Patterns of Population Structure and Historical Dispersal in Squaloid Sharks: a Species-Level Approach Using Molecular Markers

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ABSTRACT

Squaloids (Order Squaliformes) are a highle diverse group of mostly deepwater habitats (> 200 m). Many species are regularly caught in commercial fisheries worldwide but their low productivity and correspondingly low intrinsic rebound potentials make them particularly vulnerable to population depletion and overexploitation. Of special concern to fisheries management and conservation efforts are the spiny dogfish *Squalus acanthias*, the leafscale gulper shark *Centrophorus squamosus* and the Portuguese dogfish *Centroscymnus coelolepis*. These taxa have recently been declared overfished in several parts of each species' distribution but management efforts have been made to recover the "stocks" without a good understanding of the species' population structure and the level of connectivity among populations.

As such, the goal of this dissertation is to elucidate the intraspecific patterns of population structure of each species, for future inclusion in fisheries management efforts, and to infer the patterns of historical dispersal of the three species of squaloid sharks. To this end, I have developed a suite of highly polymorphic molecular markers (including nuclear microsatellites and nucleotide sequences of mitochondrial DNA gene regions) and have collected tissues samples from throughout each species geographic range.

The genetic population structure of *S. acanthias* was characterized by high genetic divergence across the equatorial Pacific, and by comparatively higher genetic homogeneity among the sample collections from the South Pacific and Atlantic oceans. Nevertheless, small but significant genetic differentiation was detected by both nuclear and mitochondrial markers among spiny dogfish collections from either side of the equatorial Atlantic. Genetic differentiation in the spiny dogfish occurred across low latitude regions characterized by warm-temperate and tropical waters, suggesting that such regions may act as effective barriers to gene flow among populations.

Regarding *C. coelolepis* and *C. squamosus*, the pattern of genetic population structure uncovered for the eastern Atlantic was similar between species. Within this region, no evidence of genetic differentiation was found among sample collections ranging from off Ireland to South Africa, and including the Azores, consistent with the existence of a single genetic stock for each species within the sampled region. Furthermore, evidence for inter-oceanic dispersal between Atlantic and New Zealand populations was also found for the leafscale gulper. These results strongly suggest that both *C. coelolepis* and *C. squamosus* have high dispersal potential and no major barriers to gene flow within the deep eastern Atlantic.

Overall, high genetic homogeneity was observed over large geographic areas (i.e. in the order of thousands of miles) in all three target species consistent with long-distance dispersal with gene flow. However, there were differences between the coastal and the deepwater squaloids regarding the regions of genetic discontinuity, which may be associated with adaptations to their respective habitats. In the coastal spiny dogfish, environmental factors such as unsuitable water temperatures appear to exert a strong influence in the species' distribution and in its genetic population structure. In the deepwater squaloids, stable environmental conditions but limited food supply may result in widely distributed populations as a strategy to maximizing resource partitioning while minimizing resource competition among individuals.

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Patterns of Population Structure and Historical Dispersal in Squaloid Sharks: a Species-Level Approach Using Molecular Markers

INTRODUCTION

The squaloid sharks (Class Chondrichthyes: subclass Elasmobranchii: Order Squaliformes) are a group of highly diverse species and the second most speciose order of extant sharks (23% of all living sharks; Compagno 1990). The group has a relatively long evolutionary history with its origin dating back to the Early Cretaceous (*c.* 145 Ma; Adnet and Capetta 2001; Adnet et al. 2008). According to the fossil record, the earliest squaloid forms were coastal in habit but it was in the deep sea (> 200 m) that the group underwent its most extensive radiation and where it still shows the highest diversity of extant taxa (Adnet and Capetta 2001; Musick et al. 2004). A total of seven families and over 130 species have been included within the Squaliformes (Compagno et al. 2005), with new species being added recently (e.g. White et al. 2008; Last et al., 2007). The squaloid families (*sensu* Compagno et al. 2005) include the bramble sharks (Family Echinorhinidae), the dogfish sharks (Family Squalidae), the gulper sharks (Family Centrophoridae), the lantern sharks (Family Etmopteridae), the sleeper sharks (Family Somniosidae), the roughsharks (Family Oxynotidae) and the kitefin sharks (Family Dalatiidae).

The diversity within Squaliformes can be illustrated by the range of maximum sizes, from less than 20 cm adult size as in *Etmopterus carteri*, to over 700 cm as in *Somniosus pacificus*. There is also considerable variability of ecomorphotypes, from more primitive, littoral types (e.g. most squalids) to highly derived, deepwater-restricted forms (e.g. etmopterids) (Compagno 1990). Nevertheless, some generalizations are possible: the bulk of the squaloid taxa is between 1 to 3 m in length, is mostly benthopelagic in habit and has a preference for cold water habitats (Musick et al. 2004). Their life histories are broadly similar to those of other elasmobranchs: they have slow growth rates, high longevities, attain sexual maturation late in life and have low fecundities (Compagno 1990; Camhi et al. 1998). However, squaliform sharks are at the extreme end of the characteristic k-strategy of elasmobranchs: age and growth studies have estimated life expectancies up to 70 or 80 years in species whose maximum sizes are between 1 to 2 meters in

length (Clarke et al. 2001; Saunders and MacFarlane 1993) with sexual maturity occurring at 70-90% of the maximum length and at or beyond 50% maximum age (Kyne and Simpfendorfer 2007). These characteristics make squaloid sharks some of the most unproductive elasmobranch taxa, with correspondingly very low intrinsic rebound potentials and population doubling times of a few decades to over a century (Simpfendorfer and Kyne 2009). They are consequently highly vulnerable to population depletion and overexploitation (García et al. 2008; Simpfendorfer and Kyne 2009).

Despite this fact, many squaloid shark species are regularly caught in several commercial fisheries around the globe, mostly as by-catch of trawls, longlines or gillnets (Gordon 1999; Kyne and Simpfendorfer 2010). As there are several problems associated with the quality and accuracy of the available landings data, such as correct species identifications, lack of species-specific landings data or unknown levels of by-catch and discards (Gordon 1999; Smith 2007; Kyne and Simpfendorfer 2010), the current levels of fishing-induced mortality might be grossly underestimated. Most shark and ray populations can only support very low levels of fishing mortality without undergoing depletion and stock collapse, either as target species or as by-catch (Walker 1998; Musick et al. 2000; Irvine et al. 2006). It is then not surprising that several squaloids are listed as vulnerable, near threatened or endangered in the IUCN SSG Red List Assessment 2000-2006. In addition, given the position of most sharks as top predator species, the removal or significant depletion of their populations through fishing, as already seen for many species, can lead to severe disruptions of trophic interactions at the ecosystem-level (Koslow et al. 2000; Stevens et al. 2000).

Of special concern to both fisheries management and conservation efforts are three squaloid species: the spiny dogfish *Squalus acanthias*, the leafscale gulper shark *Centrophorus squamosus* and the Portuguese dogfish *Centroscymnus coelolepis*. Recently, the "stocks" of the latter two

species in the eastern North Atlantic were considered depleted and likely to be below any candidate limit reference point (ICES 2006). In the case of the spiny dogfish, the western North Atlantic "stock" was declared overfished in 2002 (ASMFC 2002) and has recently rebuilt to above the target spawning stock biomass (SSB) (ASMFC 2008). However, due to consistently low recruitment since 1997, the SSB is estimated to decline sharply around 2017 (ASMFC 2008). In the eastern North Atlantic, the "stock" is considered depleted and in danger of collapse (ICES 2009). Unfortunately, our current understanding of these and other squaloid sharks' biology, ecology and population dynamics do not match the current levels of exploitation, a fact that has compromised the design of adequate and successful management plans (Gordon 1999; Kyne and Simpfendorfer 2007). In the particular case of the currently exploited species, it is of utmost importance to have a good understanding of their population structure and of the level of connectivity among populations from geographically adjacent regions as well as from throughout the species range. This kind of information is essential for the accurate identification of management units/stocks and to the effective management and conservation of any fish resource (Graves 1998; Musick et al. 2000). In turn, management plans often delineate stocks based on the most practical geographical unit without regard for their biological and genetic integrity (Ward 2000).

Population structure studies

It is widely recognized that almost all species are comprised of discrete groups of individuals or populations that are reproductively isolated from each other, and as a result exhibit some level of intraspecific genetic differentiation (Hartl and Clark 1997; Avise 2000). The intraspecific patterns of population structure are shaped by the interaction and integration of multiple events and processes ultimately affecting individual dispersal (or gene flow) and/or survival. For instance, the spatial and temporal distribution of any group of organisms is dependent on the

occurrence of a suite of biotic (e.g. competition, predation, parasitism) and abiotic variables (e.g. temperature, humidity, salinity, etc.) that allow for their survival and reproduction (Lomolino et al. 2006). Unsuitable environmental conditions, particular oceanographic features such as gyres or currents, or conflicting interactions among species can act as boundaries to individual dispersal and/or affect their survival (e.g. Shaklee and Bentzen 1998; Araújo and Luoto 2007; Johansson et al. 2008; Limborg et al. 2009).

Another important suite of factors influencing the patterns of population structure are the species' natural history and behavior (Bohonak 1999; Avise 2000). For instance, some species have high dispersal abilities and exhibit concomitant high levels of gene flow among distant locations, leading to reduced levels of population differentiation across large spatial scales (e.g. blue marlin *Makaira nigricans*, Graves and McDowell 2003; scalloped hammerhead *Sphyrna lewini*, Duncan et al. 2006; whale shark *Rhincodon typus*, Castro et al. 2007). However, certain features of a species' life history, such as natal homing or female phylopatry to nursery areas, can lead to strong population differentiation even in highly migratory species (e.g. green turtles *Chelonia mydas*, Bowen et al. 1992; blacktip shark *Carcharhinus limbatus* Keeney et al. 2005).

Finally, neither the spatial distribution of individuals nor the species' home range are static through time but are rather dynamic in nature. Throughout evolutionary time, populations can experience range expansions or contractions as environmental conditions change on a global scale (e.g. Grant and Bowen 1998), or became fragmented by newly arisen topographic or oceanographic barriers (Duncan et al. 2006). The temporal integration of all the events and processes occurring in the past play an important role in shaping the species' distribution and population structure in the present (Avise 2000).

The reconstruction of the patterns of intraspecific population structure can tell much about the species' life history strategy, ecology and evolutionary history. Additionally, because the

delineation of population units implies some level of isolation among them, the existence of barriers to dispersal/effective mixing can also be identified. This approach is particularly useful in the case of species that are difficult to access, such as most deepwater squaloid sharks, and where traditional methods based on direct observation, sampling and/or tracking of individuals are not feasible.

Several different methods have been used to elucidate the population structure and dispersal patterns of marine fish species. These have been described in detail by Pawson and Jennings (1996) and by Begg and Waldman (1999), and include the use of natural marks (e.g. parasites, otolith microchemistry) and artificial tags; meristic characters (e.g. counts of fin rays and spines) and morphometric descriptors (e.g. body measurements); molecular genetics (e.g. allelic composition and frequency); and life history parameters (e.g. growth parameters or size at maturity). Molecular genetic markers, and in particular DNA sequence data, allow the interpretation of the *contemporary* patterns and processes affecting the distribution and dispersal of individuals, and provide insight into the *historical* component of population structure (Templeton 2004; Avise 2009).

Molecular genetics and the study of populations

Molecular genetics are of universal application in living organisms and make use of the information stored in the genome of every cell (Avise 2000). Different molecular genetic markers allow inferences regarding the genealogical/phylogenetic relationships at different taxonomic levels, from pedigree analysis (e.g. nuclear microsatellites) to evolutionary genealogies (e.g. mtDNA) (Sunnucks 2000; Avise 2004, 2009). Molecular population genetics considers the patterns of genetic diversity among populations and the underlying evolutionary forces behind them, namely mutation, migration, random genetic drift and selection (Hartl and Clark 1997). The establishment of reproductive isolation between groups of individuals leads to divergence in their

genetic composition, as new genetic diversity created by mutation is restricted to its population of origin, and some of the previously existing variation is lost through random genetic drift or selection (Hartl and Clark 1997). The basic question in population structure studies is whether or not samples from different geographic regions share the same genetic composition or, in other words, if they are part of the same population (Ward 2000).

Several molecular markers and techniques are available for studies of genetic population structure (e.g. Sunnucks 2000; Silva and Russo 2000; Avise 2004). Of particular relevance are nuclear microsatellite loci, also called short tandem repeat loci (STR). These are typically highly polymorphic regions in the nuclear genome where a short sequence of nucleotides, usually 2 to 4 base pairs in length (e.g. CA or TAGA), is repeated several times. The polymorphic nature of these markers is due to their very high mutation rates (10^{-5} – 10^{-2} per locus per generation), whereby new alleles are formed by the addition or deletion of one or more repeat units (Goldstein and Schlötterer 1999; Calabrese and Sainudiin 2004). Different alleles are distinguished by their repeat number and, hence, by size (Goldstein and Schlötterer 1999), allowing their discrimination by electrophoretic separation (Avise 2004). However, direct sequencing of any given genome region provides the most detailed analysis of genetic diversity since the nucleotide composition at each position in a sequence is known (Avise 2004). Nucleotide polymorphism is created through mutation, which can take the form of nucleotide insertions, deletions, or substitutions at any given position in the genome, thereby generating new haplotypes or sequences.

In addition to population differentiation studies, molecular genetic data can be used in the reconstruction of intraspecific phylogenies (Sunnucks 2000; Avise 2004). These phylogenies allow inferences of the demographic history of populations (Nee et al. 1995; Emerson et al. 2001; Kuhner 2008) as different demographic scenarios (e.g. exponential growth, stability, or declines in population sizes) leave a signature in the genetic composition of populations (Slatkin and

Hudson 1991; Rogers and Harpending 1992). Also, several inferences can be made regarding the historical and contemporary events and processes shaping the patterns of contemporary population structure by analyzing the geographic distribution of alleles in association with their phylogenetic relationships (i.e. phylogeography, Avise 1987, 2000; Templeton et al. 1995; Templeton 1998). These analyses can provide important clues concerning past dispersal pathways (e.g. Blackburn and Measay 2009), vicariant isolation (e.g. Shaklee and Bentzen 1998), or historical range contraction due to major glaciation events (e.g. Provan and Bennett 2008).

Case-study species

My dissertation research aims to elucidate the intraspecific patterns of population structure and historical dispersal of three species of squaloid sharks: the spiny dogfish *S. acanthias*, the leafscale gulper shark *C. squamosus* and the Portuguese dogfish *C. coelolepis*. These case-study species were chosen for several reasons. First, the collection of relatively large numbers of individual samples required for population level analyses was relatively feasible in a large fraction of their ranges. Second, the need for studies concerning the patterns of population structure and individual dispersal in the three shark species is supported by their current overexploited status in some areas within their respective ranges. Little is known about the stock structure of these species and current management plans define "stocks" based on the most practical geographic unit without considering their biological and genetic integrity. Third, the spiny dogfish, the leafscale gulper shark and the Portuguese dogfish are among the most intensively studied squaloid sharks and data on their general biology and ecology is available from the literature. Additionally, all three species have similar reproductive strategies, dispersal potential and have generally overlapping geographic distributions. However, they differ in their bathymetric distribution: the spiny dogfish is a coastal species while the remaining two are bathyal sharks. As such, contrasting patterns of intraspecific population structure and

phylogeography can provide some insights into the differences and similarities in the evolutionary events and processes occurring in the different habitats.

a) Spiny dogfish *Squalus acanthias* (Family Squalidae)

The spiny dogfish *Squalus acanthias* Linnaeus, 1758 is a common coastal, cold temperate species usually found in waters over the continental shelf down to 900 m (Burgess 2002). These habitat preferences are reflected in its anti-tropical geographic distribution in the Atlantic and Pacific oceans (Burgess 2002; Compagno et al. 2005; ICES 2006). Spiny dogfish are active swimmers and voracious opportunistic predators (Hanchet 1991; Avsar 2001; Burgess 2002), and are often found in schools segregated by size and/or sex (Holden 1965; Nammack et al. 1985; Stenberg 2005).

Like all other squaloid sharks, *S. acanthias* is an yolk-sac viviparous species (Musick et al. 2005) with a synchronous reproductive cycle where two "gestational-cohorts" of mature females can be found at any time during the year, exhibiting pregnancy stages about 12 months apart (Templeman 1944; Hisaw and Albert 1947; Hanchet 1988). The gestation period lasts between 20 and 22 months (Templeman 1944; Hisaw and Albert 1947; Hanchet 1988; Jones and Ugland 2001) and the vast majority of the mature females begin a new reproductive cycle soon after the previous cycle has been completed, giving birth every other year (e.g. Hisaw and Albert 1947; Holden and Meadows 1964; Ketchen 1972). Fecundity estimates vary between 1 to 20 pups per litter (Nammack et al. 1985; Menni 1986; Hanchet 1988; Avsar 2001; Tribuzio 2004; Ellis and Keable 2008). Age and length at sexual maturity are generally attained between 10 – 16 yr and 72 – 82 cm total length for females, and between 6 - 10 yr and 60 – 64 cm in males, with maximum sizes of 110 - 117 cm (Templeman 1944; Holden and Meadows 1964; Holden 1965; Nammack et al. 1985; Hanchet 1988; Campana et al. 2007; Ellis and Keable 2008). However, in the North Pacific maturity is achieved later and at a larger size (29 – 35 yr and 92-100 cm for females and

16 - 19 yr and 70 – 80 cm for males) and individuals also attain larger maximum sizes up to 122 - 130 cm (Jones and Geen 1977; Ketchen 1972, 1975; Saunders and McFarlane 1993). Life expectancies are up to 40 years in the western North Atlantic (Nammack et al. 1985) and up to 81 years in the eastern North Pacific (Saunders and McFarlane 1993).

Studies addressing the population structure of *S. acanthias* have been conducted mostly at regional levels (see below for details), and used either molecular genetic markers or artificial tags. Overall, no population structure was detected at the regional scale with any type of molecular marker. No genetic differentiation was found among locations in Atlantic Canada with either allozymes (Annand and Beanlands 1986) or nuclear microsatellites (Campana et al. 2007). Off the western North Pacific, similar findings were obtained when comparing several locations along the coast and inshore waters using nuclear microsatellites (Hauser et al. 2007). In contrast, two independent studies provide some evidence of population genetic structure in *S. acanthias* on a worldwide scale. Hauser et al. (2007) retrieved two major groups of mtDNA control region sequences in a sample of 44 individuals from throughout the species' range: one group included only North Pacific individuals, and another group included all remaining locations including the North Pacific. Ward et al. (2007) also found two groups of mtDNA cytochrome oxidase I sequences for a total of 31 individuals: an exclusive North Pacific group and another group including only non-North Pacific individuals. These results warrant confirmation with a more robust sampling strategy but suggest the potential for population structure to exist over larger geographic distances.

Results from several tagging studies conducted to date reveal a complex migratory behavior in spiny dogfish. The species occurs mainly in water masses between 6 to 10°C over shelf areas (Shepherd et al. 2002) and its distribution appears to be intimately tied to these environmental conditions. Seasonal latitudinal variations in bottom water temperatures trigger north-south

migrations along coastal areas or inshore-offshore movements in association with optimal water temperatures (Hisaw and Albert 1947; Holden 1965; Burgess 2002; Stenberg 2005; Campana et al. 2007). Where environmental conditions remain stable throughout the year, individuals show more sedentary habits with little or no migration beyond the tagging areas, and may form year-round resident populations (McFarlane and King 2003; Campana et al. 2007). Long distance migrations have also been recorded including both eastward and westward trans-Atlantic movements and westward trans-Pacific movements, and seem to occur along suitable cold temperate coastal areas (Holden 1967; Templeman 1976; McFarlane and King 2003). It is noteworthy that among thousands of individuals tagged to date, no trans-equatorial migration has been recorded, despite the species' high dispersal ability.

b) Leafscale gulper shark *Centrophorus squamosus* (Family Centrophoridae)

The leafscale gulper shark *Centrophorus squamosus* (Bonnaterre 1788) is a medium sized, benthopelagic shark, usually found in association with continental slope waters at bathyal depths between 300 and 1500 m (Clarke et al. 2001a, b; Bañón et al. 2006). The species is geographically distributed in the eastern Atlantic from Iceland to South Africa, in the western Pacific from Japan to New Zealand, and in the western Indian Ocean off the Seychelles (Compagno et al. 2005).

The reproductive mode of *C. squamosus* is yolk sac viviparity with likely limited mucoid histotrophy, as described in the congeneric *C. granulosus* (Guallart and Vincent 2001) and commonly found in many Squaliformes (Musick et al. 2005). However, very little is known about the species' reproductive cycle and all the data on its reproductive biology have been obtained from a limited geographic region on the eastern North Atlantic. On the continental slope of western Europe, commercial catches are comprised mostly of mature males, with a small fraction of immature fish and even smaller numbers of mature females (Girard and Du Buit 1999; Clarke

et al. 2001a; Bañón et al. 2006; Figueiredo et al. 2008). The few mature females often show no signs of reproductive activity but some have ripe oocytes in their ovaries or uteri in the spent condition (Girard and Du Buit 1999; Clarke et al. 2001a; Bañón et al. 2006; Figueiredo et al. 2008). Pregnant females have rarely been caught in this region, totaling three individuals caught off the Iberian Peninsula bearing 1 to 6 embryos/litter (Bañón et al. 2006; Figueiredo et al. 2008). Recently, pregnant females were reported off Madeira and the Canary Islands where they comprised 34% and 10% of all the females sampled in each location, respectively (Severino et al. 2009; Pajuelo et al. 2010).

Figueiredo et al. (2008) proposed the existence of a seasonal breeding cycle for *C. squamosus* but the data are not conclusive. In fact, there is no seasonal variation in the frequencies of different spermatogenic stages and a third of each male gonad was composed of spermatocysts containing spermatozoa (Girard et al. 2000). These observations indicate that males are reproductively active year round and that there is no defined mating season. Length at 50% maturity in the eastern North Atlantic ranges from 98 to 102 cm in males and from 125 to 128 cm in females (Girard and Du Buit 1999; Clarke et al. 2001a; Bañón et al. 2006; Figueiredo et al. 2008), and maximum sizes are 122 cm for males and 145 cm for females (Clarke et al. 2001b). However, only part of the male and female population is present in the studied areas so the values might change as more data are added (Clarke et al. 2001b). Life expectancies are up to 70 years for females and 52 yr for males and sexual maturity is estimated to occur between 30 to 40 years in females and after 20 years in males (Clarke et al. 2002).

Population structure patterns of the leafscale gulper sharks are very poorly know. As described above, males comprise the vast majority of the individuals sampled in the eastern North Atlantic, where individuals smaller than 70 cm (i.e. juveniles) have been consistently absent. Sampling bias due to insufficient bathymetric coverage is not likely because the sampled depth

range extends beyond the species' depth of occurrence (Girard and Du Buit 1999; Clarke et al. 2001a). Gear selectivity issues have also been ruled out (Clarke et al. 2001a, b). One possible explanation for the absence of small fish and the differential distribution of adult and juvenile males and females is geographical segregation of maturity stages. Mating probably occurs along the continental slopes of western Europe, where mature males and ripe females co-occur. After mating, females appear to move out of this region for the duration of the gestation period, potentially moving southward along the continental slope (e.g. towards Madeira and the Canary Islands), migrating back northward after parturition. The location of nursery/pupping areas remains unknown, but juveniles and immature fish appear to recruit to the major fishing grounds on continental slope waters as they approach sexual maturity.

c) Portuguese dogfish *Centroscymnus coelolepis* (Family Somniosidae)

The Portuguese dogfish *Centroscymnus coelolepis* Bocage and Capello 1864 is a wide ranging species distributed in the Atlantic, southern Indian and western Pacific oceans (Yano and Tanaka 1984; Nunan and Senna 2004; Compagno et al. 2005). Its bathymetric range extends from middle to lower bathyal depths, between 500 – 1700 m (Clarke et al. 2001a, b; Nunan and Senna 2004), but varies with region (Yano and Tanaka 1984, 1988; Carrassón et al. 1992; Clò et al. 2002).

