Journal of Heredity 2012:103(2):174–185
doi:10.1093/jhered/esr131
Advance Access publication December 15, 2011

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Population Structure and Phylogeography of the Short-Tailed Stingray, *Dasyatis brevicaudata* (Hutton 1875), in the Southern Hemisphere

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Abstract

There is accumulating evidence that the degree of vagility explains little of the extent of population subdivision found within elasmobranch species. Instead, patterns of gene flow in elasmobranchs appear more closely linked to the presence of dispersal barriers, either physical or biological. Here, we investigate the potential role of some of these isolating mechanisms in shaping the population structure of a member of the stingray family Dasyatidae (*Dasyatis brevicaudata*) at various scales (southern hemisphere vs. coastal New Zealand). Analyses of the mitochondrial DNA control region from 176 individuals revealed significant genetic structure between South Africa, Australia, and New Zealand populations (analysis of molecular variance [AMOVA], overall $\Phi_{ST} = 0.67$, $P < 0.001$), although New Zealand and Australia shared some haplotypes. Surprisingly, significant population differentiation was found among several coastal New Zealand locations (AMOVA, overall $\Phi_{ST} = 0.05$, $P < 0.05$). However, data did not support the genetic differentiation between individuals from an offshore breeding area and mainland individuals. Comparisons suggest that these stingrays exhibit similar levels of population differentiation as other coastal elasmobranchs, with high divergence across oceanic basins and lower differentiation along continuous coastal habitats. Differences in coastal population structuring in elasmobranch species studied to date may be attributed to species-specific preferences for coastal habitats, which may be linked to life history functions (e.g., feeding and pupping).

Key words: *ecological niche, elasmobranch, disjunct distribution, dispersal, mitochondrial DNA control region, population genetics*

Elasmobranchs (sharks and rays) are likely to display very different patterns of population connectivity, in comparison to the far better studied teleost fishes. Elasmobranchs possess a number of distinctive life history characteristics, such as (in general) long lives, slow growth rates, late reproduction, and low reproductive output (Wourms and Demski 1993). Furthermore, as predominantly live-bearers, the dispersal of sharks and rays is not dependent on the release of pelagic eggs and larvae, thus their dispersal is mainly dependent on the vagility of adults. Stingrays (Dasyatidae) are an excellent group to examine the relative importance of potential isolation factors (i.e., distance, coastal habitat preference, and site fidelity) on marine dispersal. Due to their mostly benthic coastal lifestyle, their potential for dispersal is expected to be limited compared with that shown in the more mobile shark species predominantly studied to date. In light of the current disjunct transoceanic distribution of many stingray species (Last and Stevens 2009), this raises many questions as to how these distributions arose. Only a handful of tagging studies have looked at movements and dispersal abilities in rays and in other elasmobranchs with predominantly coastal niches (Smith and Merriner 1987; Walker et al. 1997; Matern et al. 2000; Hunter et al. 2005; Klimley et al. 2005; Vaudo and Lowe 2006; Le Port et al. 2008; Carlisle and Starr 2009). The general pattern seen in these species seems to be that of seasonal movement patterns, which involve local scale movements during warm months (presumably to mating or pupping grounds), with mesoscale (~30 km) to larger scale migrations (>100 km) between summer and winter grounds in some species (Smith and Merriner 1987; Walker et al. 1997; Vaudo and Lowe 2006; Le Port et al. 2008). However, whether such movements drive population divergence in stingrays largely remains to be determined. One study

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suggests that stingray populations may not be structured over scales of several hundreds of kilometers (Plank et al. 2010), even when tagging suggests much smaller movements (Vaudo and Lowe 2006).

