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DNA from historical and trophy samples provides insights into white shark population origins and genetic diversity

Chrysoula Gubili[1,9,*], Cory E. C. Robinson[1,*], Geremy Cliff[2], Sabine P. Wintner[2], Eleonora de Sabata[3], Sabina De Innocentiis[4], Simonepietro Canese[4], David W. Sims[5,6,7], Andrew P. Martin[8], Leslie R. Noble[1,], Catherine S. Jones[1,**,***]**

[1]Institute of Biological and Environmental Science, School of Biological Sciences, University of Aberdeen, Zoology Building, Tillydrone Avenue, Aberdeen AB24 2TZ, UK

[2]KwaZulu-Natal Sharks Board, Private Bag 2, Umhlanga Rocks 4320, and Biomedical Resource Unit, University of KwaZulu-Natal, Durban 4000, South Africa

[3]MedSharks, via Ruggero Fauro 82, 00197 Rome, Italy

[4]Italian National Institute for Environmental Protection and Research, Marine Molecular Biology Lab (BMM), via Vitaliano Brancati 60, 00166 Rome, Italy

[5]Marine Biological Association of the United Kingdom, The Laboratory, Citadel Hill, Plymouth PL1 2PB, UK

[6]Marine Biology and Ecology Research Group, School of Biological Sciences, University of Plymouth, Drake Circus, Plymouth PL4 8AA, UK

[7]Ocean and Earth Science, National Oceanography Centre Southampton, University of Southampton, Waterfront Campus, European Way, Southampton SO14 3ZH, UK

[8]Department of Ecology and Evolutionary Biology, University of Colorado, N122 Ramaley, Boulder, Colorado 80309, USA

[9]*Present address:* Coordenação de Biodiversidade, Instituto Nacional de Pesquisas da Amazônia, Av. André Araújo 2936, Manaus, AM 69060-001, Brazil

ABSTRACT: Characterizing genetic variation by retrospective genotyping of trophy or historical artifacts from endangered species is an important conservation tool. Loss of genetic diversity in top predators such as the white shark *Carcharodon carcharias* remains an issue, exacerbated in this species by declining, sometimes isolated philopatric populations. We successfully sequenced mitochondrial DNA (mtDNA) D-loop from osteodentine of contemporary South African white shark teeth (from 3 jaws), and from 34 to 129 yr old dried cartilage and skin samples from 1 Pacific Ocean and 5 Mediterranean sharks. Osteodentine-derived sequences from South African fish matched those derived from an individual's finclips, but were generally of poorer quality than those from skin and cartilage of historical samples. Three haplotypes were identified from historical Mediterranean samples (n = 5); 2 individuals had unique sequences and 3 shared the contemporary Mediterranean haplotype. Placement of previously undescribed mtDNA haplotypes from historical material within both the Mediterranean and Pacific clades fits with the accepted intraspecific phylogeny derived from contemporary material, verifying our approaches. The utility of our methodology is in its provision of additional genetic resources from osteodentine (for species lacking tooth pulp) and cartilage of rare and endangered species held in often uncurated, contemporary and historical dry collections. Such material can usefully supplement estimates of connectivity, population history, and stock viability. We confirm the depauperate haplotype diversity of historical Mediterranean sharks, consistent with founding by a small number of Pacific colonizers. The consequent lack of diversity suggests serious challenges for the maintenance of this top predator and the Mediterranean ecosystem.

KEY WORDS: Teeth · White shark · *Carcharodon carcharias* · Museum specimens · Mitochondrial DNA · Genotyping · Mediterranean · Cartilage · Osteodentine

* Authors contributed equally
** Joint last authors
*** Corresponding author: c.s.jones@abdn.ac.uk

