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# Population genetics of the deep-sea bluntnose sixgill shark, *Hexanchus griseus*, revealing spatial genetic heterogeneity

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ABSTRACT

*Hexanchus griseus* is a globally distributed deep-water shark species. It inhabits tropical and temperate waters throughout the world, including the Mediterranean Sea where it is by-caught by small-scale fisheries in the region. In this study, we analysed the genetic variation of *H. griseus* specimens collected from different areas within and outside the Mediterranean region, to assess its genetic connectivity. The mitochondrial DNA (mtDNA) sequence analysed in this study ranged from cytochrome *b* to 16S rRNA genes including the control region, the 12S rRNA gene and the interspersed tRNA genes in the region, covering a total of 3731 to 3914 nucleotides. Results have shown that this species exhibits geographically distinct maternal lineages, indicating population structure along geographical ranges. These findings reveal population subdivisions not only between the Pacific Ocean and the Atlantic Ocean, but also within the oceans and on a smaller scale within the Mediterranean Sea. This highlights the need to consider each population subdivision separately when designing management plans for the conservation of this species.

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## 1. Introduction

The bluntnose sixgill shark, *Hexanchus griseus* (Bonnaterre, 1788), is one of the largest deep-water circumglobal shark species which is found to occur mostly along the continental shelves of tropical and temperate regions. Its geographical range encompasses semi-enclosed seas, such as the Mediterranean Sea and the Sea of Marmara (Serena, 2005; Froese and Pauly, 2017). Its depth preferences range between 600 m and 1100 m, although it is known to occur at depths ranging from few meters down to 2490 m, with diurnal vertical movements (Stefanescu et al., 1993; Dunbrack and Zielinski, 2003; Andrews et al., 2010). The wide occurrence in both locations and depth preferences indicate that *H. griseus* has evolved a versatile mode of life to live in a variety of habitats, where it plays the important role of top predator (Ebert, 1994; Cortés, 1999; McNeil et al., 2016) that maintains the balance within the ecosystems it resides in. However, although *H. griseus* is widely distributed, with known small-scale fisheries around the Mediterranean (Vella and Vella, 2010a, 2010b, in press), most of its landing records are sporadic, and is usually neglected from data collection due its rareness and low economic importance (Dalli, 2004; Vella and Vella, 2010a; FAO, 2017). This species has been evaluated by IUCN as Nearly Threatened on a global scale (Cook and Compagno, 2005) and as Least Concern at European (Walls et al., 2015) and Mediterranean level (Soldo et al., 2016).

In the past decades, several direct and indirect threats have placed shark species at a high risk of becoming endangered or extinct (Ferretti et al., 2008; IUCN, 2017), with the number of species within higher IUCN risk categories constantly increasing. At the same time, the proportion of top predators in the oceans are decreasing (Ferretti et al., 2008; Tremblay-Boyer et al., 2011; Kleisner et al., 2013). The lack of accurate landing data collection for certain species such as elasmobranchs, linked with lacunae in biological knowledge make it difficult to pinpoint the first warning signs of stock decline. Within this scenario, the aim of the current study is to use mtDNA sequencing as a tool to evaluate the spatial genetic population structure of *H. griseus* that can be integrated into sustainable international fisheries management plans for better coherence between biological and fisheries management units (Reiss et al., 2009). All this is done in the wake of international concerns to safeguard elasmobranchs, where conservation genetics is playing an important role in understanding the population structure and identifying stocks for better management to safeguard both the species and small-scale fisheries that make a living from these resources. The applications of various molecular genetics tools for conservation is increasing the detail and the necessary knowledge for effective species specific conservation plans.

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## 2. Materials and methods

### 2.1. Sample collection and DNA extraction

146 specimens of *H. griseus* were collected from the: Eastern Mediterranean (EM: n = 8); Central Mediterranean (CM: n = 86); Western Mediterranean (WM: n = 34); North-Eastern Atlantic Ocean (NEA: n = 4); South-Eastern Atlantic Ocean (SEA: n = 7); North-Eastern Pacific Ocean (NEP: n = 4); and the South-Western Pacific Ocean (SWP: n = 5) (Fig. 1; Supplementary data – Table 1). Four specimens of *H. nakamurai* were collected from Taiwan and utilized as outgroup. Most of the specimens' tissue samples were collected from fisheries landings, including small-scale fisheries in the Mediterranean that target this species. Upon collection, the tissue samples were stored in 20% dimethyl sulfoxide solution saturated with sodium chloride or in 100% ethanol. The genomic DNA was extracted from 15 mg of tissue using proteinase K, phenol-chloroform extraction following Milligan (1998), and the purified DNA was stored in TE buffer at −20 °C.

