, at least for this species, by counter current phenomena that cause sporadic transport of water and plankton from Madeira to Azores (Santos et al. 1995). This colonization route is very well supported by the historical migration trend shown by our data. Migration between these two regions is shown to occur in the Madeira-Azores direction only, for both *T. pavo* and *S. cretense*. Given the probable post-glacial origin of *T. pavo* and *S. cretense* in the Azores, we would expect low genetic diversities for this population. However, this does not seem to be the case. Our data shows that Madeiran and Azorean populations of both species have extremely high levels of gene flow and exhibit no genetic differentiation (Fst and pairwise p distances are zero). Thus, the reason for the high genetic diversity of the Azores could be the present day intense connection of the two populations. For marine organisms with high dispersal potential, colonization of new areas can be achieved in a short period of time, when physical and ecological conditions are suitable. These rapid movements promote genetic homogenization and can easily erase the typical genetic signs of strong population reductions.

It is very interesting to note that the two species examined in this study share common phylogeographic patterns and a similar historical demography. Several studies have attempted to establish a relation between population structure and species ecological characteristics, especially egg types and pelagic larval duration (eg. Shulman and Bermingham 1995; Riginos and Victor 2001). Results however have not been consistent and a clear relationship between dispersal ability and population structure cannot be generalized. Other factors can influence the phylogeographic pattern of coastal fishes, particularly in regions that have experienced strong climatic and habitat changes such as the ones caused by the Pleistocene glaciations. The two species studied here are active demersal (or benthic) littoral fishes and have similar geographic distributions and thermal affinities. These characteristics seem to have triggered a similar response to the effects of Pleistocene glaciations, resulting in identical phylogeographic patterns.

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# Discussion

## 1. Phylogeographic patterns

Instead of a concordant phylogeographic pattern for all species studied, four distinct phylogeographic patterns emerged. In the first pattern, taxa like *Chromis chromis/ C. limbata*, *Parablennius sanguinolentus/ P. parvicornis* and the two lineages of *Tripterygion delaisi* showed two distinct groups of populations (sometimes considered different species), one including the Mediterranean and the Atlantic coast of western Europe, and another comprising the Atlantic archipelagos of Canaries, Madeira and Azores. A second pattern characterized by no appreciable genetic differentiation between any of the populations was revealed by *Sparisoma cretense*, *Thalassoma pavo* and *Diplodus sargus*. *Lipophrys pholis* and *Coryphoblennius galerita* conformed to a third pattern, showing a marked differentiation of the Azorean population, and, in the case of *C. galerita,* a clear divergence between Mediterranean and western European locations as well as Madeira and Canaries (*L. pholis* does not form stable populations in the Mediterranean, Zander 1986). Finally, the species pair *Parablennius ruber/ P. gattorugine* showed one form in the Mediterranean and in the northeastern Atlantic coast (P. *gattorugine*) and another one in the Atlantic islands (*P. ruber*), the latter being also present in the European coasts, thus in sympatry with *P. gattorugine.* These distinct phylogeographic patterns can be explained by a combination of differential effects of the Pleistocene glaciations in several areas of the Atlantic and Mediterranean with the particular thermal tolerances and dispersal capabilities of the species.

Taking into account the present day geographical distribution of warm water species like *C. limbata/ C. chromis*, *P. parvicornis/ P. sanguinolentus*, *T. delaisi*, *D. sargus*, *T. pavo* and *S. cretense*, we can speculate that these species would not have been able to survive the colder glacial periods in the more affected areas of the Atlantic and the Mediterranean. Ice-rafting depositions were found off the western Iberian margin, documenting extreme cooling events during the Pleistocene (de Abreu et al. 2003). Although the drop in sea surface temperatures (SSTs) was moderate in the Azores region (Crowley 1981), it might have been enough to promote local extinction of warm water fishes that have their northern distribution limit in the area. Located further south, the Madeira archipelago, the western islands of the Canaries and the tropical western coast of Africa were only slightly affected, while the eastern Canary islands were also somehow affected due to their proximity to the coast (CLIMAP 1976). Except for some warmer-water pockets in southern and eastern regions, SSTs of the Mediterranean Sea have also been severely reduced (Thiede 1978; Hayes et al. 2005). In this context it is interesting to note that the Azorean populations of *C. limbata*, *P. parvicornis* and *T. delaisi* are genetically less diverse than those of the other islands. This was also shown to be the case for *Ophioblennius atlanticus* (Muss et al. 2001). Mismatch analysis of *P. parvicornis* and *P. sanguinolentus* showed that the Azores and the western Mediterranean (represented by Cape of Gata) suffered an accentuated population expansion after the last glacial maximum, probably reflecting a recovery from the impoverishment of the cold periods. In contrast, fish from the less

