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Global phylogeography and seascape genetics of the lemon sharks (genus *Negaprion*)

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Abstract

Seascapes are complex environments, and populations are often isolated by factors other than distance. Here we investigate the role of coastal habitat preference and philopatry in shaping the distribution and population structure of lemon sharks. The genus *Negaprion* comprises the amphiatlantic lemon shark (*N. brevirostris*), with a relict population in the eastern Pacific, and its Indo-West Pacific sister species, the sicklefin lemon shark (*N. acutidens*). Analyzing 138 individuals throughout the range of *N. brevirostris* ($N = 80$) and *N. acutidens* ($N = 58$) at microsatellite loci (nine and six loci, respectively) and the mitochondrial control region, we find evidence of allopatric speciation corresponding to the Tethys Sea closure (10–14 million years ago) and isolation of the eastern Pacific *N. brevirostris* population via the emergence of the Isthmus of Panama (~3.5 million years ago). There is significant isolation by oceanic distance ($R^2 = 0.89$, $P = 0.005$), defined as the maximum distance travelled at depths greater than 200 m. We find no evidence for contemporary transatlantic gene flow (m, $M = 0.00$) across an oceanic distance of ~2400 km. *Negaprion acutidens* populations in Australia and French Polynesia, separated by oceanic distances of at least 750 km, are moderately differentiated ($F_{ST} = 0.070$–0.087, $P \leq 0.001$; $\Phi_{ST} = 0.00$, $P = 0.99$), with South Pacific archipelagos probably serving as stepping stones for rare dispersal events. Migration between coastally linked *N. brevirostris* populations is indicated by nuclear ($m = 0.31$) but not mitochondrial ($m < 0.001$) analyses, possibly indicating female natal site fidelity. However, philopatry is equivocal in *N. acutidens*, which has the lowest control region diversity ($h = 0.28$) of any shark yet studied. Restricted oceanic dispersal and high coastal connectivity stress the importance of both local and international conservation efforts for these threatened sharks.

Keywords: dispersal, elasmobranchs, fish, marine landscape ecology, vicariance

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Introduction

Phylogeographical patterns in sharks may be fundamentally different from those of bony fishes. Because elasmobranchs do not have pelagic eggs and larvae, dispersal potential is dependent upon adult vagility, which is highest in sharks that are large (> 1.5 m), pelagic (active water-column swimmers), and oceanic (habitat extending beyond the shelf break; Musick *et al*. 2004). Global surveys of genetic structure in pelagic species confirm connectivity within and between ocean basins; however, the combined analyses of nuclear and mitochondrial markers reveal that gene flow is often male-mediated while females may be philopatric (Heist *et al*. 1996; Pardini *et al*. 2001; Schrey & Heist 2003). Coastal sharks, whose habitat is bound by the continental

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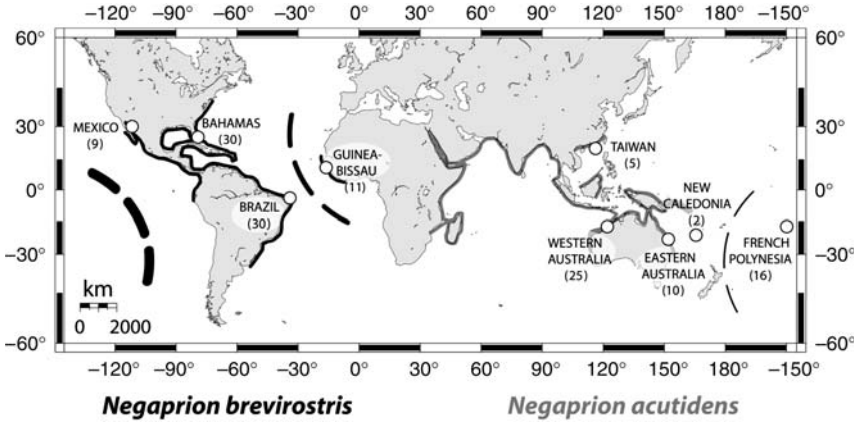

Fig. 1 Sampled populations of the lemon and sicklefin lemon shark. Dark shaded areas represent the range of *Negaprion brevirostris* and light shaded areas represent the range of *Negaprion acutidens*. Numbers in parentheses represent the number of samples collected from each location. Dashed lines represent barriers to gene flow as revealed using microsatellite and mtDNA distance matrices; line thickness increases with relative impermeability. Map created using Online Map Creation (www.aquarius.geomar.de/).

shelf break, usually at the 200-m isobath, are thought to be less vagile (Musick *et al*. 2004). Mitochondrial DNA (mtDNA) analyses of two coastal species indicate significant genetic structure across ocean basins, although the extent to which this reflects female philopatry is unknown (hammerhead, Duncan *et al*. 2006; blacktip, Keeney & Heist 2006).

Lemon sharks provide an appropriate system to test the relative importance of coastal habitat preference and philopatry in defining evolutionary partitions. The genus *Negaprion* comprises two large-bodied, subtropical, benthopelagic species (Fig. 1). The lemon shark (*Negaprion brevirostris*) inhabits shallow, inshore waters throughout the western Atlantic with small, isolated populations in the eastern Atlantic and eastern Pacific, where their presence is tenuous due to overfishing and demand for shark fins (Compagno 1988; Gruber & Manire 1990). A long-term, mark–recapture study indicates that lemon sharks rarely travel more than 10 km from their original tagging location (Kohler *et al*. 1998; N. Kohler, personal communication). Yet, a pup tagged in Bimini, Bahamas was recaptured 10 years later in Apalachicola, Florida, requiring a minimum travel distance of 1000 km and crossing open ocean (Feldheim *et al*. 2001a). There is gene flow among four coastal populations in the western Atlantic, with microsatellite analyses indicating weak structure ($\theta = 0.016$, $P \leq 0.05$) over a range of 6000 km (Feldheim *et al*. 2001a). There is also evidence for female philopatry: long-term parentage studies at two western Atlantic nurseries demonstrate that while males do not consistently sire litters at specific sites, females typically return for parturition on a biennial cycle (Feldheim *et al*. 2004; DiBattista *et al*. 2008).

Less is known about the movements and reproductive biology of the sicklefin lemon shark (*Negaprion acutidens*), largely because of its overall scarcity. Once widely distributed throughout the coastal Indo-West Pacific (Fig. 1), the species is extirpated in India and Thailand, endangered in Southeast Asia, and considered vulnerable throughout the remainder of its range [IUCN Redlist (International Union for Conservation of Nature and Natural Resources); www.iucnredlist.org]. A tagging study of sicklefin lemon sharks indicates restricted movement patterns, with 91% of recaptures occurring within 2 km of the tagging site (Stevens 1984).