INTRODUCTION

Life-history characteristics of elasmobranchs (long life span, slow maturation, long gestation periods, and low fecundity) make them highly vulnerable to fishing pressure (Baum et al. 2003). Recent estimates suggest that 25% of described sharks and rays are threatened with extinction (according to IUCN Red List criteria; Dulvy et al. 2014). This makes the development of responsible sustainable stock exploitation and conservation strategies difficult (Dulvy & Forrest 2009), especially as only 8% of threatened shark and ray species are currently protected (McClenachan et al. 2012). Such difficulties may be exacerbated in species known to exhibit some form of natal philopatry. Here widespread protection in conjunction with local conservation efforts is required to preserve the management unit (MU; Avise 1995), as defined by connectivity sufficiently low that each population should be monitored and managed separately. In these instances, and where top predators or keystone species are established from small founding propagules, there is particular urgency to identify the tipping point where anthropogenic pressures begin to impact genetic diversity, and so the resilience of a stock. Supporting this view, Spielman et al. (2004) suggest there is significant erosion of genetic diversity in advance of apparent demographic declines, and a recent meta-analysis demonstrated a significant effect of fishing pressure on genetic diversity (Pinsky & Palumbi 2014). Hence, development of sustainable management strategies may benefit from incorporating longitudinal assessments of regional fishing pressure and declines in genetic diversity derived from a comparison of historical and contemporary material.

The great white shark *Carcharodon carcharias* (Linnaeus, 1758) is an apex predator, capable of long-distance migrations (Bonfil et al. 2005), displaying complex segregation by size and sex (Domier & Nasby-Lucas 2013, Jewell et al. 2013, Kock et al. 2013), and natal philopatry (Pardini et al. 2001, Jorgensen et al. 2010). It is classified as 'Vulnerable' in the IUCN Red List, and in 2004 was placed on CITES Appendix II. Many populations have undergone dramatic declines (Baum et al. 2003), and first estimates of white shark abundance in Californian waters seemed to suggest substantially smaller numbers than other large marine predators (Chapple et al. 2011), prompting urgent calls for protection. However, a recent study refuted this, indicating a greater estimated population size in the eastern Pacific (Burgess et al. 2014), while historic abundance trends in the western North Atlantic suggest recovering populations (Curtis et al. 2014). Nonetheless, genetic analysis of Australian white shark populations suggests estimates of contemporary effective population sizes approach levels at which adaptive potential may be lost (Blower et al. 2012). A similar concern was expressed following the observation that several contemporary white sharks sampled from across the Mediterranean all had the same Pacific clade mitochondrial haplotype (Gubili et al. 2011). In Turkish waters white sharks are considered extinct in the Sea of Marmara, although contemporary records of neonates in the northern Aegean Sea suggest nearby breeding grounds (Kabasakal 2014). These conflicting views and observations illustrate that the impact of anthropogenic effects on connectivity, and consequently genetic diversity and effective population size, are probably complex and currently poorly known for this species throughout most of its range.

Population genetic analysis has been useful for shark management and conservation efforts (Dudgeon et al. 2012). However, the veracity of population and demographic parameters estimated from analysis of DNA sequences depends to a large degree on sampling a reasonable number of individuals. For example, between-population migration estimates from molecular data improve with large sample sizes (~50 individuals) (Paetkau et al. 2004). Unfortunately, because white sharks are rare, large, and difficult to sample in an unpredictable marine environment, tissue for DNA analysis is difficult to obtain, hindering application of molecular genetics to address some conservation questions. Yet, in common with other apex predators, the many trophy artifacts, as well as jaws and teeth of white sharks, held in public and private collections may permit retrospective population genetic analysis, provided these dried specimens still contain intact DNA fragments of sufficient size to be reliably and routinely recovered and characterized.

Here we explore the potential of contemporary white shark teeth, containing only osteodentine (Vennemann et al. 2001) and no pulp cavity (filled with living connective tissue and odontoblasts), as a source of DNA. While Ahonen & Stow (2008) successfully extracted DNA from the pulp cavity of teeth and jaws of several shark species from the Carcharhinidae family, DNA recovery from dentine presents some technical challenges, but has been successful from mammal teeth (Pääbo 1989, Höss & Pääbo 1993, Pfeiffer et al. 1998). Additionally, we extended our investigations to skin and cartilage recovered from trophy specimens collected from the Mediterranean and Pacific Ocean.

In this study, we report on the first attempt to extract and amplify mitochondrial DNA (mtDNA) of a series of small overlapping contiguous sequences (Fulton & Stiller 2012) from the osteodentine of contemporary white shark teeth from South Africa. We have also used this method to recover sequences from dried skin and jaw cartilage of Mediterranean and Pacific Ocean white sharks collected 34 to 129 yr ago. This resource was used to test whether the prevailing Pacific origin hypothesis (Gubili et al. 2011) of Mediterranean white sharks can be refuted by sequencing the D-loop of more individuals.

