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Historical DNA as a tool to genetically characterize the Mediterranean sand tiger shark (*Carcharias taurus*, Lamniformes: Odontaspididae): a species probably disappeared from this basin

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ABSTRACT

1. The sand tiger shark (*Carcharias taurus*) is a coastal species distributed in temperate and sub-tropical waters, classified as “Vulnerable” at global level and “Critically endangered” in Eastern Australia, Southwestern Atlantic Ocean and Mediterranean Sea. Six populations (Northwestern Atlantic, Brazil, South Africa, Japan, Eastern Australia and Western Australia) with low genetic diversity and limited gene flow were identified worldwide, but genetic information for many other geographic areas are still missing. Specifically, this species is listed in several reports as part of the Mediterranean fauna, even if there is a lack of catches and sightings in recent years in this basin. In order to clarify the origin of *C. taurus* individuals caught in the past in the Mediterranean Sea, historical samples were genetically analysed.

2. Nine samples with a certain Mediterranean origin were collected from different European museums. Genomic DNA was extracted and ~ 600 bp of the mitochondrial DNA control region was amplified using eight overlapping species-specific primer pairs. Sequences obtained were aligned with all the haplotypes globally known so far.

3. Genetic analysis revealed the misidentification of one museum specimen. Among the remaining Mediterranean historical samples, three different haplotypes were recovered. Two of them previously observed only in South Africa and one described in both South African and Brazilian populations.

4. Results suggest a genetic relationship between Mediterranean sand tiger sharks and those from the Western Indian Ocean. According to previous studies, we hypothesized that during the Pleistocene the cold Benguela upwelling barrier was temporarily reduced allowing the passage of *C. taurus* individuals from the Indian to Atlantic Ocean. After the restoration of this phylogeographic barrier some individuals were trapped in the Atlantic Ocean and probably migrated northward colonizing the Western African coasts and the Mediterranean Sea.

KEYWORDS

ancient DNA, *Carcharias taurus*, endangered species, genetics

1. INTRODUCTION

The sand tiger shark (*Carcharias taurus* Rafinesque, 1810) is a lamniform shark characterized by a burly body and protruding teeth. It can be found in coastal temperate and sub-tropical areas, except in the Eastern Pacific Ocean, usually swimming in shallow waters close to sandy or rocky bottoms or submerged reefs (Compagno, 2001). Tracking and tagging studies have demonstrated that, despite the presence of some differences depending on the geographic area examined, this is a phylopatric species and undertakes north-south seasonal migrations (Lucifora *et al.*, 2002; Dicken *et al.*, 2007; Bansemer and Bennett, 2011; Kneebone *et al.*, 2014; Teter *et al.*, 2015; Haulsee *et al.*, 2018). *C. taurus* reaches sexual maturity at the age of six-seven years in males and nine-ten years in females (Goldman *et al.*, 2006). Gestation lasts between nine-twelve months and, together with intra-uterine cannibalism, leads to the birth of only two newborns every two years (Gilmore, 1993). As for many other sharks, the features of its life cycle (i.e. late sexual maturity, long gestation, low fecundity) make it extremely prone to the risk of extinction (García *et al.*, 2008). This risk is exacerbated by the drastic population decline observed in some areas as a direct consequence of coastal habitat degradation and overexploitation, due to by-catch and intentional fisheries (Pollard *et al.*, 1996; Otway *et al.*, 2004). For these reasons, in 2000, the IUCN classified the sand tiger shark as "Vulnerable" at global level (Pollard and Smith, 2000) and it is currently considered "Critically endangered" in Eastern Australia (Pollard, Gordon, Williams, Flaherty, & McAuley, 2003), Southwestern Atlantic Ocean (Chiaramonte *et al.*, 2007) and Mediterranean Sea (Walls and Soldo, 2016).

It is well-known that a reduction in size of wild populations leads to a loss of genetic diversity (Frankham, 1996), with a consequent decrease in the ability to adapt to future environmental changes and an increased probability of extinction (Frankham, 2005). In this context, to shed light on the conservation status of threatened sharks, such as *C. taurus*, genetic population analyses are necessary (Dudgeon *et al.*, 2012). Currently, there are a limited number of studies describing levels of genetic variation and connectivity between different populations of this species. The first was performed at regional scale (South Africa, Eastern and Western Australia) by Stow et al. (2006) using AFLP loci and the mitochondrial DNA control region (mtDNA CR) as molecular markers. The second one was performed at global level using a longer sequence of the mtDNA CR and six microsatellite loci (Ahonen *et al.*, 2009). Low levels of genetic diversity were demonstrated, probably related to historical processes rather than recent human-mediated bottleneck events (Stow *et al.*, 2006; Ahonen *et al.*, 2009). In addition, a genetic structure with six distinct populations corresponding to different geographic areas (Northwestern Atlantic, Brazil, South Africa, Japan,

Eastern Australia and Western Australia) was revealed, with a low gene flow shown only between Southern Africa and Brazilian populations. These results highlighted the necessity to manage the populations of this shark as distinct Evolutionary Significant Units (ESUs; Waples, 1991) for a better conservation of this species (Ahonen *et al.*, 2009).