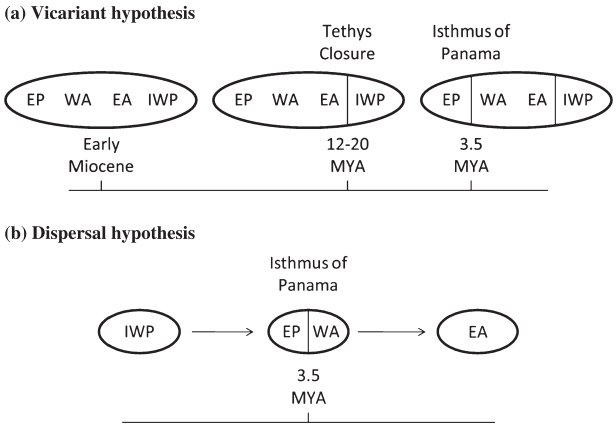

Fig. 2 Alternative phylogeographical hypotheses. Ovals represent populations; vertical lines reflect the emergence of land barriers; arrows represent dispersal; horizontal lines represent time. IWP, Indo-West Pacific; EP, East Pacific; EA, Eastern Atlantic; WA, Western Atlantic.

An ongoing issue in biogeography and evolution is how coastal species attain a global distribution. Musick *et al*. (2004) propose a classic vicariant hypothesis (Fig. 2a): the coastal range of a cosmopolitan lemon shark was sundered by closure of the Tethys Sea, a coastal link between Indian and Atlantic Oceans (Ricou 1987), isolating ancestors of lemon and sicklefin lemon shark species; subsequently, emergence of the Isthmus of Panama (~3.1–3.5 million years ago; Coates & Obando 1996; Coates *et al*. 2004) isolated eastern Pacific and Atlantic *N. brevirostris* populations. An alternative hypothesis (Fig. 2b) is dispersal across the East Pacific Barrier, 4000–7000 km of uninterrupted deep water between

Table 1 Genetic indices for all *Negaprion* populations. Sample size in parentheses

	Microsatellite DNA					Mitochondrial DNA								
	H_O	H_E	A_R	A	Private alleles	Hap	h	π	τ	Years (95% CI)	Θ_0	N_{f0}	Θ_1	N_{f1}
N. brevirostris (80):	0.73	0.81	8.5	19.8	85	11	0.78	0.00585						
Pacific Mexico (9)	0.81	0.77	7.1	7.1	12	1	0.00	0.00000						
Bahamas (30)	0.77	0.75	7.9	14.0	26	6	0.54	0.00075	0.95	81 000 (0–280 000)	0.23	1600	1.94	14 000
Brazil (30)	0.73	0.76	6.9	11.0	14	3	0.34	0.00037	2.98	260 000 (0–9 000 000)	0.90	6000	3.60	26 000
Guinea Bissau (11)	0.56	0.53	6.1	6.8	12	3	0.62	0.00099	1.87	160 000 (0–380 000)	24.3	13	1.76	13 000
N. acutidens (58):	0.58	0.67	2.6	10.5	31	4	0.28	0.00056						
Taiwan (5)	0.63	0.63	2.5	4.2	2	1	0.00	0.00000						
French Polynesia (16)	0.45	0.44	1.9	2.8	1	1	0.00	0.00000						
New Caledonia (2)	0.33	0.39	1.7	1.7	1	2	1.00	0.00185						
Eastern Australia (10)	0.56	0.69	2.8	4.7	1	2	0.33	0.00061	2.98	260 000 (0–9 000 000)	0.90	6000	3.60	26 000
Western Australia (25)	0.64	0.65	2.6	8.7	22	3	0.60	0.00154	3.55	300 000 (45 000–650 000)	0.00	0	2.39	17 000

For microsatellite data: observed heterozygosity (H_O), expected heterozygosity (H_E), average allelic richness (A_R), number of alleles (A), and number of private alleles in reference to other populations within species or other species. For mitochondrial data: number of haplotypes (Hap), haplotype diversity (h), nucleotide diversity (π), tau (τ), years since population expansion (with 95% confidence intervals), initial theta (Θ_0), initial female effective population size (N_{f0}), contemporary theta (Θ_1), contemporary female effective population size (N_{f1}).

the central Pacific and the Americas (Briggs 1974). Other 'coastal' sharks with origins in the Indo-West Pacific likely colonized the eastern Pacific using South Pacific archipelagos as stepping stones (Duncan *et al*. 2006; Keeney & Heist 2006). While both hypotheses would produce a similar phylogeny, dispersal across the eastern Pacific could have occurred more recently than the closure of the Tethys Sea.

Here we assess the relative roles of vicariance, dispersal, and female philopatry in shaping the genetic architecture of the genus *Negaprion*, using both nuclear (microsatellite) and mitochondrial analyses. We modify methods in seascape genetics (Galindo *et al*. 2006) to illuminate the impact of depth and distance on population structure within *N. brevirostris* and *N. acutidens*. Our results contribute to a small but growing literature on the phylogeography of sharks. Given the depleted status of many species, including the lemon sharks, these findings have immediate applications in conservation.

Materials and methods

Sampling

A total of 138 lemon shark specimens (*Negaprion brevirostris* = 80; *Negaprion acutidens* = 58; Table 1) were collected between 1995 and 2006. *Negaprion brevirostris* specimens represent the western Atlantic (Bimini, Bahamas and Atol das Rocas, Brazil), the eastern Atlantic (Bijagos, Guinea-Bissau), and the eastern Pacific (Mexico; Fig. 1). We used DNA extracts from previously collected fin clips from Brazil (N = 30) and the Bahamas (N = 30), representing a subset of samples originally included in the microsatellite study of western Atlantic population structure (Feldheim *et al*. 2001a, b). DNA extracts were chosen to avoid sampling siblings, as determined by Feldheim *et al*. (2002, 2004). In Guinea-Bissau (N = 11), no lemon sharks were caught via fishing or gill netting during 2 weeks of effort; 11 specimens were purchased opportunistically over 2 years from a local fishing market, reflecting their scarcity in this region likely due to overfishing (P. Sebile, personal communication). In the eastern Pacific, *N. brevirostris* samples were collected from the Gulf of California, Mexico (N = 9). One juvenile was caught in 3 weeks of gill netting off Isla Tiburon, Sonora; eight additional sharks were purchased from a fisherman working off the Islas Tres Marias (D.R. Robertson, personal communication). This population has also been exposed to high fishing pressure.

We acquired a total of 58 *N. acutidens* tissue specimens from several sources. The reference collection at the Western Australian Fisheries and Marine Research Laboratories provided 25 samples. Collections at the James Cook University and juveniles caught opportunistically at Heron Island provided a total of 10 samples from eastern Australia. Two specimens were acquired from the fish markets of New Caledonia (Iglesias *et al*. 2005). We received tissue samples from juveniles caught at Pratas Island, Taiwan (N = 5). In French Polynesia (N = 16), tissue samples were obtained using a biopsy tip at shark feeding dive sites in Moorea and Rangiroa. Sequences from the whitetip reef shark (*Triaenodon obesus*) and blacktip shark (*Carcharhinus limbatus*; obtained from GenBank, Benson *et al*. 2005) were used as outgroups for phylogenetic analyses (see Iglesias *et al*. 2005).