MATERIALS AND METHODS

Sample preparation and extraction protocols

Jaw samples and small pieces of fins were taken from 3 individuals caught in beach protection nets (Cliff et al. 1989) at different locations along the east coast of South Africa (SA; KwaZulu-Natal) between 2004 and 2005 (Table 1). In addition, we assembled a collection of museum jaw cartilage and uncurated, dried tissue samples from 7 white sharks collected 33 to 128 yr ago from the Mediterranean Sea and Atlantic and Pacific Oceans (Table 1).

Teeth (n = 3–4) from each of the South African jaws were detached, cleaned, and washed overnight with Virkon (Day-Impex Limited). After drying, teeth were cut by a rotary power tool (FMTC 140HTK, Performance TM). Tool, cutting parts, surfaces, and vice were washed with 70% ethanol and 10% sodium hydroxide. Enamel was removed, and the osteodentine was placed in a 1.5 ml microcentrifuge tube. Osteodentine from each tooth (averaging 0.12 g) was initially washed, and subsequently crushed to powder on a Spex 6750 freezer mill (Spex SamplePrep).

Fragments (0.10 to 0.25 g) of historical tissue and jaw cartilage from Mediterranean, Atlantic and Pacific Ocean white sharks were rehydrated for 24 h in 1× TE buffer (10 mM Tris pH 7.5, 1 mM EDTA) to reduce aerosol contamination and increase rate of digestion. A standard phenol extraction protocol (Sambrook et al. 1989) was employed to obtain genomic DNA from all samples, modified by an additional 40 µl of Proteinase K added prior to final overnight incubation (55°C). Finclip DNA extractions for verification of tooth-derived sequences from contemporary SA individuals were performed in a different laboratory.

Contamination controls

Stringent measures employed in successful ancient/historical DNA projects (Valentine et al. 2008, McMenamin & Hadly 2012) were instigated to eliminate contamination risks. All DNA extractions, including those from teeth, took place in laboratories free of contemporary shark material, and separate tools were designated for use on historical tissue and cleaned with 25% sodium hypochlorite solution between samples. Three tissue extractions were performed for each sample, with the addition of a negative control consisting of only reaction reagents. PCR took place in a separate DNA-free location and included 2 negative controls. Replicate amplifications (n = 3–5) were performed for each sample, of which 2

Table 1. *Carcharodon carcharias*. Details of each sampled South African white shark (supplied by the KwaZulu-Natal Sharks Board), and historical material (obtained from museum and private collections). Tissue type used in analysis is also provided. Haplotype refers to the 749 bp partial mitochondrial DNA D-loop sequence haplotype referred to in Fig. 2. F: female; M: male; TL: total length; –: unknown

Sample ID	Capture date	Capture location	Sex	TL (mm)	Mass (kg)	Tissue type	Haplotype
Contemporary samples							
DUR04039	4 Nov 2004	Durban, South Africa	F	2210	100	Teeth & finclip	–
RB05086	24 Oct 2005	Richards Bay, South Africa	M	2598	170	Teeth & finclip	–
ISP05004	8 Sep 2005	Ispingo, South Africa	M	2570	134	Teeth & finclip	–
Historical samples							
GWMD3	1 May 1900	Atlantic	–	–	–	Jaw cartilage	–
GWMD10	1885	Port Jackson, NSW, Australia	M	–	–	Chondocranium	H20
GWMD11	10 Dec 1891	Liguria, Monterosso, Italy	F	~6000	–	Jaw cartilage	H24
GWMD12	1900	Toscana, Lucca, Viareggio, Italy	F	–	–	Finclip, full specimen	H1
GWMD20	29 May 1953	Tunara, Favignana, Italy	F	–	–	Dried fin, trophy board	H2
GWMD21	4 Apr 1980	Tunara, Favignana, Italy	F	–	–	Dried fin, trophy board	H2
GWMD15	–	Palermo, Sicily, Italy	–	–	–	Jaw cartilage	H2

to 3 products were sequenced in both forward and reverse directions to ensure the veracity of results. Sequence chromatograms were screened independently by eye and verified by colleagues to ensure accuracy of base calling.