### 2.2. PCR amplification and sequencing

The mtDNA region between the 3′ end of the cytochrome *b* gene and the 16S rRNA gene was amplified via four primer sets (Table 1). Amplification was carried out in 50 μL reaction volume using ~100 ng DNA template, 1× FIREPol® Master Mix [2.5 mM $Mg^{2+}$; 200 μM each dNTP; FIREPol® DNA polymerase] (Solis BioDyne, Estonia), and 0.5 μM of each primer. The PCR reactions were subject to an initial denaturation of 95 °C for 5 min; followed by 30 cycles of 95 °C for 45 s, 60 °C for 45 s, 72 °C for 1 min; and a final extension 72 °C for 10 min. In reaction 4, the PCR was run for 34 cycles using a $T_a$ of 51 °C. 2 μL aliquots of the PCR reaction mixtures were electrophoresed on 2.0% agarose gels stained with ethidium bromide, to check the PCR product sizes and to note the presence of multiple PCR products that might arise from size heteroplasmy should there be more than one length control region variant per individual.

PCR products were sequenced using 3730XL Genetic Analyzer (Life Technologies) using the respective forward and reverse primers, while PCR product 1 was also sequenced via an internal primer (Table 1) to ensure good coverage of domain 1 of the mtDNA control region.

### 2.3. Statistical analyses

Sequences were trimmed and contiguous sequences were assembled using Geneious R6 (Kearse et al., 2012). All sequences were manually checked for consistencies. Sequences were then aligned with Geneious Alignment using 1000 iterations. The smallest homologous sequence was chosen for analyses. This included 72 bp from the 3′ end of cytochrome *b* gene up to the first 848 bp of the 16S rRNA gene. Sequences were deposited in GenBank with accession numbers KF894454 - KF894490 (Supplementary data – Table 2). Genetic variability was identified through Geneious R6 (Kearse et al., 2012) and MEGA v5 (Tamura et al., 2011). FaBox DNACollapser (Villesen, 2007) was used to identify and group individuals on the basis of their respective haplotype. The assignment of haplotypes to individuals was carried out using the whole sequenced region. Haplotypes that differed from each other in the sequence outside the variable nucleotide tandem repeat (VNTR) region, noted in domain 1, were identified from each other by numbers, while letters were added to these haplotype numbers to distinguish them further on the basis of their VNTR sequence. This facilitated the identification of haplotypes, since the VNTR region mutates differently from the rest of the sequence and thus it had to be omitted from some of the analyses that follow.

The genetic diversity was measured by Arlequin v3.5 (Excoffier and Lischer, 2010) by calculating the haplotype diversity ($h$) and the nucleotide diversity ($\pi$) indices, which were estimated without the VNTR region. Chi-squared test was used to determine whether the haplotypes were randomly distributed or whether they were clustered according to locations. Pairwise $\phi_{ST}$ values between different sampling locations were calculated using 99,999 permutations, via Arlequin v3.5 (Excoffier and Lischer, 2010). The latter software was also used to carry out Analyses of Molecular Variance (AMOVA) to test for genetic partitioning (Excoffier et al., 1992; Excoffier and Lischer, 2010), using 99,999 permutations, while SAMOVA v1.0 (Spatial Analyses of Molecular Variance)