The reproductive cycle of the Portuguese dogfish has been described in detail by Yano and Tanaka (1988), Girard and Du Buit (1999) and Veríssimo et al. (2003). Average fecundity estimates vary between 10-14 pups/litter in the eastern North Atlantic (Girard and Du Buit 1999; Clarke et al. 2001a, b; Veríssimo et al. 2003), and 22 pups/litter off Japan (Yano and Tanaka 1988). Length at 50% maturity is 85-90 cm for males and 99-102 cm for females off the eastern North Atlantic (Girard and Du Buit 1999; Clarke et al. 2001b; Bañón et al. 2006; Figueiredo et al. 2008) and 72 cm for males and 95 cm for females off Japan (Yano and Tanaka 1988). Maximum

sizes are 118 cm for males and 122 cm for females (Girard and Du Buit 1999; Clarke et al. 2001b). All female reproductive stages were found throughout the year, suggesting the lack of a breeding season (Girard and Du Buit 1999; Girard et al. 2000; Veríssimo et al. 2003). The duration of the gestation period is thought to be over two years (Figueiredo et al. 2008) and length at birth is around 30 cm (Girard and Du Buit 1999; Clarke et al. 2001b; Veríssimo et al. 2003; Figueiredo et al. 2008). No oocyte development or maturation occurs during gestation or immediately after parturition and some mature females showing no signs of pregnancy or ovarian development are found throughout the year, attesting for the presence of a resting phase between consecutive reproductive cycles (Veríssimo et al. 2003). As such, a complete reproductive cycle may take three or more years. No age estimates are available for this species even though attempts have been made to age spines (Irvine 2004).

All maturity and reproductive stages of both males and females of the Portuguese dogfish were found in all areas studied, except small juveniles (< 70 cm). Bathymetric segregation of the sexes as well as of reproductive stages are described in the literature: immature fish are found deeper while ripe females co-occur with mature males at intermediate depths and pregnant females are only found on the uppermost limit of the depth range (Yano and Tanaka 1988; Girard and Du Buit 1999; Clarke et al. 2001). As also noted for the leafscale gulper shark, the absence of juvenile fish from the fishing grounds (which coincide with sampled areas) is not due to a deficient bathymetric coverage or to gear selectivity (Clarke et al. 2001b). Small males and females must occur elsewhere in the beginning of their life and recruit into the fishing grounds close to attaining sexual maturity (Veríssimo et al. 2003). Also, the existence of a distinct Mediterranean population of *C. coelolepis* was suggested by Clò et al. (2002) based on the distinct size range of individuals (males: 32 – 56 cm; females 32 – 66 cm) as well as on different sizes at maturity (between 40 and 50 cm for both sexes) and depth distribution (exclusively >1350 m).

Main research objectives

As stated earlier, we know little about the biology and ecology of most squaloid sharks. This situation is of great concern in the case of heavily exploited species, such as the spiny dogfish *S. acanthias*, the leafscale gulper shark *C. squamosus*, and the Portuguese dogfish *C. coelolepis*, because of their low productivity and corresponding low rebound potentials. One of the major objectives of this dissertation research is to elucidate the intraspecific patterns of population structure of each species, for future inclusion in fisheries management efforts. Another goal of this project is to infer the patterns of historical dispersal of the three species of squaloid sharks, and examine the consistency of these patterns across species in order to elucidate the events and processes occurring at geological time scales in the different habitats occupied by these taxa.

Highly polymorphic molecular markers were used to infer the patterns of population structure and historical dispersal of each species. Chapter 1 describes the development and optimization of novel nuclear microsatellite loci for each of the three species. Chapter 2 presents the study of the genetic population structure and historical phylogeography of the spiny dogfish *S. acanthias*. For this purpose, samples were collected over the whole species' range (excepting southern Africa) and eight nuclear microsatellite loci and the mtDNA ND2 gene region were used as the molecular markers. Chapter 3 deals with the study of the genetic population structure of the Portuguese dogfish *C. coelolepis* within the eastern Atlantic based on eight nuclear microsatellite loci and the mtDNA control region. The genetic population structure of the leafscale gulper *C. squamosus was* studied within the eastern Atlantic and off New Zealand, using six nuclear microsatellite loci and the mtDNA ND2 gene region, and presented in Chapter 4.

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Chapter 1

Isolation and characterization of nuclear microsatellite loci in three squaloid sharks: *Squalus acanthias*, *Centroscymnus coelolepis* and *Centrophorus squamosus*

Abstract:

Several nuclear microsatellite markers were developed *de novo* for three species of squaloid sharks, namely the spiny dogfish *Squalus acanthias*, the Portuguese dogfish *Centroscymnus coelolepis*, and the leafscale gulper shark *Centrophorus squamosus*. Species-specific microsatellite-enriched libraries were constructed from high quality genomic DNA and screened for di-, tri- and tetranucleotide repeats. Additional loci were obtained from publicly available Expressed Sequence Tag libraries for *S. acanthias*, and from previously published markers. A total of eight nuclear microsatellite loci were optimized for *S. acanthias* (allele number: 5-15; n=48) and for *C. coelolepis* (allele number:3-29; n=40-41), and six loci were optmized for *C. squamosus* (allele number:4-36; n=48). Genotypic distributions of all loci conformed to expectations of Hardy-Weinberg equilibrium and there were no evidence of linkage disequilibrium among loci within species.

Nuclear microsatellite markers were developed *de novo* for all three species by constructing species-specific microsatellite-enriched genomic libraries. Initial extraction of high quality, high molecular weight genomic DNA (gDNA) was obtained from one individual per species, by grinding muscle tissue in liquid nitrogen and using a phenol-chloroform extraction following the protocols of Sambrook and Russell (2001). The isolation of nuclear microsatellite loci from the gDNA of each species was conducted according to the protocols of Hamilton et al. (1999) and of Glenn and Schable (2005), with modifications. Briefly, gDNA was digested with the restriction enzyme *Rsa*I (New England Biolabs) according to the manufacturer's instructions. The resulting restriction fragments were aproximately 500 bp in length and were ligated to double stranded Super-SNX24 linkers (SuperSNX24 forward: 5'GTTTAAGGCCTAGCTAGCAGAATC 3'; SuperSNX24+4P reverse: 5'GATTCTGCTAGCTAGGCCTTAAACAAAA 3'; Glenn and Schable, 2005) in the presence of T4 DNA ligase and the restriction enzyme *Xmn*I (New England Biolabs). The presence of *Xmn*I prevents self-ligation of linkers thereby freeing the latter to ligate to the restriction fragments. The resulting linker-ligated DNA was hybridized to 3' biotinylated oligo probes at 65 °C overnight. Several oligo probes were used for each species as follows: *S. acanthias* - $(GT)_{12}$, $(GA)_{15}$, $(TAGA)_{10}$ and $(ACAT)_8$; *C. squamosus* and *C. coelolepis* – $(TAGA)_{12}$, $(GT)_{12}$ and Mix 4 [$(AAAT)_8$, $(AACT)_8$, $(AAGT)_8$, $(ACAT)_8$, $(AGAT)_8$]. Hybridization reactions were incubated with 100 mg Strepavidin Magnesphere Paramagnetic beads (Promega) at 43 °C for 3 hours in a rotating hybridization chamber. Non-hybridized products were washed (2x per step) in decreasing concentrations of salt solution (2X to 1X SSC buffer with 0.1% SDS) and increasing temperatures (room temperature to 45°C) and the final product recovered in preheated TdotE after incubation at 95 °C for 10 min. The recovered single-stranded enriched DNA was amplified via PCR using the forward SuperSNX24 linker as primer and incorporated into bacterial vectors in the case of the *S. acanthias*, or used in a double enrichment process in the case of the other two species. This differential procedure is explained by the fact that the initial

efforts in developing microsatellite markers for *C. squamosus* and *C. coelolepis* proved fruitless, and the procedure was therefore repeated *a posteriori* using a double-enrichment process to increase the likelihood of success.

Double-stranded, enriched DNA was ligated into pCR 2.1-TOPO® plasmids (Invitrogen) and transformed into Top10 One Shot® *Escherichia coli* cells (Invitrogen) following the manufacturer's protocol. The cells were grown in Luria Bertani agar plates, and colonies with inserts were selected according to manufacturer's protocol and suspended in 100 μl of sterile water. The suspensions were boiled for 10 minutes and centrifuged at 16 000 *g* for 2 minutes to extract plasmids. The supernatant (0.05μl) was amplified via PCR in 10 μl reactions containing 1 μM (each) M13 forward and reverse primers, 5μM each dNTP, 1X Taq buffer with $MgCl_2$ and 0.25 units Taq polymerase according to the following conditions: initial denaturation at 95 °C for 5 min followed by 30 cycles at 94 °C for 30 s, 52 °C for 30 s and 72 °C for 1 min, followed by a final extension step at 72 °C for 5 min. Amplicons were run on a 2.5% agarose:Nusieve agarose (1:1) gel, and plasmids with inserts over 100 bp in length were selected for sequencing. The selected plasmids were reamplified in 25 μl reactions as described above, cleaned with QIAquick PCR purification kit (Qiagen) and used as templates for sequencing using the ABI Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems). Reactions were run on an ABI Prism 3130*xl* genetic analyzer (Applied Biosystems). Inserts exhibiting di- and trinucleotide repeats over 7 units in length were selected for primer design using MacVector 8.0 (MacVector, Inc.) and Primer3 (Rozen and Skaletsky 2000).

A total of 182 inserts were sequenced for *S. acanthias*, of which 9 had inserts and 9 primer pairs were tested. Fifty seven inserts were sequenced for *C. coelolepis* of which 34 contained repeats, and 18 primer pairs were tested. In *C. squamosus*, 288 inserts were sequenced of which 18 contained repeats, and 16 primer pairs were tested. Initial testing for consistent amplification

and screening of polymorphism levels for the putative nuclear microsatellite loci was conducted on 16-18 individuals per species. Those loci considered as polymorphic (≥ 2 alleles) were genotyped in 40-48 individuals of each species and from one sampling location. In case of the spiny dogfish *S. acanthias,* additional nuclear microsatellite loci were obtained from the publicly available *S. acanthias* Expressed-Sequenced Tag (EST) library available on GenBank (dbEST ID 47209646). The EST library was screened for dinucleotide repeats over 7 units in length using the Sequence Repeat Identification Tool (Temnykh et al. 2001) and 19 primer pairs were designed and tested for consistent amplification and polymorphism levels as described above. I also tested 8 previously developed primers for amplification of nuclear microsatellite loci in *S. acanthias* (McCauley et al. 2004), for consistent amplification and polymorphism levels.

Microsatellite genotyping was done via PCR amplification in 5 µl reactions containing 5-15 ng of gDNA, 0.0375 µM of forward primer labeled with a T3 tail, 0.15 µM of reverse primer, 0.1 µM of color (PET, VIC or 6FAM), 0.025 units *Taq* DNA polymerase, 0.2 mM each dNTP, 1.5 mM $MgCl_2$ and 1× *Taq* buffer and mili-Q autoclaved water. PCR conditions consisted of 3 min at 94°C, followed by 35-45 cycles of 1 min at 94°C, 35 s – 1 min at the corresponding annealing temperature and 35 s – 1 min s at 72°C, and a final extension step for 7 min at 72°C. The products were run on an ABI Prism 3130*xl* and scored manually with the software GeneMarker version 1.3 (Softgenetics; LLC).

The web-based software Genepop (Raymond & Rousset, 1995; Rousset, 2008) was used to provide estimates of the number of alleles per locus (A) as well as to test for deviations from Hardy-Weinberg Equilibrium (HWE) expectations and presence of linkage disequilibrium (LD) among loci. The expected heterozygosity (H_E) and inbreeding coefficient (F_{IS}) per locus was calculated in FSTAT v. 2.9.3 (Goudet 2002). Micro-Checker 2.2.3 (Van Oosterhout et al. 2004) was used to test for the presence of scoring errors and null alleles.

A total of eight nuclear microsatellite loci were successfully and consistently amplified in *S. acanthias*. One locus (*SacaGA11*) was obtained from the enriched genomic library; three loci (*Saca3853*, *Saca4234* and *Saca6396*) were obtained from the publicly available EST-library; and four loci (*DFU285, DFT289, DFJ445* and *DFJ451*) were optimized from McCauley et al. (2004). Based on a sample of 48 individuals collected off Massachusetts, U.S.A., the number of alleles per locus varied between 5 and 15 (average: 7.6) and the expected heterozygosities varied between 0.38 and 0.90 (Table 1). There was no evidence of LD among loci and no significant deviations of genotypic distributions from HWE expectations. There was also no evidence of allele scoring errors or the existence of null alleles for any of the eight loci.

Four nuclear microsatellite loci were obtained for *C. coelolepis* from the double-enriched genomic library (*Ccoe25, Ccoe55, Ccoe61* and *Ccoe75*), and on additional locus was obtained from the EST-library developed for *S.acanthias* (*Ccoe7551*). The loci developed for *S. acanthias* and for *C. squamosus* (see below) were cross-amplified in *C. coelolepis* in order to increase the number of markers available, and three of these loci (*DFJ451, SacaGA11* and *Saca3853*) were polymorphic in *C. coelolepis*. All loci were consistently amplified in 40-41 individuals collected off Portugal, and showed between 3 and 29 alleles per locus (average 11.1) and expected heterozygosities between 0.64 and 0.95 (Table 2). There was no evidence of LD among loci and no significant deviations of genotypic distributions from HWE expectations were found except for locus *CcoeGT55*. However, when the alternative hypotheses of heterozygote excess or deficiency were tested on Genepop 4.0.10 (Raymond and Rousset 1995; Rousset 2008), the more powerful one-tail tests did not reject the null hypothesis. There was no evidence of allele scoring errors or the existence of null alleles for any of the loci.

Four nuclear microsatellite loci were obtained for *C. squamosus* from the double-enriched genomic library (*CsquMx31, CsquMx59,CsquMx104* and *CsquGT64*) and consistently amplified

in 48 individuals collected off Portugal. Given the few microsatellite markers developed, the loci developed for *S. acanthias* and for *C. coelolepis* were also cross-amplified in *C. squamosus* in order to increase the number of loci available for the study of the genetic population structure in the leafscale gulper. Two of these loci (*SacaGA11* and *Saca7551*) were polymorphic in *C. squamosus* and were screened for the same 48 individuals indicated above. The number of alleles per locus varied between 4 and 36 (average: 13.3) and the expected heterozygosity varied between 0.56 and 0.92 (Table 3). There was no evidence of LD among loci or of significant deviations of genotypic distributions from HWE expectations.

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TABLE 1- Characterization of eight nuclear microsatellite loci for the spiny dogfish *Squalus acanthias* (n=48 individuals). Ta (ºC): annealing temperature; A: number of alleles per locus; HE: expected heterozygosity; FIS: inbreeding coefficient.

Marker name	Repeat motif	Primer sequence (5' – 3')	Ta (°C)	A	H_E	F_{IS}	GenBank Accession no.
SacaGA11	$[TG]_9$ C [TG]	F: TTCAAGCTCCTCACAGATTTACGGTGC	62	5	0.71	-0.08	GU553360
		R:AACACCACAACACACGAAAGCTCCTGC					
Saca3853	$[TC]_9$	F:GCAAGTCTTTTGTTTCATTGTTGTA	55.7	6	0.54	0.04	ES883853
		R: GATCTTTTATTTCCACATGAACAGG					
Saca4234	$[TG]_{14}$	F:CAGGGGTCAGGCTGTTTCTCC	58	6	0.38	0.13	ES324234
		R:ACGGTGCTGGTGTAACCTTCC					
Saca6396	$[CA]_{19}$	F:CCCTCCTCTCCCTTCCCG	55.7	15	0.90	-0.06	DV496396
		R:GCTGCTCCGCACCGTTAGATAC					
DFU285	$[CT]_{11}$	F:CTGTCCATGGTCACTTTT	54	6	0.76	0.04	AY584499
		R:GATACTTTTGTTCAGAGC					
DFT289	$[TCC]_7$	F:GGGCGTCTGTGAACGCAGAC	57.1	7	0.44	0.01	AY584501
		R:ATAGTCCAGTAACATAACCTG					
DFJ445	$[AC]_{10}$	F:ACACTCACTCGCTCACACAAA	59.2	9	0.56	0.14	AY584505
		R:TCCATTGCGGCAAACTCACT					
DFJ451	$[AC]_{10}$	F:CCAAACTGAAAAGGAGTGAGTGACC	54	7	0.66	0.05	AY584503
		R:TCCACTTACGTATCAATGATA					

TABLE 2- Characterization of eight nuclear microsatellite loci for the Portuguese dogfish *Centroscymnus coelolepis* (n=40-41 individuals). Ta (°C): annealing temperature; A: number of alleles per locus; H_E: expected heterozygosity; F_{IS}: inbreeding coefficient; n.d. – not determined.

Marker name	Repeat motif	Primer sequence (5' – 3')	Ta (°C)	A	H_E	F_{IS}	GenBank Accession no.
CcoeGT25	$[TG]_7$	F: CACTCAAAATTCCACCTGT	56	3	0.64	-0.061	HQ462567
		R: GGTTCTTTCATAAACGTAATAA					
CcoeGT55	$[CA]_{35}$	F: TCGATTGTCTGAAGAGCCAACAG	56	29	0.95	0.028	HQ462568
		R: TCGTCTAGGTGAGGATAGGATTGG					
CcoeGT61	$[TG]_{24}$	F: CAAGTCAGGCGCTGCTGG	56	17	0.89	-0.013	HQ462569
		R: GCAATATCTTTCCTCATCCTAATTCG					
CcoeGT75	$[TG]_{10}$	F: GGTCCATAACCATTAACCACACGC	62	17	0.86	0.011	HQ462570
		R: CTTGCCAATGAAATTCCCGAAG					
Ccoe7551	$[AG]_{14}$	F: TCTCTCGCTTCACATCAGGTTTG	58	6	0.70	-0.04	EG027551
		R: GCTCGTTTCCCATCTCTCAGACTG					
DFJ451	n.d.	F:CCAAACTGAAAAGGAGTGAGTGACC	55	3	0.67	-0.10	AY584503
		R:TCCACTTACGTATCAATGATA					
SacaGA11	n.d.	F: TTCAAGCTCCTCACAGATTTACGGTGC	50	9	0.74	0.04	GU553360
		R:AACACCACAACACACGAAAGCTCCTGC					
Saca3853	n.d.	F:GCAAGTCTTTTGTTTCATTGTTGTA	50	5	0.71	-0.17	ES883853
		R: GATCTTTTATTTCCACATGAACAGG					

TABLE 3- Characterization of six nuclear microsatellite loci for the leafscale gulper *Centrophorus squamosus* (n=48 individuals). Ta (ºC): annealing temperature; A: number of alleles per locus; H_E: expected heterozygosity; F_{IS}: inbreeding coefficient; n.d. – not determined.

Marker name	Repeat motif	Primer sequence (5' – 3')	Ta (°C)	A	H_E	F_{IS}	GenBank Accession no.
CsquMx31	$[AC]_{31}$	F:ACATCCAGCTTTAGCATGAGGAAG	54	36	0.90	0.03	JQ037909
		R:AGGGTACAGCAAATCAGGAGAGC					
CsquMx59	$[TGTA]_5$	F: CTGAGCCCTGTGAGAAAAGG	54	4	0.56	0.19	JQ037910
		R:TTGGGACTTTATGCTCATTGC					
CsquMx104	$[AC]_{16}$	F:AAAAGCGGCTGAAACGGC	54	13	0.88	0.05	JQ037911
		R:GATGTCAAAGCACAGGCAGGG					
CsquGT64	$[GA]_{15}$	F:CTGCCTTCGAGGAGCAATAG	57	18	0.92	0.03	JQ037912
		R:GGAGAGGGTCCTGGGATTTA					
SacaGA11	n.d.	F: TTCAAGCTCCTCACAGATTTACGGTGC	62	4	0.65	0.00	GU553360
		R:AACACCACAACACACGAAAGCTCCTGC					
Saca7551	n.d.	F: TCTCTCGCTTCACATCAGGTTTG	62	5	0.63	-0.19	EG027551
		R: GCTCGTTTCCCATCTCTCAGACTG					

Chapter 2

Global population structure of the spiny dogfish *Squalus acanthias*, a temperate shark with an antitropical distribution

Abstract:

The spiny dogfish (*Squalus acanthias*) is a temperate, coastal squaloid shark with an antitropical distribution in the Atlantic and Pacific oceans. The global population structure of this species is poorly understood, although individuals are known to undergo extensive migrations within coastal waters and across ocean basins. In this study, an analysis of the global population structure of the spiny dogfish was conducted using eight polymorphic nuclear microsatellite markers and a 566 bp fragment of the mitochondrial ND2 gene region. A low level of genetic divergence was found among collections from the Atlantic and South Pacific basins, while a high level of genetic divergence was found among Pacific Ocean collections. Two genetically distinct groups were recovered by both marker classes: one exclusive to North Pacific collections, and one including collections from the South Pacific and Atlantic locations. The strong genetic break across the equatorial Pacific coincides with major regional differences in the life history characters of spiny dogfish, suggesting that spiny dogfish in areas on either side of the Pacific equator have been evolving independently for a considerable time. Phylogeographic analyses indicate that spiny dogfish populations had a Pacific origin, and that the North Atlantic was colonized as a result of a recent range expansion from the South American coast. Finally, the available data strongly argue for the taxonomic separation of the North Pacific spiny dogfish from *S. acanthias* and a re-evaluation of the specific status of *S. acanthias* is warranted.

Introduction

During the last two decades, significant progress has been made in our understanding of the patterns of genetic population structure of elasmobranchs. Recent studies have shown that the level of intraspecific genetic differentiation in elasmobranch species appears to be highly correlated with dispersal ability. In addition, the level of population structure recovered in these studies generally agrees with that expected based on the species' maximum size and habitat preference (*e.g.* coastal, pelagic or benthic), variables intimately associated with elasmobranch vagility (Musick et al. 2004). For instance, low levels of genetic differentiation among populations were reported for highly migratory, large, oceanic species with global distributions, with population structure being detected only between ocean basins (e.g. shortfin mako *Isurus oxyrinchus* Schrey & Heist 2003; basking shark *Cetorhinus maximus* Hoelzel et al. 2000; whale shark *Rhincodon typus* Castro et al. 2007). In contrast, higher levels of intraspecific genetic differentiation across similar spatial scales have been reported for less vagile, demersal or benthic species with more restricted geographic ranges (e.g. shovelnose guitarfish *Rhinobatus productus* Sandoval-Castillo et al. 2004; thornback ray *Raja clavata* Chevolot et al. 2006; zebra shark *Stegostoma fasciatum* Dudgeon et al. 2008).

Exceptions to the low levels of genetic differentiation predicted for highly vagile elasmobranchs have nonetheless been reported. For instance, in species where females exhibit site fidelity to nursery areas, discrete populations can be found along areas of continuous habitat, as in the blacktip shark *Carcharhinus limbatus* or the sandbar shark *C. plumbeus* (Schrey & Heist 2003; Keeney & Heist 2006; Portnoy, 2008). Unsuitable environmental conditions and/or habitat discontinuities can also severely limit dispersal, even for highly vagile elasmobranch species. For example, open oceanic waters appear to be a barrier to gene flow in species such as the scalloped hammerhead *Sphyrna lewini* and the lemon shark *Negaprion brevirostris* (Feldheim et al. 2001;

Duncan et al. 2006; Schultz et al. 2008), while warm equatorial waters also seem to function as barriers to gene flow in temperate species such as the tope *Galeorhinus galeus* (Chabot & Allen 2009).

Habitat use in elasmobranch taxa is heavily influenced by water temperature conditions (Simpfendorfer & Heupel 2004). As such, changes in water temperature can alter the dispersal patterns and the distribution of populations and species. Although the Earth's climate has changed dramatically throughout geological times, high latitude regions have experienced considerably more environmental variation than regions at mid and lower latitudes (Zachos et al. 2001; Ravelo et al. 2004; Brierley et al. 2009; Liu et al. 2009). The relative stability of tropical and warm-temperate regions and their vast spatial distribution in the world's oceans have facilitated uninterrupted gene flow in highly vagile, tropical and warm-temperate shark species, effectively reducing the potential for genetic divergence. In contrast, the variability in the temperature regime at mid and high latitudes (Zachos et al. 2001; Ravelo et al. 2004; Brierley et al. 2009; Chiang 2009; Hollis et al. 2009) has likely affected the distributions of temperate and boreal elasmobranchs, and produced patterns of population structure distinct from those of their warm water relatives. However, the patterns of population structure of temperate and boreal elasmobranchs are not well known.

