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ORIGINAL ARTICLE

Characterization of 35 new microsatellite markers for the blacktip reef shark (*Carcharhinus melanopterus*) and cross-species amplification in eight other shark species

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Abstract

Background Shark species are overfished at a global scale, as they are poached for the finning industry or are caught as bycatch. Efficient conservation measures require fine-scale spatial and temporal studies to characterize shark habitat use, infer migratory habits, analyze relatedness, and detect population genetic differentiation. Gathering these types of data is costly and time-consuming, especially when it requires collection of shark tissue samples.

Methods and results Genetic tools, such as microsatellite markers, are the most economical sampling method for collecting genetic data, as they enable the estimation of genetic diversity, population structure and parentage relationships and are thus an efficient way to inform conservation strategies. Here, a set of 45 microsatellite loci was tested on three blacktip reef shark (*Carcharhinus melanopterus*) populations from three Polynesian islands: Moorea, Morane and Tenararo. The set was composed of 10 previously published microsatellite markers and 35 microsatellite markers that were developed specifically for *C. melanopterus* as part of the present study. The 35 novel and 10 existing loci were cross-amplified on eight additional shark species (*Carcharhinus amblyrhynchos, C. longimanus, C. sorrah, Galeocerdo cuvier, Negaprion acutidens, Prionacea glauca, Rhincodon typus* and *Sphyrna lewini*). These species had an average of 69% of successful amplification, considered if at least 50% of the individual samples being successfully amplified per species and per locus.

Conclusions This novel microsatellite marker set will help address numerous knowledge gaps that remain, concerning genetic stock identification, shark behavior and reproduction via parentage analysis.

Keywords Microsatellites · Genetic diversity · Sharks · Cross-species transfer · Pacific Ocean

Introduction

Over a third (37.5%) of all Chondrichthyan species are threatened with extinction [8, 15, 16, 50]. All shark species are currently threatened by overfishing and bycatch [1, 23]. Sharks are mesopredators in marine ecosystems, as they have a key role in maintaining predation pressure on lower trophic levels [35, 44]. The loss of sharks in marine ecosystems has ramifications, such as inducing trophic cascades [23].

Blacktip reef sharks (*Carcharhinus melanopterus*) live in coral reef ecosystems and have a tropical and subtropical Indo-Pacific distribution from eastern Africa to the Red Sea and in French Polynesia where they are poached for their fins used in traditional Asian meals [10]. French Polynesia is the world's largest shark sanctuary, spanning an area of 4.7 million km^2 [56]. Blacktip reef shark populations are very abundant around all islands and atolls of French Polynesia, where they were found to be genetically isolated among the different archipelagos, isolated islands, and atolls [55]. The limited gene flow combined with existing anthropogenic threats on sharks suggests the threat of depletion of isolated local populations [7, 11, 55]. In this context, understanding how to optimize blacktip reef shark conservation strategies by targeting protection of certain types of habitats such as

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shark nurseries or adult individuals that have a high reproductive output is essential. Genetic analysis tools have the potential to reveal habitat use or identify lineages to improve measures aimed at increasing juvenile shark survival, as demonstrated in other vertebrates [36, 46]. Genetic markers enable the estimation of the genetic effective sizes of populations, demonstrate inter-island gene-flow and allow parentage analyses to be conducted [9, 13].

Microsatellites are short, simple DNA sequences composed of a specific motif that is repeated between 7 to 50 times [4] and dispersed throughout the eukaryotic nuclear genome [12, 28]. While such markers were developed for particular species, because of the time and resources that are required for their development, testing the cross-species transferability of these markers is essential to facilitate their use in further studies [5, 33, 40]. For *C. melanopterus*, several previous studies used 14 microsatellites originally developed for other shark species, such as *C. limbatus* [25], *C. plumbeus* [41] and *Negaprion brevirostris* [17], to explore population structure and to conduct parentage analysis. To date, no microsatellite markers were developed specifically for *C. melanopterus* [13]. While recent studies urge the use of single nucleotide polymorphism (SNP) markers for population genetic structure analyses, microsatellite markers remain the most cost-efficient in terms of library preparation and scoring [52, 19]. Moreover, microsatellites are especially successful when focusing on individual segregation, fingerprinting and more generally in parentage analysis as they are abundant in the genome and highly polymorphic [20, 18, 26, 14, 29].

The aim of this study was to develop a microsatellite library specifically for *C. melanopterus* that would provide enough microsatellite markers to study genetic structure and parentage links in blacktip reef shark populations in French Polynesia. Further, we conducted cross-amplification tests of the newly developed markers for *C. melanopterus* on eight other shark species.