Unfortunately, the genetic characterization of *C. taurus* populations seems to be still incomplete because for some geographic areas the current presence and abundance of this species is unknown, even if their existence has been historically well documented. This is the case of the Mediterranean Sea where the occurrence of this species was known in the past. Since the 1970s, records of *C. taurus* have become more and more sporadic until they ceased in the last decade (Fergusson *et al.*, 2002). Some of the last catches were made in Sicily (Fergusson *et al.*, 2002), Tunisia (Quignard and Capapé, 1972; Capapé *et al.*, 1976), Croatia (Lipej *et al.*, 2004) and Aegean Sea (Ismen *et al.*, 2009). The lack of contemporary records makes the sampling of individuals for genetic studies impossible, however, the analysis of historical samples of *C. taurus* from the Mediterranean area could be very useful to improve the phylogeography of this species.

A first attempt to extract good quality DNA from historical shark jaws and teeth, including from *C. taurus* specimens, was made by Ahonen & Stow (2008). Two different DNA extraction methods were successfully tested. As expected, a lower amplification success of historical DNA compared to a contemporary one was observed. In fact, the PCR amplification of DNA from ancient samples is usually difficult due to the high degradation and small concentration of DNA extracted and/or by the presence of PCR inhibitors (Pääbo *et al.*, 2004). Subsequently, DNA from historical tissue and jaw cartilage was analysed to confirm the previous hypothesized Indo-Pacific origin of Mediterranean great white sharks (*Carcharodon carcharias* Linnaeus, 1758) (Gubili *et al.*, 2011, 2015). In this paper, the mtDNA CR of historical samples of Mediterranean *C. taurus* was amplified and sequenced with the aim to genetically characterize sand tiger sharks observed and caught in the past in the Mediterranean Sea. The Mediterranean haplotypes found were then compared with haplotypes known from the literature in order to assess the presence of haplotypes endemic to the Mediterranean Sea and therefore to understand if the extinction of *C. taurus* in this basin may have affected the global genetic variability of the species.

2. METHODS

2.1 Precautions to work on historical DNA

Genetic analyses on ancient and historical samples are subject to a high risk of contamination by exogenous DNA. In order to avoid this problem, pre- and post- PCR work phases were performed in two separate laboratories located in different buildings (Pääbo *et al.*, 2004; Knapp *et al.*, 2012). In particular, the pre-PCR laboratory was equipped with two hoods provided with UV lamps, the first one dedicated only to DNA extraction and the second one to reagents and PCR preparation (Knapp *et al.*, 2012). The entrance to the pre-PCR area was allowed only to qualified staff equipped with total body coverall, laboratory shoes, safety glasses, face mask and two pairs of gloves (Knapp *et al.*, 2012). All laboratory surfaces were daily cleaned with 10% bleach and wiped with ethanol 70%. In addition, they were UV irradiated for 20-30 min before and after every work session. Laboratory equipment (micropipettes, glassware, plasticware, etc…) was exposed to UV light for 20-30 min before and after their use. In contrast, the post-PCR area was dedicated only to thermocycling, electrophoretic analysis of amplicons on agarose gel and preparation of samples for sequencing. The thermocycler placed in this area was dedicated only to the amplification of ancient or historical DNA and, after each PCR cycle, was decontaminated with UV light for 30 min. Moreover, each sample was analysed separated from others to avoid cross-contamination and, extraction and PCR controls were always added to detect if contamination occurred during work phases (Pääbo et al., 2004) .

