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Forensic genetic identification of sharks involved in human attacks

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Abstract :

Each year, 75-100 unprovoked shark attacks on humans are recorded, most of them resulting in no or minor injuries, while a few are fatal. Often, shark identification responsible for attacks relies on visual observations or bite wound characteristics, which limits species determination and preclude individual identification. Here, we provide two genetic approaches to reliably identify species and/or individuals involved in shark attacks on humans based on a non-invasive DNA sampling (i.e. DNA traces present on bite wounds on victims), depending on the knowledge of previous attack history at the site. The first approach uses barcoding techniques allowing species identification without any a priori, while the second relies on microsatellite genotyping, allowing species identification confirmation and individual identification, but requiring an a priori of the potential species involved in the attack. Both approaches were validated by investigating two shark attacks that occurred in Reunion Island (southwestern Indian Ocean). According to both methods, each incident was attributed to a bull shark (Carcharhinus leucas), in agreement with suggestions derived from bite wound characteristics. Both ap-proaches appear thus suitable for the reliable identification of species involved in shark attacks on humans. Moreover, microsatellite genotyping reveals, in the studied cases, that two distinct individuals were responsible of the bites. Applying these genetic identification methods will resolve ambiguities on shark species involved in attacks and allow the collection of individual data to better understand and mitigate shark risk.

Highlights

► DNA of sharks involved in attacks on humans could be collected from bite wounds. ► Barcoding reveals shark species identification thanks to recently developed primers. ► Microsatellite genotyping reveals both shark species and individuals. ► Two studied cases in Reunion involved different bull shark (*C. leucas*) individuals.

Keywords : Human-wildlife interactions, Shark bite, Barcoding, Genotyping, Microsatellite, Bull shark(Carcharhinusleucas), Tiger shark(Galeocerdocuvier)

Introduction

Attacks on humans by predators occur worldwide, and the results may be human injury or even fatality [1]. Some human-wildlife interactions, especially shark attacks, attract widespread attention and media reports [2]. This results both into a public perception of the probability of an attack much greater than it actually is, and the implementation of measures to mitigate the risk following public concerns [3,4]. Recent data demonstrates an increase (although disputed; see [5]) of the frequency of unprovoked shark bites (*sensu* [2]; [6]), which may be linked to the better recording of incidents [1], and to many socio-ecological interacting factors, such as the increase of human nautical activities and ecotourism, changes in the abundance of shark preys, or predator and ecosystem shifts [1,2,4,6–9].

Over the last 40 years, about 75-100 unprovoked shark attacks on humans were recorded each year, from almost 60 countries and territories [10]. However, the majority (> 80%) have occurred in six of them, often referred as "global shark attack hotspots": the United States, South Africa, Australia, Brazil, the Bahamas and Reunion Island [4,6]. Although most of these interactions resulted in no or minor injuries, similar to dog bites, some caused more serious trauma or fatalities (e.g. [11]).

Although any large shark can bite humans, three species seem repetitively involved in unprovoked bites or fatalities: the great white shark (*Carcharodon carcharias*), the bull shark (*Carcharhinus leucas*), and the tiger shark (*Galeocerdo cuvier*) [6,10]. A clear identification of the species involved in the attack is important both for risk management purposes and for the victims and their close relatives. Species identification often relies on direct visual observations of the shark by the victim or witnesses. Such identifications may be ambiguous due to the lack of knowledge of the diagnostic characters used to identify shark species, and to altered or insufficient observations in a traumatic situation. They can sometimes be supported by photographs or behavioural analyses performed by shark specialists based on testimonies, but therefore rely on the quality of the photographs and the accuracy of the testimonies. Furthermore, characteristics of the wounds, through jaw size, interdental distance, or, in rare cases, teeth embedded in human tissues, can help identifying the species and the size of the shark implicated [12–20]. Assignments to the species for the sharks involved in attacks are thus difficult and often disputable (e.g. [21–23]), and may be influenced by individual experiences, and knowledge of previous attack history at the site. Additionally, observations and wound characteristics only bring limited information about the individual such as an estimate of its size and rarely discriminant marks. Only the capture of a shark, with human remains attributable to the victim in its stomach (e.g. [24]), allows *a posteriori* species (and obviously individual) identification.

