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Blacktip reef sharks, *Carcharhinus melanopterus*, have high genetic structure and varying demographic histories in their Indo-Pacific range

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Abstract

For free-swimming marine species like sharks, only population genetics and demographic history analyses can be used to assess population health/status as baseline population numbers are usually unknown. We investigated the population genetics of blacktip reef sharks, *Carcharhinus melanopterus*; one of the most abundant reef-associated sharks and the apex predator of many shallow water reefs of the Indian and Pacific Oceans. Our sampling includes 4 widely separated locations in the Indo-Pacific and 11 islands in French Polynesia with different levels of coastal development. Fourteen microsatellite loci were analysed for samples from all locations and two mitochondrial DNA fragments, the control region and cytochrome *b*, were examined for 10 locations. For microsatellites, genetic diversity is higher for the locations in the large open systems of the Red Sea and Australia than for the fragmented habitat of the smaller islands of French Polynesia. Strong significant structure was found for distant locations with F_{ST} values as high as ~0.3, and a smaller but still significant structure is found within French Polynesia. Both mitochondrial genes show only a few mutations across the sequences with a dominant shared haplotype in French Polynesia and New Caledonia suggesting a common lineage different to that of East Australia. Demographic history analyses indicate population expansions in the Red Sea and Australia that may coincide with sea level changes after climatic events. Expansions and flat signals are indicated for French Polynesia as well as a significant recent bottleneck for Moorea, the most human-impacted lagoon of the locations in French Polynesia.

Keywords: blacktip reef sharks, demographic history, French Polynesia, genetic structure, microsatellites, population genetics

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Introduction

Ecosystem health depends partially on the health of keystone species populations (Paine 1969; Barua 2011). As apex predators, sharks are keystone species, but there can be large species-specific as well as geographic differences in the ecological significance of sharks at the ecosystem level (Preisser *et al.* 2005; Myers 2007; Heithaus *et al.* 2012; Ruppert *et al.* 2013). Sharks are exploited extensively (Worm *et al.* 2013; Dulvy *et al.* 2014) despite being recognized as highly vulnerable (Rose 1996; Baum *et al.* 2003; Myers & Worm 2003; Clarke *et al.* 2006). Assessing the population health of free-swimming large marine species like sharks is easier where these species are known to be overexploited; that is, in these locations, it is probably safe to assume the populations are declining. Assessing population health/status is far harder in locations where free-swimming species like sharks are not exploited. In these locations,

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sightings of sharks even if only occasional often lead to the assumption populations are healthy. Only population genetics and demographic history analyses can be used in these instances to assess population health/status. We examine the structure and history of blacktip reef sharks throughout their range in the Indian and Pacific Oceans (the 'Indo-Pacific'). Some of the results include evidence of a population decline that highlights the critical importance of using population genetics in the marine environment.

In reef areas in the Indo-Pacific, these shark species are usually either present or abundant (among others): whitetip reef shark [*Triaenodon obesus* (Rüppell 1837)], grey reef shark [*Carcharhinus amblyrhynchos* (Bleeker 1856)] and blacktip reef shark [*Carcharhinus melanopterus* (Quoy et Gaymard 1824)]. All three species have distinctive, yet overlapping habitat associations with coral reefs (Nelson & Johnson 1980; McCauley *et al.* 2012). Whitetip reef sharks have a very specialized foraging strategy of catching prey in small caves and holes (Randall 1977) and are hence mainly found at reef crests. Grey reef sharks are mostly present in oceanic outer reef locations, including crests and passes (McKibben & Nelson 1986; Wetherbee *et al.* 1997; Vianna *et al.* 2013) and generally occupy deeper waters than blacktip reef sharks (Compagno 1984). Blacktip reef sharks inhabit shallow reef flat and sheltered lagoon habitats (Papastamatiou *et al.* 2009b, 2010). Unlike grey reef sharks and whitetip reef sharks, blacktip reef sharks are also commonly encountered in nonreef habitats like shallow inshore waters and mangrove areas (Nelson & Johnson 1980). At many coral reef locations, blacktip reef sharks are by far the most abundant generalist apex predators (Stevens 1984; Compagno *et al.* 2005) and probably have the main role in the exertion of top-down control.

For most shark species, their biology is known or well understood, but their large-scale population dynamics and dispersal patterns are largely unknown (but see review in Dudgeon *et al.* 2012) and blacktip reef sharks are no exception. Blacktip reef shark populations have been studied in French Polynesia (Mourier *et al.* 2012, 2013; Mourier & Planes 2013; Vignaud *et al.* 2013), Aldabra Atoll (Stevens 1984), Palmyra Atoll (Papastamatiou *et al.* 2009a,b, 2010), the Great Barrier Reef (Chin *et al.* 2013a) and West Australia (Speed *et al.* 2011). These studies revealed that blacktip reef sharks have a high degree of site attachment with mostly restricted movements and some temporary excursions and that they demonstrate reproductive philopatry (like both species of *Negraprion*, Feldheim *et al.* 2014). Despite indications that blacktip reef sharks have a low dispersal capacity, researchers have recently been surprised to find this species far from known habitats in the eastern Pacific (López-Garro *et al.* 2012) and in the Mediterranean Sea (Zenetos *et al.* 2005), demonstrating a potential high mobility. Previous studies of the population genetics of blacktip reef sharks have been on a single population (Mourier & Planes 2013) or at a regional scale (Vignaud *et al.* 2013), so the global genetic structure and extent of mixing among reefs in the Indo-Pacific have been unknown up to this point.

The blacktip reef shark is considered globally near threatened (NT) by the International Union for the Conservation of Nature (IUCN) Red list, with a decreasing population (Fowler *et al.* 2005; Heupel 2009). Most of the information used to grant this status was based on data collected more than two decades ago (e.g. Stevens 1984). A number of more recent surveys and observations report potentially heavy fishing pressure on reef sharks in areas like the Red Sea (e.g. Bonfil 2003; Bonfil & Abdallah 2004; Spaet & Berumen 2014), the Indian Ocean (e.g. Henderson *et al.* 2007) and the central Indo-Pacific (Robbins *et al.* 2006; Heupel *et al.* 2009; White & Kyne 2010; Field *et al.* 2012). Blacktip reef sharks are abundant, and their populations appear to be healthy and stable in remote locations with low (or no) anthropogenic impacts like the central Pacific Line Islands and the South Pacific (DeMartini *et al.* 2008; Nadon *et al.* 2012). However, these conclusions are often based on limited observations; the status of population stocks in remote locations and the degree to which these stocks are increasing or decreasing are generally unknown.