MtDNA analyses

Five pairs of primers were designed using Primer Premier 5.0 (www.PremierBiosoft.com) to amplify independently 5 overlapping fragments, from 135 to 286 bp, of the D-loop sequence (Table 2, Fig. 1). However, due to A+T-rich regions of the mtDNA, there are necessarily overlaps with 3 pairs: D-loop1Reverse with D-loop2Forward (9 bases), D-loop3Reverse with D-loop4Forward (11 bases), and D-loop4Reverse with D-loop5Forward (14 bases). Nevertheless, each overlap contained only a single polymorphic site, the remaining amplicons allowing adequate haplotypic assignment (Pardini et al. 2001). Two additional primers were designed to target polymorphisms diagnostic of potential Atlantic haplotypes as distinct from those of Mediterranean or Pacific origin (Gubili et al. 2011). Of these, only one (D-loop7) was successfully used in this analysis, yielding a 206 bp product.

Ten nanograms of genomic DNA were used for 20 µl PCRs (polymerase chain reactions) containing 1× NH_4 buffer, 200 µM of each dNTP, $MgCl_2$ (1.5–2.5 mM; Table 2), 0.3 µM of each primer, and 1.0 U of AmpliTaq Gold™ DNA polymerase (Applied Biosystems) on a Biometra T-Gradient thermal cycler. Amplification conditions consisted of initial denaturation for 5 min at 94°C, followed by 40 cycles of 30 s at 94°C; 30 s annealing (temperature dependent on the primers used; Table 2), 30 s at 72°C; and a final extension step of 10 min at 72°C. The same quantity of genomic DNA from historical material was used for an initial 20 µl PCR using the primers GWSF6 (5′ TTG GCT CCC AAA GCC AAG ATT CT 3′) and PheCaCaH (5′ CTA CTT AGC ATC TTC AGT GCC 3′) (Gubili 2009) to achieve partial amplification of the full mitochondrial D-loop. The resulting PCR products, including all negative controls, were diluted 1/10, of which 1 to 3 µl was used for subsequent semi-nested PCRs of 40 µl volume, for all 6 overlapping primer sets. Positive controls were not used, as other DNA protocols for historical studies deem them to be of little value (Fulton & Stiller 2012). All amplicons were assayed on 2% agarose gels, purified using the QIAGEN QIAquick PCR purification kit following the manufacturer's instructions, and commercially sequenced.

To confirm the suitability of teeth as a source of DNA, sequences obtained from South African tooth samples were aligned to those generated from fin tissues of the same individuals, using ProSeq 3.2 (Filatov 2002). To determine their similarity to other available haplotypes, sequences derived from historical Mediterranean samples were aligned to white shark sequences available on GenBank, in-

Table 2. *Carcharodon carcharias*. Primer sequences and PCR (polymerase chain reaction) conditions for amplification of overlapping fragments of the white shark mtDNA D-loop

Primer	Primer sequence (5′–3′)	Annealing temp. (°C)	Amplicon size (bp)	$MgCl_2$ (mM)
D-loop1	ACA CGC ACG TAT ATT GCT AAC TG CCA AAA CTG AAA GGG ATA GAG AG	54	135	2.5
D-loop2	ATT ATG GCG TCA ATC TCT CTA TC GAG GCT CAT CTG GGA CAC TAA G	54	135	2.5
D-loop3	TAG AAG AGT GTC GAG GGG AGT AC AAT CCT CAT CAA CTG AAC AAA CC	54	286	2.5
D-loop4	TAA ATG TCA GGT TTG TTC AGT TG ATC CCC ATT CAT CTA CTT ACA GC	48	228	1.5
D-loop5	AAT GAA ATT GCT GTA AGT AGA TG CTG AAT GCT GTC AAA ACA TG	48	245	2.5
D-loop7	CGT ATC CAT TAT GGC GTC AAT CTC T GCG TCA AGA TTT ATT TTC CAC	60–63	206	2.5

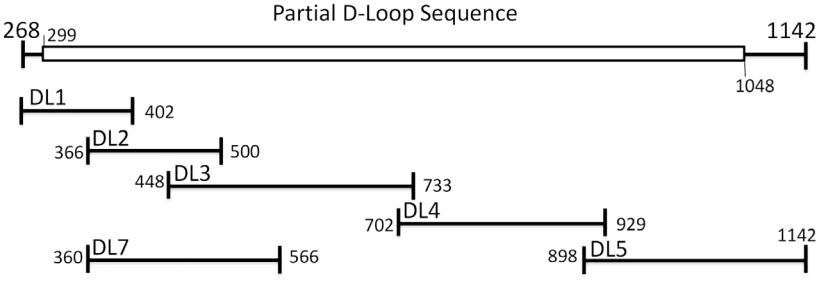

Fig. 1. *Carcharodon carcharias*. Schematic of partial mitochondrial D-loop sequence. Black bars show primer positions of Amplicons 1 to 5 and 7 of the D-loop (DL). White box along the partial sequence represents 749 base pairs of the sequence used in this analysis. Start and stop positions are provided for each primer amplicon and sequence used, and correspond to regions of the full mitochondrial DNA (mtDNA)