2.2 Sampling and DNA extraction

An overview of the ichthyological collections of the main European museums was done through on-line resources and personal contact with curators in search of *Carcharias taurus* Mediterranean specimens. A total of nine historical samples of *C. taurus* with a certain Mediterranean origin (Table 1) were found and collected. Five samples were powder from jaw cartilage, two were pieces of cartilage and two were teeth (Table 1). All samples were decontaminated prior to DNA extraction to reduce the presence of exogenous DNA and inhibitors from their surface, thus reducing the risk of contamination and the probability of PCR failure (Rohland and Hofreiter, 2007). In the case of cartilage powder, the decontamination phase was performed before sampling. Specifically, the sampling area was chosen from an internal portion of the jaws and was previously scratched using sandpaper, washed with bleach and then rinsed with ultrapure sterile water. When the surface was perfectly dry, the cartilage powder was obtained using a drill equipped with a sterile drill bit at very low speed to avoid overheating and additional damage to DNA (Rohland and Hofreiter, 2007). The powder obtained was recovered in a sterile 1.5 ml microcentrifuge tube and the hole produced on the jaws was closed with dental restoration paste to make them invisible for museum visitors. For pieces of cartilage, the decontamination phase was the same as described

above for the jaw surface, while teeth were decontaminated using the protocol proposed by Rohland and Hofreiter (2007) with an additional final step consisting in the exposure to UV light for 30 min for each side of the tooth. After decontamination, small pieces of the root were cut using a serrated blade previously washed with DNA AWAY™ Surface Decontaminant (Thermo Scientific) and UV irradiated for 30 min per side. The root was chosen for DNA extraction because it was more accessible than the inner part. In addition, *C. taurus* teeth do not contain a pulp cavity, that usually has a higher quantity of DNA, but both the root and the inside of the tooth are made of osteodentine (Whitenack *et al.*, 2010).

Genomic DNA was extracted using the protocol developed for ancient bones by Yang, Cannon, and Saunders (2004) with some modifications. Samples were put in 4 ml of lysis buffer (0.5 M EDTA pH 8.0, 0.5% SDS, 0.5 mg/ml proteinase K) and were incubated overnight at 50°C in a washing bath with gentle orbital oscillation. After incubation, samples were centrifuged to facilitate the deposition of undigested materials, 3 ml of supernatant were recovered and transferred on Amicon Ultra-15 centrifugal filter units (MWCO 30kDa, Merck Millipore) to concentrate samples up to 125 µl. Finally, the recovered volume was purified using QIAquick PCR purification kit (Qiagen) and DNA was eluted in 100 µl of ultrapure sterile water.

2.3 Amplification and Sanger sequencing

A fragment of ~ 600 bp of the mtDNA CR (Ahonen *et al.*, 2009) was analysed in this study. In order to avoid amplification problems related to the low quality and quantity of DNA extracted from historical samples, eight overlapping primer pairs were designed (Table 2, Figure 1) using the software Primer3Plus (Untergasser *et al.*, 2012) and the complete mtDNA genome of *C. taurus* deposited in GenBank (Accession number: KF569943, Chang, Jabado, Lin, & Shao, 2015) as reference sequence.

PCRs were performed in a 25 µl reaction volume containing 1X PCR buffer, 1.5 mM $MgCl_2$, 0.08 mM of each dNTP, 0.48 µM of each primer, 4U of Platinum Taq DNA Polymerase (Invitrogen) and 3 µl of genomic DNA. All amplifications were performed in a BioRad T100™ Thermal Cycler (BioRad) with an initial denaturation step at 94°C for 7 min, followed by 60 cycles of 20 s at 94°C, 30 s at 54°C and 40 s at 72°C, with a final extension at 72°C for 7 min.

PCR products were checked on 2% agarose gel stained with GelRed™ (Biotium). All amplicons were sent to BMR Genomics (Padua, Italy) for Sanger sequencing, purified by exoSAP-IT™

(Thermo Scientific) and sequenced in both directions using an automated sequencer, ABIPRISM 3730XL (Applied Biosystems).

2.4 Alignment and data analysis

For all the samples, sequences obtained using each primer pair were checked by eye and assembled to have the complete sequence of interest. All historical sequences were checked with BLAST (Altschul *et al.*, 1990) and aligned using CLUSTALW (Larkin *et al.*, 2007) with the 11 haplotypes described so far at the global level (Ahonen *et al.*, 2009; Chang *et al.*, 2015; Wynne and Wilding, 2018). When necessary, the alignment was manually edited on BioEdit (Hall, 1999). For the sample PA002, the very low amplification success and the lack of a correspondence after the alignment with *C. taurus* sequences suggested a mislabelling of the museum specimen. For this reason, the short and not contiguous sequences obtained from this sample were checked using BLAST (Altschul *et al.*, 1990) and a morphological analysis of the teeth on the jaws was carried out (Compagno, 2001) using pictures taken during the sampling phase.

Excluding the PA002 sample, evolutionary relationships between all haplotypes were shown on a Median-Joining Network (Bandelt *et al.*, 1999) using Network 5 (Fluxus Technology Ltd., www.fluxus-engineering.com), considering also gaps and missing nucleotides. The ε parameter was set to zero and information from previous studies about sampled individuals and sampling locations (Ahonen *et al.*, 2009; Chang *et al.*, 2015; Wynne and Wilding, 2018) were added to the analysis.