affected islands of Madeira and Canaries revealed a weaker expansion. *Diplodus sargus* from the Azores, Madeira and Barcelona did not show trends of a demographic expansion indicating that this species might have been able to survive the moderate Pleistocene cooling there. However, the Azores appeared as a less diverse population occupying a peripheral position. A clear population expansion was revealed by mismatch analysis for western Iberia, Canaries, Mauritania and the Greek islands.

Almada et al. (2001) suggested the western Tropical coast of Africa and the Mediterranean as refuges for the warm water blenniids during the Pleistocene glaciations. According to the same authors, these regions would have acted as diversification centers from which fishes would have invaded other Atlantic regions. The phylogeographic pattern described above for the warm water *C. limbata/ C. chromis*, *P. parvicornis/ P. sanguinolentus* and *T. delaisi*, with two highly divergent groups, fits very well in this scenario. Fishes surviving the glaciations in the western Tropical coast of Africa would have expanded northwards colonizing the northern coast of Africa and the Macaronesian islands, while fishes from the south of Mediterranean invaded the entire Sea and the adjacent European Atlantic coast. Since the two refuges were isolated from each other due to the existence of cooler water masses between them, divergence and subsequent speciation, in the case of *C. limbata/ C. chromis* and *P. parvicornis/ P. sanguinolentus,* may have happened. In this regard, it is important to note that the colonization of the Azores would have been possible by fishes that survived in Madeira, and also in the western coast of Africa, with the intermediate islands of Canaries and Madeira acting as stepping stones. This would have occurred following the sporadic transport of eggs and larvae carried by the currents and eddies flowing from Madeira and the African coast towards the Azores (Santos et al. 1995). Indeed, this colonization route was identified by historical migration estimates of *C. limbata*, *T. pavo* and *S. cretense*. High gene flow and a strong genetic connection between Canaries, Madeira and Azores were also identified for *P. parvicornis* and *D. sargus*.

Since *D. sargus*, *T. pavo* and *S. cretense* are warm water species, we would expect them to show a phylogeographic pattern similar to the one observed for *Chromis*, *Parablennius* and *Tripterygion*, which shows two highly divergent groups of populations. However, this does not seem to be the case. A possible explanation for this discrepancy relies on the higher dispersal ability of *D. sargus*, *T. pavo* and *S. cretense*, which might have promoted a very fast mixing of the populations after SSTs were restored. In this regard it is interesting to note the high levels of genetic diversity of *T. pavo* and *S. cretense* from the Azores. These species have a clear tropical affinity, which makes their survival in the Azores during the Pleistocene glaciations highly improbable. The high levels of genetic diversity might then be explained by several rapid migration fluxes of fishes from southern refuges towards the islands, importing genetic variability. Importantly, while blennids, tripterygiids and pomacentrids show male parental care of demersal eggs, *D. sargus*, *T. pavo* and *S. cretense* have planktonic eggs and larvae, which enhances their potential for dispersal. Moreover, the three fishes that show genetic differentiation are rocky littoral or sub-littoral species that as adults show very restricted movements and are confined to the upper meters of the water column (*P. sanguinolentus* 1 m, Zander 1986; *T. delaisi* 3-40 m,