Genetic tools offer the possibility of accurately identifying both the species and the individual, should DNA of the shark be collected directly on the victim [25]. In terrestrial environments, non-invasive samples, such as hair, lost teeth, scat, and saliva, are already widely used to collect DNA of various taxa (mainly mammals [26–31], but also snakes [32]), from which barcoding or microsatellite genotyping approaches are applied to identify the species or the individual. DNA from these samples tends to be in low quantity and degraded, especially when collected late after the deposit [25,33]. Additionally to these constraints, aquatic environments tend to leach the samples, making the applications of DNA techniques difficult on surfaces that have settled into water or sea. However, two recent studies [34,35] have demonstrated that swab collection around bite wounds on depredated marine fishes allows collecting enough genetic material (i.e. DNA from cells left during the bite) to reliably identify the predator species (sharks or bony fishes), using barcoding approaches.

Based on results from these recent studies, genetic identification of shark species involved in attacks on humans from DNA traces present on bite wounds should be possible. While barcoding approaches cannot discriminate individuals, microsatellites should, but require an *a priori* of the species potentially involved. Therefore, we report here both barcoding and microsatellite genotyping approaches that can be used combined or independently to genetically identify species and/or individuals involved in shark bites on humans and were successful in identifying sharks involved in two fatalities in Reunion Island (southwestern Indian Ocean).

Materials and Methods

Sample collection

Swab samples were collected from bite wounds on two victims of shark attacks (referred hereafter as Cases A and B) that occurred in Reunion Island (southwestern Indian Ocean) between 2015 and 2020 (dates are inaccurate to preserve victims anonymity) and have been attributed to *C. leucas* (bull shark) based on wound shape observations during autopsies and supported by G. Cliff (personal communication). For Case A, sampling was performed in the hour following the incident, while for Case B, in the 12 hours due to the availability of coroners. In both cases, six samples were collected individually using dry sterile cotton swabs, rubbed around the edge and into the wound, and stored individually at -18°C until further laboratory processing (six months to one year after collection).

DNA extraction and quantification

For each case, total genomic DNA of three swabs (the three others were sent to collaborators for other experiments) was extracted individually, using the DNeasy Blood & Tissue kit (Qiagen^{TM}) following manufacturer's protocol, with few modifications: to be fully immersed in lysis buffer, each cotton tip was cut and incubated in 360 µL of ATL buffer and 40 µL of proteinase K, at 56°C during 90 min. Then, 400 µL of AL buffer and 400 µL of 96% ethanol were added. The three replicates were then pooled to increase DNA yield, and all mixture was transferred sequentially into a single DNeasy

Mini spin column, with several centrifugation steps to filter the whole volume. Next steps followed the manufacturer's protocol, except the elution, which was performed in 130 µL to minimize DNA dilution but to get a sufficient volume of final extract for subsequent PCR. Extraction quality was assessed through whole DNA concentration estimation in the two resulting extracts (i.e. one for each case) with a Qubit® 2.0 fluorometer and the Qubit® dsDNA BR Assay kit (Invitrogen™). Additionally, shark DNA was quantified with qPCR performed with specific primers.

Barcoding approach

The complete mitochondrial cytochrome oxidase c subunit I (COI) was amplified using the fish specific primer cocktails C_FishF1t1/C_FishR1t1 [36], and a shorter fragment (25-315) was amplified with the shark specific CO1shark25F/CO1shark315R primers [35]. PCR reactions were performed in a total volume of 25 µL with MasterMix Applied 1X (Applied Biosystems), 0.2 µM (primer cocktails) or 0.4 µM (specific primers) of each primer and ~2 $ng.\mu L^{-1}$ of genomic DNA, and with the following thermocycling program: 94°C for 5 min + 40 × [94°C for 30 s, 52°C (primer cocktails) or 64°C (specific primers) for 40 s, 72°C for 60 s] + 72°C for 7 min. PCR products were sent to GenoScreen (Lille, France), for sequencing on an ABI 3730XL DNA Analyzer (Applied Biosystems) in both directions. Sequences were quality checked and edited using Geneious 8.1.2 [37], then queried in BOLD Identification System [38].

Microsatellite approach

In Reunion Island, two of the three species of sharks repetitively involved in global attacks [6] are present year round: the bull shark (*C. leucas*) and the tiger shark (*G. cuvier*) [39]. For the microsatellite approach, based on the history of attacks [10] and the identification derived from bite wound characteristics, we hypothesized, independently from barcoding results, that individuals involved in our two cases might belong to these two species. Therefore, DNA samples were genotyped using 47 microsatellite loci, of which 19 were reported to be specific to *C. leucas*, 20 to *G. cuvier*, and eight cross-amplified in both species (see Table S1 in the supplements). To verify the species specificity of the primers, eight identified individuals from each species were genotyped along with the samples from the studied cases.