Here, we attempt to fill the two key knowledge gaps identified above. We assess the genetic structure of blacktip reef sharks among and within the Pacific and Indian Ocean basins and use demographic history analyses to check for expansions and bottlenecks. The results help to better characterize blacktip reef sharks with respect to their population connectivity and level of sensitivity to fishing impacts and coastal development.

Materials and methods

Sampling and laboratory procedures

DNA was obtained from skin samples collected from free-swimming sharks and on rare occasion from dead specimens for a total of 1022 individuals. Sampling locations are spread across the Indian and Pacific Oceans including the large open coral reef systems of the Red Sea, West and East Australia and New Caledonia and 11 locations from the fragmented coral reef environments of French Polynesia (Fig. 1). Microsatellite loci are analysed for samples from all 15 locations. Sample sizes for the analyses of genetic diversity and structure ranged from 18 (New Caledonia) to 116 for the microsatellite DNA (subsampling of the 380 from

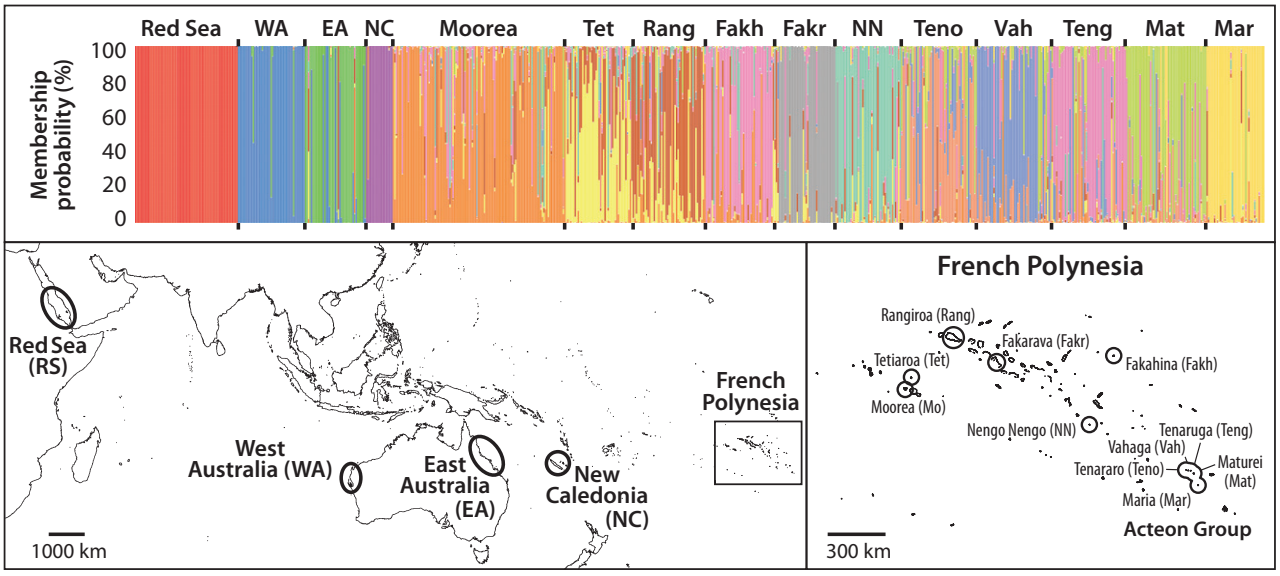


Fig. 1 Genetic structure diagram produced by the DAPC analysis—each vertical bar represents an individual, and each colour represents the probability of belonging to one of the genetic clusters (top). Sampling locations are shown on the bottom in the Indian and Pacific oceans (left) and in French Polynesia (right).

Table 1 Indices of genetic diversity for each sampling site for both microsatellites (left) and mtDNA (right)

Microsatellites (14 loci)	*N*	*A*	He	Ho	AR	mtDNA	*N*	Hn	*H*	π	Bp used
Pop											
Red Sea	69	9.93	0.63	0.63	6.7	CR	30	3	0.131	0.00026	765
						CytB	22	5	0.338	0.0005	725
West Australia	45	10.07	0.61	0.59	7.4	CR	26	1	0		807
						CytB	26	2	0.077	0.0001	757
East Australia	41	10.5	0.69	0.62	7.99	CR	21	6	0.552	0.00236	665
						CytB	22	7	0.671	0.0097	762
New Caledonia	18	5.29	0.55	0.55	5.08	CR	10	7	0.867	0.002	800
						CytB	9	2	0.222	0.00086	773
Moorea	116	7.79	0.60	0.61	5.38	CR	30	1	0		806
						CytB	30	4	0.395	0.00054	774
Tetiaroa	45	7.79	0.53	0.53	5.87						
Rangiroa	48	8.14	0.57	0.56	6.13	CR	15	3	0.362	0.00081	798
						CytB	15	1	0		780
Fakarava	50	6.71	0.60	0.62	5.18	CR	29	5	0.589	0.00103	803
						CytB	30	5	0.308	0.00042	777
Fakahina	38	4.5	0.48	0.48	3.66	CR	30	3	0.246	0.00068	803
						CytB	30	1	0		780
Nengo Nengo	44	6.21	0.53	0.52	4.92	CR	28	4	0.492	0.00071	799
						CytB	22	3	0.091	0.00036	758
Tenararo	51	5.36	0.51	0.50	4.31	CR	30	2	0.46	0.00057	806
						CytB	28	8	0.802	0.00279	708
Vahanga	51	4.93	0.52	0.50	3.91						
Tenarunga	50	5.57	0.54	0.55	4.43						
Maturei	53	5.29	0.47	0.48	3.94						
Maria	39	5.14	0.46	0.47	4.09						

The diversity indices used are as follows: *A*, number of alleles; He, expected heterozygosity; Ho, observed heterozygosity; AR, allelic richness; Hn, number of haplotypes; *H*, haplotype diversity; π, nucleotide diversity.

Moorea to the sampling undertaken within a single year, see Table 1). Mitochondrial DNA was analysed for six rather than all 11 of the locations in French Polynesia due to the geographic proximity of some islands. We selected one of the five islands in the Actéon island group (Tenararo) and excluded Tetiaroa due to proximity with Moorea (~60 km) and low genetic differentiation with Rangiroa. Thirty samples were analysed for each of the 10 locations excepting New Caledonia ($n = 18$); of a total of 298 samples, 49 samples were excluded for the control region (leaving 249) and 66 were excluded for cytochrome *b* (leaving 234) due to poor quality DNA or incomplete sequences.