Material and methods

Sampling

A total of 120 individuals sampled around three Pacific islands were used in this study: Moorea (an island located in the Society archipelago; 17° 29′ 31″ S, 149° 50′ 08″ O), Morane (an atoll located in the south of the Tuamotu and Gambier archipelago; 23° 10′ 0″ S, 137° 8′ 0″ W) and Tenararo (an atoll located in the Acteon Group, in the south of the Tuamotu; 21° 18′ 0″ S, 136° 45′ 0″ W). Moorea is separated from Morane and Tenararo by 1460 km and 1435 km, respectively. Morane and Tenararo are separated by 208 km. Samples from each of the respective islands were collected to ensure the representativeness of the genetic diversity of blacktip reef sharks across French Polynesia (Table S2).

For cross-species amplification tests, eight species of sharks were selected to test the transferability of the microsatellite markers, including: **15** *C. amblyrhynchos,* **5** *C. longimanus,* **15** *C. sorrah,* **15** *Galocerdo cuvier,* **8** *Negaprion acutidens,* **8** *Prionace glauca,* **15** *Rhincodon typus* and **15** *Shyrna lewini.* Samples were selected from the shark fin-clip collection at the Center for Island Research and Environmental Observatory (CRIOBE), in Moorea. Members of the CRIOBE collected *C. melanopterus* and *N. acutidens* fin clips using a gillnet with a 5 cm mesh size that was set perpendicular to shore around Moorea Island. Samples of adult sharks were collected using a fishing rod with a barbless hook or with a biopsy probe [34]. Other samples used for cross-species amplification were collected by international partner institutions (see Acknowledgments).

DNA extraction and design of *C. melanopterus*-specific microsatellites

Total DNA was extracted from eight *C. melanopterus* individuals with the QIAcube HT DNA extraction robot (QIAGEN, Hilden, Germany) and sent to GenoScreen (Lille, France) for microsatellite library preparation and sequencing. A total of 1 µg from an equimolar DNA pool (41.69 ± 0.035 ng/µL) of the eight specimens was used for the development of Illumina MiSeq Nano library, sequenced on the MiSeq platform Illumina (San Diego, CA). The obtained sequences were merged with the software PrinSeq [47], and the final analysis and primer design were completed with the software QDD v.3 [31]. Among 1,650,409 raw sequences, 867,286 merged sequences were obtained, 9355 primer sets were designed, and 1675 primer sets were selected. Based on this dataset, 50 primer pairs were selected, according to their repeat number (≥ 9), motif, and PCR product size (≥ 100 bp), and then tested. Among the 50 primer pairs, 35 loci were successfully amplified on *C. melanopterus* DNA through PCR amplification. Moreover, ten previously published primer pairs initially developed for *Carcharhinus limbatus* (Cli111, Cli103, Cli102, Cli12, Cli107), *Negaprion acutidens* (LS20, LS75, LS32, LS54) and *C. plumbeus* (Cpl128) were acquired from previously published studies (Table 1) and tested for PCR amplification on *C. melanopterus*.

Molecular analysis

DNA was extracted from *C. melanopterus* samples to characterize and test the newly developed microsatellites, using the QIAcube HT DNA extraction robot (Qiagen, Hilden, Germany) from small (< 0.5 cm^3) shark fin clips. PCR amplifications were performed using theType-it Microsatellite PCR

Table 1 Characterization of the 35 microsatellite markers developed for *Carcharhinus melanopterus*