PCR were performed differently depending on whether forward primers were directly or indirectly fluorochrome labelled (with a 19 bp M13 tail; see Table S1). All PCR were conducted in a total volume of 10 µL, with 1X of MasterMix Applied (Applied Biosystems) and ~2 $ng.\mu L^{-1}$ of genomic DNA, but with 0.5 µM of each primer if forward primers were directly labelled or 0.025 µM of forward primer tagged with the M13 tail, 0.25 µM of reverse primer and 0.25 µM of fluorescent dyed M13 tail if indirectly labelled. The thermocycling program was the following: 94°C for 5 min

+ 7 × (94°C for 30 s, 62°C [-1°C at each cycle] for 30 s, 72°C for 30 s) + 35 × (94°C for 30 s, 55°C for 30 s, 72°C for 30 s) + 8 × (94°C for 30 s, 56°C for 30 s, 72°C for 30 s) + 72°C for 5 min. PCR products were genotyped in simplex using an ABI 3730XL DNA Analyzer (Applied Biosystems) at the Plateforme Gentyane (INRAE, Clermont-Ferrand, France). Allelic sizes were determined with GeneMapper 4.0 (Applied Biosystems) using an internal size standard (Genescan LIZ-500; Applied Biosystems), and signal strengths were noted.

Species identification

To identify the species involved in both cases, Bayesian assignment tests were performed using Structure 2.3.4 [40], on the 18 individuals (eight known as *C. leucas*, eight as *G. cuvier* and the two investigated) genotyped with the 47 loci. However, as species specific loci induce a high proportion of missing data which can biased the analyses, assignment tests were performed both considering all 47 loci and removing those with more than 25% missing data among the identified individuals (i.e. at most four individuals did not amplify). Five iterations at $K = 2$, with 10^6 MCMC generations after an initial burn-in of 10^5 generations, were run and then combined and visualised with Clumpak [41].

Individual identification

Microsatellite genotyping also allows the identification of the individual involved. Therefore, once the species identified, the genotypes of both cases were compared with each other and with those of individuals of the same species already genotyped (from [42] for *C. leucas* or from [43] for *G. cuvier*; available at https://doi.org/10.5061/dryad.kp32qr6 and at https://doi.org/10.5061/dryad.3161qp0, respectively), to identify repetitive Multi-Locus Genotypes (MLGs), and eventually identify individuals repeatedly involved in attacks or individuals previously captured and genotyped. The R 3.3.1 [44] package '*allelematch*' [45] was used to compute matching probabilities (following [46]).

Results and Discussion

DNA concentrations

DNA concentrations were similar between both extracts (Case A: 25.1 ng.µL^{-1}; Case B: 27.9 ng.µL^{-1}). However, these measures reflect the whole DNA concentration, and are not representative of the sole shark DNA. Indeed, we roughly estimated by qPCR that shark genomic DNA represented 20% of total genomic DNA (data not shown).

Barcoding approach

The complete COI sequences obtained with the fish primers [36] did not correspond to shark mtDNA. Indeed, for Case A, BOLD assigned the sequence at 100% to *Homo sapiens* (all top 100 matches from BOLD were 100% similar to the queried sequence), while for Case B, at 100% to *Pseudomonas* sp. (99.24% similarity with *P. putida* COI; GenBank accession n°AOUR02000103). However, the shorter COI fragments obtained with the shark specific primers [35] were identical for both cases (GenBank accession n°MW205905), and were assigned at 100% to *C. leucas* in BOLD. This suggests that both attacks were carried out by a bull shark, supporting identifications derived from bite wound characteristics.

Carcharhinus leucas mtDNA was not amplified and sequenced using the fish primers, possibly because these primers are not specific enough and the extracted DNA is predominantly human, or because they target too long a fragment. Indeed, Jo et al. [47] demonstrated that long environmental DNA fragments of the Japanese Jack Mackerel (*Trachurus japonicus*) decay faster than short ones. Similarly, even if mtDNA is present in many more copy numbers than nuclear one, short fragments would have been better preserved (and sequenced) in our samples. This suggests that the success of the barcoding approach to identify sharks from DNA collected on wounds primarily depends on the strict specificity of the primers, and then the size of the targeted fragment.