cluding 4 contemporary Mediterranean sequences (HQ540294 to HQ540296, Gubili et al. 2011; JF715925) and 91 sequences from worldwide locations (AY026196 to AY026224, Pardini et al. 2001; GU00 2302 to GU002321, Jorgensen et al. 2010; HQ414073 to HQ414086, Blower et al. 2012; KC914387, Chang et al. 2014; KC511601 to KC511626). These specifically included sequences from proximal populations (such as South Africa and North West Atlantic) which may be considered as potential source populations for the Mediterranean. Base pair positions with gaps/missing data were excluded from analysis. Haplotypes, haplotypic diversity, and average pairwise sequence differences were obtained using DnaSP 5.10.1 (Librado & Rozas 2009). A haplotype genealogy was constructed in HAPVIEW following the method of Salzburger et al. (2011) using a phylogenetic tree derived in PhyML v3.0 (Guindon & Gascuel 2003, Guindon et al. 2010) following 10 000 bootstraps using GTR+G+I as the evolutionary model inferred by JMODELTEST (Posada 2008).

RESULTS

DNA amplification from contemporary teeth

D-loop PCR products were recovered for each SA individual, although not all amplifications were equally successful. All sets of primers yielded a PCR product of the expected size in at least 1 individual. PCR using primer sets D-loop1, D-loop2, and D-loop4 yielded a product of the expected size in all samples (100%). Larger fragments produced by D-loop3 (286 base pairs [bp]) and D-loop5 (245 bp) primers were successful in only 1 (Durban) in 3 samples (33.33%), indicating that amplification success is dependent upon size of the target fragment due to the poor quality of template DNA ($p < 0.05$; $X^2_{27} = 40.4$). When aligned, the 5 overlapping sequences amounted to 874 bp (from Position 268 to 1142) of the D-loop. Sequences obtained from tooth samples were identical to those generated from the fin tissues.

MtDNA amplification from historical material

DNA was extracted from 7 historical samples (Table 1). The semi-nested PCR protocol was 74% successful across all amplicons, improving on the much lower success rate (26%) and poor reproducibility of non-nested reactions across all D-loop primer sets. D-loop2 was initially used on historical material, but discarded due to the poor quality of the sequence produced. No correlations between the age of sample and semi-nested PCR success rate were detected for each primer pair ($R = -0.356$ to 0.373). All forward and reverse sequences were identical and confirmed as *Carcharodon carcharias* by BLAST searches. Low-quality amplification for the D-loop4 amplicon from the historical Atlantic sample (GW MD3) meant it was excluded from the final analysis. However, when a smaller segment (510 bp) of sequence was analysed, this sample displayed a unique haplotype found within the Atlantic/South African grouping (data not shown). Assembled contigs from each of 6 remaining historical samples produced in each case a 749 bp partial sequence of the mtDNA control region (Fig. 1).

The historic samples were aligned to the 96 known contemporary white shark mtDNA sequences available in GenBank, revealing 88 polymorphic sites distinguishing 55 different haplotypes. These haplotypes were assembled into a network with 2 main lineages separated by a minimum of 30 nucleotide substitutions: one lineage was composed mainly of North West Atlantic and South African sequences, while the other included all Pacific haplotypes (Fig. 2). Contemporary Mediterranean samples (n = 4; GenBank HQ540294 to HQ540296, Gubili et al. 2011; JF715925) exhibited a single haplotype (H2), shared with 3 historical Mediterranean samples (GWMD15, 20, and 21; Fig. 2). Two additional historical Mediterranean haplotypes (H1, GWMD12, Toscana, Italy; H24, GWMD11, Monterosso, Italy) were identified from single individuals. Mediterranean haplotypes showed little differentiation from those of Pacific sharks; for example, only 3 mutational steps separate the common Mediterranean haplotype (H2) from the Northeast Pacific/Australia/New Zealand (H19) haplotype, and 6 steps separate it from the Southwest Pacific (Taiwan) haplotype (H6) (Fig. 2). The newly described historical Mediterranean haplotype H24 was separated from the common Mediterranean haplotype (H2) by only 3 mutational steps, and from Northeast Pacific/Australia/New Zealand (H19) and Southwest Pacific (Taiwan) haplotypes (H6) by 4 steps and 7 steps, respectively (Fig. 2). However, the historical Mediterranean haplotype H1, while separated by 6 mutational steps from the common Mediterranean haplotype H2, was only 2 steps removed from contemporary Australian/New Zealand sequences (H9); placing it firmly with contemporary Pacific haplotypes. Estimates of ave-