Laboratory procedures

Total genomic DNA was extracted from all samples using the QIAGEN DNeasy Tissue kit following the manufacturer's protocol. We sequenced the entire mitochondrial control region (approximately 1100 bp) following the protocol of Keeney & Heist (2006). DNA sequences were edited using SEQUENCHER version 4.52b (Gene Codes Corporation). Sequences are available in GenBank (www.ncbi.nlm.nih.gov; accession nos FJ008696–FJ009710). Specimens were genotyped at nine microsatellite loci (LS11, LS15, LS22, LS30, LS48, LS52, LS54, LS75, and LS82) following Feldheim *et al*. (2001a, 2002). Fifty per cent of the extracted DNA samples were amplified and scored separately by J. Schultz and K. Feldheim to evaluate polymerase chain reaction and scoring error rate.

Population structure, effective migration and philopatry

Edited mtDNA sequences were aligned in PROALIGN 1.2 (Loytynoja & Milinkovitch 2003) and evaluated using MODELTEST 3.06 (Posada & Crandall 1998) to identify the most appropriate model of DNA evolution via the Akaike information criterion. Control region sequences were used to construct a statistical parsimony network in TCS 1.21 (Clement *et al*. 2000) using the default settings. Haplotype diversity (h), nucleotide diversity (π) and genetic distances (d) were calculated in ARLEQUIN 3.1 (Excoffier *et al*. 2005).

Microsatellite data from the nine loci were screened to ensure high quality (see Selkoe & Toonen 2006). The software MICRO-CHECKER 2.2.3 (van Oosterhout *et al*. 2004) was used to check for null alleles and large allele dropout. Mendelian inheritance was assessed by confirming pup–mother allele sharing of known litters (Feldheim *et al*. 2001b, 2002), but no pup–mother pairs were included in the population analyses. Molecular diversity indices and tests for Hardy–Weinberg equilibrium (100 000 iterations and 1000 dememorization steps) and linkage disequilibrium (10 000 permutations) were performed in each population and globally using Arlequin and FSTAT 2.9.3.2 (Goudet 1995).

To assess population subdivision, we performed an analysis of molecular variance (θ, AMOVA; Weir & Cockerham 1984; Excoffier *et al*. 1992) between transisthmian (Pacific Mexico compared with Bahamas, Brazil and Guinea-Bissau) and transatlantic populations (Bahamas and Brazil compared with Guinea-Bissau). Pairwise population Φ_{ST} and F_{ST} comparisons were performed in Arlequin (10 000 permutations each). Sicklefin lemon shark populations were similarly analysed using AMOVA (French Polynesia compared with eastern and western Australia). While mitochondrial analyses were performed on all samples, Mexico, Taiwan and New Caledonia were not included in microsatellite analyses due to small sample sizes of nine, five and two, respectively.

To distinguish between recent isolation and low levels of contemporary migration as explanations for observed values of mtDNA genetic divergence, we applied a Markov chain Monte Carlo approach as implemented in MDIV, where $M = 2N_e m$ and $T = t/2N_e$ (m = migration rate and t = time since divergence; Nielsen & Wakeley 2001). Isolation is inferred when $M = 0$ and the distribution of T is unimodal; migration is inferred when $M > 0$ and $T = 0$. Posterior probability distributions (5 000 000 replicates with a burn-in of 500 000) were used to estimate each parameter (mode) and 95% credibility intervals. Recent migration rates were also estimated from the microsatellite data with BAYESASS+ 1.0 (Wilson & Rannala 2003) using posterior probabilities to estimate migration rates (mean) and 95% confidence intervals. A recent population bottleneck (or recent colonization) may produce a signature pattern of excess heterozygosity in relation to that expected by a given number of alleles. Therefore, we analysed microsatellite data for the presence of a bottleneck using 1 000 000 iterations of a Wilcoxon test, using an infinite alleles model (IAM), two phase model (TPM) and a stepwise mutation model in BOTTLENECK 1.2.02 (Cornuet & Luikart 1997).

Differences in mitochondrial and microsatellite results may be attributed to several factors, including sex-biased dispersal, mode of inheritance, and amount of variation. Therefore, we calculated standardized measures of AMOVA using the programs FSTAT and RECODEDATA 0.1 (Meirmans 2006) to correct for differences in genetic variation among markers. We compared the difference between standardized mitochondrial and microsatellite markers to evaluate female philopatry.

Seascape genetics

To describe the genetic seascape spatially, mitochondrial and microsatellite (D_c, chord distance; Cavalli-Sforza & Edwards 1967) matrices were plotted on a map created via Delaunay triangulation and Voronoi tessellation using the software BARRIER 2.2 (Manni *et al*. 2004). We applied the default settings to plot up to 10 barriers.

To determine whether the data fit an isolation-by-distance model, we performed a simple linear regression of microsatellite and mitochondrial genetic divergence against total geographical (Euclidean) distance between populations, oceanic distances (calculated as the maximum distance travelled across depths > 200 m), and maximum depth. We plotted pairwise-population mitochondrial and microsatellite distances (D_c) against three measures of geographical separation (total, oceanic and depth); we evaluated regression (R^2) using MINITAB 14.12.0. Oceanic distances were mapped in GEOMAPAPP 1.6.6 (www.geomapapp.org) to reflect the minimum distance and depths required to travel between two locations.

Phylogeography

We used the software PAUP* 4.0b10 (Swofford 2000) to build maximum likelihood (ML) and maximum parsimony (MP) trees, bootstrapping over 1000 replicates. We also applied a Bayesian approach, using MRBAYES version 3.1.2 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003) with priors set according to the best-fit model as determined above. The Markov chain Monte Carlo search was run with four chains for 1 000 000 generations, trees sampled every 100 generations, and a burn-in of 10 000 trees. To test genealogical concordance in nuclear and mtDNA analyses, a microsatellite distance matrix among populations was created using the chord distance (D_c option) in the program MICROSAT 2.0 (Minch *et al*. 1995) to construct a neighbour-joining (NJ) tree in NEIGHBOR/PHYLIP 3.5 (Felsenstein 1989) modifying Luo *et al*. (2004).

Divergence times between species were obtained using a molecular clock which incorporates a penalized maximum likelihood method (software R8S 1.70; Sanderson 2003), calibrated with the emergence of the Isthmus of Panama, 3.5 million years ago. We compared these values to divergence times following a coalescent method described by Gaggiotti & Excoffier (2000) as implemented in Arlequin. We used the same software to construct and assess mismatch distributions (using 16 000 replicates), testing population-level expansion, where $\tau = 2\mu T$ and $\theta = 2N_f\mu$ (Rogers & Harpending 1992; Schneider & Excoffier 1999), and applying a generation time (T) of 20 years, given that females mature at 12–14 years of age and can live to over 50 (Brown & Gruber 1988; Feldheim *et al*. 2002; Barker *et al*. 2005).