Locus	Primer sequence	Repeat motif	Source species	Ta (°C)	Size range	GenBank accession
Cm14931	F:TGGGAGGTGTTACCACAACA R:ACATCCTGTTAGAGATCGACCT	(AGAT)6	*C. melanopterus (C. m.)*	55	112	OM803093
Cm14813	F:GCGATGTCAGCTTTCACAGT R:TCCTTGGCTATTCATTCACTCAGT	(AGG)6	*C. m*	55	165	OM803094
Cm7408	F:AAACCACTGGTCAGAATTCTATGT R:GCTGGAGAGGGTGACAAAC	(AAT)8	*C. m*	55	188–194	OM803095
Cm12415	F:TCCATCTGCTGCAAATCCGT R:GTGATGGTGGCACTGGACTC	(AAT)7	*C. m*	55	242	OM803096
Cm18389	F:GCAGAAGCAGGTTTAGCTGAA R:AAGTGCCCAAGTTTCCACGA	(AC)14	*C. m*	55	258–264	OM803097
Cm11004	F:TGCCGAACACTCCTATTCCG R:AGGGATGAATTGAATTGATGTCACA	(AC)6	*C. m*	55	292–294	OM803098
Cm16808	F:GCTTCTCCCTCCACTTTGCA R:CCAGCTGGGTAGAAACCTCA	(AC)16	*C. m*	57	114–132	OM803099
Cm17515	F:GGGATGGTGGGAGTGTGAAT R:TGAAAGATAGACGGAGGGAAAG	(AGAT)7	*C. m*	57	138–150	OM803100
Cm20205	F:GCCCTGGTTCAGTGGAAGAA R:GCTGATGGCTCTCCTCACTC	(AGGC)5	*C. m*	57	222	OM803102
Cm13438	F:TGCAATCCACTCCAGAATGTCT R:CCTCACCCTCTGTCATCTGC	(AG)17	*C. m*	57	229–243	OM803103
Cm27428	F:TGTTCAAGTGTTCTGTAGTTTGGT R:TTGCATTGACTCGAACAGGG	(AC)16	*C. m*	57	291–303	OM803104
Cm18780	F:GTATCCTCCTGGCTGGTTCC R:AGGCTTTGAAACATGGAGGGA	(AGGG)5	*C. m*	57	124	OM803105
Cm19919	F:CACGTGGTATAGCTGGGCAA R:TAATTCCCTGGCGCCTCATG	(AC)13	*C. m*	57	135–141	OM803106
Cm12656	F:TGGGCACAGCTCTATTTAGGT R:CGCTCCTTAAAGCAATGCACA	(AC)14	*C. m*	57	184–192	OM803107
Cm15761	F:TCGCTTCACCCTTCAAGAGC R:CGCTAGCTCAATCATGGGACT	(AC)12	*C. m*	57	223–235	OM803108
Cm11610	F:CCACCCTTGTTGGTACATCCT R:TCCTTACAGGAATGCGCTGG	(ACT)6	*C. m*	57	265	OM803109
Cm20294	F:TTTGAAAGGAGGAAGGGAACA R: GGGATTTATGATGGTGGTGTCC	(AGG)8	*C. m*	57	292	OM803110
Cm21351	F:TGCTTTCAGCTCTTACCGCT R:GAGAGGCTGTGTACTGGCAG	(AG)17	*C. m*	60	113–119	OM803111
Cm13817	F:ATGGGAAGACGGTCAAAGCT R:CAGTGAGGTTTCAGTGCGTTT	(AC)16	*C. m*	60	192–218	OM803112
Cm7137	F:AGACACTCGTACGCACATACC R:GGACGACGCATTGTATTATTCGT	AC(16)	*C. m*	60	207–215	OM803113
Cm5929	F:TCCGCCTCAATGTTAAATCGC R:AAGATGCGTGGGTTAGGTTGA	(ACT)9	*C. m*	60	256–259	OM803114
Cm14866	F: TGAAAGTTTATTTGGTGCCTGCT R:GTCACTGCCATTACAACAAGCT	(ACT)7	*C. m*	60	144	OM803115
Cm16825	F:AACCTACACATCTCGGGACA R:CCTGAACGGTTAGTGGAAGC	(AT)11	*C. m*	60	286–294	OM803116
Cm 11927	F:TGAACAGGTGAATGGACTTGGA R:AAACTGTGAAACTTATCTGCAGGC	(AC)18	*C. m*	57	133–151	OM803117
Cm21278	F:TGAATTGGCAGCGGGCTATA R:GACCACCAGTGTCCGTGTAC	(AC)16	*C. m*	57	178–194	OM803118
Cm16167	F:GCACCTTCAATGTAGTTTGTCCC R:ATCTGTGTCAGCTGTGCCTC	(ATC)8	*C. m*	57	211–223	OM803119
Cm11359	F:TTTGACCATTAGGGCCCTGC R:TGCCTGACCTGCTGAAATGT	(AC)13	*C. m*	57	217–221	OM803120

Table 1 (continued)

Locus	Primer sequence	Repeat motif	Source species	Ta (°C)	Size range	GenBank accession
Cm17458	F:GAGCATTTACAGCTGCAGTGG R:CCCAATTCTCATCCCATTGAACC	(AT)14	*C. m*	57	278–330	OM803121
Cm24291	F: ACAGTAAGAATATGACTGCGGAT R:CCAGCTGGGTAGAAACCTCA	(AC)15	*C. m*	57	245–265	OM803122
Cm18013	F:CCTGAACCTGATCTGTGCCT R: CAATGAGCCAAGGATCTGCC	(ATC)9	*C. m*	57	115–125	OM803123
Cm16105	F: AGATGAAACTTTGATGTGCAGGA R: AGCTGAGTTTCTGGATTAATAGCG	(AC)13	*C. m*	57	127–141	OM803124
Cm9161	F: TGGGAAGACAGTGACAAGGG R: ACGGGACAGGGTAGGTGTAG	(AAAC)7	*C. m*	57	184–188	OM803125
Cm10864	F: TGTGCACCTCTGACATCACC R: CAAGACGGAAGCATGGCCTA	(AC)28	*C. m*	57	243–255	OM803126
Cm19878	F: TGGCCCTTGGTGATGTCATC R: TCTGACACTCCCTTGTCCTT	(AAGG)10	*C. m*	57	259–283	OM803127
Cm17408	F: TGGTTAGGGTCACAAAGGTTCT R: GGTAGATCTGCTGTGTACTGAGG	(AC)16	*C. m*	57	282–296	OM803128