DNA was extracted using the QIAGEN® DX Universal Tissue Sample DNA Extraction protocol. PCR amplification and the microsatellite loci used are as in Mourier & Planes (2013). Control region amplifications were performed using primers Isp Pro-L (proline tRNA) and hsp 282 (12S rRNA) (Keeney *et al.* 2003). Cytochrome *b* amplifications were performed using primers GLUDG-L and CB3-H (Palumbi *et al.* 1991). All fragments were amplified following the PCR protocol described in Williams *et al.* (2012) and had maximum sizes after editing of 811 bp for the control region and of 782 bp for cytochrome *b* (Table 1).

Data analysis

Genetic diversity and structure. Microsatellite alleles were scored using GENEMAPPER version 3.7 software (Applied Biosystems, Foster City, CA, USA). The data were tested for the presence of null alleles and deviations from Hardy–Weinberg equilibrium using MICROCHECKER v2.2.3 (van Oosterhoot *et al.* 2004). Based on these results, three of the 17 microsatellite loci originally selected for the study (and used in Mourier & Planes 2013) were excluded from this analysis: Cpl169, Cli107 and Cli12. Indices of diversity (mean number of alleles, expected heterozygosity, observed heterozygosity and allelic richness) were analysed using GENEPOP 4.2 (Rousset 2008), and the rarefaction method was used in the HP-RARE software (Kalinowski 2005) to calculate allelic richness because this approach takes into account differences in sample size. AMOVA was calculated using ARLEQUIN 3.5 (Excoffier & Lischer 2010), and pairwise F_{ST} (Weir & Cockerham 1984) values and 95% confidence intervals (CIs) were calculated using the diveRsity (Keenan *et al.* 2013) package for R (R Development Core Team 2013). F_{ST} comparisons are considered significant if both these conditions are met: the lower CI is >0, and *P*-values are <0.05 following a false discovery rate (FDR) correction (see Narum 2006). The genotypic differentiation test (G-based, Goudet *et al.* 1996) and associated significance were computed using the GENEPOP 4.2 software. GENECLASS2 (Piry *et al.* 2004) assessed: (i) the percentage of individuals from locations in French Polynesia assigned back to their original sampling location and (ii) the percentage of all individuals assigned back to their original sampling location with and without French Polynesia locations grouped (per Rannala & Mountain 1997). ADEGENET (Jombart 2008) for R (R Development Core Team 2013) was used to perform discriminant analysis of principal components (DAPC, Jombart *et al.* 2010). This analysis maximizes among-population variation, using predefined groups and linear discriminant analysis on principal components (see Jombart *et al.* 2010; Horne *et al.* 2011 for full description of the method). Here, the number of principal components (as predictors for the discriminant analysis) was set to 30 following alpha-score indication, which finds a trade-off between power of discrimination and overfitting (Fig. 1).

MtDNA sequences were read using GENEIOUS 6 (Drumond *et al.* 2010) and aligned using the ClustalX method (Larkin *et al.* 2007) followed by manual corrections. Indices of diversity (number of haplotypes, haplotype diversity and nucleotide diversity) and haplotype matrices as well as F_{ST} were analysed using DNASP v5.10.01 (Librado & Rozas 2009; following method in Hudson *et al.* 1992).

Maximum-likelihood trees were developed using the maximum-likelihood method implemented in MEGA 5.2.2 (Tamura *et al.* 2011) with 2000 bootstrap replications. The trees with the highest log likelihood are presented [−1224.2658 for CytB (HKY model) and −1318.9420 for the CR (HKY+G model)]. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach and then selecting the topology with superior log-likelihood value.

Using location names instead of individual names reduced the tree size. In particular, French Polynesia and New Caledonia subgroups have been grouped into one single lineage following low branch weight and low number of mutations differentiating individuals from these locations.

Demographic history

Demographic history was explored on microsatellite data only because of the low number of mutations and nucleotide diversity seen in the mtDNA. The software MIGRAINE was used (http://kimura.univ-montp2.fr/~rousset/Migraine.htm) along with the newly developed model of a single population with past variations in population size (Leblois *et al.* 2014). To infer model parameters, MIGRAINE uses the class of importance

sampling algorithms developed by de Iorio & Griffiths (2004a,b) and de Iorio *et al.* (2005) and extended in Leblois *et al.* (2014) and employs a generalized stepwise-mutation model (GSM). This method is particularly powerful compared with other frequently used alternatives (Leblois *et al.* 2014). MIGRAINE provides estimates of ancestral θ and actual θ values and *D* by producing point estimates together with the 95% coverage confidence interval, each point being a set of values for all canonical model parameters. *D* is used to calculate expansion/reduction timing if mutation rates are chosen. Here, the formula to obtain the timing of the expansions or bottlenecks (if found) in generations is $T = 2 \times D \times 2\text{Ne}$ (Leblois *et al.* 2014). Actual and ancestral effective population size follows $\text{Ne} = \theta/(4 \times \mu)$. The demographic model used by MIGRAINE follows an exponential change of population's size continuing to the present. The significance of the demographic event is tested on: *N*ratio = ancestral Ne/actual Ne and is <1 in the case of a bottleneck and >1 in the case of an expansion. All runs in MIGRAINE were done for microsatellites using 20 000 trees, 2400 points and 3–9 iterations. For Moorea, 200 000 trees and 5000 points are used as the number of individual (380) is far higher than the other sampling locations (see Leblois *et al.* 2014).

To translate the parameters inferred from MIGRAINE into effective population size (Ne) and timing of the event in generation (*T*), two mutation rates (μ) were chosen. A common mutation rate used for microsatellites is 5×10^{-4} (Yue *et al.* 2007; Sun *et al.* 2012) and we applied that rate here, but mutation rates may be especially slow for sharks so we also applied 1×10^{-5} (as in Nance *et al.* 2011, following Martin 1999). The chosen rates were applied to the lowest and highest range for each parameter inferred by MIGRAINE and to the value of highest probability. The mutation rates were used only for locations where a demographic event was detected by MIGRAINE and was significant.

Results

Genetic diversity

Expected heterozygosity and allelic richness for microsatellites both show genetic diversity to be higher for the large open coral reef systems of the Red Sea, Australia and New Caledonia than for the fragmented coral reef habitats of French Polynesia. Expected heterozygosity is highest for the Red Sea, West and East Australia (He = 0.63, 0.61 and 0.69, respectively). Within French Polynesia, expected heterozygosity is higher for the large islands of Moorea (0.60), Rangiroa (0.57) and Fakarava (0.60) than for the small atolls of Maria (0.46) and Tenarunga (0.54) (Table 1). Allelic richness is also highest for the Red Sea, West and East Australia (6.7, 7.4 and 7.99, respectively). As was the case for expected heterozygosity, in French Polynesia, allelic richness values were lower for the small atolls (3.66–4.92, Table 1) than for the large islands with the exception of Tetiaroa (5.87), which is close to Moorea and Rangiroa relative to the distances between the other sampled atolls.