3. RESULTS

DNA was successfully extracted and amplified from all the historical samples of *Carcharias taurus* (Table 1). The complete mtDNA CR sequence of 574 bp in length, previously analysed also by Ahonen et al. (2009), was obtained for five samples (FI002, PA001, PA003, PR004, XL002). Amplification failures produced 550 bp for PA004, 507 bp for PR001, 495 bp for XL001 and only 198 bp for PA002. Specifically, the primer pairs CtCR2 and CtCR3 failed the amplification of the samples PR001 and XL001, respectively. The sequence produced by the primer pair CtCR6 was not obtained for two samples, PA004 and PR001. For PA002, only CtCR4, CtCR7 and CtCR8 provided a PCR product.

Undoubtedly, the primer pair and the sample with the worst amplification success were CtCR6 and PA002, respectively. The comparison of the short not contiguous sequences obtained from PA002

with those of *C. taurus* and with all the sequences deposited in data banks did not show any perfect match. The morphological analysis of the jaws showed a probable misidentification of the museum specimen; teeth on the museum jaws have two lateral cusplets on each side of the main cusp, a characteristic of the small-tooth sand tiger shark (*Odontaspis ferox* Risso, 1810) (Compagno, 2001). The lack of the complete mitochondrial genome and/or the mtDNA CR sequence of this species in data banks makes the corroboration of morphological observations impossible and this sample was precautionarily excluded from the following analysis.

All sequences obtained from historical samples have been submitted to the GenBank database under accession numbers: MK434273-MK434280. The alignment of all *C. taurus* sequences known so far and those obtained in this study have allowed, on the basis of 18 polymorphic sites, the classification of Mediterranean historical samples into three previously described haplotypes: Haplotypes A, B and I (Table 3). Of the five samples for which the complete sequence of interest was obtained, four belonged to Haplotype A (FI002, PA001, PA003, PR004) and one to Haplotype I (XL002) (Table 3). The affinity to a specific haplotype was also clearly defined for two of the three Mediterranean incomplete sequences. Sample PA004 seems to belong to Haplotype A also in absence of the diagnostic site in 356 and, sample XL001 to Haplotype B also in absence of the diagnostic site 182 (Table 3). The classification of the sample PR001 was more difficult. The amplification failure of the primer pair CtCR2 did not mask any known polymorphic sites (Table 3), while the failure of the primer pair CtCR6 did not allow us to obtain the diagnostic site 356. This latter failure prevented us from understanding if sample PR001 belonged to Haplotype B or Haplotype J (Table 3).

The alignment result was also confirmed by the Median Joining network performed to visualize haplotypes relationships (Figure 2). In addition, the inclusion of information about sampling locations from other previous studies (Ahonen *et al.*, 2009; Chang *et al.*, 2015; Wynne and Wilding, 2018) was very useful because it showed that Mediterranean historical samples have the same haplotypes as *C. taurus* individuals sampled in South Africa (Western Indian Ocean) and Brazil (Western Atlantic Ocean). Specifically, five Mediterranean samples (FI002, PA001, PA003, PR004, PA004) belonged to Haplotype A and one (XL002) to Haplotype I, previously observed only in individuals sampled in South Africa (Figure 2). The sample XL001 was identified as Haplotype B, which was found in both South Africa and Brazil (Figure 2). The Network 5 software also included the PR001 sample within Haplotype B cluster, on the basis of the maximum parsimony principle (Figure 2).

4. DISCUSSION

The sand tiger shark (*Carcharias taurus*) is considered as "Critically endangered" within the Mediterranean Sea by the IUCN (Walls and Soldo, 2016). However, its presence in this basin is currently uncertain due to the lack of sightings and catches over the last decade, which suggest a probable extinction at regional scale (Fergusson *et al.*, 2002; Walls and Soldo, 2016). The use of DNA extracted from historical samples has allowed us to genetically characterize, for the first time, *C. taurus* individuals that inhabited the Mediterranean waters in the past and to suggest a possible route of colonization of this basin. Only eight specimens of certain Mediterranean origin were sampled and analysed. It was not possible to obtain a larger sample mainly because of the lack of information about the original catch location for most museum specimens and because some institutions do not allow samples to be taken from their collections.