Results

Sequencing 1090 bp of the mitochondrial control region revealed a total of 81 polymorphic sites and fifteen haplotypes, including eleven in *Negaprion brevirostris* and four in *Negaprion acutidens* (Table S1, Supporting information). No haplotypes were shared between the two species, which differed by a minimum of 62 polymorphic sites; and the single haplotype from Pacific Mexico differed from other *N. brevirostris* haplotypes by a minimum of 25 nucleotide sites (Fig. 3). There were multiple haplotypes in each Atlantic *N. brevirostris* population (six in Bahamas, three in Guinea-Bissau and three in Brazil; Table 1). The most common haplotypes were shared between the coastal populations of Bahamas and Brazil, but none were shared across the Atlantic. We found only four mtDNA control region haplotypes in 58 sicklefin lemon sharks, exhibiting the lowest haplotypic ($h = 0.28$) and nucleotide ($\pi = 0.00056$) diversity of any shark tested to date (Hoelzel *et al*. 2006). A single haplotype was shared by 49 individuals, including all sharks from French Polynesia ($N = 16$), 23 from Western Australia, nine from eastern Australia and one from New Caledonia. The five individuals from Taiwan shared a single, unique haplotype.

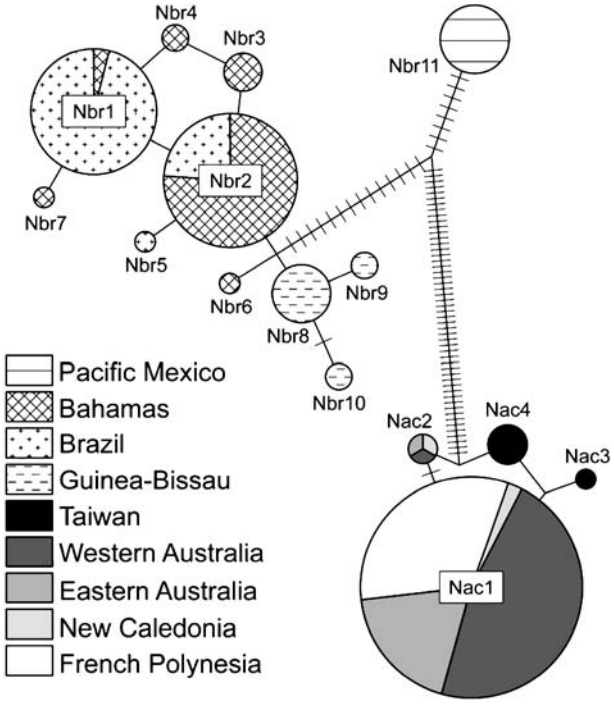

Fig. 3 Parsimony network of *Negaprion* (*N. brevirostris*, Nbr; *N. acutidens*, Nac) control region haplotypes, relative area of circles reflects sample sizes, uninterrupted lines (between dashes) represent missing haplotypes.

In *N. acutidens*, microsatellite loci LS22 and LS30 were monomorphic, and LS48 primers appeared to amplify two regions of the genome; these loci were excluded from the analyses of this species. Nine loci for *N. brevirostris* and six loci for *N. acutidens* were analysed further (Table S2, Supporting information). Comparing microsatellite allele calls between two laboratories and researchers revealed consistency in 97% of the scores. No loci showed evidence for null alleles, and there was no evidence of allelic dropout. All populations appeared to be in Hardy–Weinberg equilibrium at each locus and globally, after a Bonferroni correction ($\alpha = 0.005$). No linkage disequilibrium was observed over all conspecific populations, and none were found locally after Bonferroni correction. Of 245 segregation events in known *N. brevirostris* litters, we observed one mismatch due to a presumed stepwise mutation between mother and pup, confirming Mendelian inheritance.

Negaprion brevirostris *mitochondrial analyses*

For *N. brevirostris*, mitochondrial AMOVA analyses and pairwise comparisons between Pacific Mexico and all other populations revealed genetic isolation ($\theta = 0.96$, $P \leq 0.001$;

Table 2 Pairwise comparisons of genetic differentiation and effective migration among conspecific populations. Above diagonal, microsatellite estimates of pairwise F_{ST} and m (BAYESASS+; Wilson & Rannala 2003) migration rates with 95% confidence intervals in bold; assymetric migration (from column to row) noted with an asterisk. Below diagonal, control region Φ_{ST} and m (MDIV; Nielsen & Wakeley 2001) migration rates with 95% credibility limits in italics. All pairwise estimates significant ($P < 0.001$) unless specified not significant (NS)

(a) *Negaprion brevirostris*

	Bahamas	Brazil	Guinea-Bissau
Bahamas	—	0.057 **0.31 (0.27, 0.33)***	0.24 **0.0069 (0.00, 0.028)**
Brazil	0.45 *< 0.001 (0.0, < 0.001)*	—	0.23 **0.0036 (0.00, 0.020)**
Guinea-Bissau	0.69 *0.0 (0.0, 0.0)*	0.83 *0.0 (0.0, 0.0)*	—

*From Bahamas to Brazil m = **0.0039 (0.00, 0.022)**.

(b) *Negaprion acutidens*

	Western Australia	Eastern Australia	French Polynesia
Western Australia	—	NS **0.19 (0.057, 0.31)****	0.070 **0.011 (0.00, 0.060)**
Eastern Australia	NS *0.011 (0.001, 0.01)*	—	0.087 **0.011 (0.00, 0.050)**
French Polynesia	NS *0.005 (0.001, 0.01)*	NS *0.011 (0.001, 0.03)*	—

From eastern Australia to western Australia m = **0.015 (0.00, 0.06).

$\Phi_{ST} = 0.99$, $P \leq 0.001$). There is greater partitioning between eastern and western Atlantic *N. brevirostis* populations ($\theta = 0.79$, $P \leq 0.001$) than over all populations ($\theta = 0.67$, $P \leq 0.001$). Pairwise population estimates also indicated high transatlantic structure, with $\Phi_{ST} = 0.69$ ($P \leq 0.001$) between the Bahamas and Guinea-Bissau, and $\Phi_{ST} = 0.845$ ($P \leq 0.001$) between Brazil and Guinea-Bissau, as compared to $\Phi_{ST} = 0.454$ ($P \leq 0.001$) between coastally linked Bahamas and Brazil (Table 2a). MDIV analyses revealed no migration ($M = 0$) across the Atlantic and divergence times of 100 000 years ago (Guinea-Bissau and Bahamas; $T = 2.1$) and 480 000 years ago (Guinea-Bissau and Brazil; $T = 4.4$), with wide credibility intervals ($T = 0.2$–9.5; 95% CI = 100 000–500 000 years for both comparisons). In contrast, analyses between the Bahamas and Brazil could not be used to estimate divergence (due to a flat distribution) but provided estimates of gene flow ($M = 0.6$; 95% CI = 0.02–3.5) corresponding to low migration rates ($m = 0.000011$; 95% CI = 0.00–0.000063).

