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Forensic genetic identification of sharks involved in human attacks

Oury Nicolas ^{1,2}, Jaquemet Sébastien ^{1,3,*}, Simon Gwenola ⁴, Casalot Laurie ⁴, Vangrevelinghe Géry ⁵, Landron Fanch ⁵, Magalon éeène ^{1,2}

¹ Univ La Reunion, Univ Nouvelle Calédonie, UMR ENTROPIE, IRD,IFREMER,CNRS, St Denis, La Reunion, France.

² Lab Cogitamus, Paris, France.

³ Ctr Securite Requin, St Leu, La Reunion, France.

⁴ Aix Marseille Univ, Univ Toulon, MIO, CNRS,IRD, Marseille, France.

⁵ Squaldees, St Leu, La Reunion, France.

* Corresponding author : Sébastien Jaquemet, email address : sebastien.jaquemet@univ-reunion.fr

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 approaches 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*)

24 **Introduction**

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

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

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

56 Genetic tools offer the possibility of accurately identifying both the species and the individual,
57 should DNA of the shark be collected directly on the victim [25]. In terrestrial environments, non-
58 invasive samples, such as hair, lost teeth, scat, and saliva, are already widely used to collect DNA of

59 various taxa (mainly mammals [26–31], but also snakes [32]), from which barcoding or microsatellite
60 genotyping approaches are applied to identify the species or the individual. DNA from these samples
61 tends to be in low quantity and degraded, especially when collected late after the deposit [25,33].
62 Additionally to these constraints, aquatic environments tend to leach the samples, making the
63 applications of DNA techniques difficult on surfaces that have settled into water or sea. However,
64 two recent studies [34,35] have demonstrated that swab collection around bite wounds on depredated
65 marine fishes allows collecting enough genetic material (i.e. DNA from cells left during the bite) to
66 reliably identify the predator species (sharks or bony fishes), using barcoding approaches.

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

74

75 **Materials and Methods**

76 **Sample collection**

77 Swab samples were collected from bite wounds on two victims of shark attacks (referred
78 hereafter as Cases A and B) that occurred in Reunion Island (southwestern Indian Ocean) between
79 2015 and 2020 (dates are inaccurate to preserve victims anonymity) and have been attributed to
80 *C. leucas* (bull shark) based on wound shape observations during autopsies and supported by G. Cliff
81 (personal communication). For Case A, sampling was performed in the hour following the incident,
82 while for Case B, in the 12 hours due to the availability of coroners. In both cases, six samples were
83 collected individually using dry sterile cotton swabs, rubbed around the edge and into the wound, and
84 stored individually at -18°C until further laboratory processing (six months to one year after
85 collection).

86

87 **DNA extraction and quantification**

88 For each case, total genomic DNA of three swabs (the three others were sent to collaborators
89 for other experiments) was extracted individually, using the DNeasy Blood & Tissue kit (Qiagen™)
90 following manufacturer's protocol, with few modifications: to be fully immersed in lysis buffer, each
91 cotton tip was cut and incubated in 360 µL of ATL buffer and 40 µL of proteinase K, at 56°C during
92 90 min. Then, 400 µL of AL buffer and 400 µL of 96% ethanol were added. The three replicates were
93 then pooled to increase DNA yield, and all mixture was transferred sequentially into a single DNeasy

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

100

101 **Barcoding approach**

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

112

113 **Microsatellite approach**

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

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

129 + 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
130 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
131 products were genotyped in simplex using an ABI 3730XL DNA Analyzer (Applied Biosystems) at
132 the Plateforme Gentyane (INRAE, Clermont-Ferrand, France). Allelic sizes were determined with
133 GENEMAPPER 4.0 (Applied Biosystems) using an internal size standard (Genescan LIZ-500; Applied
134 Biosystems), and signal strengths were noted.

135

136 *Species identification*

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

145

146 *Individual identification*

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

155

156 **Results and Discussion**

157 **DNA concentrations**

158 DNA concentrations were similar between both extracts (Case A: 25.1 ng.μL⁻¹; Case B:
159 27.9 ng.μL⁻¹). However, these measures reflect the whole DNA concentration, and are not
160 representative of the sole shark DNA. Indeed, we roughly estimated by qPCR that shark genomic
161 DNA represented 20% of total genomic DNA (data not shown).