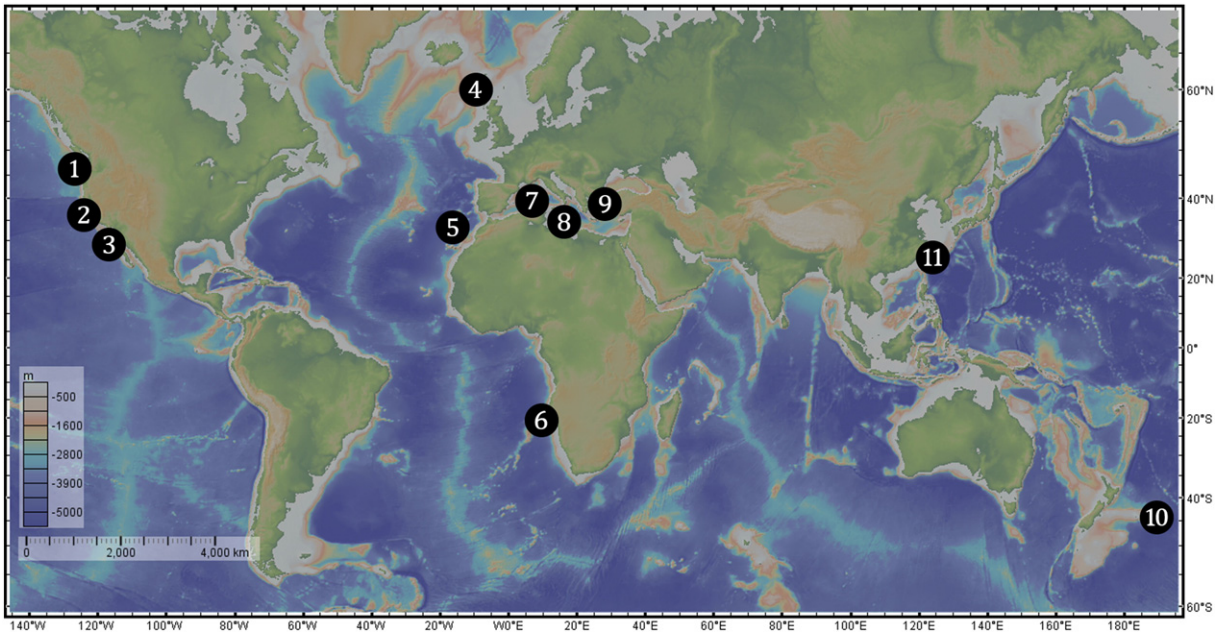


**Fig. 1.** A map (GeoMapApp, Ryan et al., 2012) showing the geographical sampling areas from where specimens were collected. **North-East Pacific Ocean (NEP)** [❶ Washington, ❷ California, ❸ Mexico]; **North-East Atlantic Ocean (NEA)** [❹ Scotland, ❺ Madeira]; **South-East Atlantic Ocean (SEA)** [❻ Namibia]; **Western Mediterranean Sea (WM)** [❼ various locations within the western basin]; **Central Mediterranean Sea (CM)** [❽ Southern Sicily and Malta]; **Eastern Mediterranean Sea (EM)** [❾ Sea of Marmara and Aegean Sea]; **South-West Pacific Ocean (SWP)** [❿ New Zealand]; **Taiwan** [⓫ *Hexanchus nakamurai*].

**Table 1**
Primer sets for PCR amplification.

| Reactions | Forward primer sequence | Reverse primer sequence | Internal primer sequence |
|---|---|---|---|
| 1. | *CytB-1040mod* 5′CGGAGGACAACCAGTTGAACAACCC (modified from Ramírez-Macías et al., 2007) | *1235R* 5′AATGAATTACCGTCCCACAAACCGT (current study) | *346F* 5′ATCCCACGTCTACCTGCCAAACC (current study) |
| 2. | *890F* 5′ACGGGCTTTTCACACATTCAATAACG (current study) | *1900R* 5′GGACCAAACCTTTTGTGCTTGTGG (current study) | |
| 3. | *1780F* 5′ACCCCCAAAACGAGGACCGA (current study) | *2910R* 5′AGGCTAGTTTTGAGGTTCAAAGTG (current study) | |
| 4. | *12Sa* 5′AAACTGGGATTAGATACCCCACTAT (Palumbi, 1996) | *16Sa* 5′ATGTTTTTGATAAACAGGCG (Palumbi, 1996) | |

(Dupanloup et al., 2002) was used to assess the genetic patterns noted without *priori* assumptions of their group identity. The configuration with the largest $\phi_{CT}$ was chosen as the best configuration that explains the given set of data.

The association between haplotypes was estimated using the 95% confidence limit through TCS v1.21 (Clement et al., 2000) which makes use of the parsimony algorithm for constructing the minimum spanning network. In this analysis the various individuals were marked according to their sampling location to illustrate patterns linked with population structure. A Maximum Likelihood (ML) tree using 1000 bootstraps was constructed using PhyML (Guindon and Gascuel, 2003) within Geneious R6 (Kearse et al., 2012) utilizing unequal base frequencies 3-parameter model, TPM1uf + I (Kimura, 1981) which was chosen as the best nucleotide substitution model via jModelTest v2.0.1. (Darriba et al., 2012) with both AICc and BIC. A Maximum Parsimony (MP) tree was also constructed using MEGA v5 (Tamura et al., 2011). Both phylogenetic trees were run using 1000 bootstrap replicates and were both rooted with *H. nakamurai*. The correlation between genetic distance and geographical distance amongst all the sampled specimens was calculated using the Mantel tests (Mantel, 1967) within IBDWS v3.23 (Jensen et al., 2005), with the geographic distance being measured via GeoMapApp (Ryan et al., 2012). The latter was estimated as the shortest distance along the continental shelves or along islands between any two locations.

The migration rate (M) between various sampling locations was calculated using MDIV (Nielsen and Wakeley, 2001), which makes use of a Bayesian Markov Chain Monte Carlo method. For each data set, the HKY model was used and the migration rate was estimated for values from 0.01 to 5 in increments of 0.01. MDIV analyses were run with a $5 \times 10^6$ generation MCMC and a burn-in period of 500,000 generations. Each data set was analysed five times to ensure convergence at the same posterior distribution.