kit (Qiagen, Hilden, Germany) in 12 µL total volume reactions containing 4 µL Type-it Multiplex PCR Master Mix 2X (contains HotStarTaq® *Plus* DNA polymerase, Type-it Microsatellite PCR buffer with 6 mM $MgCl_2$ and dNTPs), 6µL RNase-free water, 1 µL of primers (2 µM forward and reverse primers diluted in TE pH 8 buffer) and 1 µL of DNA template at 40 ng/µL. Forward primers were labelled with a fluorescent dye (YAKYE, 6FAM, ATTO550, or ATTO565, Applied Biosystems). Amplifications were carried out as follows; 5 min at 95 °C; followed by 45 cycles of 30 s at 95 °C, 1 min 30 s at optimal annealing temperature (53–60 °C, depending on locus, Table 1), and 30 s at 72 °C; and a final extension step of 30 min at 60 °C. The 45 loci were combined into eight multiplexes according to their size range and primer annealing temperature to perform PCR (Table 1). Each PCR product was run through a 1.5% agarose gel and visualized using ethidium bromide to verify amplifications. PCR products were sent to GenoScreen (Lille, France) and allele sizes were assessed using an Applied Biosystems 3730 Sequencer. For accurate sizing, an internal size ladder (GeneScan 500 LIZ, Applied Biosystems) was used. The same protocol was used to amplify each of the 45 markers on the eight other shark species. An additional low temperature was tested per multiplex (multiplexes 1 and 5 were amplified at 60 °C; multiplexes 2, 3, 7 and 8 were amplified at 53 °C, multiplexes 4 and 6 were amplified 55 °C).

Data analysis

The resulting electropherograms were scored using the program Geneious® v2022.1 (Geneious Prime, San Diego, CA). For the 120 blacktip reef shark samples, the software MICRO-CHECKER v.2.2.3 [53] was used to test for the presence of null alleles, scoring errors and large-allele dropouts, as the number of samples was sufficient. The R package LEA v.3.9.0 [21] was used to analyze population structure among the three islands by estimating the least-square estimates of ancestry proportions. The *K* ancestral populations were determined through an entropy criterion that evaluates the quality of fit of the statistical model with the data and helps select the number of ancestral populations that best explain the genotypic data [21]. The software GENODIVE v.3.0 [32] was used to validate the *K* number of ancestral populations found with the package LEA v.3.9.0. The software GENODIVE defines the optimal clustering by using the sum of squares to calculate the Bayesian Information Criterion (*BIC*), the lowest *BIC* value (within the clustering analysis from $K=2$ to $K=10$) indicates the optimal *K* number of ancestral populations [32]. The software GENODIVE v.3.0 allowed for the calculation of the *F*st index (population structure indicator, p value ≤ 0.001, [32]) using a pairwise differentiation analysis. Allele frequencies, the total number of alleles (*Na*) and the number of private alleles (N_{PA}) were estimated through the software GenAlEx v.6.503 [38]. The estimations of the observed (*Ho*) and expected (*He*) heterozygosities and the inbreeding coefficient (*Fis*) were performed in GENETIX v4.05.2 [6]. GENETIX also was used to compute the linkage disequilibrium (LD), after a sequential Bonferroni correction. The R package strataG v.2.4.905 [2] was used to test for the effects of bottlenecks in the populations in which significant linkage disequilibrium was found. For population where significant LD was detected, the *m*-ratio [22] was calculated to test for recent bottlenecks [49]. For the other shark species, selection of the highest number of individuals that were successfully amplified between the two amplification temperatures was used to compute the number of alleles, number of private alleles and optimal annealing temperature for each locus (Table S1).

Results

A total of 44 loci showed clear amplification profiles in *C. melanopterus* (Table S4 and Table S5), while Cli107 presented ambiguous or no peak profiles for all individuals. According to MICRO-CHECKER analysis, loci Cm7137 and Cli12 were likely to include null alleles along with low heterozygosity levels and above-average fixation indexes for Moorea and Tenararo samples (Table 2). Morane samples showed the same results for Cm7137, but Cli12 did not seem to present null alleles. Shark individuals from Morane showed null alleles for locus LS75, presenting a high fixation index and a large difference between the observed and expected heterozygosities. No evidence was found for null alleles, scoring errors or large allele dropout at any other loci. Slight linkage disequilibrium was detected among all loci in *C. melanopterus* individuals from Moorea, as only 3.8% of pairwise locus combinations had a significant disequilibrium after a sequential Bonferroni correction (Table S3). For loci Cm7137 and Cli12, which presented null alleles, a significant linkage disequilibrium was recorded in 14% of the pairwise loci combinations after sequential Bonferroni correction for Moorea (Table S3). For the blacktip reef shark samples from Moorea, 35 loci were polymorphic and presented 2 to 20 alleles, while 9 loci (Cm14931, Cm14813, Cm12415, Cm20205, Cm18780, Cm11610, Cm20294, Cm14866 and Cm16167; Table 2) appeared monomorphic. The shark samples from Tenararo and Morane atolls were also monomorphic at these 9 loci and presented an additional monomorphic locus (Cm5929) (Table S5). For *C. melanopterus* individuals from Moorea, microsatellite markers presented a total of 195 alleles with an average of 4.4 ± 3.5 alleles per locus. Dinucleotide markers had a total of 162 alleles with an average of 5.5 ± 3.7 alleles. Trinucleotide repeats represented a total of 18 alleles with an average of 2.2 ± 1.6 alleles. Tetranucleotide repeats represented a total of 15 alleles and an average of 2.1 ± 1.3 alleles per locus.