MtDNA was successfully extracted from all the nine historical samples using a protocol developed for ancient bones (Yang *et al.*, 2004) and, in contrast to Ahonen and Stow (2008), a higher amplification success was achieved. Ahonen and Stow (2008) tried the DNA extraction and PCR amplification on 34 historical samples (20-40 years old) from different shark species, including of *C. taurus* (cartilage and teeth). The PCR amplification failed for 19 of them highlighting that the use of a single primer pair to amplify a region of ~ 700 bp of the mtDNA CR (Stow *et al.*, 2006) is unsuitable to analyse historical DNA. Instead, the use of overlapping primer pairs delimiting a region of 150-200 bp in length has been able to improve the amplification success of both historical and ancient DNA (Barnett *et al.*, 2014; Splendiani *et al.*, 2016, 2017; Cole *et al.*, 2018) and was successful also in this study. However, for three samples, an incomplete sequence was obtained probably due to the degradation of DNA extracted and/or to the presence of PCR inhibitors (Pääbo *et al.*, 2004). The primer pairs with the lowest amplification success was CtCR6 because it failed the amplification in two *C. taurus* samples. It was designed to amplify a sequence of 213 bp in length, while it is widely known that DNA molecules extracted from ancient samples rarely exceed 200 bp (Pääbo *et al.*, 2004). The presence of repeated motifs and a high AT content in the region encompassed by these primers have limited us in primer design. The repetition of a single base or dinucleotide motifs for many times in a DNA sequence can cause the incorrect pairing of the primers on the DNA template. In addition, the presence of AT rich sequences leads to primers with a very low melting temperature (Tm). A low Tm is responsible for pairing of the primers even in

regions with several mismatches, thus leading to the amplification of aspecific PCR products (Dieffenbach *et al.*, 1993).

Excluding the sample PA002, due to the probable misidentification of the museum specimen, all the other historical jaws and teeth undoubtedly belonged to *C. taurus* individuals. The mtDNA CR sequences obtained here were attributed to two different haplotypes (Haplotype A and I) previously reported only for South Africa and one (Haplotype B) shared by both South Africa and Brazil (Ahonen *et al.*, 2009). The incomplete sequence of PR001 could be attributed to two distinct haplotypes (Haplotype B and J) however, the presence of another Haplotype B among the Mediterranean historical samples (XL001) and the distribution of the Haplotype J only in Abu Dhabi waters (Chang *et al.*, 2015) suggest that the sample PR001 bears Haplotype B, as indicated also by the Median Joining network. The lack of new haplotypes from Mediterranean historical samples was probably due to the limited number of samples analysed or to the low rate of molecular evolution estimated for this species (Stow *et al.*, 2006; Ahonen *et al.*, 2009). Instead, the observation of haplotypes mainly described for South African individuals suggests a genetic relationship between Mediterranean sand tiger sharks and those from the Western Indian Ocean.

Ahonen et al. (2009) observed the deepest genetic divergence between the Northwest Atlantic population and all the others, while the lowest divergence was identified between South Africa and Brazil, which also share some haplotypes. In the first case, the major divergence was traced back to the formation of the Isthmus of Panama (~ 3 million years ago), which has definitively separated Atlantic and Pacific Oceans (Toonen *et al.*, 2016). On the other hand, the low differentiation between South African and Brazilian populations indicates a relatively recent connection (Ahonen *et al.*, 2009). The belonging of historical samples analysed here to haplotypes already described in the Western Indian Ocean highlights a recent origin also in the case of the Mediterranean sand tiger sharks excluding an ancient origin due to the separation between the Mediterranean Sea and the Indo-Pacific Ocean by the rising of the Isthmus of Suez (11-18 million years ago) (Toonen *et al.*, 2016). The Mediterranean Sea was separated many years before the formation of the Isthmus of Panama indicating that if the Mediterranean *C. taurus* are descendant from those trapped after the raising of the Isthmus of Suez, they should have a greater genetic divergence than observed.

The connection between the Red and Mediterranean seas was re-established in 1876, after the opening of the Suez Canal, and promoted the entry of Indo-Pacific species into the Mediterranean basin, a phenomenon known as “Lessepsian migration” (Por, 1978). However, this route for colonization by Lessepsian migrants of *C. taurus* is rejected as several evidences indicate that this

306 species was already present in the Mediterranean Sea before the opening of the Suez Canal: i) the
307 species was described for the first time by Rafinesque in 1810, based on an individual caught in
308 Sicilian waters (Compagno, 2001; Fergusson *et al.*, 2002), ii) other catches and sightings were
309 reported in the Mediterranean basin before the 1876 (Fergusson *et al.*, 2002) and iii) our historical
310 samples were mainly from the Western Mediterranean and the collection dates are earlier or close to
311 the date of the opening of the Suez Canal opening. A migration through the Red Sea can also be
312 hypothesized in the opposite direction (anti-Lessepsian migration), from the Mediterranean Sea to
313 the Western Indian Ocean, but anti-Lessepsian migrants are very rare (Por, 1978). In addition, the
314 low genetic diversity observed in the Mediterranean historical samples could be due to a "founder
315 effect" suggesting that the South Africa, characterized by the highest genetic diversity (Ahonen *et*
316 *al.*, 2009), was probably the origin of the Mediterranean population.