Wirtz 1978; *C. limbata* 5-45 m Allen 1991). *Diplodus sargus*, *T. pavo* and S. *cretense* attain a size that is much larger than the species mentioned above and both juveniles and adults are active swimmers. Although precise data on the extent of their movements could not be found, it is likely that they can undergo extensive movements along the shores, a possibility that is absent for the adults of blenniids and tripterygiids and even probably small pomacentrids like *Chromis*. This mobility of the adults would allow rapid mixing between Mediterranean and Atlantic fish, erasing any sign of divergence that might have emerged while the two regions were isolated. This is supported by the historical migration trend from the Mediterranean towards the Atlantic revealed for *T. pavo* and also by the marked proximity of *D. sargus* from western Portugal and the Mediterranean. On the other hand, not being strictly benthic fishes, movements of *D. sargus*, *T. pavo* and *S. cretense* between European mainland coast and the Azores archipelago might have been facilitated by the numerous submarine banks and seamounts that have been mapped between the two regions (Kitchingman and Lai 2004; Kitchingman et al. 2007). These seamounts have summits at depths varying from 100m to 4000m, some of them being even shallower (Gorringe- 40 m, Lagabrielle and Auzende 1982; Àmpere- 18 m, Josephine-50 m, D. João de Castro- 13 m, Cardigos et al. 2005). *Diplodus sargus*, *T. pavo* and *S. cretense* are active demersal (or benthic) fishes that can bee found below 50 m (Bauchot and Hureau 1986; Quignard and Pras 1986 a,b), thus being able of reaching these seamounts. With sea level drops of 120-140m in the glacial maxima (Lambeck et al. 2002) many of these seamounts were above sea water level, making the number of available stepping stones higher than today.

The two phylogeographic scenarios outlined above fit well all the available data for warm water species, but is unlikely to hold for species that tolerate cooler waters, like *L. pholis* and *C. galerita*. These fishes might have persisted during the Pleistocene cooling episodes at the less affected areas, among which are the Azores. The long term persistence of these blenniids coupled with their limited dispersal ability would have promoted the genetic differentiation of the more isolated locations such as the Azores and the Mediterranean (in the case of *C. galerita*). A similar pattern is found for the temperate clade *Symphodus trutta* and *S. caeruleus* (Almada *et al.* 2002). While the former species occurs in Madeira and Canaries, the latter was described for the Azores (Azevedo 1999). It is very likely that *S. caeruleus'* ancestors persisted in the Azores during the glaciations giving rise to a new species. Because of the polar conditions felt in the Iberian margin during the Pleistocene, it is very unlikely that *L. pholis* and *C. galerita* were able to persist there. Survival of *C. galerita* in the western Mediterranean was supported by our data. A colonization of the Atlantic coast of Europe from the Mediterranean is however not likely, given the high degree of genetic differentiation between the two basins. The relatively cold-water tolerance of the species may have allowed their survival in the shores of northwest Africa from which they could have easily re-invaded Europe.

The warm water species discussed so far seem to have evolved according to the biogeographical scenario proposed by Almada et al. (2001) and Wirtz (1978) in which the tropical western coast of Africa and the Mediterranean acted as speciation centers. An

alternative hypothesis proposed by Zander (1980) considered the Macaronesian islands, especially the Azores, as speciation centers for blenniids, from which the new species have colonized the European shores. *Parablennius ruber* is apparently one of the species conforming to this model. This blennid is very abundant in the rocky subtidal of the Azores and has also been reported, although in lower abundances, from the European shores, where it is sympatric to its sister species *P. gattorugine*. Although definite conclusions will only be possible with population surveys, it seems likely that *P. ruber* evolved in the Azores or Madeira and accumulated sufficient differences to become a different species, being then transported to the European shores. Although *P. ruber* has been reported for the European coast since the 19$^{th}$ centuary (Valenciennes 1836) the status of the species in the area is not known. This blenniid my form stable populations there or it can also hybridize with *P. gattorugine*. Alternatively, it is also possible that *P. rubber* does not form stable population in the European coast, and that larvae are constantly being brought from the islands by ocean currents.