In this study, we investigated the global population structure of a highly vagile, temperate elasmobranch - the spiny dogfish *Squalus acanthias*. The spiny dogfish is a common coastal shark with an anti-tropical distribution in the Pacific and Atlantic oceans, where it is generally found in continental shelf waters ranging from 6 to 10°C (Burgess 2002; Compagno et al. 2005; Shepherd et al. 2002). The species is known to undergo seasonal north-south migrations along coastal areas or inshore-offshore movements triggered by changes in bottom water temperatures (Hisaw & Albert 1947; Holden 1965; Burgess 2002; Stenberg 2005; Campana et al. 2007). Long

distance migrations, including both eastward and westward trans-Atlantic movements and westward trans-Pacific movements, have also been reported for the spiny dogfish, confirming its high dispersal ability (Holden 1967; Templeman 1976; McFarlane & King 2003). Considering the wide geographic distribution and potential for long distance dispersal of the spiny dogfish, we hypothesize that restricted gene flow/genetic divergence along the species' range is associated with regions of unsuitable environmental conditions, such as warm waters at low latitudes. To test this hypothesis, we used highly polymorphic molecular markers, nuclear microsatellite loci and nucleotide sequences from a mitochondrial DNA gene region (ND2), to estimate the levels of genetic diversity and genetic differentiation among sample collections from thoughout the geographic range of the species. We also inferred the contemporary and historical patterns and processes responsible for the current distribution of genetic variation.

Materials and Methods

Sample collection, DNA extraction and analysis

Spiny dogfish collections were obtained from throughout the species' range and include locations in the North Atlantic: Irish Sea (UK), Ireland (IRE), Massachussets (MA) and Virginia (VA); the western South Atlantic: Argentina (ARG); the North Pacific: California (CA), Washington-Oregon border (WAOR) and Japan (JA); and the South Pacific: Chile (CH) and New Zealand (NZ) (Table 5). Despite our best efforts, no collections were obtained from southern Africa, or the Mediterranean or Black seas. Individual samples consisted of muscle tissue or fin clips and were preserved in 95% ethanol and stored at 4° C, or in a 20% solution (v/v) dimethyl sulfoxide (DMSO) buffer saturated with NaCl (Seutin et al. 1991) and stored at room temperature. Total genomic DNA (gDNA) was extracted from each individual sample using one of two methods: Chelex® resin according to the protocol of Estoup et al. (1996), or the Qiagen DNeasy Tissue kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

All individuals were genotyped for a total of eight nuclear microsatellite loci. Four loci (*DF U285*, *DF T289*, *DF J451* and *DF J445*) were obtained from McCauley et al. (2004) and were chosen after testing for consistent amplification and conformation of genotypic distributions to the expectations of Hardy-Weinberg Equilibrium (HWE). The remaining four microsatellite loci were developed *de novo* as described below. One microsatellite locus, *Saca GA11* (GenBank accession number GU553360), was obtained through the development and screening of a GA-repeat enriched genomic library according to the protocol of Glenn & Schable (2005). Clones with DNA fragments exhibiting GA motifs over six repeat units in length were selected and the corresponding flanking regions used for primer design. Three additional microsatellite loci were obtained from the *S. acanthias* Expressed-Sequenced Tag library available on GenBank (dbEST ID 47209646). The library was screened for dinucleotide repeats over 7 units in length using the Sequence Repeat Identification Tool (Temnykh et al. 2001), and primer pairs were designed for 19 potential loci. All loci were screened for high levels of polymorphism and conformation of genotypic distrbutions to HWE expectations, and three loci were chosen: *Saca3853*, *Saca4234* and *Saca6396* (accession nos. ES883853, ES324234, DV496396, respectively). Annealing temperatures and polymerase chain reaction (PCR) conditions were optimized for each microsatellite locus (see Table 4 and below for details). Microsatellite genotyping was conducted by amplifying each individual sample via PCR in 5 µl reactions containing 5 to 15 ng of gDNA, 0.0375 µM of forward primer labeled with a T3 tail, 0.15 µM of reverse primer, 0.1 µM of T3 primer fluorescently label (e.g. NED, PET, VIC or 6FAM), 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 0.025 units *Taq* DNA polymerase, and 1× *Taq* buffer (Qiagen, Valencia, CA) and autoclaved mili-Q water. PCR conditions consisted of an initial denaturation of 3 min at 94° C, followed by 35-45 cycles of 1 min at 94° C, 35 sec – 1 min at the corresponding annealing temperature and 35 sec – 1 min at 72° C, and a final extension step for 7 min at 72° C. The products were run on an ABI Prism 3130*xl* (Applied Biosystems, Warrington, UK). Genotypes were scored manually with

the software GeneMarker version 1.3 (Softgenetics, LLC, State College, PA). The presence of null alleles or of scoring errors in genotyping was tested for each locus using Micro-Checker (van Oosterhout et al. 2004). Conformation of genotypic distributions to HWE expectations for each locus within each population, tests of linkage disequilibrium between each pair of loci within and among all populations, number of alleles per locus and population, and observed and expected heterozygotes were calculated in Genepop version 4.0 (Raymond & Rousset 1995; Rousset 2008).

Mitochondrial DNA sequences of the NADH dehydrogenase 2 (ND2) gene region were obtained for each individual via PCR amplification using species-specific primers designed based on the complete mitochondrial genome sequence of *S. acanthias* available in GenBank (Accession no. NC_002012): ND2_F 5'TTCCTCACACAAGCAACCGC 3' and ND2_R 5'GATGGTGGCTGGGATGGC 3'. PCR master mixes of 25 µl reactions included 10 – 20 ng gDNA, 1 µM of each primer, 200 µM each dNTP, 0.025 units *Taq* polymerase and 1X *Taq* buffer with 1.5 mM $MgCl_2$ (Qiagen, Valencia, CA), and autoclaved mili-Q water. PCR conditions consisted of an initial denaturation of 5 min at 94° C, followed by 35 cycles of 1 min at 94° C, 1 min at 58° C and 1 min at 72° C, and a final extension step for 7 min at 72° C. The amplicons were cleaned with the QIAquick PCR Purification kit (Qiagen, Valencia, CA) according to the manufacturer's protocol, and the forward strands were sequenced using the ABI Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Warrington, UK). Reactions were run on an ABI Prism 3130*xl* genetic analyzer (Applied Biosystems, Warrington, UK). The resulting DNA sequences were imported into Sequencher version 4.8 (Gene Codes Corp., Ann Harbor, MI) and checked for quality and accuracy in nucleotide base assignment. All individual sequences were aligned in Macvector version 8.0 (MacVector, Inc., California, USA) using the ClustalW multiple alignment algorithm (Thompson et al. 1994). Haplotypes were confirmed by sequencing the reverse strand of one individual of each haplotype.

Statistical analysis

The total number of alleles and observed (H_O) and expected heterozygosities (H_E) were calculated for all microsatellite loci in Genepop version 4.0 (Raymond & Rousset 1995; Rousset 2008). Allelic richness (R_S, an estimate of the number of alleles standardized for unequal sample sizes) was estimated in FSTAT version 2.9.3.2 (Goudet 2002). Mitochondrial DNA ND2 sequence diversity indices were calculated in DnaSP version 5 (Librado & Rozas 2009) including number of polymorphic sites, number of haplotypes (H), haplotype diversity (*h*), nucleotide diversity (π), average number of nucleotide differences within (k), as well as number of fixed differences between haplotypes. Levels of among-population genetic differentiation were estimated by pairwise F_{ST} (microsatellites), or Φ_{ST} (mtDNA), in Arlequin version 3.11 (Excoffier et al. 2005). In the case of the F_{ST} tests, the statistical power (i.e rejection of the H_0 of genetic homogeneity among two subpopulations when it is false) and the alpha level (i.e. rejection of H_0 when it is true) were estimated with the POWSIM software (Ryman & Palm 2006) using a sampling scheme of 9 subpopulations with 45 individuals each, and one subpopulation with 20 individuals. The analyses were conducted using 1000 dememorizations, 100 batches and 1000 iterations per batch. A visual representation of among population differentiation based on microsatellite allele frequencies was constructed using a principal component analysis (PCA) as implemented in PCA-GEN version 1.2 (J. Goudet, http://www2.unil.ch/popgen/softwares/pcagen.htm). The significance of total inertia and each axis' inertia was tested by 10000 randomizations of the data.

The Structure software version 2.2 (Pritchard et al. 2000; Falush et al. 2003) was used to infer the population structure of spiny dogfish using nuclear microsatellite data. An initial analysis was conducted for K values between 1 and 10, using a 'no-admixture model' with independent frequencies and default parameters since individuals are expected to belong to only one of

population with each population having different allelic frequencies. A second round of analysis was conducted to look for structure within the major groups recovered by the first analysis. For this purpose, the total dataset was divided into groups comprising the collections assigned to each of the population clusters retrieved in the first analysis. The K values tested ranged from 1 to the maximum number of different collections in each subset, and the admixture ancestry model with correlated allelic frequencies was used as some mixing between subpopulations (i.e. admixed individuals) is expected. Ten replicates were obtained for each K value tested, with each replicate including 15000 steps of burn-in followed by 35000 steps. Criteria for choosing the best K values followed those indicated in the software manual.

Hierarchical population structure was evaluated for each type of marker through an analysis of molecular variance (AMOVA) as implemented in Arlequin. For microsatellite data, the AMOVA was performed on a locus-by-locus basis and integrated over all loci, based on allelic frequency data; for mtDNA ND2 data, the AMOVA was performed based on a distance matrix of pairwise differences. In either case, significance was estimated using 10 000 iterations (Excoffier et al. 1992). Maximum parsimony ND2 haplotype networks (Polzin & Daneschmand 2003) were constructed using the median joining algorithm (Bandelt et al. 1999) with default parameters and a transition to transversion ratio of 11:1 (as estimated by DnaSP) using the Network 4.5.1.0 software (fluxus-engineering.com). Only haplotypes occurring in more than 2 individuals were included in the haplotype network to highlight the geographic distribution of shared haplotypes. Divergence from an ancestral population of size N_0 at T generations in the past was estimated in Arlequin for each of the recovered populations groups. The value of T, scaled by the mutation rate μ, *i.e.* $\tau_D = 2\mu T$, was estimated assuming isolation after divergence and constant but unequal daughter populations sizes (Excoffier et al. 2005).

Past reduction of effective population size (bottleneck) was investigated with nuclear microsatellites using the ratio (M) test of Garza & Williamson (2001), for each locus and each population independently, and compared to the critical values (M_c). The M_c was calculated based on 7 loci, θ=10 and the conservative parameters of 90% one-step mutations and a mean size of non-one step mutations of 3.5 (Garza & Williamson 2001). The excess heterozygosity test implemented in Bottleneck version 1.2.02 (Piry et al. 1999) was also used to infer past bottleneck events. The discrepancy between heterozygosity values was tested using a Wilcoxon's test under the null hypothesis of no significant heterozygosity excess (Piry et al. 1999). The two methods were used to infer the relative timing (older vs. recent) and severity (long or short duration) of detected bottleneck events (Williamson-Natesan 2005). Demographic analyses conducted using mtDNA mitochondrial sequences were performed in Arlequin. Mismatch distributions were obtained for each population unit (10 000 permutations) to infer changes in population size based on the frequency of pairwise differences among haplotypes (Schneider & Excoffier 1999). Relative time since lineage expansion (*t*) was obtained from the mismatch distribution analysis and estimated from $\tau_G = 2\mu t$ where τ_G is obtained from the mismatch distribution, and μ is the mutation rate assumed to be equal for all populations. Deviations from selective neutrality were also tested for the mitochondrial ND2 sequences with Tajima's *D* (Tajima 1989) and Fu's *F* (Fu 1997) (10000 permutations, alpha=0.05) based on an infinite-site model without recombination.

Results

Genetic diversity

A total of 467 individuals was screened for variation at eight nuclear microsatellite loci. Complete genotypes were generated for 454 individuals while the remaining 13 individuals had missing data at one of the eight loci. Genotypic distributions conformed to HWE expectations for all locus/population combinations. Only one test of linkage disequilibrium in one collection

remained significant after Bonferroni correction (JA, *DFT289* vs. *DFJ445*) but no loci were in linkage disequilibrium globally (data not shown). The number of alleles per locus varied between 9 (*DF J451* and *Saca GA11*) and 23 (*DF J445*) (mean: 13.5) and the mean allelic richness (R_s) varied between 4.85 (CA) and 6.25 (CH) (Table 4 and 5). The North Pacific collections had lower mean heterozygosities (H_O: 0.54-0.56; H_E: 0.55-0.56) and mean R_s (4.85-5.10) compared to those of other regions (H_O: 0.62-0.69; H_E: 0.62-0.65; R_s: 5.51-6.25).

A 566 bp fragment of the mtDNA ND2 locus was sequenced for 412 spiny dogfish, resulting in 103 haploypes (GenBank accession no.s JQ035532-JQ035634). There were 83 polymorphic positions of which 40 were parsimony informative and 43 were singleton mutations. The absolute number of pairwise differences between distinct haplotypes ranged from 1 to 13 (mean: 4.84). Overall nucleotide diversity was 0.0086 and haplotype diversity was 0.839. Singleton haplotypes occurred in 63% of the individuals, while 36% to 60% of haplotypes in each collection occurred in less than 3 individuals. Only 33 haplotypes were shared by two or more collections, with none being shared between North Pacific and non-North Pacific collections. North Pacific collections showed higher haplotype diversities (0.89 – 0.93) and divergences (2.64 – 3.02) compared to other regions (0.56 – 0.73 and 0.84 – 1.54, respectively).

Genetic differentiation and population structure analysis

Power analysis of microsatellite data indicated that a pairwise F_{ST} level of 0.01 could be detected 100% of the time and for an alpha value of 0.057. Pairwise F_{ST} and Φ_{ST} tests indicated low, non significant genetic differentiation among collections within the Atlantic, South Pacific and North Pacific as well as among those from the Atlantic and South Pacific (Table 6). Conversely, strong and significant genetic differentiation was found between collections from the Atlantic and the North Pacific as well as between those from the North and South Pacific. These results are consistent with the results of the PCA analysis, in which North Pacific collections were

clearly separate from all others (PCA 1: F_{ST}=0.03624, 79% of total inertia, P=0.001; Fig. 1). Differentiation of North Atlantic collections from the southern NZ, CH and ARG collections is also suggested by the PCA but the axis separating these two groups was not significant (PCA 2: F_{ST}=0.00317, 7% of total inertia, P=1.00). This result holds true when considering only those collections outside the North Pacific, and the former without the ARG collection which showed an intermediate position between North Atlantic and South Pacific collections (data not shown).

The Structure analysis showed that the log probability of the full microsatellite dataset had the largest rate of change at K=2, while Ln Pr(D) increased only slightly for K>2 (see Supplemental Material). Moreover, the assignment percentages of each collection to one of the clusters when K=2 exceeded 95% in all replicates (see Supplemental Material) while assignment percentages were roughly equal among clusters for K>2 (data not shown). The above results indicated K=2 as the best estimate of the true K value, corresponding to one cluster including only North Pacific collections (JA, WAOR and CA), and another cluster including Atlantic and South Pacific collections. Separate analyses of these two subsets of data provided no evidence of further population sub-structuring (data not shown).

Results of the AMOVA analysis were generally consistent between nuclear microsatellite loci and the mtDNA ND2 gene region (Table 7). The null hypothesis of global panmixia was rejected due to significant genetic heterogeneity among collections (F_{ST}: 0.040, Φ_{ST}: 0.676, P<0.001). Strong genetic divergence was detected between Pacific and Atlantic collections (F_{CT}: 0.034, Φ_{CT}: 0.477, P<0.01) as well as among collections within the Pacific Ocean: the North Pacific group including JA, WAOR and CA, was significantly different from the South Pacific group including NZ and CH (F_{CT}: 0.081 & Φ_{CT}: 0.753, P <0.001). Given the continuous distribution of the spiny dogfish around the tip of South America and the absence of significant differentiation between any of the southern collections (ARG, CH and NZ), a "North Atlantic-only" group (MA, VA,

IRE and UK) and a "Southern" group (ARG, CH and NZ) were compared. Low but significant genetic heterogeneity was detected between these groups with both types of markers (F_{CT}: 0.005, Φ_{CT}: 0.009; $P< 0.001$), while no differentiation was detected within groups (F_{ST} and Φ_{ST} were both non-significant). When the ARG collection was removed, genetic differentiation among North Atlantic and South Pacific collections was still recovered (F_{CT}: 0.05, Φ_{CT}: 0.008; $P< 0.01$), although there was a slight decrease in the Φ_{CT} and P values. Small but significant differentiation was found between CA and WAOR (F_{SC}: 0.007, $P<0.05$) but this result was driven by only one locus (*DF T289*).

Phylogeographic analysis

Examination of the haplotype network based on the mtDNA ND2 region recovered two lineages separated by a minimum of 5 nucleotide substitutions: one lineage was exclusively represented by Atlantic and South Pacific sequences while the other lineage included all North Pacific haplotypes. Haplotypes in the North Pacific lineage were most closely related to haplotypes found in the NZ and ARG collections (Fig. 2). In addition, several common and geographically widespread haplotypes were found in the North Pacific clade, separated by 1 to 5 substitutions ($k_{NP} = 2.79$), from which other less frequent haplotypes were derived (Fig. 2). In contrast, the South Pacific and Atlantic lineage had a star-shaped network with one very common, central haplotype (39% of all individuals in the group) and with only the three most common haplotypes being shared across collections. Other shared haplotypes had lower frequencies and were geographically constrained: six additional haplotypes were found only in New Zealand and South America collections while three additional ones were present only in the North Atlantic. Estimates of time since population divergence from a common ancestor were in general agreement with the results described above: relative divergence times were largest for the North Pacific group, with slightly larger estimates from the North Atlantic group than from the Southern

group (τ_D = 5.35 vs. τ_D=5.05, respectively). The estimated divergence time between the later two groups produced a negative value (τ_D = -0.88).

Demographic Analysis

The geographic groups recovered in the AMOVA (*i.e.* North Pacific, North Atlantic and Southern Group) were used for all demographic analyses described below. Past events of population size reduction, or bottlenecks, were not detected by either ratio tests (mean observed M >0.7, $M_c \approx 0.675$) or excess heterozygosity tests (overall P values >0.37), except for the Southern group in which the ratio M (M=0.673, variance=0.054) was lower than the critical value. Tajima's D (NP: -1.66, P=0.018; SG: -2.49 and NA: -2.50, P <0.001) and Fu's F statistics were significantly negative in all cases (NP: -26.6; SG: -28.7; NA: -32.1, P <0.001) in conformance with a model of population growth, while mismatch distributions indicated that the sudden expansion model of population growth could not be rejected for any of the three geographic groups. Assuming an equal mutation rate for all populations, the relative time since population expansion was three times larger in the North Pacific (τ_G=3.0, 95%CI:1.55 – 3.88) than in the other two populations, with the Southern group (τ_G=1.1, 95%CI:0.66 – 1.59) having very similar values to the North Atlantic (τ_G=1.0, 95%CI:0.24 – 1.88).

Discussion

Global population structure

The null hypothesis of global panmixia in the spiny dogfish *S. acanthias* was rejected by analyses of both mtDNA ND2 nucleotide sequence (Φ_{ST} = 0.676, P<0.001) and nuclear microsatellite data (F_{ST}=0.04, P<0.001). Significant genetic differentiation was consistently detected by both types of molecular markers between geographic regions, and corresponded to two distinct genetic groups: a North Pacific group, and a non-North Pacific group including

Atlantic and South Pacific collections. The strong genetic divergence between the groups was supported by the existence of fixed nucleotide differences at the mtDNA level, and very distinct allelic frequencies and the presence of private alleles (North Pacific: 10, Atlantic & South Pacific: 31) at the nuclear microsatellite loci. A small but statistically significant level of differentiation was also detected by both mtDNA ND2 sequences and nuclear microsatellites when comparing North Atlantic (MA, VA, IRE and UK) and southern collections (NZ, CH and ARG).

The high level of genetic divergence detected across the equatorial Pacific is in agreement with results from previous studies on the molecular genetics and general biology of the spiny dogfish. Analyses of the mtDNA control region (CR) and cytochrome oxidase I (COI) gene region also showed marked genetic differences between North Pacific and non-North Pacific collections (Franks 2006; Ward et al., 2007; Hauser 2009). Furthermore, strong genetic differentiation was detected at nuclear microsatellite loci between eastern North Pacific collections and those from Chile and the northeastern U.S. (Maine) (Franks 2006). The pronounced genetic divergence found across the equatorial Pacific is coincident with regional differences in the life history of the spiny dogfish. For instance, the age and length at sexual maturity of North Pacific spiny dogfish is attained between 29 - 35 yr and 92-100 cm in females, and between 16 - 19 yr and 70 – 80 cm in males, while maximum sizes and ages are up to 122 - 130 cm and 81 years, respectively (Jones & Geen 1977; Ketchen 1972, 1975; Saunders & McFarlane 1993). Outside North Pacific waters, the age and length at sexual maturity is attained earlier and at smaller sizes, between 10 – 16 yr and 72 – 82 cm in females, and between 6 - 10 yr and 60 – 64 cm in males, with maximum sizes and ages reaching only 110 - 117 cm and 40 yr, respectively (Templeman 1944; Holden & Meadows 1964; Holden 1965; Nammack et al. 1985; Hanchet 1988; Campana et al. 2007; Ellis & Keable 2008). Overall, these results are consistent with long term isolation of spiny dogfish populations across the Pacific equator.

In contrast, Atlantic and South Pacific collections of the spiny dogfish were not significantly different (Table 6, Fig.s 1-2). The lack of marked genetic differences across such a large area is remarkable and suggests that some level of gene flow is occurring, or has occurred until recently, among locations separated by hundreds to several thousands of miles. Several independent tagging studies have reported multiple cases of long distance movements in *S. acanthias*, including trans-oceanic migrations in the North Pacific and North Atlantic (Holden 1967; Templeman 1976; McFarlane & King 2003). A few long distance migrants per generation are enough to prevent the genetic differentiation between geographically distant locations. This fact, combined with the long generation times found in Atlantic and South Pacific spiny dogfish (10-16 yr), means that the hypothesis of ongoing gene flow throughout the Atlantic and South Pacific cannot be dismissed. However, a small but highly significant level of genetic differentiation between collections on either side of the Atlantic equator were found in our study (F_{ST}=0.005 and Φ_{ST} = 0.009, P<0.001). Moreover, we also found that low frequency haplotypes were geographically limited to either the southern collections or the North Atlantic collections. These particular haplotypes are relatively derived (Fig. 2) and have probably originated recently; therefore, their spatial distribution should reflect recent dispersal events which, to the exception of one haplotype shared between ARG and IRE, appear not to include trans-equatorial crossings. Franks (2006) also found small but significant differentiation between Chilean and northeastern U.S. collections at nuclear microsatellite loci (F_{ST}=0.013, P<0.001). These results support the hypothesis that gene flow has historically occurred between locations on either side of the Atlantic equator but it has recently become restricted.

Previous studies of genetic population structure of widely distributed and highly vagile marine elasmobranchs with warm temperate and/or tropical distributions have generally found limited gene flow and/or genetic differentiation across areas of habitat discontinuity (see Introduction for more details and Pardini et al. 2001; Schrey & Heist 2003; Hoelzel et al. 2000;

Castro et al. 2007). In the case of cold temperate and boreal species, regions of constricted gene flow/genetic differentiation may coincide with warm-temperate and tropical waters at low latitudes. Indeed, the pronounced differences in life history and genetic diversity between North Pacific and non-North Pacific spiny dogfish is consistent with the existence of a barrier to gene flow at low latitudes. Similarly, and despite the comparatively smaller levels of genetic divergence between North Atlantic and southern hemisphere collections of spiny dogfish (NZ, CH and ARG), our results also suggest a recent restriction of gene flow across the equatorial Atlantic.