317 Thus, the most probable biogeographic way used by the sand tiger sharks to colonize the
318 Mediterranean Sea is along the Western African coasts. Ahonen et al. (2009) explained the low rate
319 of genetic differentiation and the gene flow observed between South African and Brazilian
320 populations by the establishment of a recent connection between Indian and Atlantic Ocean. The
321 Southwestern African coast is characterised by the presence of an upwelling zone, caused by the
322 northward flow of the cold Benguela Current, that acts as a phylogeographic barrier (Benguela
323 barrier) (Dudgeon *et al.*, 2012; Toonen *et al.*, 2016). During Pleistocene interglacial periods, the
324 northward cold Benguela current was reduced with a simultaneous expansion of the south-westward
325 warm Agulhas current (Peeters *et al.*, 2004) that seems to have promoted the passage of *C. taurus*
326 individuals from the Western Indian to Atlantic Ocean (Ahonen *et al.*, 2009). A similar pattern of
327 dispersion was also proposed to explain the genetic similarities observed for South Atlantic and
328 Indo-Pacific populations of other shark species such as *Carcharinus limbatus* (Keeney and Heist,
329 2006), *Carcharhinus longimanus* (Camargo *et al.*, 2016) and *Carcharhinus falciformis* (Domingues
330 et al., 2018).

331 A relatively recent colonization of the Mediterranean Sea by individuals of Indo-Pacific origin was
332 also suggested for the great white shark *Carcharodon carcharias* (Gubili *et al.*, 2011) and
333 confirmed by the analysis of historical samples (Gubili *et al.*, 2015). Contrary to what observed for
334 the Mediterranean sand tiger shark, the great white shark haplotypes from the Mediterranean Sea
335 were more similar to North-Eastern Pacific/Australia/New Zealand haplotypes and not to South
336 African (Western Indian Ocean) ones (Gubili *et al.*, 2011, 2015). This discrepancy is probably
337 related to the life history characteristics of the two species. Both species are characterized by natal
338 philopatry but shows a different migratory behaviour. *C. taurus* is a coastal species that usually

accomplish short migration, for example in the South-eastern coast of South Africa a seasonal north-south migration between mating, gestating and parturition areas was observed (Dicken *et al.*, 2006). *C. carcharias* instead has a high migratory capacity as documented by the observation of a trans-oceanic migration from South Africa to Western Australia (Bonfil *et al.*, 2005). Gubili et al. (2011) estimated that the separation between Mediterranean and Indo-Pacific white shark populations occurred during the Late Pleistocene, a period characterized by climate instability. During a trans-oceanic migration some Indo-Pacific white sharks reached South Africa and, following the expansion of the Agulhas current, were driven to the Eastern Atlantic Ocean. The chase of prey, such as Atlantic bluefin tuna and swordfish, that showed a similar dispersion pattern (Alvarado Bremer *et al.*, 2005) and the propensity to swim eastward to return to natal areas have forced them within the Mediterranean Sea.

In the case of *C. taurus*, an immediate colonization of the Mediterranean area seems unlikely because this species usually undertakes short migrations, only in one case a distance travelled of ~ 2000 km was observed (Dicken *et al.*, 2007). We propose that South African individuals have reached the Atlantic Ocean during the Pleistocene, when the cold Benguela Current was temporarily attenuated and the Agulhas current enhanced. The restoration of the cold Benguela upwelling barrier probably trapped some individuals of sand tiger shark along the Southeast African coasts from which they migrated northward to reach warmer habitats. In fact, *C. taurus* rarely tolerates temperature lower than 15°C (Lucifora *et al.*, 2002; Otway and Ellis, 2011; Smale *et al.*, 2012; Kneebone *et al.*, 2014; Teter *et al.*, 2015). The coastal behaviour of this species together with the propensity to accomplish north-south seasonal migrations probably allowed, following a stepping stone model of dispersion, the colonization of Western African coasts and finally entry into the Mediterranean basin. However, the lack of unique haplotypes among the Mediterranean historical samples and the lack of genetic data for Western Atlantic Ocean do not allow us to understand if Mediterranean sand tiger sharks belonged to a distinct population or if they were visitors from African Atlantic coasts (Fergusson *et al.*, 2002).