Negaprion brevirostris *microsatellite analyses*

Microsatellite AMOVA analyses across the Atlantic (combined Bahamas/Brazil and Guinea-Bissau; $\theta = 0.23$, $P \leq 0.001$) and over the entire Atlantic ($\theta = 0.14$, $P \leq 0.001$) indicated elevated transoceanic structure. Pairwise estimates of genetic differentiation across all populations reveal greater structure between transatlantic Guinea-Bissau/Bahamas ($F_{ST} = 0.24$, $P \leq 0.001$) and Guinea-Bissau/Brazil ($F_{ST} = 0.23$, $P \leq 0.001$) than the coastal comparison between the Bahamas and Brazil ($F_{ST} = 0.057$, $P \leq 0.001$; Table 2a). Furthermore, loci LS11 and LS54 were monomorphic in eastern Atlantic sharks, while there were 24 (LS11) and 5 (LS54) alleles observed in the western Atlantic populations. Thirty-four private alleles on either side of the Atlantic were observed in frequencies greater than ten percent. Transatlantic migration rate estimates derived from the microsatellite data were low, with the variance equal to the mean of the posterior probabilities ($m < 0.01$; Table 2a). However, the Bayesian estimate of migration from Brazil to Bahamas was high ($m = 0.31$; 95% confidence interval = 0.27–0.33), although small sample sizes and/or unsampled populations could produce such a signal. Tests for bottlenecks were not significant. The standardized genetic differentiation in Atlantic *N. brevirostris* computed using microsatellite data ($\theta = 0.47$) was approximately half that using mitochondrial data ($\theta = 0.92$).

Negaprion acutidens *mitochondrial analyses*

Mitochondrial AMOVA and pairwise analyses of Taiwan compared to South Pacific populations (Australia, New Caledonia, and French Polynesia) revealed strong genetic differentiation ($\theta = 0.86$, $P \leq 0.001$; $\Phi_{ST} = 0.77$–1.00, $P \leq 0.001$). Pairwise comparisons of New Caledonia and other South Pacific populations also indicated strong structure ($\Phi_{ST} = 0.21$–0.78, $P \leq 0.001$). Taiwan and New Caledonia were not included in further analyses because of small sample sizes of five and two, respectively. Mitochondrial AMOVA analyses of French Polynesian and Australian

N. acutidens populations (combined or separate) were not significant ($\theta = 0.00$, $P = 0.99$). Pairwise estimates were also not significant in any comparison ($\Phi_{ST} = 0.00$, $P = 0.99$; Table 2b). MDIV analyses revealed no divergence ($T = 0$), with elevated migration estimates among all population comparisons: within Australia ($M = 732$, 95% CI = 80–1000; $m = 0.011$; 95% CI = 0.0012–0.014); French Polynesia and western Australia ($M = 374$, 95% CI = 80–974; $m = 0.0054$; 95% CI = 0.0012–0.014); and French Polynesia and eastern Australia ($M = 418$, 95% CI = 50–950; $m = 0.011$; 95% CI = 0.0013–0.025). We found no evidence for female philopatry in the three *N. acutidens* populations, as mitochondrial analyses revealed no divergence among populations ($\theta = 0.0$).

Negaprion acutidens *microsatellite analyses*

The microsatellite AMOVA analyses over three *N. acutidens* populations (Western Australia, eastern Australia, and French Polynesia $\theta = 0.0544$, $P \leq 0.001$) and between combined Australian populations and French Polynesia ($\theta = 0.0664$, $P \leq 0.001$) indicated significant structure. Pairwise estimates of genetic differentiation indicated no structure between eastern and Western Australia, and moderate structure between French Polynesia and western ($F_{ST} = 0.0701$, $P \leq 0.001$) or eastern Australia ($F_{ST} = 0.0871$, $P = 0.0118$; Table 2b). Migration rates estimated using microsatellite data indicated high migration from western to eastern Australia ($m = 0.19$; 95% confidence interval = 0.057–0.31); however, in all other comparisons, the variance was equal to or greater than the mean of the posterior probabilities (Table 2a). Tests (Wilcoxon one-tailed test for heterozygosity excess) for bottlenecks were significant for the French Polynesian population under the infinite allele model only (IAM; $P = 0.03$). For this population, the two-phase model ($P = 0.07$) and stepwise mutation model ($P = 0.40$) were not significant. However, the IAM may be the most robust and reliable model for loci that do not conform to the stepwise mutation model (Selkoe & Toonen 2006).

Seascape genetics

For *N. brevirostris*, there was high concordance between the barriers revealed by mitochondrial and microsatellite (D_c) distance matrices analysed in BARRIER (Fig. 1). The most effective barrier identified for both matrices was that dividing the two species. The data indicated a more permeable barrier across the Atlantic than across the Isthmus of Panama. Within the N. *acutidens* populations, a barrier isolating French Polynesia was indicated by microsatellite data only.

Combining data for both species, genetic distance was not correlated to overall geographical distance (Fig. 4a) or maximum depth (Fig. 4b). However, regression of oceanic distances against microsatellite distances revealed a highly significant relationship ($R^2 = 0.89$, $P = 0.005$; Fig. 4c). The regression of oceanic distances against mitochondrial distances was not significant ($R^2 = 0.64$, $P = 0.057$; Fig. 4c), due to a lack of differentiation among *N. acutidens* populations. When these samples were removed, mitochondrial genetic distances and oceanic distances were highly correlated ($R^2 = 0.87$, $P = 0.001$).