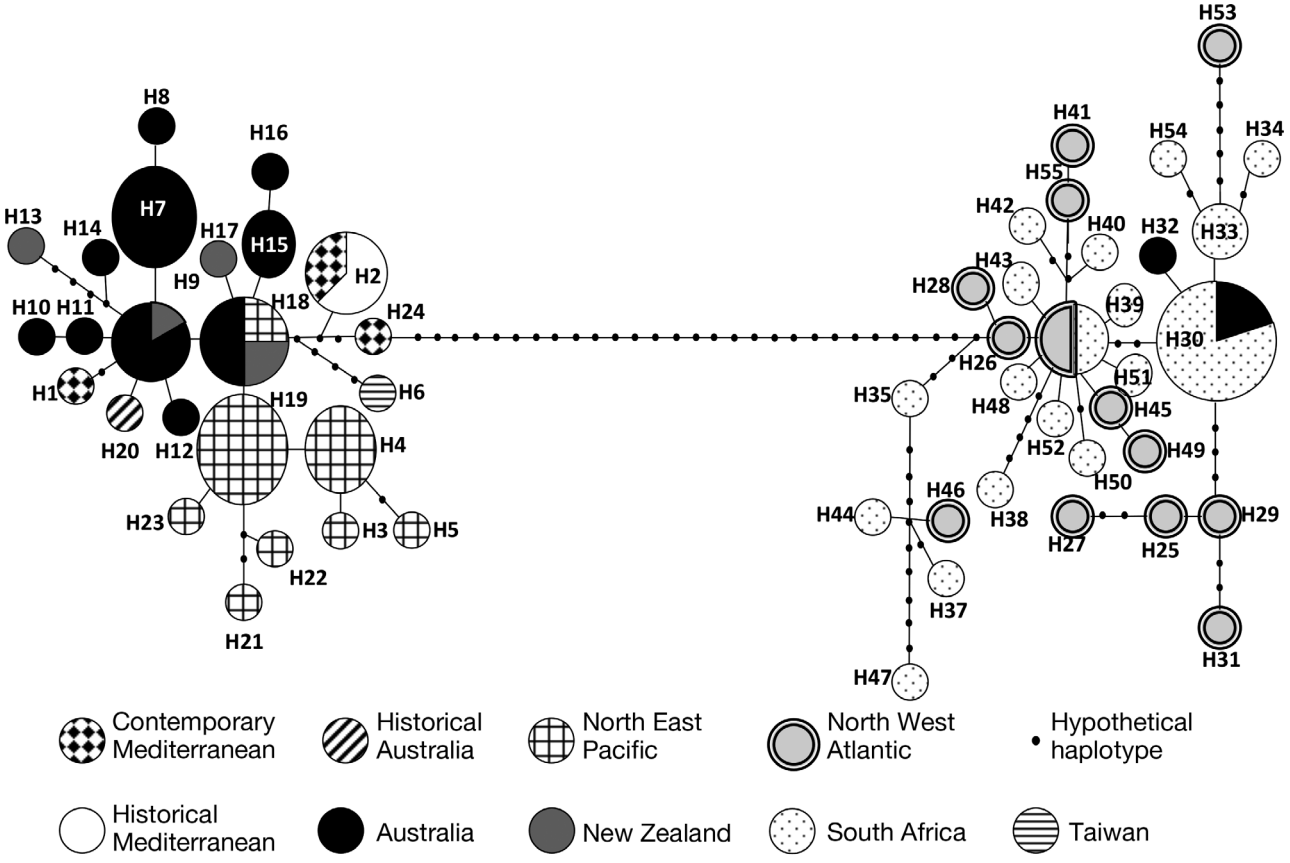

Fig. 2. *Carcharodon carcharias*. Median-joining network from a 749 bp partial mtDNA D-loop sequence consisting of 55 haplotypes derived from 6 historical and 96 contemporary white shark sequences, showing the low genetic differentiation of contemporary (H2) and historical (H1, H2, and H24) Mediterranean samples from Pacific (North East Pacific, Australia, and New Zealand) sharks. Circle size is proportional to the frequency of each haplotype; shading represents capture locality; small black circles represent hypothetical haplotypes; single mutational steps are assumed between haplotypes