Microsatellite approach

Locus species specificity

Among the 19 loci initially thought to be specific to *C. leucas*, 10 successfully cross-amplified in at least one *G. cuvier* individual, of which five were polymorphic. However, three of these loci amplified in only one to four individuals, suggesting allele dropout in *G. cuvier*. Considering the 20 *G. cuvier*-specific loci, four cross-amplified in at least one *C. leucas* (two in at least five individuals), of which one was polymorphic (Fig. 1; see Table S2 in supporting information). Thus, nine of the 47 loci appear strictly specific to *C. leucas*, 16 to *G. cuvier* and 22 cross-amplify in both species (of which eight show a low amplification rate in one species or the other; Fig. 1; Table S2).

Cases genotyping

Among the 47 microsatellite loci, 28 and 17 amplified for Cases A and B, respectively, with signal strengths varying from 2,410 to 32,639 RFU and from 152 to 32,433 RFU, respectively (Fig. 1; Table S2). For Case A, among the 28 successfully amplified loci, eight were strictly specific to *C. leucas*, two to *G. cuvier*, and 18 were cross-amplifying loci, while for Case B, all 17 amplified loci were *C. leucas*-specific (seven loci) or cross-amplifying ones (10 loci; Fig. 1; Table S2). However, for this last case, three loci (Cl03, Cl06 and Cl19; Fig. 1) were found poorly reliable (weak signal strengths and odd peak morphologies; see Fig. S1 in supporting information) and were not readable. Additionally, signal strengths from Case B were inferior to 1,000 RFU for 10 loci, suggesting lower shark DNA availability or higher shark DNA degradation than Case A, though presenting similar whole DNA concentrations. Observed differences in amplification rates and signal strengths between both cases are likely due to delayed sample collection in Case B (12 hours after the incident *vs.* one hour in Case A). Sampling should therefore be carried out as soon as possible after the attack to reduce DNA degradation and increase microsatellite amplification rate.

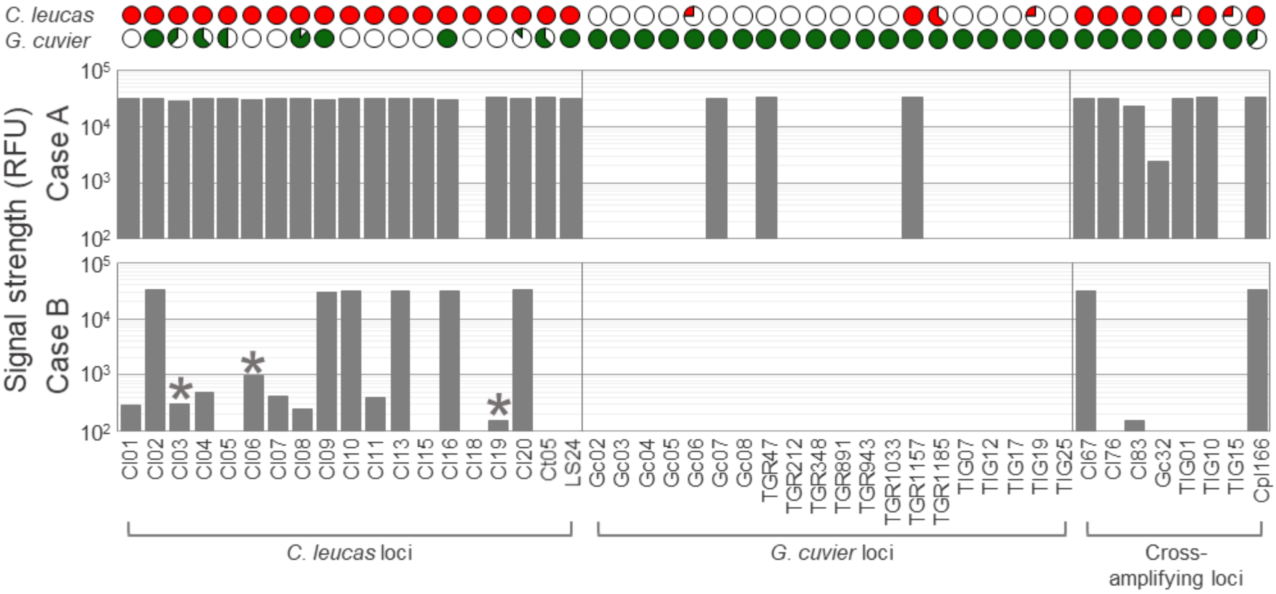

Fig. 1. Signal strength analysis and locus species specificity. Signal strength (log scale; $N = 1$) of the 47 *Carcharhinus leucas* and *Galeocerdo cuvier* loci for both cases. The proportion of amplified identified individuals from both species (over eight) is indicated above for each locus (red: *C. leucas*; green: *G. cuvier*). * indicates ambiguous amplified locus.