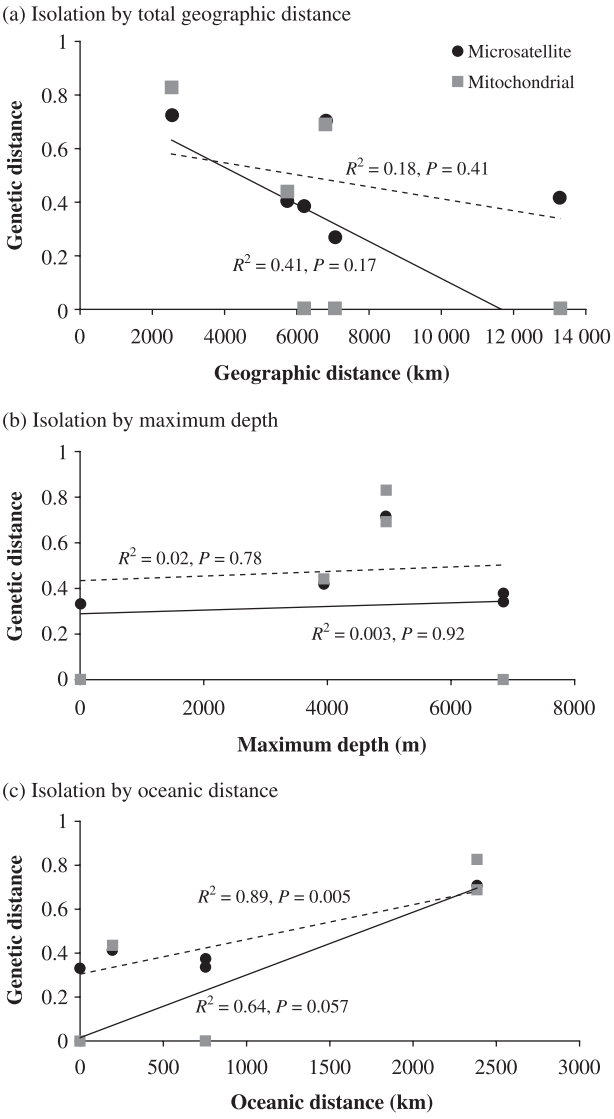

Fig. 4 Isolation-by-distance graphs comparing (a) genetic distance and total geographical distance between populations, (b) genetic distance and maximum depth between populations, and (c) genetic distance and maximum oceanic distance between populations, measured as longest distanced travelled at depths greater than 200 m. Mitochondrial analyses represented by grey squares and solid regression lines; microsatellite chord distance analyses represented by black circles and dashed lines.

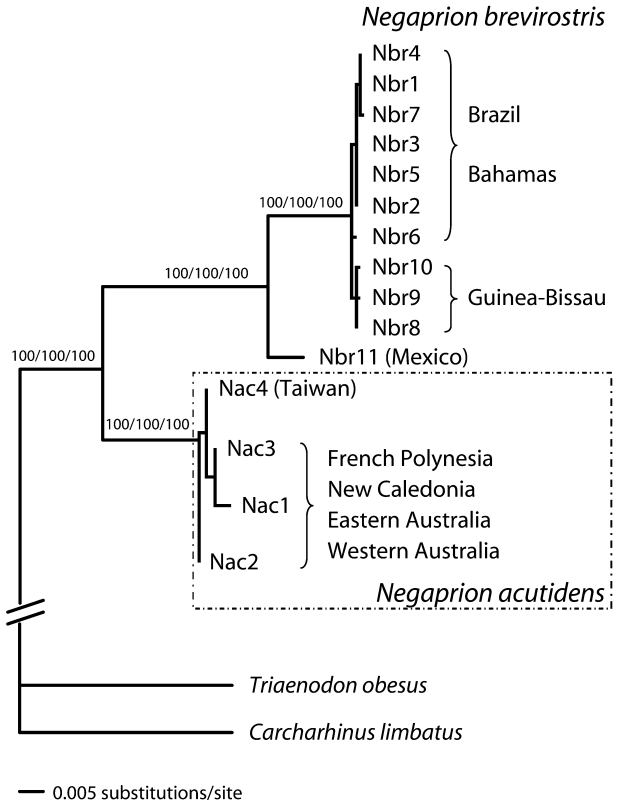

Fig. 5 Maximum likelihood (ML) tree of the mitochondrial control region showing relationships between two *Negaprion* species and two Carcharhinid outgroups. Values on branches reflect ML, maximum parsimony and Bayesian support, respectively.

Phylogeography of the genus Negaprion

Using the Akaike information criterion, the best-fit model was HKY + I (Hasegawa *et al*. 1985). Maximum likelihood, maximum parsimony, and Bayesian analyses of the mitochondrial control region sequence data produced congruent topologies and high bootstrap values reflecting the deepest divergence between species (d_{ave} = 0.060) and across the Isthmus of Panama (d_{ave} = 0.024; Fig. 5). We found shallow divergence across the Atlantic (d_{ave} = 0.003). Although we recognize the limitations of using microsatellite loci for phylogenetic analyses due to homoplasy, neighbour-joining analysis of the data produced a tree (not shown) topologically identical to the mitochondrial phylogeny with additional resolution clustering the Taiwan and Australian populations separately from the French Polynesia and New Caledonia populations.

Transisthmian genetic distance (d_{ave} = 0.024) corresponded to 0.67% divergence between lineages per million years, a rate similar to the calibration for scalloped hammerheads (0.8%; Duncan *et al*. 2006) providing further support for slow rates of mtDNA evolution in sharks (Martin *et al*. 1992; Martin 1995). Using the maximum likelihood molecular-clock estimation, *N. acutidens* and *N. brevirostris* diverged ~14 million years ago, with isolation across the Atlantic ~0.29 million years ago. Following Gaggiotti & Excoffier (2000), we estimated that species divergence (τ = 68) occurred ~10 million years ago with an ancestral female effective population size of 50 000 (θ = 6.94). All populations exhibited evidence of population expansion, with none showing significant deviation from a unimodal mismatch distribution ($P > 0.05$). Population level expansions ranged from 81 000 to 300 000 years ago (τ = 0.95–3.55), with wide 95% confidence intervals (0–9.0 million years). Contemporary female effective population sizes (N_f) ranged from 13 000 to 26 000 (Table 1).

Discussion

Seascape genetics

Ranking and mapping barriers to gene flow in lemon sharks (Fig. 1), we find that oceanic expanses are much more effective than coastal distances. The greatest genetic differentiation occurs across the East Pacific Barrier, an oceanic expanse of 6400 km dividing the two species. The mid-Atlantic barrier (~2400 km) hampers contemporary gene flow: mitochondrial and microsatellite AMOVA analyses of *Negaprion brevirostris* indicate high genetic structure across the Atlantic (θ = 0.79, $P \leq 0.001$; θ = 0.23, $P \leq 0.001$, respectively), with isolation ~100 000–480 000 years ago (Table 2) and no subsequent gene flow (M, m = 0.0; Table 2a). When we consider pairwise estimates of genetic differentiation relative to total geographical distance between populations, we find incongruent results. Extensive geographical distance between Australia and French Polynesia (6200 km) merely limits genetic connectivity among *Negaprion acutidens* populations, with significant microsatellite differentiation (θ = 0.066, $P \leq 0.001$), reflecting low levels of recent migration (m = 0.010) and no genetic differentiation using mitochondrial analyses (θ = 0.00, P = 0.99). Neither microsatellite nor mitochondrial analyses indicate genetic structure between Australian *N. acutidens* populations, separated by 7070 km (Table 2b). While we find evidence for nuclear gene flow (m = 0.31) between western Atlantic *N. brevirostris* populations separated by 5740 km, mitochondrial analyses indicate strong structure ($m < 0.010$; Φ_{ST} = 0.45, $P \leq 0.001$). Clearly *Negaprion* spp. do not fit a simple isolation-by-distance model, in which genetic differentiation increases as geographical distance between populations increases (Fig. 4a). We suggest two behaviours to explain the discrepancy: coastal habitat preference and female philopatry.