Recent studies have shown that intraspecific genetic differentiation in elasmobranchs may result from a variety of mechanisms, such as vicariant events (Schultz et al. 2008), limited dispersal capabilities (Palumbi 1992), habitat discontinuities (Duncan et al. 2006), and fidelity to breeding areas (Keeney and Heist 2006). In the marine environment, reproductive modes and dispersal capabilities vary widely between benthic, coastal, and pelagic species. That is, large oceans may represent barriers to gene flow for benthic and coastal species relying on the short dispersal distances of adults, whereas species that rely on long-distance swimming capabilities of adults, or the dispersal of highly mobile larvae, may not show strong genetic heterogeneity across ocean basins (e.g., whale shark, *Rhincodon typus* [Schmidt et al. 2009]). However, there is accumulating evidence that the genetic structure of populations may reflect not only contemporary restrictions to dispersal (e.g., distance and depth) but also past vicariant events (e.g., rise of Isthmus of Panama and Tethys Sea closure). In many large vagile shark and ray species capable of high dispersal, population genetic structure between ocean basins has been attributed to the presence of one or more barriers to their dispersal. Historical barriers have been inferred in the tope (*Galeorhinus galeus*) (Chabot and Allen 2009), spotted eagle ray (*Aetobatus narinari*) (Richards et al. 2009), and lemon shark (*Negaprion brevirostris*) (Schultz et al. 2008), whereas contemporary barriers have been inferred in the scalloped hammerhead shark (*Sphryrna lewini*) (Duncan et al. 2006) and the blacktip shark (*Carcharhinus limbatus*) (Keeney and Heist 2006).

Although the ability for long-distance dispersal is important in establishing new populations (Graves 1998), at smaller scales (within regions) other factors may come into play, and there are likely to be differences between taxa within ecological niches (i.e., benthic shark vs. benthic stingray). Recent population genetic studies have revealed that many highly mobile circumglobal shark species which display genetic structure between ocean basins may also display population structure within ocean basins and along continental margins (Keeney and Heist 2006; Chapman et al. 2009), similar to that reported in less mobile benthic species with more limited geographic ranges, for example, shovelnose guitarfish (*Rhinobatus productus*) (Sandoval-Castillo et al. 2004). This pattern has mainly been attributed to species-specific behaviors (coastal habitat preferences and/or reproductive behavior), as opposed to physical barriers to movement or limited dispersal abilities. For example, significant genetic structure for maternally inherited mitochondrial DNA (mtDNA) among both coastal and oceanic sharks have been attributed to females exhibiting fidelity to specific sites for reproductive purposes (pupping and breeding), thereby limiting their dispersal relative to males. Coastal lemon sharks (*N. brevirostris*) exhibit this pattern (Feldheim et al. 2002; Schultz et al. 2008), as well as more oceanic species like blacktip sharks (*C. limbatus*) (Keeney et al. 2003, 2005; Keeney and Heist 2006) and scalloped hammerhead sharks (*Sphyrna lewini*) (Duncan and Holland 2006; Quattro et al. 2006). Even if they are not loyal to specific reproductive areas, reproduction in many species is strongly affiliated with sheltered coastal habitats (Duncan et al. 2006). This may explain both the philopatric behavior increasingly reported in females of many wide ranging sharks, as well as the resulting population structure seen in these species.

The short-tailed stingray (*Dasyatis brevicaudata*) is a warm temperate stingray with a disjunct southern hemisphere distribution (Last and Stevens 2009). There are at least 3 known geographically distinct populations: the southwest Pacific (New Zealand/eastern Australia), the eastern Indian Ocean (western Australia), and western Indian Ocean/southeast Atlantic (South Africa). The species has not been reported from South American waters, even though the distantly related *D. hypostigma* has been described from Brazil and Argentina. Although common both in New Zealand and Australian coastal waters, the genetic structure of *D. brevicaudata* has not yet been studied and little is known about the individual movements, reproductive biology, or habitat requirements of this species. This benthopelagic stingray has populations separated by thousands of kilometers, which begs the question: Are these populations linked by contemporary gene flow or by historical dispersal? The distribution of *D. brevicaudata* in waters around the major southern hemisphere temperate coasts suggests historical dispersal along coastlines when corridors were available (e.g., during glaciations when shallower waters provided additional stepping-stones, or when cooler waters allowed access to more tropical coastlines), but it is also possible that geographically disjunct populations are linked by contemporary gene flow. In the latter case, individuals would need to cross substantial barriers (i.e., deep oceans and large distances or unsuitably warm coastal waters) to maintain gene flow. This seems unlikely in the light of the short distance movements (<100 km) and relatively shallow maximum depths recorded (200–250 m) for *D. brevicaudata* (Le Port et al. 2008). Thus, strong population subdivision is expected in these rays, both at regional and perhaps also at local scales. Information about the existence or whereabouts of coastal breeding or nursery areas, as has been reported in sharks (Keeney et al. 2003), is lacking in this species. However, the seasonal aggregation of mainly large mature females at the Poor Knights Islands marine reserve, an offshore breeding area in New Zealand (Le Port 2009), provides an ideal setting to test the level of gene flow between this and nearby coastal areas and by extrapolation, the potential for philopatric behavior in *D. brevicaudata*.