162

163 **Barcoding approach**

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

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

180

181 **Microsatellite approach**

182 *Locus species specificity*

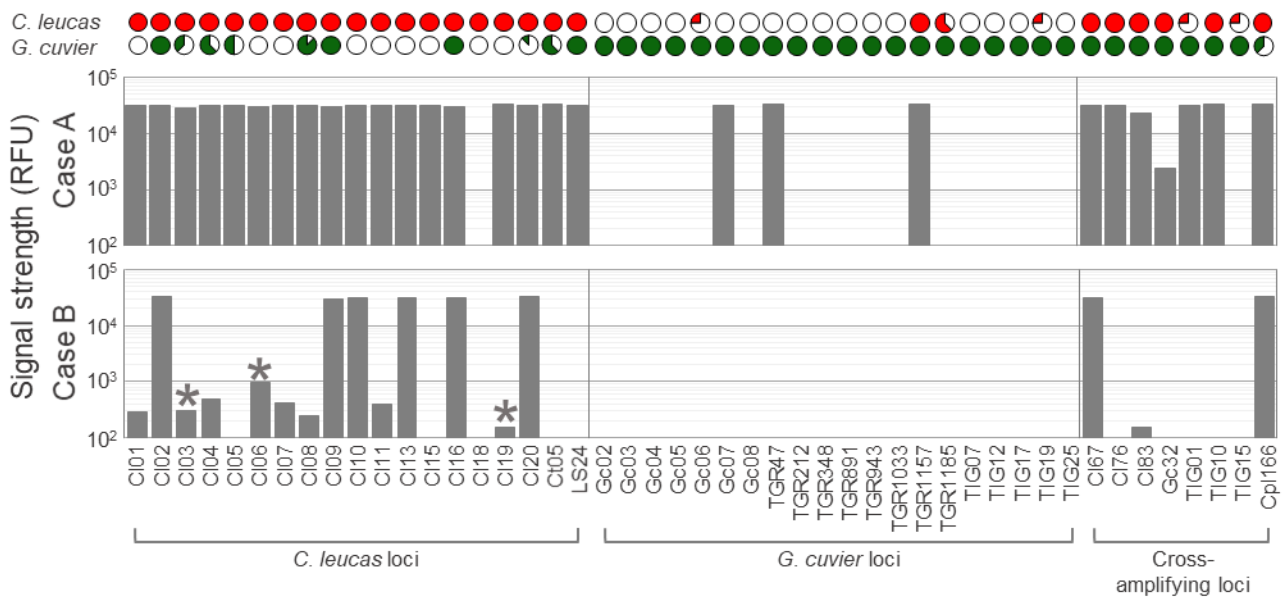
183 Among the 19 loci initially thought to be specific to *C. leucas*, 10 successfully cross-amplified
184 in at least one *G. cuvier* individual, of which five were polymorphic. However, three of these loci
185 amplified in only one to four individuals, suggesting allele dropout in *G. cuvier*. Considering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

190

191 *Cases genotyping*

192 Among the 47 microsatellite loci, 28 and 17 amplified for Cases A and B, respectively, with
193 signal strengths varying from 2,410 to 32,639 RFU and from 152 to 32,433 RFU, respectively (Fig. 1;
194 Table S2). For Case A, among the 28 successfully amplified loci, eight were strictly specific to
195 *C. leucas*, two to *G. cuvier*, and 18 were cross-amplifying loci, while for Case B, all 17 amplified loci
196 were *C. leucas*-specific (seven loci) or cross-amplifying ones (10 loci; Fig. 1; Table S2). However,
197 for this last case, three loci (CI03, CI06 and CI19; Fig. 1) were found poorly reliable (weak signal

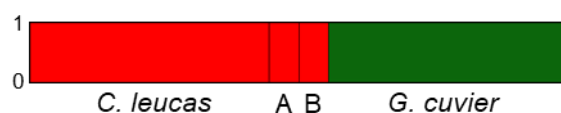
198 strengths and odd peak morphologies; see Fig. S1 in supporting information) and were not readable.
 199 Additionally, signal strengths from Case B were inferior to 1,000 RFU for 10 loci, suggesting lower
 200 shark DNA availability or higher shark DNA degradation than Case A, though presenting similar
 201 whole DNA concentrations. Observed differences in amplification rates and signal strengths between
 202 both cases are likely due to delayed sample collection in Case B (12 hours after the incident vs. one
 203 hour in Case A). Sampling should therefore be carried out as soon as possible after the attack to
 204 reduce DNA degradation and increase microsatellite amplification rate.
 205



206 **Fig. 1.** Signal strength analysis and locus species specificity. Signal strength (log scale; $N = 1$) of the
 207 47 *Carcharhinus leucas* and *Galeocerdo cuvier* loci for both cases. The proportion of amplified
 208 identified individuals from both species (over eight) is indicated above for each locus (red: *C. leucas*;
 209 green: *G. cuvier*). * indicates ambiguous amplified locus.
 210

211 *Species identification*

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

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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.

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Individual identification

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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.

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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

254 shark risk, responding to public concerns, and reducing captures of species or individuals not involved
255 in attacks.

256

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

267

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414 **Supplementary materials**

415 **Table S1.** Microsatellite loci used in this study.

416 **Table S2.** Microsatellite genotypes.

417 **Fig. S1.** Electrophoregrams.