As most of the sampling effort was focused on the Mediterranean Sea, the specimens from this area were evaluated further to assess other population genetic features in this region. Rarefaction analyses using Analytic Rarefaction (UGA Stratigraphy Lab, 2013) was carried out to check whether enough specimens were sequenced from the various sampling locations in the Mediterranean based on the haplotype frequencies identified per region against the simulated expected number of haplotypes. An estimate of past population expansion was carried out using mismatch distributions (Schneider and Excoffier, 1999) between the various haplotypes identified from the Mediterranean Sea. The fit of the simulated mismatch distribution was assessed with the sum of square deviations (SSD) and Harpending's Raggedness Index (Harpending et al., 1993; Excoffier and Lischer, 2010) where a non-significant test would indicate a good fit with the expansion models and support past expansion. These analyses were computed via Arlequin v3 (Excoffier and Lischer, 2010). The same software was used to test for deviations from neutrality via Fu's Fs estimator (Fu, 1997) where significant negative values are indicative of population expansion. The age of the Mediterranean population was estimated to provide an insight on the time of its origin and better understand which geographical events might have influenced the population structure in the area. The age of the population was estimated using $\tau = 2\mu t$ (Rogers and Harpending, 1992), with 't' being the time in generations since the commencement of population expansion, and 'μ' being the mutation rate per sequence and per generation. The latter two components are not known for Hexanchidae species and thus 'μ' was estimated to be $0.2\%/10^6$ years/nucleotide for the sequence analysed (cytb to 16S rRNA gene excluding the VNTR region) using the mutation rate noted in the control region of other shark species (Duncan et al., 2006; Schultz et al., 2008) and taking into consideration the nucleotide diversity of the overall studied sequence in proportion to the nucleotide diversity noted in the control region analysed, while a generation time of 20 years similar to that noted in other large long-lived shark species (Duncan et al., 2006; Hoelzel et al., 2006; Schultz et al., 2008) was used for the analysis. The initial ($N_{f0}$) and contemporary ($N_{f1}$) effective female population sizes were estimated using $\Theta_0$ and $\Theta_1$ through $\Theta = 2N_f\mu$ (Fu, 1994; Hoelzel et al., 2006; Castro et al., 2007; Schultz et al., 2008; Nance et al., 2011).

## 3. Results and discussion

### 3.1. The studied sequence

The mtDNA region analysed in this study ranged from 3731 bp to 3914 bp and included the last 72 bp from the 3′ end of cytochrome *b* gene, 72 bp from tRNA-Thr gene, 69 bp from tRNA-Pro gene, 1570–1752 bp from the control region, 70–71 bp from tRNA-Phe gene, 959–960 bp from 12S rRNA gene, 72 bp from tRNA-Val gene and the first 848 bp from the 5′ of the 16S rRNA gene. No genetic differences were noted in cytochrome *b*, tRNA-Thr and tRNA-Pro genes, while the rest of the genes exhibited some degree of genetic variation (Supplementary data – Table 3). The most variable was the control region, which contained a VNTR region within domain 1 (Fig. 2). The complete control region alone exhibited 26 haplotypes when including the VNTR region and 10 haplotypes when excluding the VNTR region. The latter has shown low global haplotype diversity ($h = 0.724$) even when compared to sharks with similar life-histories (Castro et al., 2007; Ahonen et al., 2009; Gubili et al., 2010; Mendonça et al., 2010; Portnoy et al., 2010; Daly-Engel et al., 2012), consequently the region under study was expanded up to the 16S rRNA gene to encompass a larger sequence leading to more genetic differences and better segregation of haplotypes with a higher level of confidence. The inclusion of the RNA genes increased the haplotype diversity to 0.834 and the number of variable sites from 14 bp to 31 bp.

This study identified 22 haplotypes (H1 to H22), which on considering the VNTR region, they differed further from each other producing a total of 37 different haplotypes (Supplementary data – Tables 1, 2). Fifteen haplotypes (29 including VNTR) were noted for the Mediterranean Sea specimens, 1 haplotype for the NEA (shared with the Mediterranean Sea), 3 haplotypes (4 including VNTR) for the SEA, 3 haplotypes for the NEP and 1 haplotype for the SWP (Supplementary data – Tables 1, 3).

### 3.2. The mtDNA control region, VNTRs and sampling locations

Domain 1 of the control region exhibited a 45 bp motif (Fig. 2) that was imperfectly repeated between seven and eleven times leading to major length variation between haplotypes. The imperfect repetition of the motif has yielded fourteen 45 bp sequences which differed from each other solely through transitions (Supplementary data – Table 4). MFold DNA (Zuker, 2003) has shown that when single stranded most

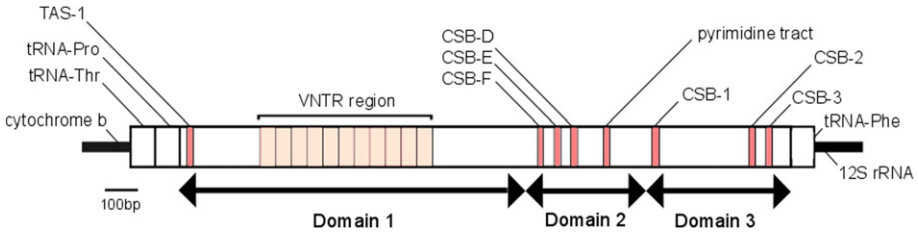


**Fig. 2.** The mtDNA control region (including the various domains and conserved sequence blocks) and neighbouring genes for *Hexanchus griseus*, with Mediterranean specimens exhibiting a maximum of 11 repeats in the VNTR region which is composed of 45 bp motif repeats.

of these sequences, especially the most common ones, can fold on themselves to form highly stable secondary structures leading to the conclusion that this VNTR region is probably the result of repeated strand slippage during DNA replication through stem-loop formation as noted in Mundy et al. (1996) and in Ray and Densmore (2003). No length heteroplasmy was noted in the *H. griseus* specimens analysed, as during gel electrophoresis only one band was produced per individual and also one product was noted for each PCR product sequenced.