Both control region and cytochrome *b* sequences showed a small number of mutations, and varying levels of haplotype diversity among locations. This low variability of the mtDNA is not surprising as sharks are known to have a slow rate of mitochondrial evolution (Martin *et al.* 1992; Martin 1999). Twenty-four haplotypes were found for the control region, and 22 haplotypes were found for cytochrome *b*. For the control region, the number of haplotypes ranged from 1 (Western Australia and Moorea) to 7 (New Caledonia) and from 1 (Fakahina) to 8 (Tenararo) for the cytochrome *b* (Table 1). Nucleotide diversity was lower than 0.003 for both genes and all locations excepting cytochrome *b* for East Australia (0.009). Values for nucleotide diversity for both genes are presented in Table 1, but comparing nucleotide diversity among sampling locations is uninformative as the number of mutations was very low for both genes for nearly all locations. These results contrast with those found for microsatellite loci, in which there is no clear pattern of haplotype diversity being greatest for the large open systems of the Red Sea, Australia and New Caledonia or greater for the large islands of French Polynesia than for the small atolls.

Genetic structure

Discriminant analysis of principal components results visually show high level of genetic structure, particularly at large scales (see Fig. 1). In French Polynesia, structure is found to be moderate to high; each sampling location has specific colour dominance, even in the Actéons group where islands are only a few kilometres apart. The AMOVA performed on the 14 microsatellites loci and for 15 sampling locations indicated that the source of variation was 18.81% among sampling locations and 81.18% within individuals. GENECLASS2 reassigned 83.2% of individuals back to their original locations when using our 15 sampling locations separated, and 79.5% when analysing only the 11 separated locations within French Polynesia. The percentage reassigned increased to 99.1% when our sampling was separated into five location regions (the Red Sea, West Australia, East Australia, New Caledonia and French Polynesia), so global re-assignment for the 15 locations of 83.2% was brought down by French Polynesia. Individuals incorrectly re-assigned ($n = 7$) when separating

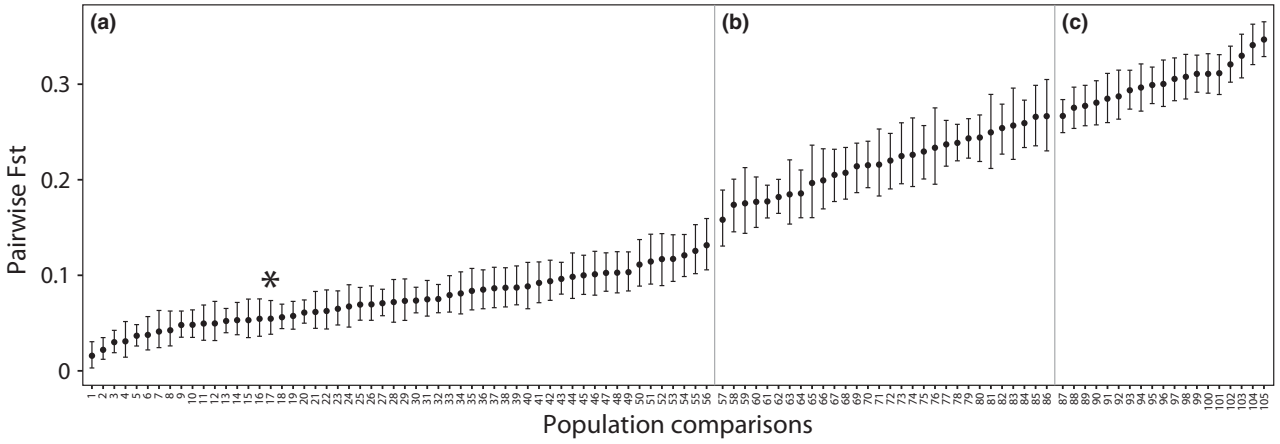
sampling into five regions were from West Australia and were assigned to East Australia ($n = 4$) or vice versa ($n = 1$) or were from French Polynesia and were assigned to East Australia ($n = 1$) or vice versa ($n = 1$).

The pairwise genotypic differentiation analysis results were all significant for the microsatellite DNA with *P*-values <0.001, and the pairwise F_{ST} values were also significant (all lower 95% confidence intervals >0, and *P*-values <0.05 after FDR corrections). These results indicate a moderate to high level of structure among the sampling locations. F_{ST} values ranged from 0.016 to 0.132 for comparisons within French Polynesia (Fig. 2). The highest values were found for comparisons between French Polynesia sampling locations and the Red Sea (0.244–0.351), West Australia (0.243–0.335), East Australia (0.180–0.267) and New Caledonia (0.160–0.269).

The F_{ST} value between West and East Australia was 0.0579. This value is lower than for some of the comparisons of sampling locations from French Polynesia despite West and East Australia being much further apart (~5500 km) than any of the locations in French Polynesia (see * in Fig. 2). Most of the lowest F_{ST} values are for comparison between locations located in French Polynesia (Fig. 2a). The highest F_{ST} values are for comparisons of sites separated by the greatest geographic distances; comparisons of all locations with the Red Sea have among the highest F_{ST} values (Fig. 2c). The largest CIs are associated with New Caledonia as the number of samples from this location is lower (18).

Mitochondrial DNA F_{ST} values are based on very limited information in comparison with the microsatellite loci and so are less reliable and not used here to examine structure. Rather, we examine the extent to which individuals from the sampling locations share haplotypes. Only three of the 24 control region haplotypes and four of the 22 cytochrome *b* haplotypes are shared between two or more sampling locations—another line of evidence that genetic structure is high. One haplotype for both the control region (CR04) and for cytochrome *b* (Cyt04) is shared by New Caledonia and all of the French Polynesia locations. This shared haplotype is dominant among the South Pacific locations and is not seen in the Red Sea, but was found in one individual from East Australia for cytochrome *b* (Table 2).