Phylogeographic reconstruction

The current distribution of genetic diversity observed for the spiny dogfish appears to have resulted from a series of discrete range expansion events followed by population divergence across areas of unsuitable environmental conditions (e.g. warm, low latitude waters). The clear break in both genetic diversity and life history strategies found for fish on either side of the Pacific equator, and the large estimate of time since population divergence calculated for the North Pacific lineage suggest a relatively old divergence between the northern and southern Pacific populations. Moreover, evidence of an old and severe bottleneck (significant M-test, Williamson-Natesan 2005) in combination with sudden population expansion in the Southern group are consistent with a scenario of population size reduction and long-term maintenance of small effective sizes (Rogers & Harpending 1992), such as would result from a dispersal (founder) event of fish from the North to the South Pacific. Our data therefore point to a North Pacific origin of the current spiny dogfish populations with subsequent dispersal into South Pacific waters. Dispersal into Atlantic waters appears to have occurred along the South American coast as indicated by the presence of both genetic lineages in the Pacific basin and the closer interclade relationship exhibited by North Pacific and NZ and ARG collections. Dispersal into the

North Atlantic basin appears to be the last step in the population history of *S. acanthias* apparently occurring relatively recently and in association with a rapid increase in population size in the South Pacific and Atlantic.

A North Pacific origin and subsequent dispersal into the Atlantic and South Pacific was also proposed by Franks (2006) based on the observation of non-North Pacific mtDNA control region haplotypes nesting within North Pacific ones. However, and contrary to our findings, Franks (2006) proposed a northern link between the Pacific and Atlantic oceans with subsequent range expansion into the South Atlantic and South Pacific, although no data was provided to support this hypothesis. Our alternative hypothesis of a southern link between the Pacific and Atlantic oceans is in agreement with the results of Jones & Geen (1976), in which a "South American stock", mostly represented by Chilean samples in their study, presented values of mean total number of vertebrae that were intermediate between North Pacific and North Atlantic spiny dogfish. A very different perspective on the population history of the spiny dogfish is provided by the fossil record, which argues for a North Atlantic origin and later dispersal into the North Pacific. The oldest unambiguous fossil of *S. acanthias* in the North Atlantic dates back to the Early Pliocene of Belgium (Herman 1974 *in* Capetta 2006), while the oldest record in the North Pacific is of a younger age (Pleistocene of California; Fitch 1967 & 1968 *in* Capetta 2006). Nevertheless, the possibility of an incomplete fossil record in the North Pacific cannot be discounted.

In the absence of a well calibrated molecular clock for *S. acanthias* or any of its squaloid relatives, there are considerable limitations to inferring an approximate timing of events. The only estimates of mutation rate for elasmobranch taxa refer solely to the non-coding mtDNA CR (in the order of 10^{-5} mutations per generation for the whole mtDNA CR) of two warm-temperate sharks, the bonnethead *Sphyrna lewini* and the blacktip shark *Carcharhinus limbatus* (Duncan et

al. 2006; Keeney & Heist, 2006). The equivalent mutation rates are likely considerably lower in the temperate spiny dogfish due to its potentially lower metabolic rate and longer generation time (Avise et al. 1992; Martin & Palumbi 1993; Martin 1999). Nevertheless, the application of the above estimate should serve as an indication of the most recent time of events in the history of *S. acanthias*. As such, population divergence across the equatorial Pacific must have occurred earlier than 7.8 MY (i.e. before the late Miocene). On another account, the fossil record places *S. acanthias* in the North Atlantic by the Early Pliocene (5.3 – 3.6 MY; Herman 1974 *in* Capetta 2006). If the presence of spiny dogfish in the North Atlantic was indeed preceded by range expansion from the south, dispersal into the North Atlantic must have occurred before the Pliocene.

Taxonomic status of the North Pacific spiny dogfish

The North Pacific spiny dogfish was originally given species status and designated as *Squalus suckleyi* Girard 1854. Later, it was considered a subspecies of the cosmopolitan *S. acanthias* and designated *S.a.suckleyi* (see Jones & Geen 1976 and references therein). Its taxonomic validity was subsequently addressed by Bigelow & Schroeder (1948) and Jones & Geen (1976), who did not find evidence supporting the separation of the North Pacific spiny dogfish into a distinct taxonomic unit based on morphological and meristic characters. Since then, only one species has been considered as valid, namely *S. acanthias*.

Recently, molecular studies have consistently found strong genetic divergence between North Pacific and non-North Pacific spiny dogfish at three different mtDNA gene regions (control region, COI and ND2) and at nuclear microsatellite loci (Franks 2006; Ward et al. 2007; our study). In addition, two reciprocally monophyletic lineages have been found with two of the three mitochondrial (COI and ND2, but not CR) and with nuclear DNA markers, corresponding to a North Pacific-only clade and a "rest of the world" clade (Ward et al. 2007, this study). All these

data are consistent with long term cessation of gene flow between the North Pacific spiny dogfish and those from other regions. In addition, the genetic divergence among groups is coincident with distinct life history strategies: North Pacific fish reach maturity at an older age, have larger maximum sizes and live longer than fish occurring outside North Pacific waters (see above for references). Based on the above data, the North Pacific spiny dogfish should be considered as an independent management and/or conservation unit, as proposed by Hauser (2009). From a conservation genetics' perspective, the removal of genetic diversity from the North Pacific will result in the loss of unique genetic variation. Ultimately, the available data strongly argue for the taxonomic separation of the North Pacific spiny dogfish from *S. acanthias* and, as such, a re-evaluation of the specific status of *S. acanthias* incorporating molecular and morphological analyses is warranted.

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TABLE 4 – Nuclear microsatellite loci of the spiny dogfish *S. acanthias*, with respective motif and repeat units, and total number of alleles scored at each locus (A).

Marker name	Repeat motif	A
SacaGA11	$[TG]_9$ C [TG]	9
Saca3853	$[TC]_9$	14
Saca4234	$[TG]_{14}$	11
Saca6396	$[CA]_{19}$	22
DFU285	$[CT]_{11}$	11
DFT289	$[TCC]_7$	11
DFJ445	$[AC]_{10}$	23
DFJ451	$[AC]_{10}$	9

TABLE 5 – Genetic diversity indices for the spiny dogfish, *S. acanthias*, integrated over all microsatellite loci and all mtDNA ND2 haplotypes from each sampling location. N: number of samples; H0: observed mean heterozygosity; HE: expected mean heterozygosity; Mean A: mean number of alleles; Mean Rs: mean allelic richness; H: number of haplotypes (unique haplotypes); *h*: haplotype diversity; π: nucleotide diversity; k: mean number of nucleotide differences between haplotypes.

		Nuclear microsatellites						Mitochondrial ND2 sequences				
		N	H0	HE	Mean A	Mean Rs		N	H	*h*	π	k
North Pacific												
Japan	JA	49	0.56	0.56	6.25	4.87		46	21 (8)	0.93	0.0047	2.64
Washington/Oregon	WAOR	50	0.55	0.55	6.88	5.10		49	24 (9)	0.93	0.0054	3.02
California	CA	49	0.54	0.55	6.50	4.85		45	21 (9)	0.89	0.0048	2.73
South Pacific												
New Zealand	NZ	46	0.69	0.65	7.00	5.51		40	18 (9)	0.73	0.0022	1.23
Chile	CH	18	0.64	0.64	6.25	6.25		18	8 (3)	0.64	0.0016	0.88
North Atlantic												
Virginia	VA	55	0.65	0.65	8.25	6.19		43	11 (4)	0.57	0.0015	0.84
Massachussetts	MA	48	0.62	0.62	7.00	5.66		41	13 (6)	0.68	0.0017	0.95
Ireland	IRE	56	0.62	0.62	8.00	5.89		39	15 (9)	0.71	0.0024	1.33
Irish Sea	UK	48	0.63	0.64	7.38	5.88		48	14 (7)	0.56	0.0016	0.89
South Atlantic												
Argentina	ARG	48	0.64	0.64	7.63	5.93		43	16 (6)	0.71	0.0027	1.54

TABLE 6 – Levels of genetic divergence among populations of *S. acanthias*. Pairwise F_{ST} below diagonal; pairwise Φ_{ST} above diagonal. Numbers in bold have *P* values < 0.001.

		Φ_{ST}									
		JA	WAOR	CA	NZ	CH	VA	MA	IRE	UK	ARG
F_{ST}	JA		-0.008	0.011	**0.170**	**0.198**	**0.249**	**0.193**	**0.178**	**0.258**	**0.178**
	WAOR	-0.005		-0.004	**0.170**	**0.198**	**0.248**	**0.193**	**0.178**	**0.257**	**0.179**
	CA	0.014	0.007		**0.190**	**0.220**	**0.270**	**0.213**	**0.199**	**0.279**	**0.199**
	NZ	**0.091**	**0.092**	**0.074**		-0.018	0.004	-0.002	-0.010	0.007	-0.014
	CH	**0.084**	**0.080**	**0.069**	0.001		-0.007	-0.009	-0.012	-0.010	-0.018
	VA	**0.094**	**0.092**	**0.065**	0.005	0.007		0.000	-0.002	-0.012	0.003
	MA	**0.084**	**0.083**	**0.064**	0.007	0.007	0.001		-0.001	0.002	0.002
	IRE	**0.094**	**0.091**	**0.066**	0.005	0.004	-0.002	0.001		0.003	0.000
	UK	**0.094**	**0.093**	**0.068**	0.002	0.002	-0.005	0.002	-0.003		0.002
	ARG	**0.075**	**0.073**	**0.055**	-0.002	-0.003	0.000	-0.001	-0.012	0.005	

TABLE 7 – Analysis of molecular variance (AMOVA) for *S. acanthias*. Numbers in bold have *P* values < 0.001 (***), 0.01 (**) or 0.05 (*).

		Microsatellites		mtDNA
Global panmixia				
	FST	**0.040*** **		**0.676*** **
Between oceans				
	FST	**0.055*** **		**0.744*** **
	FSC	**0.022*** **		**0.511*** **
	FCT	**0.034*** **		**0.47788**
North vs. South Pacific				
	FST	**0.085*** **		**0.752*** **
	FSC	**0.004* **		-0.005
	FCT	**0.081*** **		**0.753*** **
Southern Group vs. North Atlantic				
	FST	0.004		0.007
	FSC	-0.001		-0.002
	FCT	**0.005*** **		**0.009*** **
North Pacific vs. North Atlantic				
	FST	**0.081*** **		**0.797*** **
	FSC	0.001		-0.004
	FCT	**0.080*** **		**0.798*** **

Figure 1 – Principal component analysis (PCA) of microsatellite allelic frequencies (8 loci) for the spiny dogfish *S. acanthias*. Abbreviations of locations are as indicated in the text. Circles around locations highlight separation according to PCA1 ($P = 0.01$).

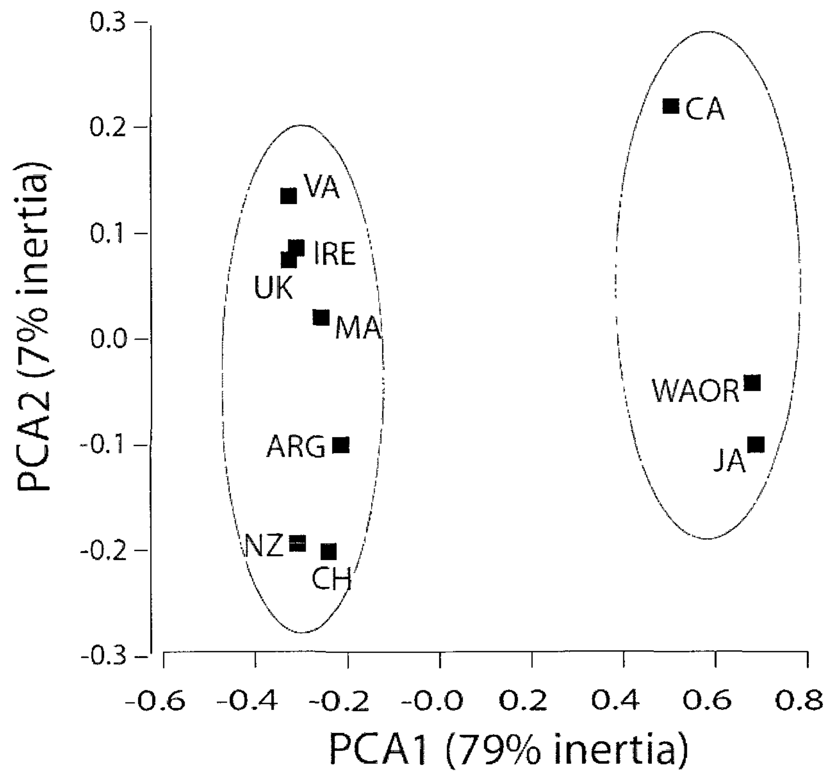
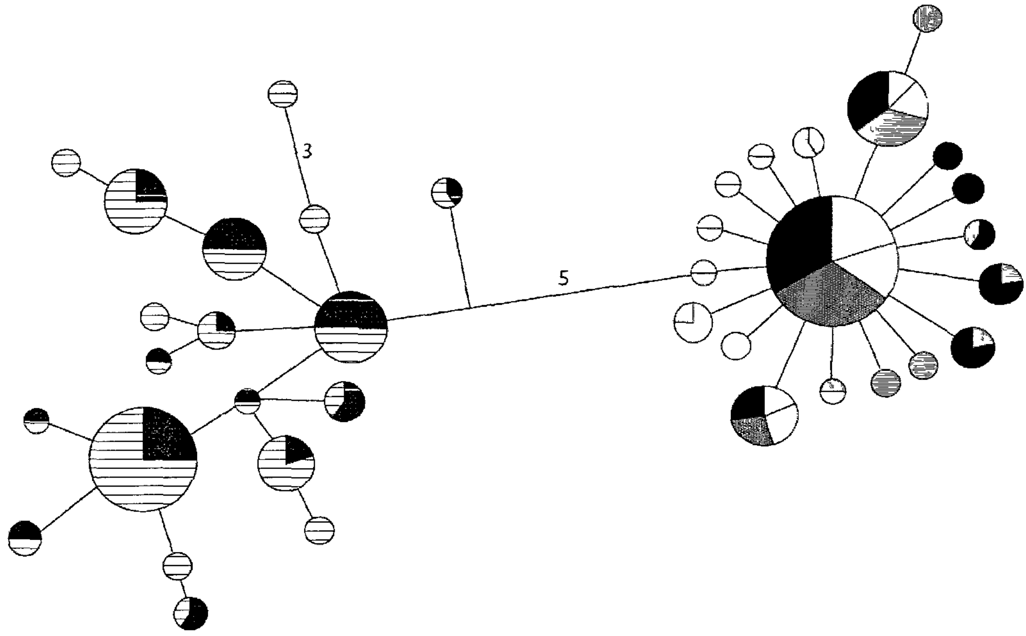

0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
PCA2 (7% inertia)
VA
IRE
UK
MA
ARG
NZ
CH
CA
WAOR
JA
-0.6
-0.4
-0.2
-0.0
0.2
0.4
0.6
0.8
PCA1 (79% inertia)

Figure 2 – Maximum parsimony haplotype network of the mitochondrial DNA ND2 for the spiny dogfish *S. acanthias*. Haplotypes are represented by circles with sizes proportional to absolute frequency in the total sample. Color codes are as follows: black with white stripes – JA; white with black stripes – WAOR and CA; white – NZ and CH; light grey – ARG; dark grey - IRE and UK; black - MA and VA. All branches correspond to one nucleotide substitution between haplotypes except where indicated with a number.


3
5

Chapter 3

Population structure of a deep-water squaloid shark, the Portuguese dogfish (*Centroscymnus coelolepis*)

Abstract:

The genetic population structure of the deep-water squaloid *Centroscymnus coelolepis* (the Portuguese dogfish) in the eastern Atlantic was investigated using eight polymorphic nuclear microsatellite markers and a 496-bp fragment of the mitochondrial DNA control region (mtDNA CR). Samples (20–50 individuals per location) were collected off Ireland, Portugal, Madeira, Mauritania, South Africa, and the Azores (mid-Atlantic ridge). High levels of genetic diversity were found at the nuclear microsatellite loci (mean $A = 17.3$; overall $H_o = 0.77$), although there was low diversity at the mtDNA CR ($h = 0.65$, $\pi = 0.0018$). Genetic diversity for the Portuguese dogfish was homogeneously distributed among sampling locations, resulting in low and non-significant indices of genetic differentiation (e.g. $F_{ST} = -0.0013$ to 0.0096, $\Phi_{ST} = -0.017$ to 0.033; $p > 0.5$), consistent with the absence of population structure within the area sampled. The results indicate that *C. coelolepis* has a high dispersal potential within the eastern Atlantic, and its apparent spatial segregation by size and maturity stage suggest large-scale migration associated with the reproductive cycle.

Introduction

The squaloids (Order Squaliformes) are the second most diverse group of sharks, mostly benthopelagic in habit, and preferentially living in cold, bathyal waters (Musick *et al.*, 2004). Squaloid taxa have conservative life-history features such as late sexual maturation, potentially long lifespans, and low lifetime fecundities (see Kyne and Simpfendorfer, 2010, for more detail). Although such features are generally shared by all elasmobranch taxa, they are particularly pronounced in squaloid sharks and even more so in those occupying deep water, rendering them particularly vulnerable to population depletion and overexploitation (Kyne and Simpfendorfer, 2010). Other than their generally conservative life histories, we know little about the biology and ecology of most squaloids, particularly those living in deep water, and almost nothing about the patterns of species distribution and population structure.

In the absence of studies on the population structure of deep-water squaloids, important clues about the factors contributing to such structuring may be drawn from shallow-water elasmobranch taxa. Recent studies suggest that vagility, environmental conditions, and life strategy exert the strongest influences in shaping population structure in shallow-water elasmobranchs. Highly mobile species such as the shortfin mako (*Isurus oxyrinxhus*) exhibit genetic homogeneity over large spatial scales (Schrey and Heist, 2003), whereas less vagile species with more restricted geographic ranges, such as the zebra shark (*Stegostoma fasciatum*), show comparatively greater genetic differentiation across similar spatial scales (Dudgeon *et al.*, 2008). Regardless of a species' vagility, genetic differentiation has been found among populations separated by unsuitable environmental conditions. For instance, warm equatorial waters coincide with areas of genetic break in temperate sharks, such as the spiny dogfish (*Squalus acanthias*; Veríssimo *et al.*, 2010), whereas open-ocean expanses separate populations of the coastal lemon shark (*Negaprion brevirostris*; Schultz *et al.*, 2008). Life-history strategy also influences the population structure of elasmobranchs. Discrete population units may be

found along areas of continuous habitat in taxa where females exhibit phylopatry to nursery areas, such as in the blacktip shark (*Carcharhinus limbatus*; Keeney *et al.*, 2003).

This work is the first approach to the study of the patterns of population structure in deep-water squaloids, using the Portuguese dogfish (*Centroscymnus coelolepis*) as a case study. The Portuguese dogfish is a medium-sized squaloid (<1.5 m) with an extensive geographic distribution including the Atlantic, southern Indian, and western Pacific oceans (Compagno *et al.*, 2005). In the eastern Atlantic, the species inhabits continental slope waters from west of the British Isles to South Africa, including the Mediterranean, as well as off Madeira and the Canary Islands, and along the Mid-Atlantic Ridge from Iceland to (at least) the Azores (Compagno *et al.*, 2005; Menezes *et al.*, 2009). Its bathymetric range extends from middle to lower bathyal depths (500–1700 m), but may vary with region (Yano and Tanaka, 1988; Clarke *et al.*, 2001). Like most elasmobranch taxa, the Portuguese dogfish exhibits bathymetric segregation according to size, sex, and maturity stage. Immature fish are usually found deeper, mature males at intermediate depth, and mature females occupying the uppermost limit of the depth range (Yano and Tanaka, 1988; Girard and DuBuit, 1999; Clarke *et al.*, 2001).

The Portuguese dogfish reproduces through yolk-sac viviparity, attains sexual maturity at a large size, usually 85–90 cm long in males (maximum length 118 cm) and 99–102 cm in females (maximum length 122 cm), and has low mean fecundities of 10–14 pups per litter (Girard and DuBuit, 1999; Clarke *et al.*, 2001; Veríssimo *et al.*, 2003; Figueiredo *et al.*, 2008). The reproductive cycle in *C. coelolepis*, as in many of its deep-water relatives, does not appear to be synchronous among mature males and females (Girard and DuBuit, 1999; Clarke *et al.*, 2001; Veríssimo *et al.*, 2003; Figueiredo *et al.*, 2008). Moreover, females undergo a resting stage between consecutive gestation periods, as indicated by the absence of ripe oocytes at parturition (Veríssimo *et al.*, 2003). Pups are born at a length of ~30 cm after a gestation period of two years

or more (Girard and DuBuit, 1999; Clarke *et al.*, 2001; Veríssimo *et al.*, 2003; Figueiredo *et al.*, 2008). No mating or nursery grounds have yet been reported for the species.

The Portuguese dogfish is taken commercially in the eastern North Atlantic and, until recently, it was caught regularly in mixed trawl and longline fisheries (ICES, 2009). Large-scale commercial exploitation off the European continental slope began for the species in the early 1990s and peaked in 2003 (10 876 t), decreasing thereafter (ICES, 2009). In 2005, the stock abundance of this and other species (e.g. *Centrophorus squamosus*) was determined to be depleted and below any candidate limit reference point, so a zero catch of deep-water sharks was recommended (ICES, 2009). Despite the state of the resources, management efforts have been developed without a good understanding of stock structure. In cases where the managed stock does not correspond to the true biological stock, e.g. where the managed stock includes several biological stocks, or excludes part of the biological stock, the effectiveness of management plans may be compromised. Our study provides data that will help elucidate the intraspecific patterns of the population structure of *C. coelolepis* for future inclusion in fishery management. Moreover, the findings may provide insights into potential structuring factors of populations of other deep-water sharks.

Material and Methods

Portuguese dogfish sample collections were obtained from six locations in the eastern Atlantic Ocean (Table 1) – off Ireland (IRE), mainland Portugal (POR), Mauritania (MAU), South Africa (SA), Madeira (MAD), and the Mid-Atlantic Ridge north of the Azores (MAR) – including most of the species' range in the region. Females were more abundant than males in all samples except SA (Table 8). The mean total length per collection was between 95.0 and 108.6 cm (i.e. adults or maturing fish), with the exception of MAU, where the mean total length was 62.3 cm (i.e. small juveniles; Table 8). The former collections (IRE, POR, MAD, SA, and MAR) are hereafter designated as adult collections and the latter (MAU) as the juvenile collection.

Samples consisted of muscle tissue or finclips and were preserved in DMSO buffer saturated with NaCl (Seutin *et al.*, 1991). Total genomic DNA (gDNA) was extracted from each individual sample using the Qiagen DNeasy Tissue kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

All fish were genotyped for a total of eight nuclear microsatellite loci (Table 9). Four new microsatellite loci (*CcoeGT25*, *CcoeGT55*, *Ccoe61*, and *Ccoe75*) were developed *de novo*, as described in Veríssimo *et al.* (in press). Two microsatellite loci, *Saca GA11* and *Saca3853*, were obtained as described in Veríssimo *et al.* (2010). Locus *Saca7555* was obtained from the publicly available expressed sequence tag library for *Squalus acanthias* (GenBank dbEST ID 47209646). Finally, one locus (*DF J451*) was obtained from McCauley *et al.* (2004). All loci were chosen after testing for consistent amplification and conformation of genotypic distributions to the expectations of Hardy–Weinberg equilibrium (HWE). Annealing temperatures and polymerase chain reaction (PCR) conditions were optimized for each microsatellite locus (see Table 9 and below for detail). Microsatellite genotyping was conducted via PCR amplification in 5-μl reactions containing 5–15 ng of gDNA, 0.0375 μM of forward primer with an added T3 tail, 0.15 μM of reverse primer, 0.1 μM of fluorescently labelled T3 primer (e.g. NED, PET, VIC, or 6FAM), 0.025 units *Taq* DNA polymerase (Qiagen, Valencia, CA), 0.2 mM each dNTP, 1.5 mM $MgCl_2$, and 1 × *Taq* buffer (Qiagen, Valencia, CA) and autoclaved mili-Q water. PCR conditions consisted of an initial denaturation of 3 min at 94°C, followed by 35–45 cycles of 1 min at 94°C, 35–60 s at the corresponding annealing temperature, 35–60 s at 72°C, and a final extension step for 7 min at 72°C. The products were run on an ABI Prism 3130*xl* (Applied Biosystems, Warrington, UK). Genotypes were scored manually with the software GeneMarker version 1.3 (Softgenetics, LLC, State College, PA) and the presence of null alleles or scoring errors was tested for each locus using Micro-Checker (van Oosterhout *et al.*, 2004). Conformation of genotypic distributions to HWE expectations for each locus within each sample collection and

tests of linkage disequilibrium between each pair of loci within and among all collections were calculated in Genepop version 4.0 (Raymound and Rousset, 1995; Rousset, 2008).