The specimens collected from the Mediterranean Sea exhibited the longest VNTR region containing 9, 10 and 11 copies of the repeat, while all specimens from the Atlantic Ocean exhibited 10 repeats (Supplementary data – Table 5). No significant differences were detected between the number of repeats recorded for the Atlantic Ocean, Eastern, Central and Western Mediterranean specimens. All the specimens from the Pacific Ocean exhibited 7 repeats leading to a significant difference ($P < 0.001$) in the number of repeats between these specimens and those of Atlantic-Mediterranean origin. This indicates that the global distribution of the number of repeats in the VNTR region is not due to chance but is linked to the geographical region specimens originate from. However, since the mechanisms responsible for the formation/deletion of repeats within the VNTR region are not completely understood across all species (Mundy et al., 1996; Ray and Densmore, 2003; Roques et al., 2004) and given that the repeats behave as units rather than separate basepairs, then the VNTR region was omitted from the analyses that used point mutations as markers for understanding the population structure.

### 3.3. Point mutations and the global population structure

On excluding the VNTR region, a total of 31 intraspecific variable sites were identified (Supplementary data – Table 3), 64.5% of them were due to genetic differences by transitions. The overall nucleotide diversity observed was 0.0010 (SD: ±0.0006) and the haplotype diversity was 0.834 (SD: ±0.023).

Haplotypes were not randomly distributed ($\times^2$, $P = 0.004$), but rather they were clustered according to the geographical origin of the specimens as different haplotypes were confined to specific sampling locations (Table 2, Figs. 3, 4). This was also confirmed through the estimated low migration rates noted between sampling sites as in most cases the highest likelihood value for *M* was found to be very close to zero (Table 3). Haplotypes from the same geographical region had very low pairwise differences as opposed to the genetic differences between specimens collected from different and distant geographically regions. This study revealed statistically significant positive correlation between genetic and geographical distance (Mantel coefficient $r = 0.939$; P-value $< 0.001$).

The most pronounced divergence was that between the specimens of Pacific origin and those of Eastern Atlantic origin, which did not share any haplotypes, probably because the American continent impedes genetic connectivity between the two oceans while the connection between the Western Indo-Pacific Ocean and the Atlantic Ocean is limited by the African continent. Consequently, this study noted that the connectivity through maternal linages is absent between the Pacific Ocean and the Eastern Atlantic Ocean ($M < 0.01$). This is supported by a large and significant $\phi_{ST}$ value (0.799, $P < 0.001$) (Supplementary data – Table 6) and by the differences in the length of the VNTR region as discussed earlier. AMOVA analyses has also indicated that 80% of the genetic variation occurred between oceans as opposed to the 20% within oceans.

Within the Pacific Ocean itself, a large degree of genetic differentiation was noted between the NEP specimens and the SWP specimens, which had an average divergence of 0.22% and a significantly large $\phi_{ST}$ value of 0.964 ($P = 0.008$). Even though few specimens were collected from both sampling locations, such observation is significant and is in agreement with restricted transoceanic migration patterns (Schultz et al., 2008; Blower et al., 2012). This was also confirmed through SAMOVA (Dupanloup et al., 2002) which gave the maximal differentiated groupings at $K = 3$ ($\phi_{CT} = 0.823$) with the split in the population being the

**Table 2**
A table showing the number of haplotypes noted, the haplotype diversity ($h$) and the nucleotide diversity ($\pi$) at each sampling region. Twenty-two haplotypes were noted (H1 − H22), which differed further from each other at the VNTR region forming 37 haplotypes (these are identified by the letter following the haplotype number).

| Haplotype | H 1a | H 1b | H 1c | H 1d | H 1e | H 1f | H 1g | H 1h | H 2a | H 2b | H 3a | H 3b | H 4a | H 5a | H 6a | H 7a | H 7b | H 8a | H 9a | H 10a | H 11a |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| EM | | | | | | | | | | | | | | | | | | | | | |
| CM | 4 | 1 | 5 | 1 | 1 | 1 | 1 | | 25 | | 14 | 3 | 2 | 1 | 2 | 3 | 1 | 1 | 1 | 2 | 3 |
| WM | 1 | | 4 | | | | | 1 | 25 | 1 | | | | | | | | | | | |
| NEA | | | | | | | | | | | | | | | 4 | | | | | | |
| SEA | | | | | | | | | | | | | | | | | | | | | |
| NEP | | | | | | | | | | | | | | | | | | | | | |
| SWP | | | | | | | | | | | | | | | | | | | | | |

| Haplotype | H 11b | H 11c | H 12a | H 12b | H 12c | H 13a | H 14a | H 15a | H 16a | H 16b | H 17a | H 18a | H 19a | H 20a | H 21a | H 22a | $h$ | $\pi$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| EM | | | | 7 | 1 | | | | | | | | | | | | n/a | n/a |
| CM | 1 | 2 | 6 | 1 | | 1 | 1 | | | | | | | | | | 0.836 ± 0.023 | 0.0006 ± 0.0004 |
| WM | | | | | | | | 2 | | | | | | | | | 0.392 ± 0.0912 | 0.0002 ± 0.0002 |
| NEA | | | | | | | | | | | | | | | | | n/a | n/a |
| SEA | | | | | | | | | 2 | 1 | 2 | 2 | | | | | 0.762 ± 0.115 | 0.0007 ± 0.0005 |
| NEP | | | | | | | | | | | | | 1 | 2 | 1 | | 0.833 ± 0.222 | 0.0003 ± 0.0003 |
| SWP | | | | | | | | | | | | | | | | 5 | n/a | n/a |