This is surprising as the geographic distance between New Caledonia and French Polynesia is much greater (>5000 km) than between New Caledonia and East Australia (~1500 km). The maximum-likelihood trees (Fig. 3) show a similar pattern, with the Red Sea and West Australia in different branches than French



1 - Tet vs Rang
2 - Teno vs Mat
3 - Moorea vs Fakr
4 - Teno vs Vah
5 - Moorea vs Rang
6 - Vah vs Teng
7 - Teno vs Teng
8 - Rang vs Fakr
9 - Moorea vs Tet
10 - Tet vs Teno
11 - Rang vs NN
12 - Teng vs Mat
13 - Moorea vs NN
14 - Rang vs Teno
15 - Tet vs NN
16 - Fakr vs NN
17 - WA vs EA*
18 - Tet vs Mat
19 - Moorea vs Teng
20 - Moorea vs Teno
21 - Rang vs Teng
22 - Vah vs Mat
23 - Fakr vs Teng
24 - Tet vs Fakr
25 - Teno vs Mar
26 - Rang vs Mat
27 - Moorea vs Mar
28 - Tet vs Teng
29 - Rang vs Mar
30 - Moorea vs Mat
31 - NN vs Teng
32 - Moorea vs Vah
33 - Rang vs Vah
34 - Fakr vs Teno
35 - Tet vs Fakh
36 - Fakh vs Teno
37 - Rang vs Fakh
38 - Fakh vs Mat
39 - NN vs Teno
40 - Tet vs Mar
41 - Tet vs Vah
42 - Fakr vs Vah
43 - Moorea vs Fakh
44 - Fakh vs Teng
45 - Vah vs Mar
46 - Fakr vs Mar
47 - Fakh vs Vah
48 - Fakr vs Mat
49 - NN vs Mat
50 - Mat vs Mar
51 - Teng vs Mar
52 - Fakh vs NN
53 - NN vs Mar
54 - NN vs Vah
55 - Fakh vs Mar
56 - Fakr vs Fakh
57 - NC vs Moorea
58 - EA vs Fakr
59 - NC vs Fakr
60 - EA vs Rang
61 - RS vs EA
62 - RS vs WA
63 - EA vs NC
64 - EA vs Moorea
65 - NC vs Rang
66 - NC vs Teng
67 - EA vs Tet
68 - EA vs Fakh
69 - EA vs Teng
70 - EA vs Vah
71 - NC vs Vah
72 - EA vs NN
73 - NC vs NN
74 - NC vs Teno
75 - EA vs Teno
76 - NC vs Tet
77 - WA vs Fakr
78 - RS vs Fakr
79 - WA vs Moorea
80 - WA vs Rang
81 - NC vs Mat
82 - EA vs Mat
83 - NC vs Fakh
84 - EA vs Mar
85 - WA vs NC
86 - NC vs Mar
87 - RS vs Moorea
88 - WA vs Vah
89 - RS vs Rang
90 - WA vs Teng
91 - WA vs Tet
92 - RS vs NC
93 - RS vs Teng
94 - WA vs NN
95 - RS vs Vah
96 - WA vs Teno
97 - WA vs Fakh
98 - WA vs Mar
99 - RS vs NN
100 - RS vs Tet
101 - RS vs Teno
102 - RS vs Mar
103 - WA vs Mat
104 - RS vs Fakh
105 - RS vs Mat

Fig. 2 Blacktip reef shark microsatellites pairwise F_{ST} and 95% confidence interval values for all sampling locations (ordered from least to greatest value). All lower 95% CIs are >0 and all *P*-values associated with F_{ST} comparisons are <0.05 after FDR correction. The left (lower values) side represents comparisons among locations in French Polynesia (except for the comparison between West and East Australia marked with a *), and the right side represents values associated with New Caledonia/East Australia/West Australia/Red Sea (b), West Australia and the Red Sea only (c)

Table 2 Haplotype distribution for the blacktip reef sharks sampled at 10 locations for the control region (top) and cytochrome *b* (bottom). Haplotypes present only once in the whole data set (singletons) are shown in the last row for each location

Location	*N*	CR01	CR02	CR03	CR04	CR05	CR06	CR07	CR08	CR09	Singletons
Red Sea	30	28									2
West Australia	26		26								0
East Australia	21		3	14						1	3
New Caledonia	10				5						5
Moorea	30				30						0
Rangiroa	15				12	2					1
Fakahina	30				26		3				1
Fakarava	29				5			21	2		1
Nengo Nengo	28				7					19	2
Tenararo	30				20					10	0

Location	*N*	CytB01	CytB02	CytB03	CytB04	CytB05	CytB06	CytB07	CytB08	CytB09	Singletons
Red Sea	22	18									4
West Australia	26		25								1
East Australia	22		6	14	1						1
New Caledonia	9				8						1
Moorea	30				23	5					2
Rangiroa	15				15						0
Fakahina	30				30						0
Fakarava	30				26	2	1				1
Nengo Nengo	22				21						1
Tenararo	28				11	5	1	4	3	2	2

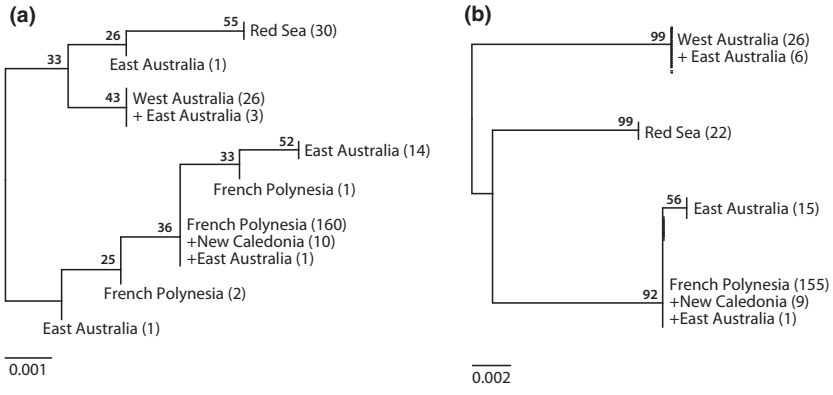


Fig. 3 Maximum-likelihood trees were inferred by using the maximum-likelihood method implemented in MEGA 5.2.2 (Tamura *et al.* 2011) with 2000 bootstrap replications. The trees with the highest log likelihood are presented [−1224.2658 for CytB (HKY model) and −1318.9420 for the CR (HKY+G model)]. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

Polynesia and New Caledonia, which have been pulled together. In the trees, East Australia is suggested to be a younger lineage coming from the Pacific. This seems unlikely at first because of the low F_{ST} values and the sharing of one haplotype (CR02 and CytB02, Table 2 and Fig. 2) between West and East Australia. The tree for the control region has a lower percentage output for this branch than others, probably due to the low number of mutations upon which it is based. However, there are more mutations on the CytB gene, which also indicates some individuals from East Australia (6) are from the same lineage as West Australia.