Three distinct populations were identified between the islands Moorea, Morane and Tenararo ($BIC = 846$, for $K = 3$, Fig. 1). The *F*st values were high and significant (p-value ≤ 0.001): 0.153 between Moorea and Morane, 0.127 between Moorea and Tenararo, and 0.112 between Morane and Tenararo.

Cross-species amplification

All 45 microsatellite markers were tested for cross-species amplification in eight additional shark species (Table 3). Each microsatellite marker was amplified at two different annealing temperatures (the temperature for which it was successfully amplified in *C. melanopterus* and one additional temperature), and the temperature that supported the successful amplification of the highest number of individuals was used to compute the results (Table 3). The percentage of successfully amplified samples per locus was considered notable above 50%. *Carcharhinus sorrah* had the highest percentage of samples that amplified at least at 50% of individuals per locus with a total of 93%, and *Sphyrna lewini* had the lowest percentage of samples amplified above 50% per locus with 36%. The average total number of alleles per species was 150.6 ± 37.5, with *C. sorrah* accounting for the highest total number of alleles (210 alleles) and *C. longimanus* the lowest (95 alleles) (Table S3). The average total number of private alleles was 38.8 ± 16.2, with *C. sorrah* accounting for the highest number of private alleles (53) and *C. longimanus* the lowest (17) (Table S3).

Discussion

Microsatellite marker amplification

Overall, a total of 35 new microsatellite markers were successfully amplified for blacktip reef sharks (*C. melanopterus*) together with 9 published markers initially designed for other shark species. Among these 35 new loci, 57% were dinucleotides, 23% were trinucleotides and 20% were tetranucleotides, which is consistent with the findings of Richards et al. [43]. The great white shark (*Carcharodon carcharias*) had similar di, tri and tetranucleotide microsatellite proportions (di = 7467 (88.9%), tri = 864 (10.3%), tetra = 73 (0.9%), [43]) as blacktip reef sharks, in contrast with other fish species (*Nothobranchius furzeri* exhibited a di- > tetra- > trinucleotide pattern, [27]) or chimpanzees (the distribution pattern in *Pan troglodytes* was di- > tetra- > trinucleotide, [58]).

While it has been shown that trinucleotides are easier to genotype than dinucleotides [43], developing microsatellite markers has proven time-consuming due to their scarcity in shark genomes [48]. Dinucleotides were found to have a higher level of polymorphism (5.58 ± 3.78 alleles) than trinucleotides (2.25 ± 1.67 alleles) and tetranucleotides (2.14 ± 1.35 alleles) in blacktip reef sharks surrounding the islands of Moorea, Tenararo and Morane. Polymorphism levels for di-, tri- and tetranucleotides seem to be variable across shark species, as *Galeocerdo cuvier* had a higher polymorphism for tetranucleotide microsatellite marker ($Na = 8$, $N = 101$, [39]) and *Carcharhinus plumbeus* for dinucleotide microsatellite markers ($Na = 4$–39, $N = 47$, [40]). Moreover, all microsatellite markers that were monomorphic in blacktip reef sharks (i.e., 9 loci) were polymorphic in at least one

Table 2 Summary statistics for genetic variation of the number of alleles (*N*a), the observed (*H*o) and expected (*H*e) heterozygosities, the *F*is fixation index [57] of the 40 *Carcharhinus melanopterus* samples analyzed per island for Moorea, Morane and Tenararo