Species identification

At $K = 2$, considering all 47 loci or only those with less than 25% missing data among the identified individuals (15 cross-amplifying loci), both species were clearly separated by STRUCTURE (Fig. 2). All identified individuals were assigned to a specific cluster with a mean probability over the five runs greater than 0.993 (Fig. 2). The two unknown individuals were assigned to the *C. leucas* cluster with a mean probability of 0.999 and 0.993 for Cases A and B, respectively, when considering all loci (Fig. 2), and of 0.998 and 0.996, respectively, when considering only the 15 loci with less than 25% missing data among the identified individuals.

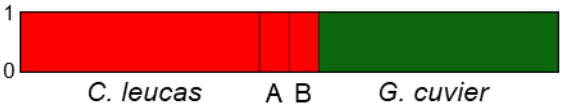

Fig. 2. Bayesian assignment analysis. Assignment probabilities for the 18 individuals [eight *Carcharhinus leucas*, eight *Galeocerdo cuvier* and the two unknown individuals (A and B; referring to the studied cases)] over the five runs of STRUCTURE at $K = 2$, based on the 47 loci. Results are similar when removing loci with more than 25% missing data among the identified individuals.

This suggests that a bull shark was responsible of each attack, consistent with the barcoding approach and coroners' identifications based on wound characteristics. Therefore, for species identification alone, one could use either the barcoding or the microsatellite approach (or both for more confidence), depending on knowledge of previous attack history at the site. Indeed, microsatellite approach alone requires an *a priori* of species identification to avoid testing hundreds of shark specific microsatellite markers, and identified individuals for the Bayesian assignment analysis (data available in public repositories for some species). Therefore, when the history of site attacks is not known, the barcoding approach seems the most suitable for species identification.

Individual identification

Considering the 14 loci (28 alleles) genotyped in both cases, individual genotypes differed from eight loci and nine alleles (see Table S2 in supporting information). Moreover, by comparing the genotypes of the two individuals involved in our studied cases with the 25-loci genotypes of the database from [42] ($N = 370$ individuals, including 126 from Reunion Island), no repetitive MLG was found. Matching probabilities of 8.17×10^{-6} and 1.15×10^{-4} were calculated for Cases A and B, respectively. This suggests that each investigated attack was performed by a distinct individual, which was apparently not previously captured and genotyped.

Identifying individuals involved in attacks and comparing their genotypes with those of previously sampled individuals as part of capture-mark-recapture programs (e.g. [39]) could allow collecting data such as sex, maturity, or size. Such data will provide a more precise portrait of the sharks involved in attacks, and will allow confirming or infirming recent theories on high-risk sharks (i.e. sharks with specific behaviours that may potentially pose a higher risk than conspecifics; [48]). It also allows population identification through individual assignment tests with existing database (this was not performed here, as all Indian and Pacific *C. leucas* individuals studied in [42] were assigned to a single genetic cluster with microsatellites therein). Finally, in Reunion Island, shark attacks trigger post-attack capture programs. Identifying both the individual involved in the attack and those captured allows evaluating the efficiency of this strategy, and possibly confirms that the individual responsible of the attack was captured. All this information will be useful in mitigating shark risk, responding to public concerns, and reducing captures of species or individuals not involved in attacks.

In conclusion, this study provides two genetic approaches to reliably identify species and/or individuals involved in shark attacks on humans, should genetic material be collected on the victim and conserved at -18°C shortly after the attack (< 24 h). Indeed the shorter the sample collection time, the higher the probability to successfully extract enough shark DNA. While the barcoding approach could be used to identify the species without any knowledge of the site attack history, the microsatellite genotyping approach identifies the individual in addition to confirming the species identification. Each approach can be used independently or conjointly, according to the degree of identification intended. Finally, applying these genetic identification methods will resolve ambiguities on shark species involved in attacks and allow the collection of individual data to better understand and mitigate shark risk.

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Supplementary materials

Table S1. Microsatellite loci used in this study.

Table S2. Microsatellite genotypes.

Fig. S1. Electrophoregrams.