Demographic history

Nine of 15 sampling locations did not show a significant population expansion or bottleneck: eight of these are in French Polynesia and the other is New Caledonia. Of the six sampling locations where a significant event was inferred (presented in Fig. 4), five are population expansions—Red Sea, West and East Australia, Tetiaroa and Rangiroa. The other event inferred is a bottleneck in Moorea. MIGRAINE produces values for the point estimate and for the 95% confidence interval. Here, the values for θ and *D* used are based on point

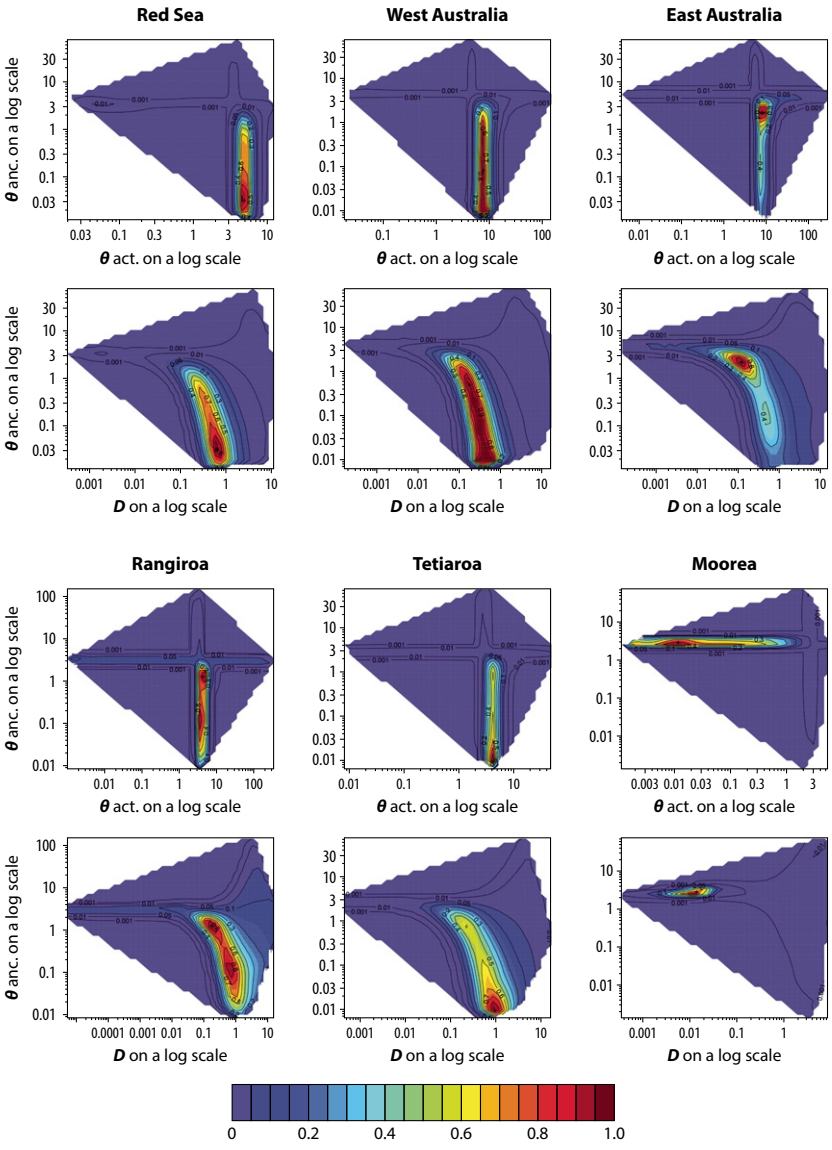


Fig. 4 MIGRAINE outputs representing likelihood surfaces for indicators of effective population size (ancestral (anc) and actual (act) θ) and timing of the demographic history events (*D*). Past expansions (all but Moorea) contrast the very recent bottleneck indicated for Moorea. The point estimates and 95% coverage confidence intervals values are shown in Table 3.

estimates derived from likelihood ratios (see italics, Table 3). Effective population sizes for the indicated expansions using point estimates are 150x greater for the Red Sea now than in the distant past, 15x for West Australia, 3x for East Australia, 390x for Tetiaroa and 3x for Rangiroa. The timing, in generations, since the beginning of those expansions are over 1500 (high μ) and over 75 000 (low μ) in all cases, indicating that the expansions are probably ancient. For Moorea, MIGRAINE indicates a highly significant decrease in the effective population size in the very recent past. For the lower mutation rate, actual effective population size is only 250 for Moorea, while the corresponding ancestral Ne value is 72 500 and the number of generations is 14 for this scenario, well more than an order of magnitude smaller than the number of generations for the indicated expansions (87 780 for East Australia). The bottleneck in Moorea likely took place in the last 150 years assuming a generation time for blacktip reef shark of 4–7 years (based on Smith *et al.* 1998). For both mutation rates, ancestral and actual θ values as well as *D* are shown in Table 3 for the six significant events inferred based on the two mutation rates applied. The likelihood ratios for ancestral and actual θ values as well as *D* from MIGRAINE are also plotted in Fig. 4 showing the stark contrast between the past expansions indicated and the very recent bottleneck in Moorea.

Discussion

Blacktip diversity is shown here to vary greatly among locations and, despite being common throughout their

Table 3 MIGRAINE outputs for locations for which a significant event is inferred based on two mutation rates (μ) for actual (act) and ancestral (anc) θ: *denote population expansions and † a bottleneck. The 95% coverage confidence interval outputs from MIGRAINE are shown. Numbers in italics represent point estimates. The full output and likelihood ratio for each of these locations is shown in Fig. 4. Effective population sizes shown are based on Ne = θ/4μ, and the time in generations shown for *D* is based on Tgeneration = 2 × *D* × 2Ne.

	Red Sea*			West Australia*			East Australia*			Rangiroa*			Tetiaroa*			Moorea†		
	μ	0.0005	0.00001	μ	0.0005	0.00001	μ	0.0005	0.00001	μ	0.0005	0.00001	μ	0.0005	0.00001	μ	0.0005	0.00001
Anc	0.013	6.5	325	0.007	3.5	175	0.72	360	18 000	0.009	4.5	225	0.0068	3.4	170	2.3	1150	57 500
θ	*0.032*	*16*	*800*	*0.49*	*245*	*12 250*	*2.17*	*1085*	*54 250*	*1.3*	*650*	*32 500*	*0.01*	*5*	*250*	*2.9*	*1450*	*72 500*
	2.1	1050	52 500	3	1500	75 000	4.7	2350	117 500	2.8	1400	70 000	2.3	1150	57 500	3.8	1900	95 000
Act	3.6	1800	90 000	5	2500	125 000	5.4	2700	135 000	2.8	1400	70 000	2.8	1400	70 000	0.0014	<1	35
θ	*4.8*	*2400*	*120 000*	*7.5*	*3750*	*187 500*	*7.7*	*3850*	*192 500*	*4.2*	*2100*	*105 000*	*3.9*	*1950*	*97 500*	*0.01*	*5*	*250*
	6.7	3350	167 500	11	5500	275 000	15	7500	375 000	6.3	3150	157 500	6	3000	150 000	1.3	650	32 500
D	0.081	1085.4	54 270	0.027	270	13 500	0.011	118.8	5940	0.027	151.2	7560	0.045	252	12 600	0.0026	<1	<1
	0.65	*6240*	*312 000*	*0.17*	*2550*	*127 500*	*0.114*	*1755.6*	*87 780*	*0.18*	*1512*	*75 600*	*0.92*	*7176*	*358 800*	*0.014*	*<1*	*14*
	2.1	28 140	1 407 000	0.99	21 780	1 089 000	0.32	9600	480 000	7.7	97 020	4 851 000	3.6	43 200	2 160 000	0.029	75.4	3770