Locus	Multiplex Number	Moorea				Morane				Tenararo			
		*N*a	*H*o	*H*e	*F*is(W&C)	*N*a	*H*o	*H*e	*F*is(W&C)	*N*a	*H*o	*H*e	*F*is(W&C)
Cm14813	1	1	–	–	–	1	–	–	–	3	0.257	0.231	− 0.097
Cm11004	1	2	0.551	0.493	− 0.109	5	0.775	0.623	− 0.231	5	0.675	0.617	− 0.082
Cm12415	1	1	–	–	–	1	–	–	–	1	–	–	–
Cm7408	1	2	0.449	0.475	0.065	2	0.225	0.199	− 0.114	2	0.175	0.159	− 0.083
Cm14931	1	1	–	–	–	1	–	–	–	1	–	–	–
CM18389	1	4	0.612	0.561	− 0.08	5	0.9	0.738	− 0.208*	5	0.775	0.708	− 0.082
Cm17515	2	4	0.612	0.638	0.05	3	0.6	0.586	− 0.011	3	0.475	0.62	0.246
Cm27428	2	6	0.674	0.723	0.078	5	0.725	0.76	0.059	5	0.675	0.726	0.084
Cm20205	2	1	–	–	–	1	–	–	–	1	–	–	–
Cm16808	2	5	0.714	0.692	− 0.022**	5	0.525	0.707	0.27*	5	0.641	0.676	0.065
Cm13438	2	4	0.592	0.565	− 0.038	4	0.4	0.568	0.307**	4	0.475	0.673	0.306*
Cm18780	3	1	–	–	–	1	–	–	–	1	–	–	–
Cm11610	3	3	–	–	–	1	–	–	–	1	–	–	–
Cm12656	3	3	0.469	0.541	0.142	2	0.139	0.15	− 0.068	4	0.15	0.185	0.203
Cm15761	3	4	0.225	0.316	0.3	4	0.175	0.207	0.168***	7	0.282	0.355	0.218**
Cm20294	3	6	–	–	–	1	–	–	–	1	–	–	–
Cm19919	3	4	0.633	0.683	0.084	5	0.625	0.578	− 0.068***	6	0.7	0.705	0.021***
Cm14866	4	1	–	–	–	1	–	–	–	1	–	–	–
Cm16825	4	2	0.265	0.23	− 0.143	2	0.4	0.348	− 0.135	2	0.325	0.409	0.219
Cm7137	4	3	0.102	0.348	0.712***	3	0.25	0.46	0.483***	5	0.043	0.371	0.888***
Cm21351	4	5	0.714	0.703	− 0.005	5	0.575	0.568	0.001	6	0.6	0.703	0.158*
Cm5929	4	2	0.082	0.078	− 0.032	1	–	–	–	1	–	–	–
Cm13817	4	10	0.571	0.646	0.125	2	0.475	0.387	− 0.215	3	0.375	0.373	0.007
Cm21278	5	5	0.225	0.225	0.013**	2	0.525	0.499	− 0.038	2	0.5	0.498	0.01
Cm17458	5	10	0.816	0.812	0.004	2	0.1	0.139	0.291	4	0.25	0.346	0.289*
Cm11359	5	3	0.489	0.503	0.037	2	0.15	0.139	− 0.068	3	0.125	0.162	0.238
Cm16167	5	1	–	–	–	1	–	–	–	1	–	–	–
Cm11927	5	9	0.816	0.829	0.025***	6	0.75	0.751	0.015	6	0.625	0.631	0.022***
Cm24291	5	9	0.653	0.696	0.072	4	0.35	0.344	− 0.005	3	0.35	0.558	0.384***
Cm18013	6	2	0.428	0.417	− 0.018	2	0.375	0.335	− 0.108	2	0.5	0.498	0.01
Cm16105	6	4	0.674	0.618	− 0.079	1	–	–	–	3	0.375	0.335	− 0.107
Cm9161	6	2	0.469	0.497	0.065	2	0.2	0.18	− 0.099	2	0.325	0.362	0.115
Cm10864	6	11	0.714	0.831	0.151	8	0.6	0.699	0.155	11	0.725	0.819	0.128
Cm19878	6	4	0.428	0.42	− 0.01	1	–	–	–	2	0.05	0.487	− 0.013
Cm17408	6	6	0.65	0.67	0.03	4	0.55	0.607	0.108	3	0.3	0.391	0.245
LS20	7	2	0.408	0.408	0.01	2	0.525	0.429	− 0.21	2	0.45	0.455	0.024
Cli103	7	2	0.511	0.497	− 0.017	2	0.475	0.492	0.048	2	0.425	0.409	− 0.025
Cli111	7	17	0.836	0.913	0.095	11	0.725	0.769	0.071	15	0.85	0.865	0.033
LS75	7	3	0.633	0.597	− 0.05	2	0.075	0.159	0.539***	3	0.575	0.6	0.055
Cli 12	8	4	0.286	0.543	0.482**	3	0.2	0.224	0.119***	3	0.075	0.182	0.597***
Cpl128	8	14	0.811	0.768	− 0.042	10	0.579	0.715	0.204	11	0.687	0.671	− 0.009
LS54	8	3	0.367	0.397	0.084	2	0.384	0.341	0.146	3	0.3	0.262	− 0.136
LS32	8	3	0.367	0.341	−0.069	2	0.384	0.341	− 0.115	2	0.182	0.164	0.228
Cli102	8	5	0.327	0.318	− 0.016	1	–	–	–	2	0.231	0.341	0.335*

*Indicates significant departures from Hardy–Weinberg Equilibrium (*p-value < 0.05, **p-value < 0.01, ***p-value < 0.001)