## 2. The Atlantic-Mediterranean divide

As described in the introduction, coastal species show variable levels of genetic differentiation between Atlantic and Mediterranean populations. In this study only one species, the blenniid *Coryphoblennius galerita*, showed clear and strong genetic differentiation between the two basins. Although two subspecies of *Diplodus sargus* are formally recognized as Atlantic and Mediterranean forms, our analysis showed no genetic partition between the two basins, which are connected by high levels of gene flow. The same conclusion has also been advanced in other molecular surveys (Summerer et al. 2001; Bargelloni et al. 2005). The pomacentrid *Chromis chromis* showed high levels of gene flow between the western coast of Portugal and several western Mediterranean locations. Indeed, an analysis of molecular variance (AMOVA) showed that only 7% of the total variance of the data was attributable to the separation between the Atlantic and the Mediterranean. Similarly, Atlantic and Mediterranean populations of the wrasse *Thalassoma pavo* and the parrotfish *Sparisoma cretense* showed no genetic partition, and high levels of gene flow between several locations of the two basins. Results for *Tripterygion delaisi* were not straightforward. Although samples from the Portuguese coast clustered together with the ones from the Mediterranean, they were represented by a single and unique haplotype. This haplotype was, however, only a few steps different from the Mediterranean ones. Additionally, high levels of gene flow between the two locations were found for the nuclear fragment, but not for the mitochondrial one. In contrast to these results, *C. galerita* showed a very clear genetic partition between Atlantic and Mediterranean populations, which was accompanied by morphological differentiation (Mediterranean representatives possess lower and narrower crests). Divergence between these two forms might have occurred during the Pleistocene glaciations, when communication through the Strait of Gibraltar was reduced due to sea level lowering. On a more recent time scale, the complex

pattern of gyres and eddies of the Alboran sea can constitute an effective physical barrier to small coastal fish like *C. galerita*, whose dispersal is restricted to the planktonic larval phase. The Strait of Gibraltar and/or the Almeria-Oran jet have also been suggested as effective barriers to gene flow for the goby *Pomatoschistus microps* (Gysels et al. 2004) and the sparid *Diplodus puntazzo* (Bargelloni et al. 2005) as well as for several invertebrates. Other factors such as larval behavior and the superficial currents during *C. galerita*'s spawning season my also have influence in the segregation of the two divergent lineages.

### 3. Population structure within the Mediterranean

Similarly to what has been shown for the Atlantic-Mediterranean transition, contrasting levels of genetic differentiation were revealed within the Mediterranean Sea across species. *Diplodus sargus* and *Sparisoma cretense* show no genetic discontinuities along the Mediterranean basin. In the case of the sparid, individuals sampled in the western and eastern basins shared haplotypes and the two basins were connected by high gene flow. Although only three *S. cretense* individuals were sampled in the western basin, their haplotypes were shared with fish from the Greek islands, pointing to a lack of genetic partition between the two basins. In contrast to these results, *Thalassoma pavo* and *Chromis chromis* showed a restriction to gene flow south of the Greek Peloponnese, where a permanent anticyclonic gyre has been identified (Malanotte-Rizzoli and Bergamasco 1989). In the case of the pomacentrid, gene flow showed a prevailing east direction. Another coastal organism, the bivalve *Cerastoderma glaucum,* shows also a genetic partition at this region (Nikula and Väinölä 2003). This genetic brake is probably linked to sea levels changes during the Pleistocene glaciations, which might have promoted the isolation of the two basins. The intense anticyclonic eddie that exists in the region may also act as an effective barrier.

### 4. Factors that contribute to the phylogeographic patterns

The distinct phylogeographic patterns depicted from the study of these eleven coastal fishes can only be understood when we think of the multitude of factors that can influence species responses to challenging environments. Predicting the population structure of a given species is thus not an easy task. According to our results, it is clear that geographical distribution of the genetic diversity of the species studied was highly shaped by the differential effects of the Pleistocene glaciations. Thermal tolerances of the different species to cooling events seem to have been an important factor determining the survival or local extinction of populations in regions like the Azores and the Mediterranean. Importantly, demographic and genetic aspects such as the effective size of the population affected, the amplitude of a bottleneck and the genetic diversity that was preserved are also probably relevant in determining the present day phylogeographic pattern of a