4.1 Conclusion

The decline of chondrichthyan species recorded at global scale and in particular in the Mediterranean Sea as a consequence of human activities is alarming (Ferretti *et al.*, 2008; Dulvy *et al.*, 2014). In this context, the importance of genetic tools to develop beneficial management and conservation strategies has been largely demonstrated (Dudgeon *et al.*, 2012). However, the difficulty in collecting shark specimens poses a serious limit to conservation genetic studies. This

limit can be overcome by the use of historical shark jaws and teeth that represent an alternative source of DNA (Ahonen and Stow, 2008; Gubili *et al.*, 2015; Nielsen *et al.*, 2017). In this study, the genetic analysis of historical samples helped us to genetically characterize Mediterranean sand tiger sharks using historical DNA and to hypothesize a biogeographic scenario for the colonization of the Mediterranean Sea by individuals coming from Western Indian Ocean. However, the limited number of samples and the complete lack of genetic information for some geographic areas (e.g. Eastern Atlantic Ocean) did not allow us to clarify if Mediterranean individuals belonged to a distinct population currently extinct or if they were vagrants from the African Atlantic coast (Fergusson *et al.*, 2002). The identification of previously described haplotypes among historical Mediterranean samples suggests that, if a Mediterranean *C. taurus* population had been lost, there would have not been a loss in terms of global genetic variability. Regarding individuals from African Atlantic coasts, a conservation planning to reduce the threats for this species could allow the recolonization of the Eastern Atlantic coast and probably of the Mediterranean Sea. Shark species of Western Africa have long been subjected to over-exploitation by fishing activities (Diop and Dossa, 2011), this could have led to the reduction of *C. taurus* populations also in this area. Further studies are therefore necessary to clarify the status of the Mediterranean sand tiger shark and to improve the global knowledge on this species. Following the last IUCN assessment for the sand tiger shark (Walls and Soldo, 2016), trends and dynamics in the world populations of this species are still unknown. Data about its distribution range and conservation status are absent or incomplete for several geographic area, as observed for the Mediterranean Sea and Eastern Atlantic Ocean. Fragmentation and isolation are known as factors that may weak subpopulations, and in the case of a species as the sand tiger sharks such vulnerable to coastal human impact (i.e. by-catch, commercial fisheries, habitat degradation), they can strengthen a declining process. Additional information about the distribution range, size of populations, levels of genetic diversity and gene flow between different geographic areas, also by the analysis of historical samples, must be obtained. These data could favour the development of regional and inter-regional conservation policies to prevent the extinction of *C. taurus* at local and global level and, if possible, to encourage the recolonization of areas from which it seems to have disappeared.

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589

590 **TABLES**

591 **TABLE 1** Information about museum specimens of *Carcharias taurus* sampled and analysed in the
592 present study

Genetic code	Institution	Institution code	Description	Sampling location and date	Reference	Sample type
FI002	Museo di Storia Naturale, Sezione di Zoologia "La Specola", Florence, Italy	INV6136	Taxidermied specimen, male, 170 cm length	Messina, Sicily, 15th November 1879	Vanni, 1992	Cartilage powder
PA001	Museo di Zoologia "Pietro Doderlein", Palermo, Italy	ID AN 68	Jaws	Sicily, second half of the XIX century	Doderlein, 1879	Cartilage powder
PA002	Museo di Zoologia "Pietro Doderlein", Palermo, Italy	ID AN 94	Jaws	Sicily, second half of the XIX century	Doderlein, 1879	Cartilage powder

PA003	Museo di Zoologia "Pietro Doderlein", Palermo, Italy	ID AN 60	Jaws	Sicily, second half of the XIX century	Doderlein, 1879	Cartilage powder
PA004	Museo di Zoologia "Pietro Doderlein", Palermo, Italy	ID AN 38	Skeleton	Sicily, second half of the XIX century	Doderlein, 1879	Cartilage powder
PR001	Muséum National d'Histoire Naturelle, Paris, France	A-9685	Taxidermied specimen, female	Algeria, ~ 1840	Guichenot, 1850 B. Seret, pers. comm.	Piece of cartilage
PR004	Muséum National d'Histoire Naturelle, Paris, France	AB-0038	Jaws	Algeria, ~ 1840	Guichenot, 1850 B. Seret, pers. comm.	Piece of cartilage
XL001	Royal Belgian Institute of Natural Sciences, Brussels, Belgium	507β	Jaws	Algeria, end of the XIX century	O. Pauwels, pers. comm.	Tooth
XL002	Royal Belgian Institute of Natural Sciences, Brussels, Belgium	1386β	Teeth collection, erroneously classified as *Odontaspis ferox*	Tunisia, 1st October 1933	O. Pauwels, pers. comm.	Tooth