Sharks are active swimmers from birth, whereas bony fishes usually have pelagic larvae, capable of spanning deep water and oceanic expanses. While overall depth may have little impact on pelagic species (or larvae) that occur

in the upper water column, lemon sharks are benthopelagic, spending most of their time just above the substrate (Musick *et al*. 2004). At depths of less than 200 m (which we designate as the division between coastal and oceanic habitat), they encounter water temperatures similar to those at the surface at most locations. The East Pacific Barrier is characterized by depths over 5000 m, and the mid-Atlantic exceeds depths of 4500 m. Between French Polynesia and Australia lies the Tonga Trench, with a maximum depth of over 9000 m (Wright *et al*. 2000). Crossing these barriers at depth would expose subtropical sharks to temperatures approaching 3 °C. Although we find no correlation between genetic differentiation and total geographical distance (Fig. 4a) or maximum depth (Fig. 4b), there is a highly significant pattern of isolation by oceanic distance, the maximum distance travelled at depths > 200 m ($R^2 = 0.89$, $P = 0.005$; Fig. 4c). This relationship is best depicted by mapping a theoretical course between locations, minimizing total and oceanic distance travelled, and plotting depths encountered throughout the journey (Fig. 6). A route from French Polynesia to Mexico (Fig. 6c), requires crossing the East Pacific Barrier, which is characterized by great depths and the widest oceanic distance (~5000 km; Fig. 6b). Therefore, we believe this to be an impermeable barrier for a lemon shark. Within species comparisons reveal genetically isolated populations across the Atlantic Ocean. The shortest, shallowest route between Brazil and Guinea-Bissau (Fig. 6d) requires crossing an oceanic distance of ~2400 km (Fig. 6f). The relatively weak population structure among *N. acutidens* populations indicates that oceanic barriers are permeable to this coastal shark, if there are island stepping stones to facilitate dispersal. Travelling from Australia to French Polynesia requires passage over considerable depths, but no oceanic distance is greater than 800 km (Fig. 6a). Although such dispersal events may be rare, they are sufficient to prevent reproductive isolation.

There is low nuclear differentiation ($F_{ST} = 0.057$, $P \leq 0.001$; Table 2a) among western Atlantic *N. brevirostris* populations connected by contiguous coastline, shallow depths and short oceanic distances (~195 km; Fig. 6e). However, mitochondrial analyses indicate extensive population structure between the coastally linked populations of Bahamas and Brazil ($\Phi_{ST} = 0.45$, $P \leq 0.001$). In *N. brevirostis* comparisons, levels of population differentiation based upon mitochondrial analyses are consistently higher than those involving nuclear microsatellites, even after standardization. While these data corroborate previous descriptions of female philopatry based on behavioural genetic studies (Feldheim *et al*. 2002, 2004; DiBattista *et al*. 2008), faster lineage sorting in mtDNA due to maternal and haplotypic inheritance cannot be discounted. In *N. acutidens*, we see little evidence for female philopatry, except perhaps in the Taiwan population, where a single, unique haplotype is shared by five individuals. An alternative explanation is isolation of this population relative to all others, due to low sea levels exposing land barriers as recently as 6000 years ago (Barber *et al*. 2000). Mitochondrial analyses revealing no differentiation among French Polynesian and Australian populations may indicate a lack of female philopatry; yet 94% of these sharks share a single haplotype, and the French Polynesian population shows evidence of a recent population bottleneck, both of which may prevent detection of population divergence.

Tethyan divergence

Our phylogenetic analyses reflect deep divergence between *N. brevirostris* and *N. acutidens*, subsequent isolation of eastern Pacific *N. brevirostris*, and shallow phylogenetic divergence among transatlantic populations (Fig. 5). Using a coalescent method and a penalized maximum likelihood molecular clock, the species diverged ~10–14 million years ago. We regard these dates as broad estimates, and acknowledge that gene trees may not represent species trees. Nonetheless, our data support the vicariant hypothesis of a cosmopolitan ancestral lemon shark whose range was divided by two well-documented geological events: convergence of African and Eurasian Plate 12–20 million years ago, eliminating the warm, coastal Tethyan corridor between the Atlantic and Indian Oceans; and emergence of the Isthmus of Panama ~3.5 million years ago, closing the subtropical seaway connecting the Atlantic and eastern Pacific. Paleontological evidence supports the existence of a cosmopolitan ancestor, as *Negaprion eurybathrodon* teeth are reported from Pakistan and Georgia, USA (Case & West 1991; Case & Borodin 2000), and the species likely had a widespread distribution in the Miocene (Cappetta 1987; Kriwet 2005).

We cannot exclude the East Pacific dispersal model with finality but favour the vicariant Tethyan model based on two lines of evidence. First, divergence times based on molecular-clock estimates closely match expectations of the vicariant model. Notably, the molecular clock is calibrated using the second vicariant event (Isthmus of Panama); however, our divergence rate is similar to that estimated for another 'coastal' shark species (scalloped hammerhead; Duncan *et al*. 2006). Second, the comparison of eastern and western Atlantic populations indicates a genetic partition dating to ~300 000 years ago. While we cannot distinguish between cessation of gene flow and a colonization event, either explanation leads to the same conclusion: oceanic dispersal is rare. Given that the East Pacific Barrier is twice as wide as the Atlantic basin, we provisionally discount this avenue of colonization.

Phylogeography of coastal sharks

Phylogeographical analyses of the lemon sharks distinguish them from other large, 'coastal' sharks, *Carcharhinus limbatus*