	Fakh	Fakr	NN	Tena	Vah	Teng	Mat	Mar	NC
Anc θ	[0.012–29]	[0.012–57]	[0.011–88]	[0.016–2.0]	[0.013–46]	[0.03–16]	n.e.	n.e.	n.e.
Act θ	[0.002–inf]	[0–55]	[0.015–153]	[0.0045–4.75]	[0–17]	[0–20]	~1.5	~1.5	~2
D	[0–inf]	[0–inf]	[0–10]	[0.00026–inf]	[0–37]	[0–14]	n.e.	n.e.	n.e.

bio-geographic range in the tropics, blacktip reef sharks have high levels of genetic structure. We found that genetic diversity is much higher in open reefs (Red Sea and Australia) than in small islands surrounded by oceanic waters as is the case in French Polynesia. The effective population size is likely to be higher for open reefs than for smaller isolated locations for three reasons. The food chain of small islands likely only supports a limited number of predators; predator population size has an upper limit that depends on prey availability (Stevens 2012). Second, differences in life history traits across regions such as size at maturity and maximum size could influence population features in the long term (see review in Mourier *et al.* 2013). Third, locations like the Red Sea and West and East Australia have greater connectivity of shallow habitat so a larger population size can be supported. The same drivers of genetic diversity seem to be operating within French Polynesia as well because the smallest genetic diversity values are found for small atolls, rather than for the three larger islands/atolls (Moorea, Rangiroa and Fakarava). In essence, finding higher genetic diversity in larger islands in French Polynesia indicates population size is linked with genetic diversity for blacktip reef sharks.

A high level of genetic structure is found among the sampling locations, despite the potential connectivity between locations like West and East Australia. Of >700 samples, only seven were not correctly reassigned by GENECLASS2 to the five sampling location regions and F_{ST} value comparisons are significant (lower 95% CIs all >0, see Fig. 2) between all sampling locations. These results indicate that almost no direct mixing occurs between the locations. Blacktip reef shark size and ecology prevent them from swimming large distances in open ocean, although a recent unprecedented sighting in the East Pacific demonstrates they are capable of long open ocean swims (López-Garro *et al.* 2012). Such migrations are probably rare though. Even in French Polynesia, the genetic structure is moderate to strong, as was shown in Vignaud *et al.* (2013) but with far fewer samples. If mixing among locations is very low, as the results suggest, classic genetic divergence processes like genetic drift are contributing to the level of structure seen. Differences in the demographic histories of the populations from the five location regions could be another contributing factor. For example, a step-by-step colonization of islands can create a founder effect, which would contribute to genetic structure if connectivity is low as we assume must be the case for our sampling regions. Philopatry is also a major potential contributor; philopatric behaviour has been observed for blacktip reef sharks in French Polynesia (Mourier & Planes 2013) and weakens genetic dispersal (see review on sharks in Hueter *et al.* 2004; Dudgeon *et al.* 2012).

The genetic structure between West and East Australia (from F_{ST} value comparison) is very similar to that for locations within French Polynesia, despite the long swimming distance between West and East Australia. The F_{ST} value for the comparison between West and East Australia is only 0.0579, which is similar to values found between islands separated by only a few hundred kilometres within the fragmented environment of French Polynesia. The large population size seen in both Australian locations could partially contribute to the low F_{ST} values and expansion signals. An important aspect is that separation by deep oceanic water even if only by a few hundred kilometres is contributing to a higher genetic differentiation of blacktip demes in French Polynesia. Another factor likely contributing to these differences is that West and East Australia could have a more recent common history, which would lower the effect of genetic drift on the observed differentiation. This has been shown in northern Australia for many marine species (Lukoschek *et al.* 2008; van Herwerden *et al.* 2009; Horne *et al.* 2011; Blair *et al.* 2014) and is due to the development of the Torres Strait within the last 10 000 years (see Blair *et al.* 2014). Despite the level of structure seen, the ML trees for mtDNA suggested that East and West Australia have a common lineage. On the CytB tree, six individuals from East Australia were classified with the sharks from West Australia. Our sampling from East Australia could be composed of individuals from two meta-populations, one being from the Pacific and the other associated with our sampling from West Australia. However, the low number of mutations in the mtDNA genes means we have low confidence in the ML trees. Further, parallel random mutations at the same base pair in East Australia and French Polynesia could lead to their grouping. We have shown evidence of low structure for East vs. West Australia as well as some evidence of a common lineage; greater sampling from the locations in Australia and subsequent analysis will help elucidate whether the East and West Australian blacktip sharks are 1 or 2 metapopulations.

Examination of the haplotypes and the ML trees for both mitochondrial sequences shows sharing of one dominant haplotype between all French Polynesian sampling locations and New Caledonia. This is surprising considering that these locations are separated by ~6500 km of oceanic water. A greater extent of haplotype sharing between East Australia and New Caledonia was expected. The Coral Sea, east of Australia, may be a strong dispersal barrier. The Coral Sea is known for high densities of blacktip reef shark predators, like tiger, bull and great white sharks (e.g. Smith *et al.* 2008;

Werry *et al.* 2012, 2014). The linkage between New Caledonia and French Polynesia based on mtDNA is not seen in the microsatellite data, which in our case may be a stronger marker as the sequences employed show few mutations. Additional sampling in areas between New Caledonia and French Polynesia, as well as the employment of more variable sequence regions for sharks, may improve our understanding of shark connectivity between these locations. Results similar to those presented here were found for the lemon shark. Significant microsatellite differentiation was found for lemon sharks between Australia and French Polynesia, but no genetic differentiation was seen in the analysis of mitochondrial sequences (Schultz *et al.* 2008). It is possible that the main path taken by ancestral blacktip reef sharks involved colonizing Australia through Indonesia and Papua New Guinea and that a different Pacific lineage links the populations of French Polynesia and New Caledonia.