species. When we think of recolonization phenomena from glacial refuges, as well as on the capacity to overcome oceanographic barriers, dispersal abilities of the species are also very important. Several studies have attempted to relate species range and population structure to dispersal abilities, putting effort on establishing a relationship between planktonic larval duration (PLD) and extent of population structure. Comparative results across species have, however, not been consistent (Shulman and Bermingham 1995; Victor and Wellington 2000; Riginos and Victor 2001; Bay et al. 2006) and a clear relationship between dispersal ability and population structure cannot be generalized. Our study contributes to this idea. For instance, *Coryphoblennius galerita*, a blenniid with strong genetic differentiation between populations of the Portuguese coast and the western border of the Mediterranean, has a PLD of 26-27 days (Raventós and Macpherson 2001), while *Tripterygion delaisi* and *Chromis chromis*, with lower PLDs (17-18 days and 18-19 days, respectively, Raventós and Macpherson 2001) have no genetic differentiation between the two regions. On the other hand, it is also true that larvae of *Thalassoma pavo* stay in the plankton for as long as 38-49 days (Raventós and Macpherson 2001), and that this wrasse shows high levels of gene flow through the northeastern Atlantic and the Mediterranean (with a restriction only found in the eastern basin). Other aspects of the biology of the species, such as egg type and larval and adult behavior, as well as hydrological conditions of the reproductive season, may also influence their dispersal ability (Leis and McCormick 2002). As noted before, warm water species with pelagic eggs and higher dispersal ability as adults (*Diplodus sargus*, *T. pavo* and *Sparisoma cretense*), appear to have been able of recolonizing the more affected regions very rapidly, in contrast to the strictly benthic species with benthic guarded eggs (*Parablennius parvicornis*, *T. delaisi*, *Chromis limbata*). Moreover, costal species that are prone to be transported attached to algae or other objects by rafting, as was suggested for *Parablennius rubber*, have higher chances of colonizing more distant areas.

The comparative analysis of the alternative phylogeographic patterns revealed by the study of these fishes, in light of their biological and ecological characteristics, contributed to further our knowledge on the evolutionary relationships of the coastal fauna of the Atlantic-Mediterranean and also of the forces that shaped those patterns.

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# FINAL REMARKS

The papers presented in this thesis show that the warm water species present phylogeographic patterns that are consistent with the hypothesis proposed by Almada et al. (2001) where the Mediterranean and the western Tropical African coast may have acted as glacial refuges and centers of speciation. Although this idea is supported by the geographical distribution of the genetic diversity of the populations studied as well as by their historical demography and migration routes, it can only be fully tested when individuals from the western Tropical Africa are analyzed. Unfortunately, due to logistic limitations, we were not able to obtain samples from that location. If the western Tropical Africa acted as a refuge, we would expect to find high levels of genetic diversity in the region and signs of a long-term stable population. Additionally, it would be very interesting to recover historical migration directions of the warm water fishes including samples from this potential refuge. We would expect the western coast of Africa to be a population source by identifying migration from this location towards the northern regions.

Another very interesting avenue of research would be the analysis of genetic patterns of fishes from the seamounts that occur between the mainland Portuguese coast and the archipelagos of the Azores and Madeira. As mentioned in the discussion, these seamounts may have acted as stepping-stones for the colonization of these islands. If this was the case, we would expect to identify this colonization route when computing the historical migration fluxes of the species including samples from the seamounts.

The comparative analysis of the distinct phylogeographic patterns revealed by the study of several coastal fishes with different thermal tolerances and dispersal abilities, shed light on the historical and evolutionary relationships of the Atlantic-Mediterranean. Our knowledge will certainly be improved if identical analyses were conducted on other coastal taxa with similar, and also contrasting, ecological and biological characteristics. In this respect, genetic analysis of the several invertebrates that have been subject to biogeographical studies seems very promising. In addition, contrasting these results with studies on pelagic species that have high dispersal ability as adults, and that are able to reach deeper depths being exposed to different ocean currents, would allow a more complete picture of the evolutionary relationships of the marine fauna of the Atlantic-Mediterranean.