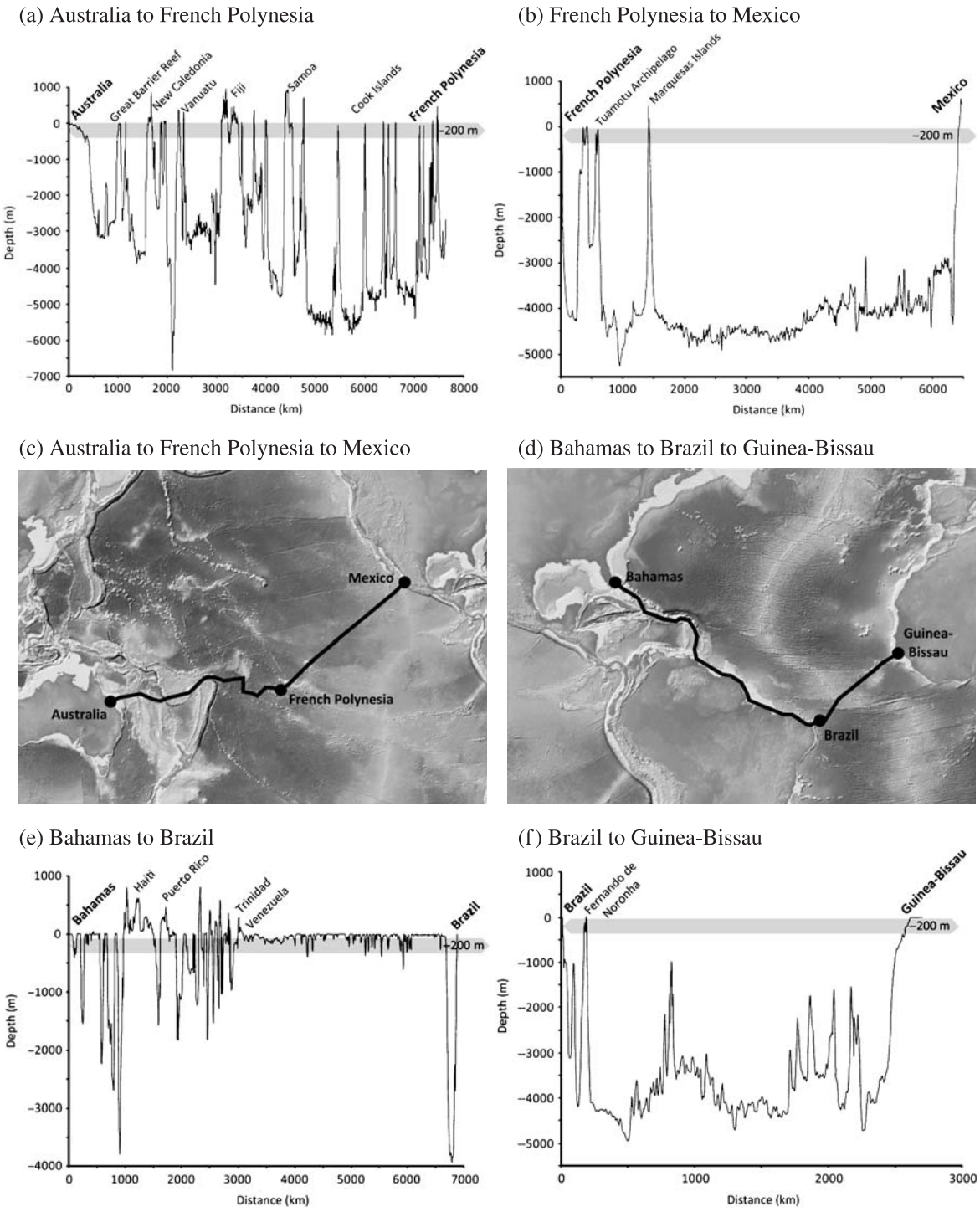

Fig. 6 Oceanic distances mapped to reflect the minimum total distance and depths required for travel between two locations. Grey bar represents boundary between coastal and oceanic waters at 200 m. Figure 6(a,e) correspond to high gene flow between coastally linked and insular populations, whereas Fig. 6(b,f) display oceanic barriers to gene flow and ancient evolutionary separations.

and *Sphyrna lewini*, which show evidence of recent dispersal across the East Pacific Barrier (Duncan *et al*. 2006; Keeney & Heist 2006). We conclude that the disparity reflects differences in coastal proclivity. The blacktip shark, *C. limbatus*, exhibits strong genetic structure across the Atlantic, similar to *N. brevirostris*; however, Pacific populations are less differentiated (Keeney & Heist 2006). Blacktip sharks have a cosmopolitan distribution and are highly mobile.

A large-scale tagging study of Atlantic sharks reveals that blacktips travelled a maximum distance of 1159 km, compared to 230 km for *N. brevirostris* and 1600 km for the coastal-oceanic scalloped hammerhead (Kohler *et al*. 1998). The scalloped hammerhead (*S. lewini*) is a common, cosmopolitan species found in both coastal and oceanic waters (Castro 1993). Duncan *et al*. (2006) conclude that this species used South Pacific archipelagos as stepping stones to colonize Hawaii and the eastern Pacific. Although the coastal *N. acutidens* likely uses South Pacific archipelagos as stepping stones for rare dispersal events between Australia and French Polynesia, the width and depth of the East Pacific Barrier are likely impenetrable for lemon sharks. For these reasons, we believe that Tethyan divergence better explains *Negaprion* distribution and evolution.

Conservation

Among sharks studied to date, *N. acutidens* has the lowest reported haplotypic diversity ($h = 0.28$), but this may not be the result of an anthropogenic population bottleneck (Hoelzel *et al*. 2006). Within-population sequences coalesce to the Pleistocene, and tests for a bottleneck are significant only for French Polynesia ($P = 0.03$). Human impact cannot be ignored, as *N. acutidens* is listed as vulnerable on the IUCN Redlist, and populations in Thailand and India have been extirpated. While female effective population sizes for *Negaprion* ($N_f = 13\,000$–$26\,000$) may seem large, our difficulty obtaining *N. brevirostris* specimens in the eastern Pacific and eastern Atlantic likely reflects the impact of local overfishing. Notably, these two populations are highly isolated by time (thousands to millions of years) and space (thousands of kilometres of oceanic habitat). Conservation of estuarine nursery habitats and protection against overfishing are imperative to prevent local extinction because, given the coastal nature of lemon sharks, it is unlikely that depleted populations will be replenished by transoceanic dispersal.

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This study was part of Jennifer Schultz's doctoral research that investigates the genetic diversity and connectivity of threatened coastal species, including sharks, reef fish and the Hawaiian Monk seal. Kevin Feldheim is interested in using genetic markers to study the evolution of mating systems in different organisms. Mary Ashley's research interests include urban ecology and restoration, land and seascape genetics, and conservation genetics. Founder of the Bimini Biological Field Station, Samuel Gruber is interested in behavioural ecology, bioenergetics, ethology and sensory physiology. Timothy McGovern's interests lie seafloor mapping, cartography and GIS. Brian Bowen works primarily on the phylogeography of bony fish, but acknowledges important lessons from the cartilaginous fishes.

Supporting Information

Additional supporting information may be found in the online version of this article:

Table S1 Geographical distribution and intraspecific polymorphic nucleotide positions of control region haplotypes in (a) *Negaprion brevirostris*, and (b) *Negaprion acutidens*. Interspecific polymorphisms ($N = 62$) not shown. Haplotype number (hap) shown at left; number of sharks with haplotype listed under corresponding collection site. Position of polymorphic nucleotide across top. Nucleotides for the first haplotype shown; matching nucleotides represented by a dot, differing nucleotides shown. Dashes represent gaps in sequence. Sample size in parentheses.

Table S2 Observed microsatellite heterozygosities/allelic richness for *Negaprion brevirostris* and *Negaprion acutidens* populations at nine and six microsatellite, respectively. Sample size in parentheses.

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