The three sampling locations that are open environments—Red Sea and East Australia and West Australia—all probably have relatively large actual population sizes (large actual θ), and MIGRAINE indicates blacktip reef sharks in each of these locations have undergone a population expansion. It is important to note that demographic history analysis may be amplified by the presence of 'ghost populations' (Slatkin 2005) connected to the Red Sea, West and East Australia groups, adding strength to the expansion signal. The expansions are all likely to have occurred over the course of many thousands of generations, according to the profile likelihood ratios from the MIGRAINE outputs. Blacktip reef sharks are well adapted to current climates and could easily have moved along coastlines, reefs and estuarine environments (e.g. Chin *et al.* 2013b) during older climatic events. Importantly, the expansions found for the Red Sea and both Australian locations do not necessarily indicate those populations are healthy. Blacktip reef sharks are known to be fished (Robbins *et al.* 2006; Chin *et al.* 2012) at all of these locations, and bottlenecks can stay undetected depending on both population and sampling characteristics (Heller *et al.* 2013). More detailed and diverse surveys and analyses of the population dynamics of blacktip reef sharks in the Red Sea and Australia would be necessary to reliably assess their health and status.

The demographic history of sampling locations in French Polynesia and especially Moorea is in stark contrast to that seen for the open environments of the Red Sea and Australia. Flat signals are found for 8 of the 11 sampling locations in French Polynesia. These locations are all small islands that are nearly pristine with no or almost no human presence. Expansion could be limited at these locations because of the availability of prey and suitable breeding and pupping habitat and because of isolation. However, demographic history analysis can be sensitive to the extreme isolation of these populations, lowering the inferred number of individuals in the present vs. the past (Marko & Hart 2011).

An expansion is found for only two of the 11 locations in French Polynesia: Rangiroa and Tetiaroa. These two islands are also the least differentiated of all our sampling locations (pairwise F_{ST} value—0.016). Blacktip reef sharks may occasionally travel between the islands and mix, so the population over time here has steadily grown. Tetiaroa is a small atoll, but Rangiroa is particularly large and open and is approximately 330 km northeast of Tetiaroa. The uniqueness of this expansion signal within French Polynesia might be explained by Rangiroa providing a refuge from low sea levels in the past and, again, by Rangiroa and Tetiaroa being connected in the past or possibly still connected now. Fakarava is a large, open atoll like Rangiroa, but no significant expansion was detected for Fakarava. There are slight but significant differences between these two large atolls in relation to the quality of habitat for blacktip reef sharks such as the inner lagoon depth or other features that relate to coastal habitat quality for pupping. The features of the two atolls that could drive differences in the ability of blacktip populations to expand warrant further research.

Moorea is the only location in our study for which a significant bottleneck is detected. The timing of the bottleneck (in generations) is an approximation based on the *D* values inferred by MIGRAINE. The approximation is a range with some values having a far higher likelihood than others. The point estimate value suggests, for the lower mutation rate (more conservative), that the bottleneck has occurred in the last ~15 generations. The effective population sizes calculated suggests the effective population has crashed in that time period and may now be as low as ~250. Very recent bottlenecks are known to be very hard to detect, mostly because of the use of mutation models that poorly represent reality often in conjunction with low sample size (Peery *et al.* 2012). MIGRAINE partly solves the mutation model issue, and we have 380 samples for Moorea so, for Moorea, neither of the common problems is an issue. However, bottlenecks may have gone undetected for other islands in French Polynesia where our sample sizes are far lower (always <100). The bottleneck detected for Moorea coincides with the expansion of the local human population and with coastal development and lagoon habitat destruction. Moorea is the sister island of Tahiti; the two are separated by only 20 km. The coastal marine habitats of Moorea that form the feeding, breeding and nursery areas for blacktip reef sharks (see Mourier

& Planes 2013) have been subjected to extensive manipulation and destruction since 1960 (Aubanel *et al.* 1999; Lecchini *et al.* 2009; Benet 2010). The resident human population has grown from 2000 in 1946 to ~17 000 in 2007 and over this last decade 100 000 tourists have visited per year. To support tourism, 1100 hotel rooms have been built and ~200 are bungalows that sit directly in the lagoon. Foundations for many of the hotels and hotel rooms, private homes and local infrastructure like roads are made from a coral soup that comes from sand and coral taken from the lagoon and reef crest using excavators. The digging has damaged reefs directly and indirectly by altering current dynamics and water quality and clarity. Coastal development activities may also have impacted shark behaviour due to sharks being repulsed by the noise. Overall, over 20% of the Moorea coast has been artificially transformed in the past two decades, resulting in 47.5% of the coastline being affected in some way by human construction as of 2001 (Polti 2001) and coastal development has not slowed since then.

Habitat destruction is a far more likely cause of the bottleneck seen in Moorea than fishing. Fishing may be a partial contributor, but the local Polynesians very rarely eat shark (if at all) for cultural reasons. Reports suggest sharks are occasionally caught for consumption by some locals and tourists, but traditionally, sharks are not a common target of fishers in the area as tuna and other more prized food fish are readily available. There are no reports of illegal fishing by outsiders in Moorea dating back as far as the early 1960s when the CRIOBE marine laboratory was established. In 2006, a total ban on shark fishing (except mako *Isurus* sp.) was established in French Polynesia. The ban on shark fishing may not prevent the blacktip population from declining further given the circumstantial evidence we present suggesting habitat destruction is the most likely cause of the bottleneck we find.

The evidence based on the demographic history analyses we present is the first to indicate that the blacktip shark population in Moorea declined recently (and may still be declining). This result emphasizes the critical importance of studies of population genetics for marine species irrespective of whether the discovered bottleneck is due to a recent human impact or to other unknown cause(s). The scientific and management community often has no indication whatsoever of the health of free-swimming marine species. This is especially true of species like sharks that can avoid humans or can artificially concentrate because they are fed to entertain tourists (e.g. Vignon *et al.* 2010; Clua *et al.* 2011). Blacktip reef sharks are seen regularly on dives around Moorea, and there is something innately human about allowing seeing large animals occasionally to result in the assumption their populations are healthy. The results presented here indicate how dangerous this assumption can be, and the results can be used locally in Moorea to support strengthening and diversifying shark protection measures. Preferred blacktip breeding, pupping and nursery habitat could easily be identified, and development activities could be limited in these areas. Population genetics can now infer demographic history with more and more power as we use more genes from greater numbers of samples and improve models that use steadily advancing computing technology. As shown here for the blacktip reef sharks of Moorea, demographic history analyses can be an important and often even the only line of evidence that populations are being impacted and can thus serve as a powerful driver of positive change.

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Data accessibility

Microsatellites genotype and both CR and CytB sequences with sampling locations are both accessible at the following doi:10.5061/dryad.th4h5.