Nucleotide sequences of the mitochondrial DNA (mtDNA) control region (CR) were obtained via PCR amplification using the forward primer Pro-L 5'-AGGGRAAGGAGGGTCAAACT-3' (Keeney *et al.*, 2003) and the reverse primer 5'-GGAGGATCTGTAAATCTTGAGACAG-3' (developed *de novo*). PCR master mixes of 25-μl reactions included 10–20 ng gDNA, 1 μM of each primer, 200 μM each dNTP, 1 × *Taq* buffer with 1.5 mM MgCl2 (Qiagen, Valencia, CA) and 0.025 units *Taq* polymerase (Qiagen, Valencia, CA), and autoclaved mili-Q water. PCR conditions consisted of an initial denaturation of 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 35 s at 58°C, and 1 min at 72°C, and a final extension step for 7 min at 72°C. The amplicons were cleaned with the QIAquick PCR Purification kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The reverse strands of the amplicons were sequenced using the ABI Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Warrington, UK) and run on an ABI Prism 3130*xl* genetic analyser (Applied Biosystems, Warrington, UK). The resulting DNA sequences were imported into Sequencher version 4.8 (Gene Codes Corp., Ann Harbor, MI) and checked for quality and accuracy in nucleotide base assignment. All individual sequences were aligned in MacVector version 8.0 (MacVector, Inc., California, USA) using the ClustalW multiple alignment algorithm (Thompson *et al.*, 1994). Haplotypes were confirmed by sequencing the forward strand of one individual per unique haplotype.

Indices of genetic diversity at the nuclear microsatellite loci were estimated in FSTAT version 2.9.3.2 (Goudet, 2002), including number of alleles per locus (A) and per sampling location, inbreeding coefficient (F_{is}), observed heterozygosity (H_o), and allelic richness (R_s). Mitochondrial DNA CR diversity indices were calculated in DnaSP version 5 (Librado and Rozas, 2009) including number of haplotypes (H), haplotype diversity (h), nucleotide diversity

(π), and the average number of nucleotide differences among sequences within each sampling location (k) and over all sequences. A visual representation of the genetic variability at the nuclear microsatellite loci within and among sampling locations was constructed using a factorial correspondence analysis (FCA) as implemented in Genetix version 4.05.2 (Belkir *et al.*, 2004). In this analysis, individual genotypes are plotted in an n-factorial dimensional space according to their allelic composition over all loci, whereby individuals with similar genetic compositions are plotted closer together. Maximum parsimony mtDNA CR haplotype networks (Polzin and Daneschmand, 2003) were constructed using the median joining algorithm (Bandelt *et al.*, 1999) with default parameters using the Network 4.5.1.0 software (fluxus-engineering.com).

Levels of among-population genetic differentiation based on nuclear microsatellite data and mtDNA sequence data were estimated by the Weir and Cockerham's F_{ST} analog (θ) in FSTAT, and by Φ_{ST} values based on the mean number of pairwise differences among sequences in Arlequin version 3.11 (Excoffier *et al.*, 2005), respectively. Statistical significance of either estimate was assessed through 10 000 permutations of the dataset. The statistical power of the F_{ST} tests (i.e. rejection of the null hypothesis H_0 of genetic homogeneity among two subpopulations when it is false) and the alpha level (i.e. rejection of H_0 when it is true) were estimated with the POWSIM software (Ryman and Palm, 2006) using a sampling scheme of 4 subpopulations with 45 individuals each, plus 2 subpopulations with 20 individuals. The analyses were conducted using 10 000 dememorizations, 100 batches, and 1000 iterations per batch. An analysis of molecular variance (AMOVA) was conducted in Arlequin with both the nuclear and mitochondrial DNA datasets, using 10 000 permutations of the data.

An isolation by distance (IBD) analysis was conducted with the program ISOLDE as implemented in Genepop version 4.0 (using the Mantel option), to test the null hypothesis of independence between genetic distances and sampling locations (p calculated using 1000 iterations). To this purpose, multilocus Cavalli-Sforza and Edwards' genetic distances were

calculated in Genetix based on the microsatellite data, and uncorrected mean *p*-distances were calculated in Arlequin based on the mtDNA CR sequence data. Geographic distances were calculated as the shortest straight-line distance between sampling locations.

Past reduction of effective population size was investigated with nuclear microsatellites using the *M*-ratio test of Garza and Williamson (2001), and comparing it to the critical value (M_c) based on seven loci, $\theta = 10$, and the conservative parameters of 90% one-step mutations and a mean size of non-one step mutations of 3.5 (Garza and Williamson, 2001). In addition, the occurrence of bottlenecks was investigated by means of the excess heterozygosity test implemented in Bottleneck version 1.2.02 (Piry *et al.*, 1999), using a Wilcoxon's test under the null hypothesis of no significant heterozygosity excess (Piry *et al.*, 1999). The two methods were used for consistency purposes and to infer the relative timing and severity of detected bottleneck events (Williamson-Natesan, 2005). Changes in population size were investigated with Arlequin using the mtDNA CR sequence data and the mismatch distribution of pairwise nucleotide differences among sequences (10 000 permutations; Schneider and Excoffier, 1999). Deviations from selective neutrality for the mitochondrial CR were also tested with Tajima's D (Tajima, 1989) and Fu's F_S statistics (Fu, 1997; 1000 permutations, $\alpha = 0.05$) based on an infinite-site model without recombination.

Results

Genetic diversity

Multilocus microsatellite genotypes were obtained for 211 Portuguese dogfish, of which 20 had data missing at one locus. Genotypic distributions conformed to HWE expectations for all locus/population combinations and there was no evidence of linkage disequilibrium among loci (data not shown). Moreover, no null alleles were detected at any of the microsatellite loci. Gene diversity and total number of alleles per locus varied between 0.61 and 0.95 (mean = 0.77), and between 3 and 46 (mean = 17.3), respectively (Table 9). The levels of genetic diversity at the

microsatellite loci were similar among sampling locations (Table 8), with mean observed heterozygosity varying between 0.76 (MAD and MAR) and 0.78 (MAU), and mean allelic richness between 7.9 (MAD) and 8.5 (MAU).

Nucleotide sequences of a 496-bp fragment of the mtDNA control region were obtained for 192 of the Portuguese dogfish sampled, resulting in 18 unique haplotypes (GenBank accession no. HQ664432-HQ664449). There were 12 polymorphic positions corresponding to 8 transitions, 4 transversions, and 1 deletion (Table 10). The mean nucleotide and haplotype diversities across all sequences were 0.0018 and 0.65, respectively, and the mean number of nucleotide differences between all sequences was 0.872. In fact, the number of haplotypes found in each sampling location was small, ranging from a minimum of six in MAD and SA, to a maximum of 9 in MAR, and the haplotype diversities were low, varying between 0.57 (POR) and 0.74 (MAD and MAR; Table 8). Ten of 18 haplotypes (56%) were shared by two or more sampling locations (Table 11), comprising some 96% of the fish sequenced. Singleton haplotypes were found in IRE ($n = 2$), MAR ($n = 3$), and MAD ($n = 3$) (Table 11).

Genetic differentiation and population structure

Individual multilocus microsatellite genotypes were used in a FCA to provide a visual representation of the underlying structure in the data matrix. The resulting analysis indicated that eigenvectors 1–3 explained 70% of the inertia and showed largely overlapping distributions of multilocus genotypes among collections. This overlap was particularly evident among individuals from IRE, MAR, MAU, and SA (Figure 3), whereas those from POR and MAD were spread over different quadrants of the bi-dimensional space. Overall, the FCA indicated great similarity among the individual multilocus microsatellite genotypes sampled from different locations. When comparing adult/maturing vs. juvenile fish genotypes (80-cm total length cut-off), the latter showed a more restricted distribution in space than the former, but there was still a broad overlap of juvenile genotypes with those of adult/maturing fish (data not shown).

Pairwise F_{ST} tests indicated no significant genetic differentiation among sampling locations (–0.0013 to 0.0096) after Bonferroni correction (Table 12). However, most pairwise F_{ST} values including the MAD collection were significant ($p < 0.05$, except for MAR) prior to Bonferroni correction. The power analysis for detecting genetic differentiation using the nuclear microsatellite loci indicated that a pairwise F_{ST} of 0.01 could be detected in 100% of the runs, whereas an F_{ST} of 0.002 could only be detected in 66% of the runs. F_{ST} values <0.02 could not be detected in most runs (data not shown). The α value (type I error) was 0.05. In terms of the pairwise Φ_{ST} tests, there was no indication of genetic differentiation among sampling locations (–0.017 to 0.033; $p > 0.05$). The null hypothesis of global panmixia considering only the adult fish collections (i.e. excluding MAU) was tested by AMOVA and was not rejected with either the nuclear microsatellite data or mtDNA sequence data ($F_{ST} = 0.00038$ and $\Phi_{ST} = 0.00153$; $p > 0.5$). This result did not change when MAU was included in the analysis ($F_{ST} = 0.00086$ and $\Phi_{ST} = -0.00259$; $p > 0.5$). There was no correlation between multilocus Cavalli–Sforza and Edwards genetic distances and geographic location, and the null hypothesis of independence between the two variables was not rejected ($a = 0.0194$, $b = 1.8 \times 10^{-4}$; $p > 0.5$). Similarly, no evidence of isolation by distance was apparent in the mtDNA sequence data ($a = 0.920$, $b = -1.6 \times 10^{-3}$; $p > 0.5$).

The haplotype network of the mtDNA CR fragment had a star-shaped conformation with two common, central haplotypes differing by only one mutation step and found in 53% and 23% of the fish, respectively. Multiple, less frequent haplotypes (<6%, or <10 fish) differed from one of the central haplotypes by a single mutation step (except haplotype 9, two mutation steps from the closest central haplotype; Figure 4). Overall, haplotypes differed by 1–4 mutation steps. In terms of haplotype distribution, no discernible geographic pattern was evident from the network. The two central haplotypes were found at all sampling locations and made up the majority of the fish at each site (from 72% to 86%). The less-frequent, more-derived haplotypes were found in only

1–2 fish per site, and were shared among locations throughout the sampling area, i.e. were not restricted to any particular geographic region (Figure 4).

Demographic analyses

All samples were included in one group, which was used in all demographic analyses described below. The occurrence of past population size reductions was rejected by the two tests applied to the nuclear microsatellite data. The Wilcoxon test did not detect excess heterozygosity ($p > 0.05$, irrespective of mutation model), and the M-ratio test indicated a mean observed M of 0.93 (variance 0.26), which was larger than the critical value of M under conservative parameters ($M_c \approx 0.675$). Alternatively, the results from the mtDNA-based population expansion analyses were rather ambiguous. Evidence of past population growth was suggested by negative Tajima's D (–1.40, $p > 0.05$) and Fu's F_S statistics (–14.60, $p < 0.01$), although only the latter, most-sensitive test (Ramos-Onsins and Rozas, 2002) was significant. In contrast, the frequency distribution of pairwise differences among sequences did not conform to a model of sudden population expansion (Harpending's raggedness = 0.11, $p < 0.01$).

Discussion

We found no evidence of genetic population structure among collections of Portuguese dogfish from the eastern Atlantic and the Mid-Atlantic Ridge north of the Azores. The genetic diversity found at the nuclear microsatellite loci and at the mtDNA CR was homogeneously distributed among sampling locations, resulting in low levels of genetic differentiation. Also, there was no indication of increased genetic divergence with geographic distance in our dataset. The observed pattern of genetic diversity is consistent with the absence of genetic population structure in the Portuguese dogfish in the above area. Moreover, our demographic analyses showed no indication of past reduction in population size, and the signal of population growth was ambiguous. These results suggest that the population of Portuguese dogfish in the eastern Atlantic has historically been relatively stable in size.

Our ability to detect the signal of genetic divergence over the noise may have been compromised by random sampling errors associated with marker variability or sample size, or by non-random sampling errors attributable to an incomplete representation of the entire population (Waples, 1998). Indeed, the low levels of genetic diversity found in the mtDNA control region of *C. coelolepis*, although not unexpected in elasmobranch taxa (Martin and Palumbi, 1993; Martin, 1999), may have reduced the power to detect genetic differentiation among locations. Similarly, the genetic diversity at the nuclear microsatellite loci resulted in limited power to detect genetic differentiation levels as low as those calculated with our dataset. On the other hand, there was the potential for an upward bias in the F_{ST} values resulting from small sample sizes (e.g. MAD) and unequal composition of sample collections, e.g. dominated by juveniles or maturing/adult fish (Waples, 1998). Nevertheless, all pairwise F_{ST} values were consistently low (in fact, most were <0.0005; Table 12), suggesting little genetic differentiation among locations. These results were in agreement with the FCA and AMOVA. All analyses are consistent in suggesting a lack of geographic partitioning of the genetic variability surveyed at the mtDNA CR and at the nuclear microsatellite loci in *C. coelolepis*.

The genetic homogeneity found among Portuguese dogfish collections separated by thousands of kilometres implies gene flow among the sampled locations either currently or in the recent past. Because long-distance dispersal and gene flow in elasmobranchs strictly depend on active swimming of the individuals (Musick *et al.*, 2004), a considerable number of migrant fish per generation must be exchanged among locations to show low levels of genetic differentiation (Waples, 1998), as found for the Portuguese dogfish. This observation strongly suggests that *C. coelolepis* has high dispersal potential, and there appear to be no major barriers to dispersal within the eastern Atlantic. However, the generation times of squaloid sharks are often of the order of decades (Kyne and Simpfendorfer, 2010), and in the case of the Portuguese dogfish, it may be close to 20 years, as estimated for its similar-sized relative *Centroselachus crepidater*

(Irvine *et al.*, 2006). Therefore, long-distance dispersal in *C. coelolepis* may take place over many years. Also, because vagility and body length appear to be intimately related in elasmobranchs (Musick *et al.*, 2004), it may be reasonable to expect that other deep-water squaloids of similar or larger size than *C. coelolepis* may be equally capable of long-distance movements within the span of a generation.

Life-history strategy is also an important factor shaping the overall pattern of population structure in elasmobranchs. Some important insights into the life strategy of *C. coelolepis* have been provided by earlier studies, but of particular relevance to our work is the maturity stage composition of the Portuguese dogfish collected at different locations (Table 13). In general, all stages of the reproductive cycle were found in any given area, although with different relative frequencies. Mature females predominated over immature females in catches from all areas surveyed, although the latter had slightly higher frequencies off Portugal and off the British Isles. Females in the first stages of the reproductive cycle, i.e. prior to ovulation (resting/maturing, or with ripe oocytes), constituted the vast majority of the mature females caught on the Mid-Atlantic Ridge and off Madeira, but roughly half of those caught off western Europe. Pregnant females accounted for 34–51% of the mature females off Portugal, but were less frequent along the Mid-Atlantic Ridge and west of the British Isles (<26%). Spent females (post-partum) were reported only off Portugal and west of the British Isles. Interestingly, mature males dominated over immature/maturing males in areas where most of the females were in early reproductive stages (i.e. Mid-Atlantic Ridge, Madeira), the opposite being true where pregnant females were more common (e.g. off Portugal). In terms of the distribution of small, juvenile Portuguese dogfish (mostly <70 cm), several studies have reported their absence in catches from deep demersal longlines and trawls operating off the British Isles and Portugal (Girard and DuBuit, 1999; Clarke *et al.*, 2001; Veríssimo *et al.*, 2003). In contrast, 88% of the *C. coelolepis* sampled off the

Mauritanian continental slope with demersal trawls were juveniles (L. Fernandéz, pers. comm.), suggesting that the area may be a nursery for *C. coelolepis*.

The above data suggest that the Portuguese dogfish may be spatially segregated according to size and maturity stage, and that mature females may undergo large-scale migration within the eastern North Atlantic associated with the reproductive cycle. For instance, mating may take place along the Mid-Atlantic Ridge and west of the British Isles, with pregnant females potentially moving along the continental slope off western Europe south to Mauritania to give birth. After parturition, spent females may move back to the feeding areas and undergo a resting period before the subsequent breeding cycle. The absence of small juveniles in catches from the western European continental slope and northern Mid-Atlantic Ridge (where adults dominate) may indicate recruitment to these areas only when individuals are nearing sexual maturation (ca. 80 cm). If that is true, the above life-history strategy of the Portuguese dogfish would be consistent with the absence of population structure within the eastern North Atlantic. Nevertheless, the low levels of genetic divergence between the SA collection and those to the north may reflect connectivity between the two areas of the eastern Atlantic.

The pattern of spatial segregation described above for *C. coelolepis* needs further confirmation because the catch composition reported by the different studies was not obtained in a standardized fashion. Nevertheless, the bias associated with inter-study comparisons was reduced by considering only data from deep-water longlines (except off western Ireland; see Table 13), which are more efficient at catching deep-water elasmobranchs and are also less size-selective for *C. coelolepis* than demersal trawls (Clarke *et al.*, 2005). Moreover, only those studies where fishing occurred throughout the whole bathymetric range of the species were compared (Table 13), to avoid bias associated with the bathymetric segregation by size, sex, and maturity stage reported for *C. coelolepis* (Girard and DuBuit, 1999; Clarke *et al.*, 2001). Future

studies should aim at providing a more detailed view of the life strategy of the species, by extending the area surveyed and by standardizing catch compositions.

Spatial segregation according to size, sex, and maturity stage has been previously reported for other elasmobranch taxa. The existence of spatially discrete nursery areas, with juveniles away from adult habitats, has been found for most of the carcharhinid and sphyrnid sharks studied to date (Grubbs, 2010). Also, spatial segregation by sex and maturity stage has been described for the blue shark (*Prionace glauca*; Nakano and Seki, 2003) and to some extent for the shortfin mako *Isurus oxyrinchus* (Stevens 2008; Mucientes et al. 2009). The complex population structures and migration patterns in these epipelagic oceanic sharks have been associated with the challenges of finding food, mates, and nursery areas (i.e. of increased productivity) in the vast and rather unproductive epipelagic marine environment (Stevens, 2010). This particular life strategy may help in maximizing resource partitioning, so reducing intraspecific competition in taxa found in habitats with limited food availability. Similarly, deep-water squaloids may benefit from adopting such a strategy in the large and food-limited deep-sea environment.

Independent evidence of spatial segregation with maturity stage in other deep-water squaloids has been suggested by several authors. For instance, few gestating females of the great lanternshark (*Etmopterus princeps*) were reported off Iceland (Jakobsdóttir, 2001), and pregnant and ripe females were dominant along the Mid-Atlantic Ridge (Cotton, 2010). Pregnant female leafscale gulper sharks (*Centrophorus squamosus*) were also rarely caught along the western European continental slope (Girard and DuBuit, 1999; Clarke *et al.*, 2001; Figueiredo *et al.*, 2008), but made up 34% of the females sampled off Madeira (Severino *et al.*, 2009). In contrast, all *Centrophorus* cf. *niaukang* caught in the western North Atlantic were adult females, and most were pregnant (Cotton, 2010). Clearly, more data are needed to confirm these trends, but the circumstantial evidence above strongly suggests that intraspecific spatial segregation in deep-water squaloid shark populations by sex and maturity stage may be found in taxa other than

Portuguese dogfish. Consequently, the spatial distribution of a given population unit may include a considerably large geographic area.

In light of our results, future fishery management plans for the Portuguese dogfish need to consider the genetic stock unit to include the whole eastern Atlantic and the northern section of the Mid-Atlantic Ridge. However, it should be kept in mind that the rates of migration between neighbouring areas may not be sufficient to ensure short-term recovery in abundance in the event of localized depletion, even though they may be enough to homogenize the gene pool between those areas (Carvalho and Hauser, 1994). Also, as a result of the apparent spatial segregation by size and maturity stage of *C. coelolepis,* fishing operations conducted in different regions of the eastern Atlantic are likely to target different components of the population, e.g. gestating females or small juveniles. This practice can have repercussions not only locally, but over the entire population, so the distribution of fishing effort needs to be managed accordingly.

To summarize, we found no evidence of genetic differentiation among sample collections of the Portuguese dogfish (*C. coelolepis*), including an area extending from west of the British Isles to South Africa, including the northern section of the Mid-Atlantic Ridge, a result consistent between nuclear microsatellite and mtDNA CR datasets. The results therefore suggest that there is no genetic population structure of Portuguese dogfish in the area studied.

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TABLE 8. Genetic diversity indices for the Portuguese dogfish (*C. coelolepis*), integrated over all microsatellite loci and all mtDNA control region haplotypes from each sampling location.

Sampling location		Nuclear microsatellites					Mitochondrial CR sequences					F/M	TL (cm)
		n	H_o	F_{IS}	A	R_s	n	H	h	π	k		
Ireland	IRE	45	0.77	0.006	11.3 (6)	8.2	38	8 (2)	0.64	0.0019	0.926	14.0	97.4
Portugal	POR	41	0.77	–0.032	11.4 (7)	8.1	42	7	0.57	0.0014	0.677	47.0	108.6
Madeira	MAD	17	0.76	–0.017	8.0 (6)	7.9	15	6 (3)	0.74	0.0020	1.009	2.0	101.6
Azores	MAR	40	0.76	0.026	11.0 (1)	8.1	35	9 (3)	0.74	0.0022	1.106	2.3	103.7
Mauritania	MAU	46	0.78	–0.041	12.0 (12)	8.5	40	7	0.63	0.0017	0.826	1.4	62.3
South Africa	SA	22	0.77	–0.008	8.9 (3)	7.9	22	6	0.69	0.0019	0.922	0.2	95.0

n = number of samples; H_o = observed mean heterozygosity; F_{IS} = inbreeding coefficient; A = mean number of alleles (number of unique alleles); R_s = mean allelic richness; H = number of haplotypes (number of unique haplotypes); h = haplotype diversity; π = nucleotide diversity; k = mean number of nucleotide differences between haplotypes; F/M = females/males; TL = mean total length.

TABLE 9. Nuclear microsatellite loci of the Portuguese dogfish (*C. coelolepis*), with the respective repeat motif, overall expected heterozygosity (H_e), and total number of alleles scored per locus (A).

Marker name	Repeat motif	H_e	A
Ccoe25	[TG]7	0.61	3
Ccoe55	[CA]35	0.95	46
Ccoe61	[TG]24	0.87	26
Ccoe75	[TG]10	0.89	26
Ccoe7551	[AG]14	0.71	7
DF J451	$[AC]_{10}$	0.67	6
SacaGA11	$[TG]_9$ C [TG]	0.76	18
Saca3853	$[TC]_9$ GC $[TC]_2$	0.69	6
Overall		0.77	17.3

TABLE 10. Haplotype list for the Portuguese dogfish (*C. coelolepis*) showing the variable nucleotide positions (n =12) and respective polymorphisms in the 496-bp fragment of the mtDNA control region.

Haplotype	Variable nucleotide positions												GenBank Access no.
	32	120	204	269	275	300	308	313	328	474	480	484	
H1	T	A	A	G	T	G	C	A	G	C	T	G	HQ664432
H2	.	.	.	.	.	.	.	.	A	.	.	.	HG664433
H3	.	.	.	.	.	C	.	.	.	.	.	.	HG664434
H4	.	.	.	.	A	.	.	.	A	.	.	.	HG664435
H5	.	G	.	.	.	.	.	.	A	.	.	.	HG664436
H6	.	.	.	.	.	.	.	.	A	.	C	.	HG664437
H7	.	G	.	.	.	.	.	.	.	.	.	.	HG664438
H8	.	.	.	.	.	.	.	G	A	.	.	.	HG664439
H9	.	.	.	.	.	.	.	G	A	.	C	.	HG664440
H10	.	.	.	-	.	.	.	.	.	.	.	.	HG664441
H11	.	.	.	A	.	.	.	.	A	.	.	.	HG664442
H12	.	.	.	A	.	.	.	.	.	.	.	.	HG664443
H13	.	.	.	.	.	.	T	.	A	.	.	.	HG664444
H14	G	.	.	.	.	.	.	.	.	.	.	.	HG664445
H15	.	.	.	.	.	.	.	.	.	.	.	A	HG664446
H16	.	.	G	.	.	.	.	.	.	.	.	.	HG664447
H17	.	.	.	.	.	.	.	.	.	A	.	.	HG664448
H18	.	.	.	.	.	.	.	.	.	.	C	.	HG664449

TABLE 11. Mitochondrial DNA control region haplotype frequencies found per sampling location of the Portuguese dogfish (*C. coelolepis*).