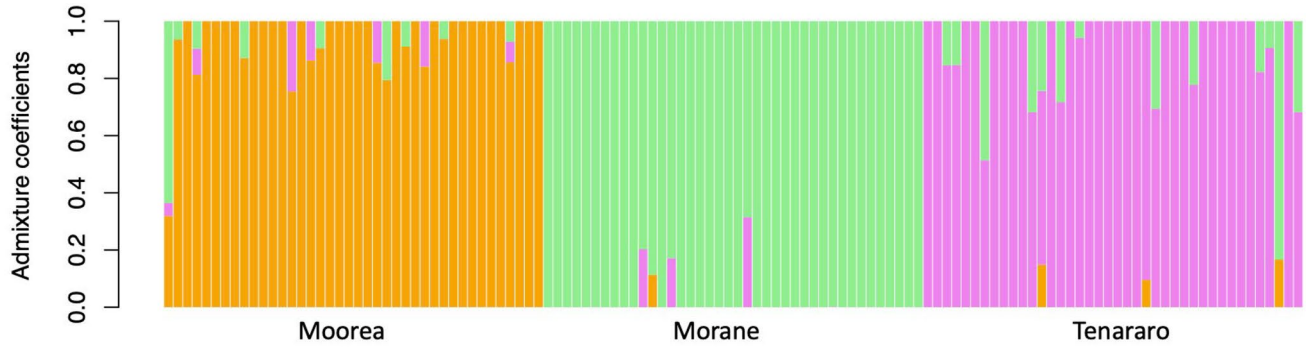

Fig. 1 Barplot of ancestry coefficients following a Bayesian clustering approach for blacktip reef shark samples from the islands of Moorea, Morane and Tenararo. Ancestral groups are reported in different colors. 40 samples per island were used with a cross-entropy criterion of $K=3$

of the eight other tested species, emphasizing the value of testing monomorphic loci on closely related species [26].

Genetic diversity

The average observed heterozygosities were 0.447 for Moorea, 0.337 for Morane and 0.351 for Tenararo population and the average expected heterozygosities were 0.442, 0.337 and 0.382, respectively. Vignaud et al. [55] analyzed the genetic structure of blacktip reef sharks across French Polynesia and found a higher expected heterozygosity for Moorea ($He=0.6$, $N=38$) and Tenararo ($He=0.51$, $N=51$), like Mourier and Planes [37] who also found a slightly higher average expected heterozygosity (Moorea, $He=0.55$, $N=247$). The number of microsatellite markers used (33 polymorphic loci in the present study vs. 14 loci in Vignaud et al. [55] and 17 in Mourier and Planes [37]) might be the reason for these differences. For the island of Moorea, the sample size of $N=247$ used by Mourier and Planes [37], might be the cause of the slightly higher expected heterozygosity. Moreover, Moorea is a larger island (132 km^2) than Morane (2 km^2) and Tenararo (2.72 km^2), with a more extensive reef area, and may thus have a larger effective population size. Further, Moorea's proximity to Tahiti and Tetiaroa might provide higher genetic exchange. In contrast, the islands of Morane and Tenararo were found to be isolated atolls ($Fst=0.112$) in the south of the Tuamotu Archipelago, limiting gene exchange with other blacktip reef shark populations.

Two microsatellite markers, Cm7137 and Cli12, repeatedly presented null alleles, leading to heterozygote deficiency and a significant departure from Hardy–Weinberg Equilibrium. Moreover, these loci had an above average level of LD, with 14% of their pairwise loci combinations exhibiting significant disequilibrium (the average LD of the sample of Moorea was of 3.8% which could originate from a bottleneck in the population as the *M*-ratio for Moorea was 0.75 ± 0.21); thus, they should be removed from further analyses. To date, no linkage disequilibrium in the pairwise locus combination was reported in other shark species (*Carcharhinus limbatus*, [25]; *Rhincodon typus*, [42]; *Carcharhinus leucas*, [39]; *Ginglymostoma cirratum*, [24]). The LD measured in blacktip reef sharks in Moorea might be caused by the structure of the population, with related subgroups within the population due to non-random mating among individuals [3, 45].

Cross-species amplification

Cross-species amplification of the 44 microsatellites had an average success of 69% (with a threshold set at 50% of the samples to define successful amplification per locus) among the eight shark species. Indeed, cross-species amplification in chondrichthyan species were often found successful due to highly conserved microsatellite flanking sequences [51]. The species that had the highest percentage of markers that were amplified in at least 50% of the samples per species were also from the genus *Carcharhinus* (between 82 and 93%, Table 3). These results are consistent with the phylogeny of sharks as these three shark species are closely related to *C. melanopterus* [30, 54]. The existing shark phylogeny includes three major clades: (i) *Carcharhinus melanopterus, C. sorrah, C. longimanus*, *Prionace glauca* and *Negaprion acutidens* are in the cluster *Galeomorphii*, in the *Carcharhiniformes* group, while (ii) *Rhincodon typus* belongs to the *Orectolobiformes* within the *Galeomorphii* group, and (iii) *Galeocerdo cuvier* and *Sphyrna lewini* belong to a separate cluster in the *Carcharhiniformes* group [54]. Phylogenetic inference using microsatellites might be limited for the species having frequent mutations and recombinations which can decrease the frequency of microsatellite loci [30].