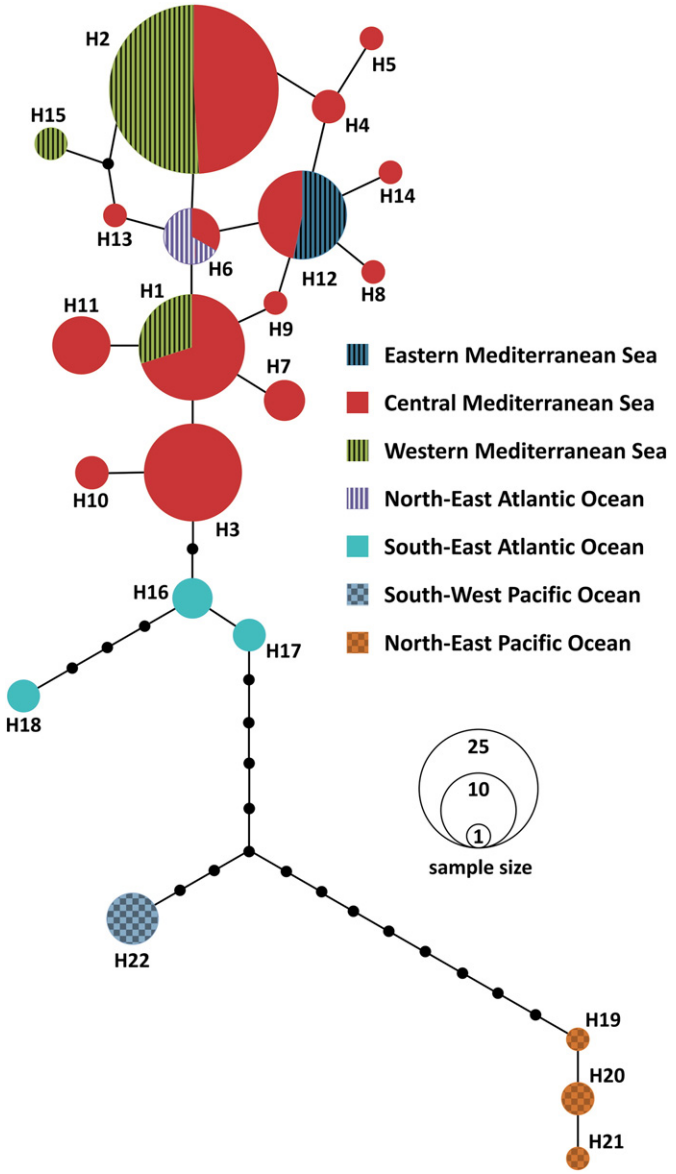


**Fig. 3.** A Minimum Spanning Parsimony Network for the 22 haplotypes identified using the complete sequenced region (*cytb* to 16S rRNA gene, excluding the VNTR region). Branches indicate one substitution/indel event each. The haplotype frequencies are proportional with the area of the circle. The black unlabelled circles represent inferred putative haplotypes that were not observed during this study.

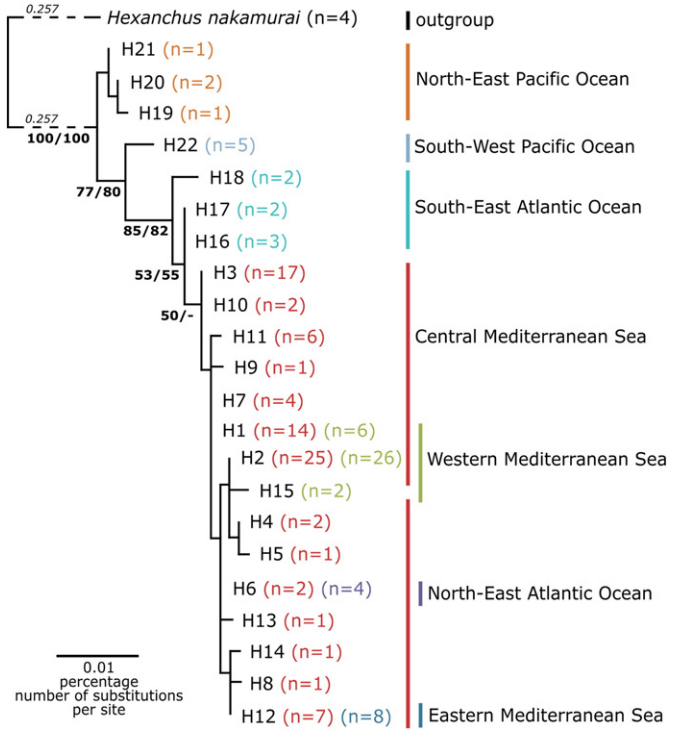


**Fig. 4.** A Maximum Likelihood tree showing the phylogeny of the 22 haplotypes identified in this study. The bootstrap values larger than 50 are shown at their respective nodes (ML/MP bootstrap values).