rage pairwise sequence differences and haplotypic diversity were higher in the historical compared to the contemporary Mediterranean samples (Table 3). Haplotype H20, recovered from a historical Australian chondrocranium (GWMD10; New South Wales), was closely related to a contemporary Australian/New Zealand haplotype (H9), differing by a single mutational step.

Table 3. *Carcharodon carcharias*. Estimates of diversity for the contemporary and historical Mediterranean samples

	Contemporary	Historical
Sample size, n	5	5
Haplotype number	1	3
Average pairwise difference	0	3.00
Haplotype diversity ($1 - \sum p^2$)	0	0.70
Nucleotide diversity	0	0.00411

DISCUSSION

This is the first successful attempt to extract genomic DNA from contemporary white shark teeth, which do not contain pulp, and from dry tissue and jaw cartilage samples collected 34 to 129 yr ago. We report attempts to reconstruct larger mtDNA sequences by combining small overlapping amplicons to confirm the veracity of this methodology on shark teeth, and demonstrate its capacity for genotyping white sharks, determining their potential provenance through phylogeographic analyses of restricted samples from as little as 0.1 to 0.25 g of dried historical cartilage or tissue. Although only partial mitochondrial D-loop sequences were recovered, characterization of haplotypes is particularly informative in the white shark, as natal philopatry leads to certain haplotypes becoming characteristic of specific geographic areas (Pardini et al. 2001). Hence,

the approach outlined here allows investigation of genetic changes between historical and contemporary local shark stocks and identification of the putative origin of individuals — aspects central to estimates of stock viability and assessment of anthropogenic impacts.

Ahonen & Stow (2008) demonstrated successful DNA extraction and PCR amplification from only 44% of historical jaws or teeth collected 20 to 40 yr earlier from whaler sharks (family Carcharhinidae), grey nurse sharks *Carcharias taurus*, tiger shark *Galeocerdo cuvier*, and school sharks *Galeorhinus galeus*; teeth of all these species, except the nurse shark, possess a pulp cavity, which generally yields better quality DNA than that recovered from osteodentine. From these species they reported a 608 bp mtDNA D-loop sequence derived from amplicons of ca. 700 bp obtained using generic primers designed from a contemporary grey nurse shark. In contrast, our approach uses species-specific primers to target smaller overlapping amplicons, ensuring product fidelity in most historical, badly adulterated, and degraded material from teeth/jaws and tissue. This strategy reliably produced 6 amplicons of between 135 and 286 bp for reconstituting a mtDNA D-loop sequence of up to 874 bp from contemporary South African shark teeth and 749 bp from historical material. Yet the poorer quality and low yield of DNA from the osteodentine of contemporary white shark teeth suggest use of historical teeth may be possible but challenging.

In contrast to poor yields from teeth, rehydration of historical dried tissue and brittle cartilage gave good yields of genomic DNA. Additionally, variable success using primers producing larger products was greatly improved by implementation of a semi-nested PCR approach, often advantageous when working with degraded historical samples. To recover the full spectrum of haplotypes from historical material for comparison with contemporary samples, it was important to use additional primers (such as those for D-loop7F), to span areas of the sequence which were inaccessible due to the strategy of using a series of small overlapping amplicons, where informative polymorphic sites differentiating haplotypes were concealed within primer sites (e.g. Positions 386 and 451 within D-loop1R and D-loop3F primers, respectively) (Fig. 1). This highlights the importance of designing many overlapping primer sets to resolve false positives, polymorphisms within the primer sequence, and PCR artefacts, thereby providing sufficient fidelity to legitimately compare haplotype diversity between historical and contemporary materials.

Consistent, reproducible amplifications were obtained using DNA extracted from dried finclips (GWMD20 and 21), jaw cartilage preserved with lacquer (GWMD15), and from dried condocranium tissue, the oldest sample used in this study (GWMD10, collected in 1885), suggesting that tissue type and mode of preservation impact amplification success. Amplification of larger products may be possible with better sample preservation and from species with teeth containing a pulp cavity (Ahonen & Stow 2008). Whilst recovery of ancient/historical DNA is technically difficult, it promises novel and potentially important data of conservation significance for rare and endangered species (Alter et al. 2012).