Study of the eight shark species targeted for cross-species amplification, as well as blacktip reef sharks, will benefit from the use of this new set of 44 microsatellite markers. These markers will support critical conservation-based research, such as estimating population sizes, deciphering

Table 3 Percentage of individuals successfully amplified for each of the eight species selected for cross-species amplification (*C. amblyrhynchos* (*N*=15), *C. longimanus* (*N*=5), *C. sorrah* (*N*=15), *Galocerdo cuvier* (*N*=15), *Negaprion acutidens* (*N*=8), *Prionace glauca* (*N*=8), *Rhincodon typus* (*N*=15), *Shyrna lewini* (*N*=15)) and for each microsatellite (0–20%: white, 21–40%: light gray, 41–60%: silver, 61–80%: grey, 81–100%: dark grey). The final row indicates the percentage of markers that were amplified in at least 50% of the samples for each species

	Grey reef shark *(Carcharhinus amblyrhynchos)*	Oceanic whitetip shark (*Carcharhinus longimanus*)	Spot-tail shark (*Carcharhinus sorrah*)	Tiger shark (*Galeocerdo cuvier*)	Lemon shark (*Negaprion acutidens*)	Blue shark (*Prionace glauca*)	Whale shark (*Rhincodon typus*)	Scalloped hammerhead (*Sphyrna lewini*)
Cm14813	100%	100%	100%	93%	88%	63%	80%	67%
Cm11004	100%	80%	93%	60%	75%	63%	53%	53%
Cm12415	33%	40%	27%	40%	38%	13%	33%	13%
Cm7408	100%	100%	100%	93%	100%	63%	67%	80%
Cm14931	40%	80%	80%	53%	100%	13%	53%	67%
Cm18389	100%	60%	100%	53%	100%	63%	53%	27%
Cm17515	100%	100%	93%	87%	100%	100%	67%	67%
Cm27428	87%	100%	100%	0%	63%	88%	27%	7%
Cm20205	100%	100%	100%	73%	100%	75%	60%	73%
Cm16808	27%	0%	60%	33%	0%	63%	27%	53%
Cm13438	100%	100%	100%	87%	100%	75%	93%	40%
Cm18780	100%	100%	100%	80%	100%	75%	67%	60%
Cm11610	100%	100%	100%	73%	100%	75%	47%	40%
Cm12656	100%	100%	93%	13%	100%	63%	47%	20%
Cm15761	93%	100%	100%	40%	100%	50%	40%	27%
Cm20294	100%	100%	73%	80%	100%	0%	67%	13%
Cm19919	100%	100%	87%	73%	100%	63%	47%	67%
Cm14866	100%	100%	100%	80%	100%	75%	33%	60%
Cm16825	100%	100%	93%	0%	0%	25%	20%	7%
Cm7137	13%	40%	7%	13%	13%	13%	47%	0%
Cm21351	100%	100%	80%	7%	100%	75%	33%	27%
Cm5929	80%	40%	73%	0%	25%	50%	80%	13%
Cm13817	0%	20%	60%	20%	0%	0%	13%	27%
Cm21278	80%	100%	87%	53%	88%	88%	100%	27%
Cm17458	100%	80%	87%	7%	38%	50%	27%	7%
Cm11359	100%	100%	87%	87%	75%	63%	7%	20%
Cm16167	100%	100%	93%	40%	100%	50%	87%	27%
Cm11927	100%	100%	73%	87%	75%	38%	20%	27%
Cm24291	100%	60%	93%	20%	75%	63%	7%	20%
Cm18013	100%	100%	93%	80%	100%	100%	53%	60%
Cm17408	100%	100%	80%	20%	75%	38%	27%	7%
Cm9161	100%	100%	100%	0%	75%	63%	27%	7%
Cm10864	73%	100%	53%	53%	63%	63%	13%	7%
Cm16105	0%	100%	100%	0%	50%	88%	7%	7%
Cm19878	80%	100%	87%	0%	100%	63%	13%	7%
LS20	93%	100%	73%	87%	100%	100%	60%	53%
Cli103	100%	100%	60%	67%	100%	63%	67%	47%
Cli111	60%	80%	80%	33%	100%	50%	33%	53%
Cli107	13%	100%	33%	33%	100%	0%	20%	33%
LS75	87%	100%	87%	53%	63%	100%	60%	73%
Cli 12	33%	100%	80%	87%	100%	88%	7%	33%
Cpl128	100%	100%	93%	60%	100%	88%	53%	33%
LS54	100%	100%	100%	80%	100%	100%	87%	80%
LS32	100%	100%	80%	87%	88%	88%	100%	100%
Cli102	82%	100%	87%	7%	100%	87%	7%	73%
Total above 50%	82%	89%	93%	58%	82%	68%	45%	36%

parentage links between individuals, and analyzing structure and demographic history of shark populations.

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Declarations

Competing interests The authors declare that they have no conflict of interest.

Ethical approval Sampling of blacktip reef sharks was conducted by the Center for Island Research and Environmental Observatory (CRIOBE) under the permit N°9524 issued by the Ministère de la Promotion des Langues, de la Culture, de la Communication et de l'Environnement of the French Polynesian government in October 2015.

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