Here, we first aim to answer 3 questions: 1) Are there significant genetic differences among regional populations of *D. brevicaudata*, and can we infer the evolutionary history of this species across its distribution, 2) What is the level of connectivity of *D. brevicaudata* along coastal New Zealand areas (i.e., are there restrictions to dispersal among New Zealand locations?), and 3) Are the (breeding) populations of *D. brevicaudata* at the Poor Knights Islands distinct from other New Zealand populations and thus genetically

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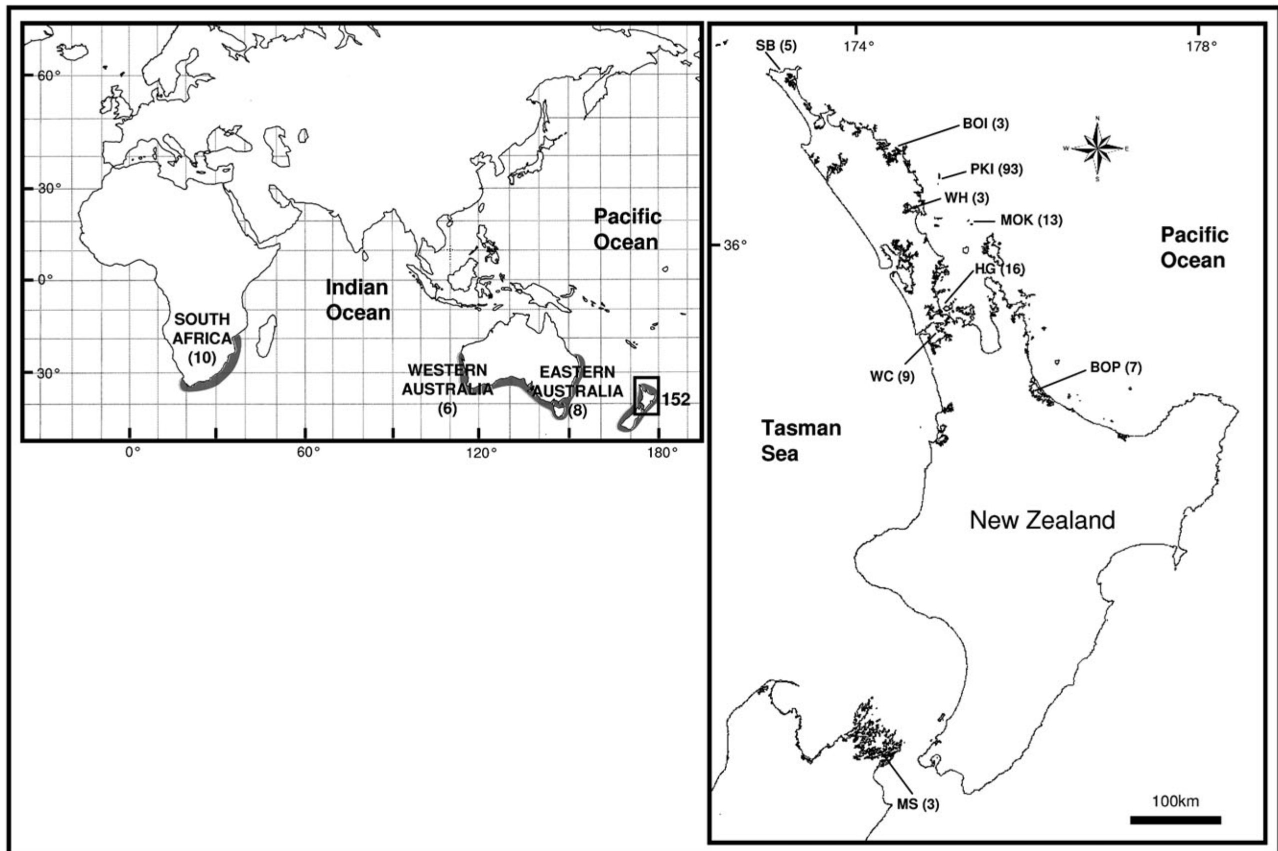