The origins, population connectivity, and genetic diversity of white sharks in the Mediterranean are poorly known. All satellite tagging expeditions to date have been unsuccessful, and current hypotheses rely on historical capture and sighting data (Fergusson 2002). The first genetic study of contemporary Mediterranean material indicated this population exhibited little genetic diversity and suggested the ancestors of these sharks came not, as might be expected, from the adjacent Atlantic, but from distant Pacific stocks — perhaps a consequence of an anomalous migratory event (Gubili et al. 2011). Our recent results do not counter this view, with 3 haplotypes apparent in the 5 historical Mediterranean samples, 3 individuals sharing the contemporary Mediterranean haplotype (H2), and 2 new haplotypes (H1 & H24) clustering with contemporary Pacific sequences. This placement of haplotypes obtained from historical material with an accepted phylogeny derived from contemporary Mediterranean and Pacific samples supports the validity of our current methodological approach. Notably, 1 historic haplotype (H1) clusters with an Australia/New Zealand haplotype (H9), differing by only 2 mutational steps. This is no more distant than other contemporary Australian haplotypes, which raises the intriguing possibility that Mediterranean white sharks have multiple, and possibly more recent, Pacific founders. However, because this analysis is based on less mtDNA D-loop sequence than reported in Gubili et al. (2011), it is impossible to differentiate between the null hypothesis of H1 evolving *in situ* in the Mediterranean from founding stock, the most likely explanation for H24, or the alternative hypothesis that it indicates multiple, and perhaps more recent, migrations from the Pacific. It is notable that these additional haplotypes are present in the oldest (19th century) samples.

If further analysis of historical Mediterranean white shark material, for both mtDNA and nuclear markers, does not refute the conjecture of a potentially small, genetically isolated, and highly inbred population, then concerns should be raised about its ability to cope with rapid environmental and ecological change (Cheptou & Donohue 2011). As a result of current declines (Cavanagh & Gibson 2007, Storai et al. 2011) and the rarity of contemporary material, it is imperative that museum and trophy specimens found throughout the Mediterranean region and Europe are utilized to refine estimates of connectivity, decline of genetic diversity, and contemporary gene flow to assess the viability of Mediterranean white shark stocks.

Recourse to historical Mediterranean material may be the only way to study the genetic diversity of this threatened population, as artisanal fisheries throughout the region complicate management strategies, and contemporary captures are probably sold at market before they can be sampled (S. Canese pers. obs.). Yet reports of pregnant females off Tunisia and of neonates in the Aegean Sea suggest key nursery sites for this species are located within highly overexploited and data-deficient regions (Saïdi et al. 2005, Kabasakal 2008). In support, as a response to large declines in predatory sharks (Ferretti et al. 2008) and rapidly shifting trophic systems, increased pressure has been placed on regional fisheries management organizations throughout the Mediterranean to improve species-specific catch and landings data, prohibit finning and encourage full utilization, and assess management needs for elasmobranch conservation (Camhi et al. 2009).

Three out of 5 Mediterranean historical samples were of the contemporary haplotype, suggesting haplotypes of the 2 oldest samples have not been seen in the contemporary population sampled to date. Hence, this retrospective analysis of tissue archives, with the caveat of very small sample sizes and partial sequences, confirms the lack of haplotype diversity in contemporary sharks and tentatively suggests a loss of genetic diversity (Table 3) in the last 100 yr — a period during which white shark stocks and habitats have suffered degradation. Necessarily, the ecological implications of a lack, possibly as a result of recent loss, of genetic diversity in an apex predator extend beyond the demise of a single species.

CONCLUSIONS

This study demonstrates the validity of our approach for extraction, amplification, and sequencing of genomic DNA from white shark teeth and historical material. Historical DNA sequences can be combined with contemporary samples to increase sample size for rare species, affording estimates of changes in population and demographic parameters across centuries. Data from this approach are particularly relevant to conservation management of an endangered K-selected species exhibiting philopatric behaviour. Our findings give no cause for complacency, suggesting haplotype diversity of contemporary Mediterranean individuals is depauperate compared with that of other populations.

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