Eastern Atlantic (including Mediterranean)/North East Pacific/South West Pacific. Both the minimum spanning network (Fig. 3) and the phylogenetic tree (Fig. 4) indicate that these two Pacific populations share common ancestry and have haplotypes with the highest similarity to *H. nakamurai*, pointing towards the possibility that the latter, which is an Indo-Pacific species, might share ancestry with *H. griseus* from the Pacific Ocean.

Specimens collected from Namibia (SEA), did not share any of the haplotypes observed with any other region (Table 2, Figs. 3, 4), not even with those from Madeira or Scotland (NEA), thus showing that there is lack of genetic homogeneity between the South and the North Eastern Atlantic Ocean. Similar heterogeneity within the Eastern Atlantic Ocean has been noted for other deep-water fish such as *Hoplostethus atlanticus* (Varela et al., 2013). The Namibian specimens of *H. griseus* were closely related to those collected from the North Atlantic Ocean (including the Mediterranean Sea), however they diverged by at least 9 bp from those collected from the Pacific Ocean, and were found to be more closely related to SWP than NEP. This indicates that the species might have spread out of the Pacific Ocean to the Eastern Atlantic Ocean through colonization across the Indo-Pacific Ocean and the southern coast of Africa (Teske et al., 2011). Such event could have occurred during interglacial periods (Peeters et al., 2004) during which strong currents might have driven Indo-Pacific species into the Eastern Atlantic Ocean (Gubili et al., 2010).

No genetic differences were noted between the four specimens collected from the NEA region as they all shared the same haplotype H6a (3869 bp). This was quite unexpected due to the fact that these specimens were obtained from two locations that are over 2800 km apart. H6a was also identified in two individuals from the CM but was absent in the WM, thus can be considered as a rare haplotype within the Mediterranean Sea, leading to a significant $\phi_{ST}$ value (0.246; P = 0.040) between samples of NEA origin to those of Mediterranean origin. These observations reveal a degree of population substructuring with the Strait of Gibraltar acting as a barrier, limiting connectivity between Mediterranean Sea from the Northern Atlantic Ocean (Fitzmaurice et al., 2005; Valsecchi et al., 2005; Chevolot et al., 2006; Patarnello et al., 2007; Meynard et al., 2012). Moreover, the haplotypes in the Mediterranean are monophyletic (Fig. 4), and the three major haplotypes identified in the Mediterranean Sea (68% of the analysed Mediterranean specimens) radiate out of H6 (Fig. 3) which is the one representing the NEA specimens. Similar patterns have been noted in other Mediterranean species such as *Raja clavata* and *Xiphias gladius* (Alvarado

**Table 3**
A table showing the highest likelihood for the migration rate (M) as estimated via MDIV (Nielsen and Wakeley, 2001).

| Sampling locations | M |
|---|---|
| Atlantic (including Mediterranean) vs Pacific Ocean | <0.01 |
| North-East Atlantic (including Mediterranean) vs South-East Atlantic Ocean | <0.01 |
| Western Mediterranean vs Central Mediterranean Sea | 0.72 |
| Central Mediterranean vs Eastern Mediterranean Sea | 0.40 |
| Western Mediterranean vs Eastern Mediterranean Sea | <0.01 |

Bremer et al., 2005; Chevolot et al., 2006), both of which branched off the North-Eastern Atlantic populations during interglacial events, when the sea level allowed easier connectivity between the two regions.

### 3.4. Population structure within the Mediterranean Sea

Geographic partitioning on small regional scale has been noted in a number of sharks such as for *Negaprion brevirostris* and *Negaprion acutidens* (Schultz et al., 2008), *Sphyrna lewini* (Nance et al., 2011) and *Triakis semifasciata* (Lewallen et al., 2007). However, given that *H. griseus* is widely distributed in the Mediterranean and given that it is a large long-lived species with a wide home range (Andrews et al., 2009, 2010), then it was expected that there would be genetic homogeneity in the Mediterranean region. Nonetheless, *H. griseus* exhibited a degree of geographic partitioning (Supplementary data – Table 6). The haplotypes were clustered together according to the specimen's origin. Specimens from the EM ($n = 8$) did not share any haplotypes with specimens of WM origin ($n = 34$) leading to a significant $\phi_{ST}$ value 0.709 ($P < 0.001$), while the CM ($n = 86$) did share some haplotypes with the other two basins, it differed greatly in the haplotype frequencies leading to significant $\phi_{ST}$ values (CM against WM 0.204, P-value < 0.001; CM against EM 0.367, $P < 0.001$). Therefore, the current data set shows strong evidence of haplotype segregation between the specimens of WM origin, CM origin and Sea of Marmara and the Aegean Sea (EM) origin. Although it cannot be excluded that there might be other haplotypes present in these regions that could have been detected using larger sample sizes, rarefaction analyses of the current data sets produced graphs that closely reached a plateau with converging 95% confidence intervals, indicating that the sampling strategy from the studied regions represents most of the haplotypes present in the respective areas. It is unlikely that the haplotype frequencies found in a particular sampling location would be present in another one.