Haplotype	IRE	POR	MAD	MAR	MAU	SA	Total
H1	22	26	7	15	21	11	102
H2	7	10	4	10	13	6	50
H3				1			1
H4				1			1
H5				1			1
H6	2	1		1		1	5
H7	3	2		3	2		10
H8				2	1	1	4
H9	1			1	1		3
H10	1						1
H11	1						1
H12	1	1	1				3
H13			1				1
H14			1				1
H15			1				1
H16					1	2	3
H17		1			1		2
H18		1				1	2
Total	38	42	15	35	40	22	192

TABLE 12. Levels of genetic divergence among populations of *C. coelolepis*, with pairwise microsatellite F_{ST} below the diagonal, and pairwise mitochondrial DNA control region Φ_{ST} above the diagonal.

		Φ_{ST}					
		IRE	POR	MAD	MAU	SA	MAR
F_{ST}	IRE		–0.017	–0.016	–0.005	–0.009	0.012
	POR	–0.001		–0.012	–0.002	0.000	0.033
	MAD	0.000	0.002		–0.016	–0.017	0.007
	MAU	0.000	0.000	0.010		–0.022	0.003
	SA	0.000	–0.001	0.000	–0.001		0.000
	MAR	0.000	0.000	–0.001	–0.010	–0.004	

No *P*-value was significant after Bonferroni correction.

TABLE 13. Maturity stage composition (% of total sample) of male and female Portuguese dogfish (*C. coelolepis*).

Sampling location	Males			Females						Depth* (m)	Reference
	TL (cm)	IM/Mat	Adult	TL (cm)	IM	Mat/Rest	Ripe	Preg	Spent		
Off British Isles	70–116	36	64	90–121	28	55[1]		14	31	500–2 000	[2]Clarke *et al.* (2001), *n* = 644
Off Portugal	68–100	85	15	82–122	26	33	25	34	7	800–1 600	Figueiredo *et al.* (2008), *n* = 2 177
	68–100	85	15	76–120	15	25	18	51	6	700–1 500	Veríssimo *et al.* (2003), *n* = 956
Off Madeira (Seine Seamount)	85–99	11	99	92–115	10	68	16	16		180–2 100	G. Menezes, pers. comm., *n* = 33
Off MAR (north of the Azores)	80–100	21	79	80–120		20	77	3		840–3 400	C. Cotton, pers. comm., *n* = 63
	85–98		100	85–119	9	70	5	25		740–2 000	G. Menezes, pers. comm., *n* = 39

TL = total length range; IM = immature; Mat = maturing; Rest = resting; Preg = pregnant.
*Depth of survey
1 Includes mature, resting, and ripe females.
2 Only 57% of individuals were from longlines, and the remainder were from deep-water trawls (M. Clarke, pers. comm.).

Figure 3. Factorial correspondence analysis of multilocus microsatellite genotypes of the Portuguese dogfish (*C. coelolepis*), according to sampling collection (see Table 1 for abbreviations). Factorial component 1 (FC1) is the x-axis (30.4% inertia) and factorial component 2 (FC2) is the y-axis (20.7% inertia).

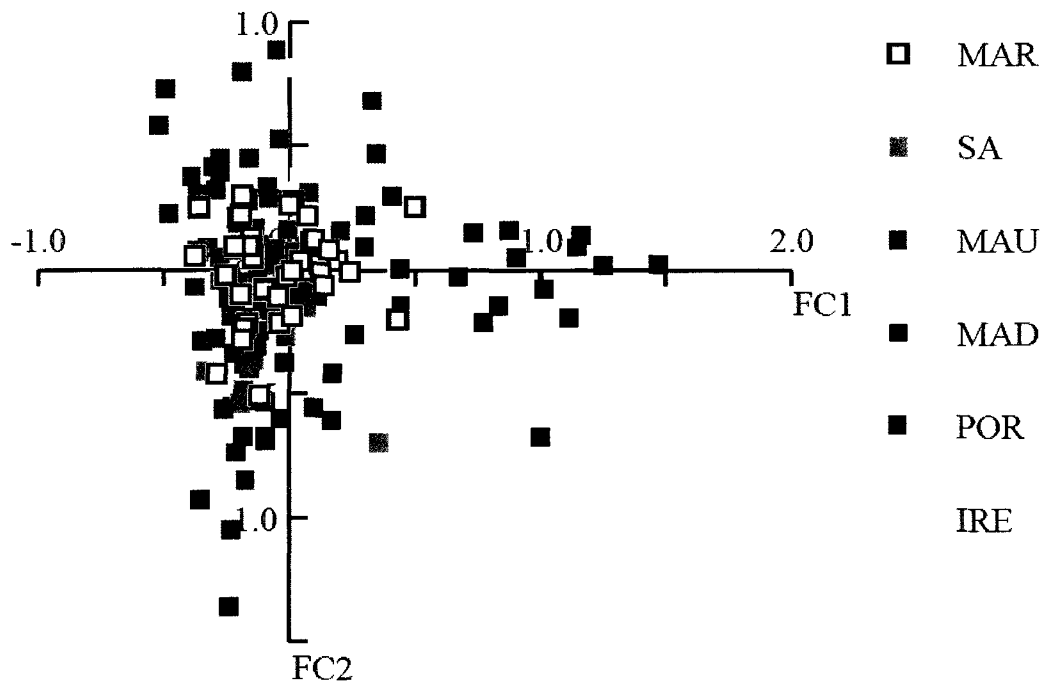
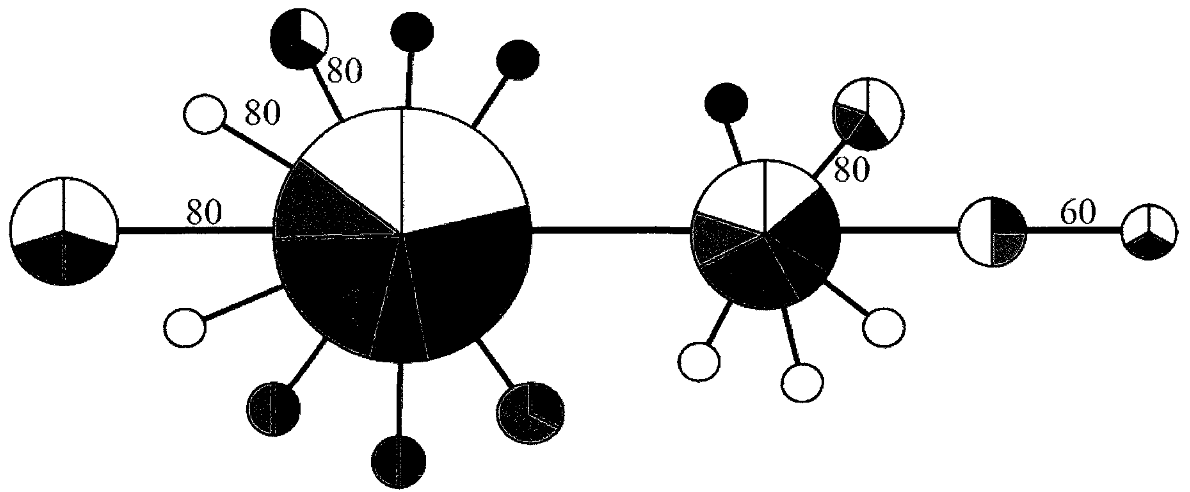

1.0
-1.0
1.0
2.0
FC1
1.0
FC2
MAR
SA
MAU
MAD
POR
IRE

Figure 4. Maximum parsimony haplotype network of the mtDNA CR of the Portuguese dogfish (*C. coelolepis*). Haplotypes (n = 18) are represented by circles with sizes proportional to absolute frequency in the total sample. All connections correspond to one nucleotide substitution between haplotypes. Support values of connections were 100% except where noted. Colour code: yellow – Ireland; dark pink – Portugal; white – Azores; purple – Madeira; green – Mauritania; orange – South Africa.


80
80
80
80
60

Chapter 4
Population structure and genetic connectivity in a wide-ranging deepwater squaloid shark, the leafscale gulper *Centrophorus squamosus*

Abstract:

Centrophorus squamosus is a deepwater squaloid shark occurring in the eastern Atlantic, and west Indian and Pacific oceans. Despite its wide geographic distribution, little is known about the pattern of population structure and genetic connectivity among regions within the species' range. To investigate this issue, samples were collected off the eastern North Atlantic, South Africa and New Zealand, and each individual was screened at six nuclear microsatellite loci and at the mtDNA ND2 gene region. We found high genetic homogeneity among eastern North Atlantic and South Africa collections, consistent with the presence of a single genetic stock and in agreement with previous studies suggesting extensive migrations associated with the reproductive cycle. In contrast, genetic divergence between New Zealand and the remaining collections was detected at the mtDNA ND2 gene region but not at the nuclear loci. Long-term divergence and sex-biased dispersal between New Zealand and eastern Atlantic/South Africa group have likely contributed to the discrepancy between marker types.

Introduction

The deep-sea, i.e. the water column extending to depths below 200 m, comprises by far the largest environment on the planet. Initially thought to be devoid of life, the accumulated evidence indicates that the deep-sea harbors an incredible diversity of life forms (e.g. Rex and Etter 2010). High species diversity is found among deepwater fish taxa (e.g. Koslow et al. 1997; Merrett 1994), which occupy a variety of habitats extending from the continental shelf break to the abyssal plains and deep trenches. A diverse chondrichthyan fauna is also found in deep water (approximately 46% of the total; Kyne and Simpfendorfer 2010). In fact, some groups of cartilaginous fishes occur almost exclusively below 200 m, such as most squaloid sharks (order Squaliformes) and the chimaeras (order Chimaeriformes).

All deepwater taxa are dependent on the input of organic matter produced in the upper layer of the world's oceans, except for the localized communities associated with hydrothermal vents and cold seeps (Van Dover 2000; Levin 2005). Not surprisingly, the patterns of deepwater biodiversity at both local and regional levels have been linked to the levels and annual regimes of primary productivity of surface waters (e.g. Corliss et al. 2009; Fock 2008; Rex and Etter 2010). For instance, diversity of local deepwater pelagic fish communities follows a "hump-shaped" curve with high diversity found in areas of intermediate levels of surface primary productivity, and lower diversity in areas of lowest and highest production (Fock 2008). At the regional level, taxa with life-history strategies best suited to take advantage of large seasonal food pulses exhibit distributions distinct from taxa adapted to exploit a more uniform food supply throughout the year (Fock 2008). Thus, it seems reasonable to expect that food supply may influence the distribution of individuals and the structure of populations of deepwater species.

During the last decade, molecular genetic studies have made considerable progress elucidating the patterns of population structure and genetic connectivity of deepwater teleosts.

Overall, these studies indicate that genetic homogeneity over large geographic regions is the norm, and that genetic population structure is intimately related to life-history strategy. For instance, high fecundities and extended larval stages allow for long-distance dispersal along major ocean currents, and appear to be determinant in shaping the population structure of the Greenland halibut *Reinhardtius hippoglossoides* and the roundnose grenadier *Coryphaenoides rupestris* in the North Atlantic (Knutsen et al. 2007; White et al. 2010b). In taxa where larval stage duration is shortened, gene flow across large geographic regions appears to be maintained by actively swimming adults, as in the orange roughy *Hoplostethus atlanticus* (White et al. 2009). Although spatial segregation of individuals according to size/maturity stage and sex has been observed, extensive mixing on the spawning grounds has been suggested to account for large-scale panmixia in the blue hake *Antimora rostrata* and in the wreckfish *Polyprion americanus* (Ball et al. 2000; White et al. 2010a). Conversely, strong intraspecific genetic divergence associated with depth-specific recruitment of larvae and juveniles to their parental depth-segregated populations has been described for species of *Sebastes* (e.g. *S. mentella* Cadrin et al. 2010; *S. miniatus* Hyde et al. 2008), and appears to be linked to extensive speciation within the genus (Ingram 2010).

Comparatively fewer studies have focused on the patterns of genetic population structure and connectivity in deepwater elasmobranchs and holocephalans (e.g. Straube et al. 2010; Veríssimo et al. 2011). Deepwater chondrichthyans are opportunistic predators and occupy high trophic positions in their communities (Cortés 1999; Ebert and Bizarro 2007), making them particularly sensitive to levels of food supply. In fact, the bathymetric distribution of deepwater sharks has been suggested to be limited to the upper 3000 m of the water column as the relatively high energy demand of these taxa cannot be sustained in the extreme oligotrophic environment of the abyss (Priede et al. 2006). Also, and contrary to most teleosts, all chondrichthyan fishes have comparatively lower fecundities and no larval stage, with dispersal being dependent on actively

swimming adults (Musick et al. 2004). Consequently, the food requirements, the life-history and the dispersal strategies of deepwater chondrichthyans are considerably different from their teleost counterparts, and may exert a distinct influence in the distribution of individuals.

This study aims at increasing our understanding of the intraspecific patterns of population structure in deepwater elasmobranchs by investigating the distribution of the genetic diversity among geographically distinct samples of the leafscale gulper shark *Centrophorus squamosus* (Bonnaterre 1788) within part of its range. The leafscale gulper is a medium sized (< 1.5 m) benthopelagic shark usually found in association with continental slope waters at bathyal depths between 300 and 1500 m (Clarke et al. 2001a, b). The species has a wide geographic distribution including the eastern Atlantic from Iceland to South Africa, the western Indian Ocean off the Seychelles, and the western Pacific from Japan to New Zealand (Compagno et al. 2005). Studies conducted on the continental slopes off western Europe suggest that *C. squamosus* is bathymetrically and spatially segregated according to size, sex and maturity stage (Bañón et al. 2006; Clarke et al. 2001a; Figueiredo et al. 2008; Girard and DuBuit 1999).

The leafscale gulper is a yolk-sac viviparous species but very little is known about the species' reproductive cycle. Pregnant females are mostly absent from the eastern North Atlantic, although a large fraction of the females off Madeira and the Canary Islands have developing embryos in their uteri (Pajuelo et al. 2010; Severino et al. 2009). A study of seasonal variation in the frequencies of different spermatogenic stages in *C. squamosus* indicates that males are reproductively active year round and that there is no defined mating season (Girard et al. 2000). Length at 50% maturity in the eastern North Atlantic ranges from 98 to 102 cm in males and from 125 to 128 cm in females (Bañón et al. 2006; Clarke et al. 2001a; Figueiredo et al. 2008; Girard and DuBuit 1999), and maximum sizes are 122 cm for males and 145 cm for females (Clarke et al. 2001b). However, only part of the male and female components of the population(s) were

present in the studied areas (indeed, juvenile fish are mostly absent; Clarke et al. 2001a; Figueiredo et al. 2008), so these values may change as more data are added (Clarke et al. 2001b). Life expectancies up to 70 years for females and 52 yr for males have been estimated for *C. squamosus*, and sexual maturity is estimated to occur between 30 to 40 years in females and after 20 years in males (Clarke et al. 2002).

The study of the genetic population structure of *C. squamosus* is also relevant from a management and conservation perspective, as the species is an important by-catch of commercial deepwater fisheries off the eastern North Atlantic (ICES 2010). Evidence from previous shark and ray fisheries clearly show that elasmobranch taxa can only support very low levels of fishing mortality without undergoing depletion and stock collapse (Walker, 1998; Musick et al., 2000). In recent years, the stock abundance of *C. squamosus* and other deepwater squaloids (e.g. *Centroscymnus coelolepis*) off the eastern North Atlantic has been considered depleted, and a zero fishing mortality has been advised since 2006 (ICES 2010). Despite concerns about resource sustainability, there is limited information regarding the stock structure of the species in this region. It is therefore of utmost importance to have a good understanding of the species' population structure and of the level of connectivity among geographically adjacent regions, as well as throughout the species range.

Materials and Methods

Sample collection and DNA analysis

Tissue samples (fin clips or muscle) were collected from leafscale gulper sharks caught by commercial fishing vessels and scientific research cruises off west Ireland (IRE), mainland Portugal (POR), Azores (MAR), eastern South Africa (SA) and northwest New Zealand (NZ). The total length of sampled specimens ranged between 47 cm and 144 cm, but varied among

collection sites (Table 14). Mature males and immature females were sampled in IRE, POR and MAR, while mature males and females in all maturity stages were sampled in SA and NZ. All tissue samples were preserved in 20% DMSO buffer saturated with NaCl (Seutin et al. 1991) and stored at room temperature. Extraction of genomic DNA (gDNA) was done using the Qiagen DNeasy Tissue kit according to the manufacturer's instructions.

A fragment of the mtDNA NADH dehydrogenase subunit 2 (ND2) gene region was amplified via the polymerase chain reaction (PCR) in 25 μl reactions containing 10 – 20 ng gDNA, 1 μM of each primer (ND2_F 5'TTCCTCACACAAGCAACCGC 3'; ND2_R 5'GATGGTGGCTGGGATGGC 3'), 200 μM each dNTP, 5 μg of bovine serum albumin (BSA), 0.025 units *Taq* polymerase, 1X *Taq* buffer with 1.5 mM $MgCl_2$ (Qiagen, Valencia, CA), and autoclaved mili-Q water. PCR conditions consisted of an initial denaturation of 5 min at 94° C, followed by 45 cycles of 1 min at 94° C, 35 sec at 58° C, 1 min at 72° C, and a final extension step of 5 min at 72° C. The amplicons were cleaned with the QIAquick PCR Purification kit (Qiagen, Valencia, CA) according to the manufacturer's protocol, and the forward strands were sequenced using the ABI Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Warrington, UK). Reactions were run on an ABI Prism 3130*xl* genetic analyzer (Applied Biosystems, Warrington, UK) and the resulting DNA sequences were imported into Sequencher version 4.8 (Gene Codes Corp., Ann Harbor, MI) and checked for quality and accuracy in nucleotide base assignment. All sequences were aligned in MacVector version 8.0 (MacVector, Inc., California, USA) using the ClustalW multiple alignment algorithm (Thompson et al. 1994). Haplotypes were confirmed by sequencing the reverse strand of one individual for each unique haplotype.

Individual fish were genotyped at six nuclear microsatellite loci, of which four (*CsquMx31*, *CsquMx59*, *CsquMx104*, *CsquGT64*; GenBank accession no.s JQ037909- JQ037912,

respectively) were developed *de novo* according to the protocol of Glenn and Schable (2005) using the biotinylated probes $(GT)_{12}$ and Mix 4. The resulting genomic libraries were screened for inserts exhibiting di- and trinucleotide repeats over 7 units in length or tetranucleotide repeats over 5 units in length. The flanking regions of the selected sequences were used for primer design using MacVector 8.1.2 (MacVector, Inc.) or Primer3 (Rozen and Skaletsky 2000). Two additional loci, namely *SacaGA11* (Veríssimo et al. 2010) and locus *Saca7551* (GenBank Accession no. EG027551), were obtained following successful cross-amplification with primers originally designed for *Squalus acanthias*. All loci were initially screened for polymorphisms and subsequently tested for consistent amplification and conformation of genotypic distributions to the expectations of Hardy-Weinberg equilibrium (HWE). Microsatellite genotyping was conducted via PCR amplification in 5 µl reactions containing 5 to 15 ng of gDNA, 0.0375 µM of forward primer with an added T3 tail, 0.15 µM of reverse primer, 0.1 µM of fluorescently labeled T3 primer (e.g. PET, VIC or 6FAM), 0.025 units *Taq* DNA polymerase (Qiagen, Valencia, CA), 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 1µg BSA, 1× *Taq* buffer (Qiagen, Valencia, CA) and autoclaved mili-Q water. PCR conditions consisted of an initial denaturation of 3 min at 94° C, followed by 35-45 cycles of 1 min at 94° C, 35 sec – 1 min at the corresponding annealing temperature (see Table 14 for details) and 35 sec – 1 min at 72° C, and a final extension step for 7 min at 72° C. The products were run on an ABI Prism 3130*xl* (Applied Biosystems, Warrington, UK). Genotypes were scored manually with the software GeneMarker version 1.3 (Softgenetics, LLC, State College, PA) and the presence of null alleles and scoring errors was tested for each locus using Micro-Checker (van Oosterhout et al. 2004). Conformation of genotypic distributions to HWE expectations for each locus within each sample collection, and tests of linkage disequilibrium (LD) between each pair of loci within and among all collections were calculated in Genepop version 4.0 (Raymound and Rousset 1995; Rousset 2008).

Statistical data analysis

Several indices of genetic diversity were obtained for the nuclear microsatellite loci using FSTAT version 2.9.3.2 (Goudet 2002), including the total number of alleles (A), observed heterozygosity (H_O), inbreeding coefficient (F_{IS}) and allelic richness (R_S, an estimate of the number of alleles standardized for unequal sample sizes). Mitochondrial DNA sequence diversity indices were calculated in DnaSP version 5 (Librado and Rozas 2009) including number of polymorphic sites, number of haplotypes (H), haplotype diversity (h), nucleotide diversity (π), and average number of nucleotide differences between sequences (k). In addition, the between-sample component of diversity (Δ_{ST}, or the effective number of populations) and its reciprocal (Δ_S / Δ_T, the proportion of total diversity present in the average subpopulation), as well as a measure of the absolute genetic differentiation (D_{est}, corrected for small sample sizes) were estimated for each microsatellite locus using SMOGD (Crawford 2010), and for the mtDNA ND2 fragment according to Jost (2008). Estimates of the 95% confidence intervals (CI) for D_{est} were obtained for each microsatellite locus by resampling individuals in 1000 bootstrap replicates (Crawford 2010).

Levels of between-population genetic differentiation were estimated in Arlequin version 3.5.1.2 (Excoffier and Lischer 2010) using pairwise F_{ST} tests based on allele frequency differences at the nuclear microsatellite loci, and pairwise Φ_{ST} values based on a genetic distance matrix of pairwise differences for the mtDNA ND2 haplotypes. The corresponding P values were estimated from 10000 permutations of alleles/haplotypes between sample collections (Excoffier et al. 1992). Pairwise values of Jost's D (Jost 2008) were also estimated to allow for between-marker comparisons of the levels of genetic differentiation among populations. Estimates of D were obtained for the nuclear microsatellite loci using the harmonic mean of the distances across loci implemented in SMOGD (Crawford 2010), and for the mtDNA ND2 fragment using the method described in Jost (2008).

The spatial analysis of shared alleles and haplotypes (SAShA) was investigated using SAShA2 (Kelly et al. 2010). The mean observed distance between co-occurring alleles/haplotypes (OM) was estimated based on a pairwise geographic distance matrix between sampling locations, and on allele/haplotype frequency matrices. The OM was tested against the expected mean distance (EM) under the null model of random distribution of alleles/haplotypes (i.e. panmixia) using 1000 permutations of the dataset (Kelly et al. 2010). This analysis was conducted for the mtDNA ND2 haplotype dataset, for each microsatellite locus independently, and for the combined alleles at the six microsatellite loci. A jack-knife sampling procedure was conducted for each analysis to detect the alleles/haplotypes that significantly deviated from expectations under a random distribution (Kelly et al. 2010).

Hierarchical population structure scenarios were evaluated for each marker type through an analysis of molecular variance (AMOVA) as implemented in Arlequin. For microsatellite data, the AMOVA was based on allelic frequencies and performed for each locus independently as well as across loci. For mtDNA sequence data, the AMOVA was based on a genetic distance matrix of pairwise differences. In either case, significance was estimated by 10 000 permutations of the data (Excoffier et al. 1992).

The Structure software version 2.2 (Pritchard et al. 2000; Falush et al. 2003) was used to infer the population structure of the leafscale gulper using nuclear microsatellite data, and to detect the presence of individuals of admixed ancestry within each sample collection. The analysis was conducted for K values (i.e. number of populations) between 1 and 5, using an admixture model with independent frequencies and default parameters. Five replicates were obtained for each K value tested, with each replicate including by 10^5 steps plus $3x10^4$ steps of burn-in. Criteria for choosing the best K values followed those indicated in the software manual.

A haplotype network was constructed in Network 4.5.1.0 (fluxus-engineering.com), in which distinct nucleotide sequences are positioned according to their overall similarity, and their geographic occurrence can be superimposed to highlight spatial patterns in haplotype distribution. The network was constructed using the maximum parsimony approach of Polzin and Daneschmand (2003) and the median joining algorithm of Bandelt et al. (1999), with default parameters and a transition to transversion ratio of 4:1 (estimated from the data).