TABLE 2 Primer pairs designed and used to amplify a portion of the mtDNA CR of *Carcharias taurus* historical samples

Primer name		Sequence 5' to 3'	Product length
CtCR1	**F**	CTTCAATCCTTGATCGCGTCA	135 bp
	R	CTTCCGGGGAATAGCGATGG	
CtCR2	**F**	TGGCATTTTCGTCCTTGATCG	146 bp
	R	TGAGTATGTTAGATAGATGTCGAGGA	
CtCR3	**F**	GGCTGAACTGGGACACTGAG	146 bp
	R	TCGAAACTTGCCGACTATGG	
CtCR4	**F**	TGTCAAGTTGACCAAAACTGAAA	118 bp
	R	CCGGATGGGGGTTAAGAGAG	
CtCR5	**F**	CCATAGTCGGCAAGTTTCGA	148 bp
	R	TGCCAGATAAAGTGAAGAATGTGT	
CtCR6	**F**	CTCTCTTAACCCCCATCCGG	213 bp
	R	GGGTTTTTCGAGGAGTCCGT	
CtCR7	**F**	ACACATTCTTCACTTTATCTGGCA	172 bp

	R	ATGTCCGGCCCTCGTTTTAG	
CtCR8	**F**	ACGGACTCCTCGAAAAACCC	141 bp
	R	TCATCTTAGCATCTTCAGTGCCA	

TABLE 3 Polymorphic sites detected after the alignment

	Polymorphic sites																	
	42	**131**	**182**	**318**	**330**	**335**	**337**	**339**	**356**	**407**	**408**	**420**	**421**	**427**	**444**	**445**	**562**	**572**
Haplotypes																		
HapA	T	C	A	C	A	G	G	G	T	A	G	-	-	G	G	A	G	A
FI002	.	.	.	.	.	.	.	.	.	.	.	-	-	.	.	.	.	.
PA001	.	.	.	.	.	.	.	.	.	.	.	-	-	.	.	.	.	.
PA003	.	.	.	.	.	.	.	.	.	.	.	-	-	.	.	.	.	.
PR004	.	.	.	.	.	.	.	.	.	.	.	-	-	.	.	.	.	.
PA004	.	.	.	.	.	.	.	.	?	.	.	-	-	.	.	.	.	.
HapB	.	.	.	.	.	A	.	.	.	.	.	-	-	.	.	.	.	.
XL001	.	.	?	.	.	A	.	.	.	.	.	-	-	.	.	.	.	.
PR001	.	.	.	.	.	A	.	.	?	.	.	-	-	.	.	.	.	.
HapJ	.	.	.	.	.	A	.	.	C	.	.	-	-	.	.	.	.	.
HapD	.	.	.	.	G	A	.	.	.	.	.	-	-	.	.	.	.	.
HapI	.	.	.	.	.	A	A	.	.	.	.	-	-	.	.	.	.	.
XL002	.	.	.	.	.	A	A	.	.	.	.	-	-	.	.	.	.	.
HapC	.	.	.	.	.	A	A	.	.	.	.	-	-	.	.	.	.	G
HapH	.	T	G	.	G	A	A	A	.	.	.	-	-	.	.	.	.	G
HapE	.	.	G	.	G	A	A	A	.	.	.	-	-	.	.	.	.	G
HapG	.	.	G	.	G	A	A	A	.	G	A	A	T	A	-	-	T	.
HapF	.	.	G	T	G	A	A	A	.	G	A	A	T	A	-	-	T	.
HapK	A	.	G	.	G	A	A	A	.	G	A	A	T	A	-	-	?	?

Haplotypes from previous studies were highlighted in grey (Ahonen *et al.*, 2009; Chang *et al.*, 2015; Wynne and Wilding, 2018). Haplotype A was used as a reference sequence. All identical nucleotides in other sequences are indicated as full stops (.), indels as dashes (-) and missing nucleotides as question marks (?). In the case of historical samples, missing data are due to amplification failures, whereas for Haplotype K (Wynne and Wilding, 2018) they are present because the sequence is shorter than the others (518 bp *vs* 574 bp).

FIGURE LEGENDS

FIGURE 1 Graphic representation of the eight overlapping primer pairs designed to amplify a portion of the mtDNA CR sequence in Mediterranean historical samples of *Carcharias taurus*. The numeration of the mitochondrial DNA started from the first base of the region studied by Ahonen et al. (2009)

FIGURE 2 Median Joining network showing the relationship between mtDNA CR haplotypes of *Carcharias taurus*. The circle size is related to the number of individuals sampled worldwide for each haplotype. Each colour indicates a different sampling location