Most of the specimens collected from the Balearic Sea (Valencia, Sete and Marseille) exhibited haplotype H2 (74%), which was also shared with those of CM origin, however its occurrence in CM was 31%. On the other hand, H1 was recorded in Tuscany (H1a), South-Eastern Sardinia (H1a) and Algeria (H1a, H1b and H1f), while H15a was a private haplotype that was noted only in the two *H. griseus* specimens collected from Milazzo (northern Sicily). Specimens from the Aegean Sea shared a common haplotype (H12), with H12b being present in all the specimens from the Sea of Marmara, North Aegean Sea and Ikara, a haplotype that was only detected in one specimen from the Central Mediterranean. While the specimen collected from the southern Aegean Sea (Crete), has shown a unique haplotype, H12c, which differed only in the VNTR region when compared to the northern Aegean Sea specimens. H12 only occurred in 8% of the CM specimens. The lack of genetic differences between the North Aegean Sea specimens and those from the Sea of Marmara, led to no population structure identification between these two areas. The CM region was characterized by a number of private haplotypes which includes H3, H11 and H12a that were present in 20.3%, 7.6% and 7.6% of the CM population and were not noted in studied specimens from any other region of the Mediterranean Sea.

Ice ages have also drastically changed the Mediterranean's marine ecosystems (Kettle et al., 2011) as a substantial reduction in the global sea level would have led the Mediterranean Sea to have a different shape from how it looks today (Lambeck and Bard, 2000). A fairly smooth unimodal mismatch distribution graph suggested that the Mediterranean population has undergone a historic population expansion. This was further corroborated by a non-significant SSD (0.0306, P-value = 0.1150) and Harpending's raggedness index (0.1142, P-value = 0.1754) together with a negative and significant Fu's Fs value (−4.6682, P-value = 0.0468) revealing that the population in the Mediterranean Sea has undergone expansion, probably after colonization from the North-Eastern Atlantic Ocean during an interglacial period around 170,000 years ago (as estimated through expansion models). Such event could have led to a population expansion in size from a few tens of individuals (estimated $N_{f0} = 37$) to a contemporary estimated effective female population size of around 100,000 individuals. This population expansion probably led *H. griseus* to colonize and make use of new breeding grounds. However, subsequent glacial maxima would have not only limited gene flow and the connectivity between the Mediterranean and the Atlantic Ocean (Strait of Gibraltar: currently ~300 m deep), but also the connectivity between the various basins of the Mediterranean itself, namely that between the EM and WM (Bozec et al., 2007). The connection between the Aegean Sea and the Sea of Marmara via the Dardanelles (currently ~55 m deep) would have become very shallow possibly closing this strait as the sea levels lowered below the strait's sill (Lericolais et al., 2011). These historic barriers, linked with female philopatric behaviour, would have later led to the genetic heterogeneity noted in the Mediterranean region. Similar scenarios have been recorded with other large migratory species, where physical barriers alone are not enough to explain the heterogeneity noted in the current population structure of cosmopolitan species (Keeney et al., 2003; Carlsson et al., 2004; Ramírez-Macías et al., 2007; Jorgensen et al., 2010; Portnoy et al., 2010; Nance et al., 2011; Tillett et al., 2012). Geographical characteristics are tightly linked to behavioural mechanisms including specific habitat preferences, female philopatry and migratory routes causing large long-lived highly migratory species, such as *H. griseus*, to form a pronounced population structure.

## 4. Conclusion

The mtDNA sequences of specimens from the Pacific Ocean and those from the Atlantic Ocean vary from each other by their number of repeats within the VNTR region and by unique haplotypes. These distinctions provide evidence for clear genetic differences between these two populations. The level of divergence between the Atlantic Ocean and the Pacific Ocean could be tightly linked to the presence of continental land masses and geographical distance that separate the populations. Moreover, specimens from each respective ocean also showed a degree of heterogeneity possibly because this shark species lacks pelagic life-stages, and its distribution is solely based on the movement and home ranges of the individuals themselves, in which case they are limited mostly to the continental shelf. Within this scenario, one cannot exclude female philopatry, as it plays an important role in shaping mtDNA phylogenetic patterns. The molecular heterogeneity noted points towards the need to manage the different subpopulations in order to conserve the species' entire genetic diversity given that its population is subdivided into genetically distinct units which are separated from each other by distance.

## Competing interests

The authors declare that they have no competing interests.

## Authors' contributions

Both authors participated equally in the project and approved the final manuscript.

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## Appendix A. Supplementary data

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