Past reductions of effective population size, i.e. 'bottleneck" events, were investigated with nuclear microsatellite data using the ratio (M) test of Garza and Williamson (2001) and the excess heterozygosity test implemented in Bottleneck version 1.2.02 (Piry et al. 1999). The M-ratio test is based on the faster reduction in allele numbers compared to allele size range after a reduction in population size. M-ratios were estimated for each locus and each population independently and compared to the critical value M_c calculated based on 7 loci, $\theta=10$ and the conservative parameters of 90% one-step mutations and a mean size of non-one step mutations of 3.5 (Garza and Williamson 2001). The excess heterozygosity test is based on the discrepancy between measured heterozygosity (based on allelic frequencies) and expected equilibrium heterozygosity (based on allele number) following a population bottleneck, and was tested using a Wilcoxon's test under the null hypothesis of no significant heterozygosity excess (Piry et al. 1999).

Demographic analyses based on mtDNA ND2 sequences were performed in Arlequin. Population size changes were inferred from the frequency of pairwise differences among haplotypes (i.e. mismatch distributions, MMD) and tested against a model of demographic and spatial expansion using 10 000 bootstrap replicates (Schneider and Excoffier 1999). Deviations from selective neutrality/demographic equilibrium were also tested with Fu's F_S (Fu 1997) using 10 000 simulations of the data ($\alpha=0.05$), assuming an infinite-site model without recombination. MDIV (Nielsen and Wakeley 2001) was used to infer the joint estimates of scaled divergence

time (T = divergence time/2*effective population size) and scaled migration rates (M = 2*effective population size* migration rate) between populations, based on mtDNA ND2 sequences. Runs were performed using the HKY model of sequence evolution (Hasegawa et al. 1985), and with lengths of $2x10^6$ steps with $5x10^5$ steps of burn-in. Convergence of estimates was evaluated by running multiple runs, using different random seeds, and using different prior intervals.

Historical migration rates M (i.e. migration rate m scaled by the mutation rate μ) among populations were estimated with MIGRATE-n v.3.2.7 (Beerli 1998; Beerli and Felsenstein 1999, 2001) using a maximum likelihood approach (ML). The microsatellite and mitochondrial datasets were run independently to compare the patterns produced by each marker type. Preliminary runs were performed with default start parameters to test for adequacy of run and burn-in length, and to estimate start parameters for subsequent runs. The final runs for microsatellite genotypes used 10 short chains for a total of $2x10^6$ visited genealogies (10^4 recorded steps), one long chain of $4x10^6$ visited genealogies (10^5 recorded steps), and a burn-in of 10^5 steps. The final runs for the mtDNA data used 10 short chains of $20x10^6$ visited genealogies (10^4 recorded steps) and one long chain of $20x10^6$ visited genealogies (10^5 recorded steps) with a burn-in of $5x10^4$ steps. The adaptive (4 chains) and static (5 chains) heating schemes were used to adequately explore the parameter space for the microsatellite and mitochondrial data sets, respectively. Consistency of parameter estimates was checked by using different start parameters, different run lengths and replicate runs.

Results

Genetic Diversity

A total of 228 leafscale gulper sharks was genotyped at six nuclear microsatellite loci, with the exception of three individuals that were missing data at one locus each. There were no significant deviations of genotypic distributions from HWE expectations and there was no evidence of LD among loci. No allele scoring errors or null alleles were detected for any of the loci. The total number of alleles per locus varied between 5 and 55 (average: 18.5) and the overall expected heterozygosity varied between 0.53 and 0.92 (average: 0.75). The genetic diversity at the microsatellite loci was similarly distributed among collections (Table 14 and 15). The proportion of total diversity present in the average subpopulation (i.e. Δ_S/Δ_T) ranged from 90% to 99%, resulting in an effective number of subpopulations close to 1 (Δ_{ST}: 1.01-1.11). Concordantly, the estimates of absolute differentiation per locus (D_{est}) resulted in negative values (which equate to zero differentiation) for all loci except *Csqu Mx31* (D_{est}=0.011), which showed the highest number of alleles. Also, little variation was found for the mean observed heterozygosity (0.73-0.77) and mean allelic richness (11.0–13.0) across loci and among samples.

A 602 bp fragment of the mtDNA ND2 gene region was amplified for 215 individuals of the leafscale gulper, exhibiting a total of 26 mutations (21 transitions and 5 transversions) at 24 polymorphic nucleotide positions, corresponding to 21 distinct haplotypes (GenBank accession no.s JQ035532-JQ035655). The vast majority of the nucleotide polymorphisms were silent mutations, with 8 non-synonymous substitutions being detected (haplotypes 5, 8, 11, 13, 16-19; Fig. 5). The overall nucleotide and haplotype diversities were 0.0018 and 0.574, respectively, and the absolute number of pairwise differences between haplotypes ranged from 1 to 9 (average: 1.1). The proportion of total diversity found at the mtDNA ND2 region that was present in the average subpopulation (Δ_S/Δ_T) was 77%, increasing to 99% when NZ was excluded from the analysis. Concordantly, the effective number of subpopulations and the estimate of absolute differentiation across collections was 1.3 and 0.29, respectively, decreasing to 1.0 and 0.01, respectively, when NZ was excluded.

The maximum parsimony haplotype network of the mtDNA ND2 region had a star-shaped conformation with one very common and ubiquitous central haplotype and several low-frequency, derived haplotypes (Fig. 5). Low haplotype and nucleotide diversities were found in each sample collection (Table 15) resulting from the predominance of a single haplotype per collection (Fig. 5): all eastern Atlantic collections and SA were dominated by the central haplotype (H2; 72% to 77% of the individuals), while NZ was dominated by a derived haplotype exclusive to this location (H17; 69% of the individuals). Low-frequency haplotypes were either restricted to New Zealand or to the eastern Atlantic/South Africa region, with only a few being found in both regions (n=3; Fig. 5).

Genetic divergence and population structure

The pairwise F_{ST} tests based on nuclear microsatellite genotypes indicated no significant genetic differentiation among collections (range: -0.0043 to 0.0012; P>0.05). A similar result was obtained from the pairwise Φ_{ST} tests among eastern Atlantic collections (i.e. IRE, POR, MAR) and SA (range: -0.0046 to 0.0094; P>0.05) (Table 16). In contrast, significant genetic differentiation was found at the mtDNA level for all comparisons including the NZ collection (range: 0.3231 to 0.0.3884, P<0.001; Table 16). The unbiased estimates of absolute genetic differentiation among markers types (D_{est}) were largely concordant with the above results for comparisons involving eastern Atlantic collections and SA, though the values were slightly higher at the mtDNA gene region (D_{est}: 0.000 to 0.010). For the comparisons including NZ, the values of D_{est} were very dissimilar between marker types: no differentiation was found at the nuclear microsatellite loci but high genetic differentiation was found at the mtDNA ND2 gene region (D_{est}: 0.732-0.734).

The analysis of the spatial distribution of shared alleles at the nuclear microsatellite loci showed that a null model of random distribution of alleles could not be rejected for any of the loci

(Table 17). The same pattern was observed on an allele-by-allele basis, regardless of locus, with the average difference of observed (OM) to expected mean distributions (EM) per allele being ±0.142% (data not shown). In contrast, the spatial analysis of the shared mtDNA ND2 haplotypes showed higher OM compared to expectations under a null hypothesis of random distribution of haplotypes ($P<0.001$; Table 17). This result was driven by the significantly higher OM estimated for the haplotype H2 (the high frequency, ubiquitous haplotype). The removal of H2 from the analysis leads to an OM significantly smaller than expected under the null model (OM: 1114 km; $P<0.001$).

When considering all collections together in one group, the AMOVA conducted on the nuclear microsatellite dataset did not reject the null hypothesis of panmixia. This result was consistently obtained when using all loci combined (FST=-0.002, $P=0.917$) as well as when considering each locus separately (FST: -0.0004 - 0.001, P: 0.33 - 0.86). Similarly, the Structure analysis showed that the log probability of the full microsatellite dataset was highest for K=1 (Table 18). Also, assignment percentages of each collection, and of each individual within each collection, to different clusters was roughly equivalent in all cases where K>2 (data not shown). These results suggest K=1 as the best estimate of the true K value, corresponding to one cluster including all collections.

The AMOVA performed on the ND2 mtDNA dataset found that a significant amount of variance was due to variation between samples when considering all samples as a single group, and rejected the null hypothesis of panmixia (FST=0.185, $P=0.000$). According to the pattern of genetic differentiation uncovered by the pairwise Φ_{ST} tests described above, the NZ collection was compared to a group including all other collections. The AMOVA results based on this hypothetical population structure indicated high genetic divergence between the two groups (FCT=0.366, $P=0.000$) but no significant genetic differentiation among collections within the

Atlantic/SA group (FSC=-0.001, P=0.445). The mean sampling depth between Atlantic/SA and NZ was also found to be significantly different (990 m vs. 733 m, respectively; P=0.000).

Demographic analyses

Demographic analyses were conducted assuming the two groups of collections defined by the AMOVA and supported by the pairwise Φ_{ST} tests and SAShA2, namely NZ and an Atlantic group including IRE, POR, MAR and SA. According to the MMD analyses, the model of past demographic growth could not be rejected for any of the population groups (τ_{At}=0.5, P=0.169; τ_{NZ}=0.7, P=0.714). Likewise, the model of past spatial expansion was also not rejected for either population unit, but the time since spatial expansion was much higher in the Atlantic compared to NZ (τ_{At}=7.0, P=0.549; τ_{NZ}=0.3, P=0.443). Evidence of past population growth was suggested by negative Fu's F_S (Atlantic: -14.64, P=0.000; NZ: -2.17, P=0.094) although the value was significant only for the Atlantic group.

No evidence of past bottleneck events was found by either the M-ratio tests or the excess heterozygosity tests for any of the population groups. Mean M-ratios across loci were higher than the critical M_C of 0.675 in the Atlantic (mean: 0.88, variance: 0.12) and in NZ (mean: 1.35, variance: 0.70). Also, the null hypothesis of no heterozygosity excess was not rejected for either group (P>0.05), regardless of the microsatellite mutation model tested.

Estimates of scaled divergence time and migration rates obtained with MDIV and based on mtDNA ND2 sequence data suggested long-term divergence ($T \rightarrow \infty$, i.e. the likelihood of T increased with increasing divergence time) with migration (M=0.9, 95% confidence interval (CI): 0.50 - 3.15) between the Atlantic and NZ. Regarding the historical migration rates based on the nuclear microsatellite genotypes, similar estimates were obtained for both directions between the Atlantic to NZ ($M_{At \rightarrow NZ}$: 2.03, 95%CI: 1.76 – 2.34; $M_{NZ \rightarrow At}$: 4.13, 95%CI: 3.65 – 4.70).

Estimation of historical migration rates using the full mtDNA dataset did not converge (i.e. estimates were not consistent between runs) even when several different length runs, start parameters and heating schemes were tested. According to the haplotype network, there appears to have been two temporally spaced migration events between the Atlantic and NZ: an older event suggested by the sharing of two highly derived, low frequency haplotypes (H1 and H4) between Atlantic and NZ collections, and a more recent event suggested by the sharing of a less derived, low frequency haplotype (H7). The existence of different migration events (with potentially different rate values) with different regions of higher likelihood within the parameter space, may have led to the lack of convergence and inconsistency in posterior parameter distributions between runs. Therefore, we ran MIGRATE-n on two mtDNA datasets, for which convergence and consistency were achieved: dataset 1 (older migration event) excluded H7, and dataset 2 (recent migration event) excluded H1 and H4. Dataset 1 recovered similar migration rate estimates for both directions ($M_{At \rightarrow NZ}$: 97.1, 95% CI: 52.7 – 160.7; $M_{NZ \rightarrow At}$: 115.5, 95%CI: 62.8 – 198.8), while dataset 2 indicated little to no migration from NZ to the Atlantic and a much higher value in the opposite direction ($M_{At \rightarrow NZ}$: 58.0, 95% CI: 30.0 – 99.6; $M_{NZ \rightarrow At}$: 0.00, 95%CI: 0.00 – 11.8).

Discussion

Our study investigated the large-scale pattern of genetic population structure of the deepwater squaloid shark *C. squamosus*, using nuclear microsatellite loci and mtDNA nucleotide sequences. The results indicated that IRE, POR, MAR and SA shared 99% of their genetic diversity and had correspondingly low and non-significant genetic differentiation, regardless of marker type. Such high genetic homogeneity is consistent with the presence of a well-mixed gene pool within the eastern Atlantic and South African waters.

Two possible explanations can be proposed to account for the above described genetic homogeneity in *C. squamosus* across such a large geographic area. On the one hand, some of the samples may have been collected from areas where individuals from different breeding populations overlap (e.g. feeding areas), and may thereby comprise a mixture of individuals with distinct genetic compositions. On the other hand, contemporary gene flow may be occurring among the sampled locations, resulting in effectively homogenous genetic compositions within the eastern Atlantic and South Africa. Although we cannot exclude the possibility that fish from different breeding populations do not overlap in some region(s) within the species range, the STRUCTURE analysis suggested that all individuals sampled in this study are fairly admixed in their genetic composition (i.e. no individual is predominantly assigned to one population or collection). In turn, these results support the hypothesis of high gene flow occurring among distant locations within the eastern North Atlantic and SA.

Previous studies have suggested that the leafscale gulper may indeed undergo large-distance movements given the spatial segregation of individuals according to size and maturity stage, as reported off the western European continental slope. For instance, the absence of *C. squamosus* juveniles of either sex has been consistently noticed in studies conducted within the eastern North Atlantic (Bañon et al. 2006; Clarke et al. 2001; Figueiredo et al. 2008; Hareide et al. 2006; Severino et al. 2009). Moreover, females were generally less abundant than males and were mostly immature, while mature males were largely predominant at each sampled location (Bañon et al. 2006; Clarke et al. 2001; Figueiredo et al. 2008; Hareide et al. 2006; Severino et al. 2009). Pregnant females, in particular, were absent to the west of the British Isles and were very scarce (<1% of the females) off the Iberian Peninsula and around the Azores (Bañon et al. 2006; Clarke et al. 2001; Figueiredo et al. 2008; Hareide et al. 2006). The patterns described above were observed throughout the year, without any seasonal variation. In contrast, pregnant females comprised 34% and 10% of the females sampled off Madeira and off the Canary Islands,

respectively (Pajuelo et al. 2010; Severino et al. 2009). The discrepancies in the composition of individual *C. squamosus* among locations in the eastern North Atlantic may reflect the occurrence of extensive migrations associated with movements from potential nursery grounds to feeding/mating areas with the onset of sexual maturation, and with the reproductive cycle (Bañon et al. 2006; Clarke et al. 2001). If this is indeed the case, the high genetic homogeneity within the eastern North Atlantic southward to southern Africa would be consistent with a single population of *C. squamosus* occupying a large geographic area.

From an evolutionary viewpoint, the spatial segregation of individuals and the complex migration patterns suggested for *C. squamosus* and supported by our data, would be consistent with a life-strategy aiming at maximizing resource partitioning in high trophic level species occupying habitats of low food availability. For instance, spatial segregation with regard to sex and maturity stage has been reported for some epipelagic sharks, such as the blue shark *Prionace glau*ca (Nakano and Seki 2003; Nakano and Stevens 2008) and the shortfin mako *Isurus oxyrinchus* (Mucientes et al. 2009). The observed spatial segregation and movement dynamics of these taxa may increase the chances of finding prey, mating partners and nursery areas, in the large and rather unproductive epipelagic marine environment (Stevens 2010). Accordingly, some deepwater squaloids could potentially benefit from adopting a similar life strategy in the vast and food-deprived deep-sea. Indeed, spatial segregation according to size and maturity stage has also been observed in other deepwater squaloids like the great lantern shark *Etmopterus princeps* (Cotton 2010; Jakobsdóttir 2001), the Taiwan gulper *Centrophorus* cf. *niaukang* (Cotton 2010; Moore et al. 2003), and the Portuguese dogfish *Centroscymnus coelolepis* (Clarke et al. 2001b; Girard and DuBuit 1999; Veríssimo et al. 2003).

In contrast to the high genetic homogeneity found with both marker types among eastern Atlantic and South Africa collections, comparisons between NZ and the remaining collections

indicated strong genetic divergence at the mtDNA ND2 region but not at nuclear microsatellite loci. Because of the different mode of inheritance of the mitochondrial (maternally inherited) and the nuclear genomes (bi-parentally inherited), genetic differentiation between populations is expected to be higher at the mtDNA locus in species where females are phylopatric or have limited dispersal compared to males (Prugnolle and de Meeus 2002). In *C. squamosus*, the strong genetic divergence at the mitochondrial locus between the Atlantic and NZ and the non-random geographical distribution of ND2 haplotypes suggests that female dispersal across the Indian Ocean may be limited or absent, while high genetic homogeneity at the nuclear microsatellite loci and random geographical distribution of alleles suggests unrestricted, male-mediated gene flow among sample locations. A similar pattern was also reported for several other shark species, such as the great white *Carcharodon carcharias* (Pardini et al. 2001), the shortfin mako *Isurus oxyrinchus* (Schrey and Heist 2003) and the sandbar shark *Carcharhinus limbatus* (Portnoy et al. 2010), where male-biased gene flow predominated over large geographic distances and females showed stronger genetic structure over similar geographic ranges.

Strong genetic differentiation at mitochondrial but not at nuclear genes may also result from recent isolation between two populations. The discrepancy in the genetic signal between markers types are due to the smaller effective size and shorter coalescence time of mtDNA compared to nuclear DNA (Zink and Barrowclough 2008). We evaluated this hypothetical scenario in *C. squamosus* using mtDNA ND2 sequences, but our results have instead suggested long term divergence between the Atlantic group and NZ in the presence of migration. Moreover, the migration rate estimates from our MIGRATE-n analyses using nuclear and mitochondrial data support the occurrence of historical gene flow between the Atlantic and NZ (in both directions) for males and females equally, although female-mediated gene flow from NZ to Atlantic waters appears to have recently become restricted. Assuming a mutation rate in the order of 10^{-10} per year for the mtDNA genome (Martin et al. 1992) and a generation time of 30 yr for *C.*

squamosus, the migration rates would be in the order of 10^{-6} females per generation. On the other hand, and assuming a mutation rate of $10^{-2} - 10^{-5}$ per locus per generation for microsatellite loci (Ellegren 2000), the migration rate accounting for both males and females would be higher and in the order of $10^{-2} - 10^{-5}$ individuals per generation. The existence of low levels of female-mediated gene flow between the long-diverged Atlantic and NZ populations may be insufficient to counteract divergence at the mtDNA locus, but low to moderate migration rates of males may effectively homogenize genetic diversity at the nuclear loci. The above observations suggest that long-term divergence between Atlantic and New Zealand coupled with potential sex-biased dispersal in *C. squamosus* have likely contributed to the discrepancy in the levels of genetic divergence between nuclear and mitochondrial markers. On another account, the above results reinforce earlier suggestions that *C. squamosus* may be capable of large-scale movements.

It is also interesting to note that the marked difference in the frequency of mtDNA ND2 haplotypes between Atlantic/SA and NZ collections of C. squamosus coincides with different sampling depths, with Atlantic/SA specimens being captured at deeper depths compared to those off NZ. The mtDNA ND2 gene codes for a subunit of the NADH dehydrogenase enzyme and is therefore under selection. The NADH dehydrogenase enzyme is responsible for the transfer of electrons from NADH to the respiratory chain, and is the "entry" enzyme to the electron transport chain. Changes in the nucleotide composition of the ND2 gene may affect the structure of this particular subunit as well as the function of the enzyme as a whole. Although the observed differences in haplotype frequency and sampling depth between the two populations are not necessarily directly related, it can be hypothesized that the above differences in ND2 haplotypic frequencies may be due to local adaptation to different bathymetric ranges. Future studies looking at the genetic population structure of C. squamosus using the mtDNA ND2 and including samples from other regions within the species' range should explore this hypothesis further by testing the

consistency among haplotype composition and frequency and bathymetric ranges among populations.

Regarding the past demographic history of *C. squamosus*, we found no evidence of past population size reduction (via bottleneck) for either population group, although past population growth was detected for the Atlantic group, consistent with spatial range expansion. This result is congruent with the star-shaped haplotype network of the eastern Atlantic and SA collections which is characteristic of populations that have experienced recent sudden population expansion (Slatkin and Hudson 1991).

From a fisheries management and conservation standpoint, our study shows genetic homogeneity in leafscale gulper collections separated by thousands of miles. As such, in the event of localized depletion (e.g. off Ireland), the loss of unique genetic diversity is potentially minimal as other regions (e.g. South Africa) will have genetic compositions similar to the one of the depleted region. Moreover, such high genetic homogeneity in *C. squamosus* also suggests high genetic connectivity among distant locations. However, genetic connectivity does not necessarily equate to demographic connectivity, and more data are needed to determine the actual relationship between the two conditions in the leafscale gulper. Future studies should therefore aim at providing detailed information about the size and sexual maturity composition of *C. squamosus* throughout the whole eastern Atlantic, and at clarifying the hypothesis of large-scale migration associated with the reproductive cycle within the eastern Atlantic.

In summary, our results indicate high genetic homogeneity within the eastern Atlantic and southern Africa at nuclear and mitochondrial markers of *C. squamosus*, suggesting the presence of a single genetic stock in this region (i.e. Atlantic group). The presence of a single genetic stock occupying such a large geographic area is congruent with the observed spatial segregation reported for the species and the potential for extensive migrations associated with sexual maturity

and reproduction. We also found evidence suggesting that the Atlantic and NZ collections have been diverging for a considerable amount of time, although migration has been occurring between the two regions.

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TABLE 14 - Nuclear microsatellite loci of the leafscale gulper *C. squamosus*, with the respective repeat motif, total number of alleles scored per locus (A), overall expected heterozygosity (H_e), effective number of populations (Δ_{ST}), proportion of total diversity contained in the average subpopulation (Δ_S/Δ_T), unbiased estimator of genetic differentiation (D_{est}) across subpopulations and respective 95% confidence intervals (D_{est} 95% CI).

Marker name	Repeat motif	A	H_e	Δ_{ST}	Δ_S/Δ_T	D_{est}	D_{est} 95% CI
CsquGT64	$[GA]_{15}$	22	0.92	1.09	0.916	-0.006	0.047-0.170
CsquMx59	$[TGTA]_5$	5	0.53	1.01	0.993	-0.004	-0.006-0.030
CsquMx31	$[AC]_{31}$	55	0.92	1.11	0.899	0.011	0.072-0.189
CsquMx104	$[AC]_{16}$	18	0.88	1.05	0.949	-0.013	0.017-0.189
SacaGA11	$[TG]_9$	9	0.62	1.01	0.990	-0.005	-0.007-0.037
Saca7555	$[AG]_{14}$	5	0.61	1.01	0.993	-0.009	-0.006-0.032

TABLE 15 - Genetic diversity indices for the leafscale gulper *C. squamosus* for all microsatellite loci combined and mtDNA ND2 gene region. N: number of samples; Ho: observed mean heterozygosity; F_{IS}: inbreeding coefficient; A: mean number of alleles; R_S: mean allelic richness; H: number of haplotypes (unique haplotypes); *h*: haplotype diversity; π: nucleotide diversity; *k*: mean number of nucleotide differences between haplotypes; Sex-ratio (percent females sampled, %F); TL range: minimum–maximum (mean total length).

		Nuclear microsatellites					Mitochondrial ND2 sequences					Sex-ratio	TL range (cm)
		N	Ho	F_{IS}	A	R_S	N	H	*h*	π	k	(%F)	(mean)
Ireland	IRE	48	0.74	0.014	11.5	11.0	44	9 (4)	0.47	0.0015	0.93	0.17	57 – 116 (103.5)
Portugal	POR	48	0.76	0.020	13.3 (4)	12.6	46	9	0.48	0.0014	0.81	0.38	93 – 140 (108.6)
South Africa	SA	47	0.77	-0.025	13.8 (10)	13.0	44	8	0.40	0.0020	1.23	0.73	95 – 144 (117.3)
Azores	MAR	45	0.73	0.040	12.7 (6)	12.2	39	9 (3)	0.41	0.0011	0.66	0.00	102 – 119 (108.8)
New Zealand	NZ	40	0.75	0.030	13 (2)	12.9	42	7 (3)	0.50	0.0017	1.02	0.82	47 – 142 (104.9)

TABLE 16 – Levels of genetic divergence among populations of *C. squamosus*. Pairwise F_{ST} values below diagonal; pairwise Φ_{ST} values above diagonal; Jost's D in parentheses. P-values significant after Bonferroni correction shown in bold ($P<0.005$).

		mtDNA ND2				
		IRE	POR	SA	MAR	NZ
Nuclear microsatellites	IRE		-0.001 (0.006)	0.009 (0.010)	-0.002 (0.009)	**0.323 (0.732)**
	POR	-0.002 (0.000)		-0.005 (0.004)	0.000 (0.004)	**0.357 (0.732)**
	SA	-0.002 (0.000)	-0.002 (0.000)		-0.004 (0.000)	**0.388 (0.732)**
	MAR	-0.003 (0.000)	-0.003 (0.000)	0.001 (0.001)		**0.371 (0.734)**
	NZ	-0.004 (0.000)	-0.002 (0.000)	-0.002 (0.000)	-0.004 (0.000)	

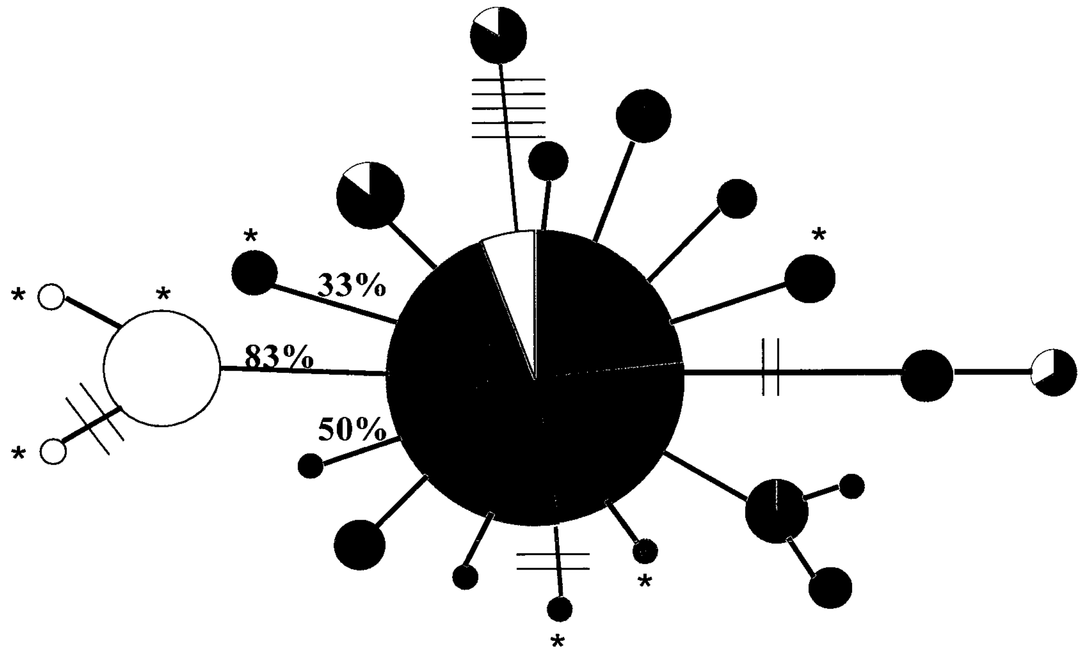
TABLE 17 – Spatial analysis of shared alleles (SAShA) at the nuclear microsatellite loci and mtDNA ND2 gene region of *C. squamosus*. OM: observed mean distance between shared alleles; EM: expected mean distance between shared alleles; *P*: proportion of permuted datasets with mean distance between shared allele more extreme then the observed values; #shared: number of shared alleles at each microsatellite locus or at the mtDNA ND2 gene region.

	OM	EM	*P*	# shared alleles
All alleles	7113	7105	0.839	92
CsquGT64	7188	7132	0.577	22
CsquMx59	7120	7101	0.673	5
CsquMx31	7162	7111	0.672	42
CsquMx104	7140	7106	0.652	14
SacaGA11	7107	7096	0.827	4
Saca7555	7076	7101	0.534	5
ND2 - all	7310	7060	**0.023**	13

TABLE 18 - STRUCTURE analysis results for the nuclear microsatellite genotype data set of *C. squamosus*. Ln P(D) - log probability of the data given K number of populations; Var - variance; Max - maximum; Min - minimum.

K	Ln P(D)	Var Ln P(D)	Min Ln P(D)	Max Ln P(D)
1	-5335.6	0.1	-5356.1	-5355.2
2	-5432.4	1931.9	-5502.1	-5376.6
3	-5406.8	1907.4	-5477.3	-5362.3
4	-5434.6	3083.4	-5508.0	-5374.3
5	-5453.6	9918.5	-5636.5	-5365.4

Figure 5 – Maximum parsimony haplotype network of the mtDNA ND2 of the leafscale gulper *C. squamosus*. Haplotypes (n=21) are represented by circles with sizes proportional to absolute frequency in the total sample. All connections correspond to one nucleotide substitution between haplotypes, except where indicated by black bars. Support values of connections were 100% except where noted. Color code: orange – Ireland; red – Portugal; brown – South Africa; green – MAR; white - NZ. * Haplotypes with non-synonimous substitutions.


*
*
*
*
33%
83%
50%
*
*
*

CONCLUSIONS

The main research objective of my dissertation research was to investigate the population structure and the historical dispersal pathways of three species of wide ranging squaloid sharks, namely the spiny dogfish *Squalus acanthias*, the Portuguese dogfish *Centroscymnus coelolepis*, and the leafscale gulper *Centrophorus squamosus*. In this general discussion section, I begin by summarizing the major findings of my work for each of the target species, and then compare the patterns observed among the three squaloid sharks. Finally, my results are discussed within the broader context of genetic population structure in elasmobranch taxa, based on previously published peer-reviewed papers.

Species-specific patterns of genetic population structure

The genetic population structure of the spiny dogfish *S. acanthias* was characterized by high genetic differentiation of North Pacific collections, and by comparatively higher genetic homogeneity among the sample collections from the South Pacific and Atlantic oceans. The strong genetic break detected in this work was consistent with results from previous studies using other mtDNA markers (e.g. Ward et al., 2007; Hauser, 2009), and with very distinct life-history strategies of North Pacific spiny dogfish (e.g. Ketchen 1972; Jones and Geen, 1977; Saunders & McFarlane, 1993). Based on the accumulated evidence, I have argued for a re-evaluation of the taxonomic status of *S. acanthias* which was recently conducted by Ebert et al. (2010). As a result, the taxon *Squalus suckleyi* (Girard 1854), originally described based on North Pacific spiny dogfish and later rejected due to a lack of distinguishing morphological features (Bigelow and Schroeder, 1948; Jones and Geen, 1976), has been resurrected.

Nevertheless, small but significant genetic differentiation was detected by both nuclear and mitochondrial markers among spiny dogfish collections from the north and south Atlantic. Thus, genetic differentiation in the spiny dogfish occurred across low latitude regions characterized by warm-temperate and tropical waters, suggesting that such regions may act as effective barriers to

gene flow among populations. This result is consistent with the preference of *S. acanthias* for cold-temperate waters, and its distribution within higher latitude coastal regions. In contrast, genetic homogeneity was found among fish from opposite ocean margins (i.e. western and eastern coasts) in the North Pacific, South Pacific and North Atlantic, implying that gene flow has been occurring or has occurred until recently across these ocean basins. Again, these results are consistent with the reported cases of trans-oceanic migrations of *S. acanthias* in the North Pacific and North Atlantic (Holden 1967; Templeman 1976; McFarlane and King 2003), and support earlier suggestions of the high vagility and dispersal potential of spiny dogfish.

Regarding the Portuguese dogfish *C. coelolepis* and the leafscale gulper shark *C. squamosus*, the pattern of genetic population structure uncovered for the eastern Atlantic was similar between species. Within this region, no evidence of genetic differentiation was found among sample collections ranging from off Ireland to South Africa, and including the Azores, consistent with the existence of a single genetic stock for each species within the sampled region. This result was surprising given the large geographic distances separating some of the sampled locations (e.g. Ireland and South Africa). Furthermore, evidence for inter-oceanic dispersal was also found for the leafscale gulper as indicated by non-zero migration rate estimates between Atlantic and New Zealand populations. These results strongly suggest that both *C. coelolepis* and *C. squamosus* have high dispersal potential and no major barriers to gene flow within the deep eastern Atlantic.

When interpreting the above results, it should be kept in mind that very low levels of gene flow (i.e. a few individual migrants per generation) can effectively homogenize the gene pools of otherwise reproductively isolated populations (Hartl and Clark 1997). Moreover, generation time in many deepwater squaloids can span several decades (Kyne and Simpfendorfer 2010). For instance, the generation time in *C. coelolepis* may be 20 years or more, as estimated for its similar-sized relative *Centroscymnus crepidater* (Irvine et al. 2006), while it may be between 30 to 40 years for *C. squamosus* (Clarke et al. 2002). Therefore, gene flow among geographically

distant locations may be sustained by a few individual migrants actively swimming considerably long distances within the period of a generation and at a relatively slow pace.

The high genetic homogeneity of *C. coelolepis* and *C. squamosus* within the eastern Atlantic may also be reflective of their particular life history strategy. Evidence from previous studies conducted at several locations in the eastern North Atlantic and mid-Atlantic ridge suggests that both the Portuguese dogfish and the leafscale gulper shark are spatially segregated according to size and maturity stage. The spatial segregation implies the existence of extensive migrations over a large geographic area during the life cycle of individual fish. First, immature males and females recruit to the sampling locations prior to the onset of sexual maturation. Once sexually mature, adult fish undergo large scale movements associated with the reproductive cycle, with different maturity stages occurring in different areas. This particular life history strategy would result in genetic homogeneity over a large geographic range for both *C. coelolepis* and *C. squamosus*.

Inter-specific patterns of population structure

The high genetic homogeneity observed over large geographic areas (i.e. in the order of thousands of miles) is consistent with long-distance dispersal with gene flow in all three target species. However, in the coastal spiny dogfish, gene flow appears to be restricted across regions of warm equatorial waters within the Atlantic and Pacific basins. In contrast, there were no apparent barriers to gene flow for either of the deepwater squaloids within the eastern Atlantic. Given the similarly high dispersal potential among species, the differences in the patterns between the coastal and the deepwater squaloids may be associated with adaptations to their respective habitats.

The shallow coastal waters at mid-latitudes, which correspond to the center of spiny dogfish distribution, experience seasonal environmental fluctuations. Water temperature, in particular, can change considerably between winter and summer months. The impact of pronounced

environmental variability on the spiny dogfish distribution is reflected in the seasonal migratory behavior along continental shelf waters towards areas of suitable water temperatures (Hisaw and Albert 1947; Burgess 2002; Stenberg 2005). Concordantly, little or no migration has been reported for spiny dogfish occurring in areas where environmental conditions are comparatively more stable throughout the year (McFarlane and King, 2003; Campana et al. 2007). These observations support my hypothesis that environmental factors, such as unsuitable water temperatures, exert a strong influence in the distribution of *S.acanthias* and are reflected in its genetic population structure.

Comparatively more stable environmental conditions are observed at bathyal depths (< 1000 m) where the Portuguese dogfish and the leafscale gulper are most commonly found. However, and contrary to the highly productive coastal shelf waters inhabited by the spiny dogfish, the amount of organic matter available to the deepwater food webs decreases dramatically with depth. Given the high trophic level of deepwater squaloids (e.g. trophic level range: 3.5 – 4.4; Cortés 1999), food availability is likely to have a major effect on the distribution and in the population structure of the Portuguese dogfish and the leafscale gulper. Thus, the development of a life-history strategy aiming at maximizing resource partitioning, while minimizing resource competition, among individuals of the same species could be advantageous in deepwater squaloids.

Increased intraspecific resource partitioning may be achieved through spatial segregation of different components of the populations over a large geographic area, for instance with juvenile fish occurring separate from the adults as in *C. coelolepis* and *C. squamosus* (this study; Clarke et al. 2001; Veríssimo et al. 2003). Given the expected long lifespan and late age at maturity of deepwater squaloids (Kyne and Simpfendorfer 2010), the transition among areas where different sizes and/or maturity stages occur can occur at a relatively slow pace and without being too demanding energetically. The evolution of this particular life-history strategy as an adaptation to

life in the vast and unproductive deep-sea would result in high genetic homogeneity over large geographic regions, as observed in the two deepwater squaloid species within the eastern North Atlantic.

Patterns of population structure in elasmobranchs

In the last two decades, the interest in the patterns of population structure in elasmobranch taxa has increased and many studies have been conducted on this topic. At present, studies on the population genetics of over thirty species of elasmobranchs from seven of the 12 extant orders (*sensu* Compagno et al. 2005) have been published (Table 19). Despite the bias towards coastal, and medium to large species (>1 m total length), the amount of information collected so far allows some preliminary considerations on the patterns of population structure across elasmobranch taxa.

Dispersal ability appears to be an important trait affecting the geographic distribution of genetic diversity within elasmobranchs. Dispersal in elasmobranchs is exclusively dependent on active swimming of individuals, since pelagic eggs or larval stages are absent in chondrichthyans (Musick et al. 2004). Body size and life habit (i.e. benthic, benthopelagic, or pelagic) have been suggested to greatly influence dispersal potential. For instance, large pelagic species appear to have higher dispersal abilities and wider geographic ranges compared to smaller benthic-oriented taxa (Musick et al. 2004). As such, gene flow is expected to occur over large geographic areas in the former, while genetic differentiation should be detected at smaller geographic scales in the latter. The above expectation is generally supported by the available literature on elasmobranchs' genetic population structure (Table 19). Genetic differentiation in large (> 3 m total length, TL) epipelagic species with wide geographic distributions (i.e. including two or more adjacent ocean basins) was detected mostly between ocean basins, as in the white shark *Carcharodon carcharias* (Pardini et al. 2001) and the whale shark *Rhincodon typus* (Schimdt et al. 2007). In contrast,

small- to medium-sized (< 3 m TL) benthic and benthopelagic species with comparatively narrower ranges exhibit genetic differentiation at the local (< 500 miles) and/or at the regional level (< 2000 miles), as in the narrownose smoothhound *Mustelus schmitti* (Pereyra et al. 2010) or the thornback ray *Raja clavata* (Chevolot et al. 2006).

However, species with high dispersal potential may exhibit genetic differentiation at a smaller geographic scale than expected based only on size and habit. For instance, highly vagile and widely distributed species may exhibit strong population structure across regions of unsuitable environmental conditions. Regions of sub-optimal water temperatures and/or of open oceanic waters have been shown to severely limit dispersal and mixing of individuals in some elasmobranch species such as in the tope *Galeorhinus galeus* (Chabot and Allen 2010) and the spiny dogfish *S. acanthias* (this study). On another account, historical events such as vicariance or sea level changes also affect the levels of gene flow among individuals across geographically close locations (i.e. at the regional scale), as reported for the scalloped hammerhead *Sphyrna lewini* (Quattro et al. 2006; Duncan et al. 2006) and the shovelnose guitarfish *Rhinobatus productus* (Sandoval-Castillo et al. 2004).

Further exceptions to the "dispersal-based" pattern of population structure in elasmobranchs are associated with particular life history strategies. For example, female site fidelity to nursery areas may result in distinct population units across a relatively small geographic region of continuous habitat, as observed in the blacktip shark *Carcharhinus limbatus* (Keeney et al., 2005). On the other hand, species that undergo long distance migrations associated with the reproductive cycle (as suggested here for *C. coelolepis* and *C. squamosus*) may exhibit high genetic homogeneity over a surprisingly large geographic region (e.g. eastern Atlantic).

Overall, the available data suggest that the genetic population structure of elasmobranch taxa is greatly influenced by the species' dispersal abilities, environmental constraints, life history

strategy and historical processes. In addition, these studies demonstrate the usefulness of molecular genetic techniques to uncover specific details of the species' life histories, such as their reproductive strategies, as well as their ecological constrains and dispersal abilities. Molecular genetics can therefore provide a potentially powerful method to address these types of intraspecific-level questions for other elasmobranch taxa.

Future research

My dissertation research focused exclusively on the study of the large scale patterns of genetic population structure for the three squaloid species, and the sampling design was adapted to the geographic scale of the question. However, little is still known about the processes and patterns of genetic diversity distribution at the regional scale. An important aspect needing further clarification is the delineation of reproductive stock units within each species range. For instance, tagging and fisheries-independent catch data suggested the existence of a spiny dogfish metapopulation along the western North Atlantic (Campana et al. 2007). Future research should aim at sampling adult fish in mating aggregations and/or juveniles in putative nursery grounds (if known), in order to discriminate the possible existence of different reproductive stocks for all three of the target species.

Other important research questions refer to the taxonomic uncertainty within *Squalus acanthias*. Our results confirmed the existence of a distinct evolutionary significant unit of spiny dogfish in the North Pacific and future studies should extend this approach to the remaining regions of the taxon range, namely the Mediterranean and Black seas. Similar to the observations of North Pacific spiny dogfish, the population in the Black Sea shows somewhat distinct life history parameters from the eastern Atlantic counterpart, such as larger sizes at age and faster growth rates particularly in females (Avsar et al. 2001; Demirhan and Seyhan 2007). Given the geographical isolation of the Black and Mediterranean seas, the populations residing in these areas might show similarly high genetic divergence from the Atlantic populations.

My study raises several interesting new questions regarding the deepwater squaloids' population structure. Is the large scale pattern of genetic homogeneity observed in *C. coelolepis* and *C. squamosus* a reflection of widespread dispersal at depth, or is it due to a particular life-history strategy that includes large-scale migrations during an individual's life cycle? More data are needed on the individual composition at different locations within each species range, particularly with respect to size and maturity stage, to test for the putative spatial segregation suggested earlier. This will require extensive spatial and temporal sampling for each of the deepwater species.

Future studies on the population structure of deepwater elasmobranchs will provide important comparisons to the observed patterns, and will test whether a deepwater habit increases the scale at which gene flow may occur within a species range, regardless of body size. Also, they will help demonstrate whether spatial segregation and large-scale migration may be a common life strategy in the large, unproductive deep sea.

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TABLE 19 – Summary of available studies on the genetic population structure of elasmobranch taxa. Only those studies that sampled the entire or the majority of each species' geographic range were considered. Size (measured as maximum total length or disc width for galeoids and batoids, respectively): small < 1 m; medium 1 to 3 m; large > 3 m. Scale of genetic differentiation (i.e. geographic distance between the closest locations for which genetic differentiation was detected): local <500 miles; regional >500 and <2000 miles; ocean/inter-ocean >2000 miles. Information on Habit, Habitat and Size for the galoid sharks were taken from Musick et al. (2004).

Taxa	Habit	Habitat	Size	Reproductive mode	Geographic range	Scale of genetic differentiation	Sampling scale	Reference
CARCHARHINIFORMES								
Carcharhinus leucas	benthopelagic	coastal	large	viviparous	worldwide	regional	regional	Karl et al 2011
Carcharhinus limbatus	benthopelagic	coastal	medium	viviparous	worldwide	regional	regional	Keeney et al 2005
Carcharhinus obscurus	benthopelagic	coastal	large	viviparous	worldwide	ocean/inter-ocean	ocean/inter-ocean	Benavides et al 2011
Carcharhinus plumbeus	benthopelagic	coastal	medium	viviparous	worldwide	regional	ocean/inter-ocean/	Portnoy et al 2010
Carcharhinus sorrah	benthopelagic	coastal	medium	viviparous	Indo-West Pacific	regional	regional	Ovenden et al 2009
Negaprion acutidens	benthopelagic	coastal	medium	viviparous	Indo-West Pacific	regional	ocean/inter-ocean/	Schultz et al 2008
Negaprion brevirostris	benthopelagic	coastal	medium	viviparous	Atlantic, central East Pacific	regional	ocean/inter-ocean/	Feldheim et al 2001, Schultz et al 2008
Prionace glauca	pelagic	open ocean	large	viviparous	worldwide	not detected	regional	Ovenden et al 2009
Rhizoprionodon acutus	benthopelagic	coastal	medium	viviparous	Atlantic, Indo-West Pacific	regional	regional	Ovenden et al 2011
Rhizoprionodon lalandii	benthopelagic	coastal	small	viviparous	western central Atlantic	local	local	Mendonça et al 2009
Rhizoprionodon porosus	benthopelagic	coastal	small	viviparous	Caribbean - Brazil	local	regional	Mendonça et al 2011
Rhizoprionodon terranovae	benthopelagic	coastal	small	viviparous	western North Atlantic, Gulf of Mexico	not detected	regional	Heist et al 1996
Sphyrna lewini	benthopelagic	coastal	large	viviparous	worldwide	regional	ocean/inter-ocean/	Quattro et al 2006, Duncan et al 2006
Galeorhinus galeus	benthopelagic	coastal	medium	viviparous	worldwide	ocean/inter-ocean	ocean/inter-ocean/	Chabot and Allen 2010

Taxa	Habit	Habitat	Size	Reproductive mode	Geographic range	Scale of genetic differentiation	Sampling scale	Reference
Mustelus schmitti	benthopelagic	coastal	small	viviparous	Southwest Atlantic	not detected	regional	Pereyra et al 2010
Triakis semifasciata	benthopelagic	coastal	medium	viviparous	Northeast Pacific	regional	regional	Lewallen et al 2008
LAMNIFORMES								
Carcharadon carcharias	benthopelagic	open ocean	large	viviparous	worldwide	ocean/inter-ocean	ocean/inter-ocean	Pardini et al 2001, Jorgensen et al 2010
Carcharhias taurus	benthopelagic	coastal	large	viviparous	Atlantic, Mediterranean, Indo-West Pacific	regional	ocean/inter-ocean/	Ahonen et al 2009
Isurus oxyrinchus	pelagic	open ocean	large	viviparous	worldwide	ocean/inter-ocean	ocean/inter-ocean	Schrey and Heist 2003
Cetorhinus maximus	pelagic	open ocean	large	viviparous	Atlantic & Pacific	not detected	ocean/inter-ocean	Hoelzel et al 2006
ORECTOLOBIFORMES								
Rhincodon typus	pelagic	open ocean	large	viviparous	worldwide	not detected	ocean/inter-ocean	Castro et al 2007, Schmidt et al 2009
Stegostoma fasciatum	benthic	coastal	medium	oviparous	Indo-West Pacific	regional	ocean/inter-ocean	Dudgeon et al 2008
SQUALIFORMES								
Centrophorous squamosus	benthopelagic	slope	medium	viviparous	Worldwide	ocean/inter-ocean	ocean/inter-ocean	This study
Centroscymnus coelolepis	benthopelagic	slope	small	viviparous	Worldwide	ocean/inter-ocean	ocean/inter-ocean	This study
Etmopterus cf granulosus	benthopelagic	slope	small	viviparous	Southern Ocean	ocean/inter-ocean	ocean/inter-ocean	Straube et al 2010
Squalus acanthias	benthopelagic	coastal	medium	viviparous	Worldwide	ocean/inter-ocean	ocean/inter-ocean	This study
PRISTIFORMES								
Pristis clavata	benthic	coastal	large	viviparous	Indo-West Pacific	regional	regional	Phillips et al 2011
Pristis microdon	benthic	coastal	large	viviparous	Indo-West Pacific	regional	regional	Phillips et al 2011
Pristis zijsron	benthic	coastal	large	viviparous	Indo-West Pacific	regional	regional	Phillips et al 2011

Taxa	Habit	Habitat	Size	Reproductive mode	Geographic range	Scale of genetic differentiation	Sampling scale	Reference
MYLIOBATIFORMES								
Aetobatus narinari	benthopelagic	coastal	large	viviparous	Worldwide	regional	ocean/inter-ocean	Schluessel et al 2010
Urobatis halleri	benthic	coastal	small	viviparous	Eastern Pacific	local	local	Plank et al 2010
RAJIFORMES								
Amblyraja radiata	benthic	coastal	medium	oviparous	North Atlantic	regional	ocean/inter-ocean	Chevolot et al 2007
Dipturus oxyrinchus	benthic	coastal	medium	oviparous	Eastern Atlantic	regional	regional	Griffiths et al 2011
Raja clavata	benthic	coastal	medium	oviparous	Eastern North Atlantic	regional	regional	Chevolot et al 2006
Rhinobatos productus	benthic	coastal	medium	viviparous	California - Sea of Cortez	local	local	Sandoval-Castillo et al 2004

VITA

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