Figure 1. Geographical distribution and sampled populations of *Dasyatis brevicaudata* specimens obtained from each geographical location ($n = 176$). Shaded areas represent the known range of *D. brevicaudata* and numbers represent the number of samples collected within areas (map adapted from Last and Stevens 2009).

differentiated? We can then determine if genetic differences among sites are explained entirely by distance or if other factors such as habitat preference or site fidelity play a role. Overall, our aim was to confirm whether stingrays conform to the emerging pattern found in coastal sharks studied to date, of high divergence across oceanic basins and lower differentiation along continuous coastal habitats, and to help determine whether vagility or barriers to dispersal are the major force driving population subdivision in this species.

Materials and Methods

Taxon Sampling

To examine mtDNA diversity within short-tailed stingrays (*D. brevicaudata*) throughout their known distribution, samples consisting of muscle, ventral tail fold, and blood were obtained nonlethally by biopsy between 2004 and 2007 from New Zealand ($n = 152$), Australia ($n = 14$), and South Africa ($n = 10$), for a total of 176 samples (Figure 1). In New Zealand, samples were collected from 9 locations (Figure 1), which are representative of this species' distribution and abundance around New Zealand. These ranged from Spirits Bay (SB) in the far north of the North Island to the Marlborough Sounds (MS) on the north coast of the South Island. All locations were sampled twice yearly during 3 years, with the exception of MS that was sampled once only and the Poor Knights Islands (PKI) marine reserve that was sampled monthly or bimonthly as part of a seasonal abundance survey (Le Port 2009). The majority of *D. brevicaudata* (82.8%) samples from PKI were collected from midsummer (January) to early autumn (March) as this is when these rays are the most abundant at this location. *Dasyatis brevicaudata* samples from other locations around New Zealand were collected in spring (8.3%), summer (55.0%), and autumn (31.7%) with only a handful of samples collected during winter (5.0%). Samples were also collected at PKI ($n = 93$) over 3 consecutive summers (2004–2007) to investigate whether females show site fidelity to a breeding location and to determine the origin of migrating individuals.

Laboratory Procedures

Total genomic DNA was extracted from an approximately 2 × 2 mm ethanol-preserved tissue biopsy (muscle and/or skin) chopped finely, using a standard phenol–chloroform–isoamyl alcohol extraction protocol (Sambrook et al. 1989). DNA extraction from 4 Australian blood samples stored on

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Whatman's FTA cards (Whatman, Part of GE Healthcare, NJ) was undertaken using a modified FTA purification protocol as described by the manufacturer.

The entire mtDNA control region (CR) was amplified (~1928 bp total) by PCR (Saiki et al. 1988) using a series of primers developed for the CR, primarily using the Pro_dasyatis1/CR_Middle_R pair and the CR_Middle_F/Phe_dasyatis1 pair (Supplementary Figure S1 and Table S1). These primer pairs produced 2 sequence fragments each approximately 700–950 bp long (Supplementary Figure S1).

Amplification reactions were carried out in 25-μl final volumes consisting of 2.5 μl of 10× PCR buffer, 1.25 μl of 50 mM $MgCl_2$, 0.3 μl of 200 μM dNTPs mix, 1 μl of each 10 μM primer, and 0.125U of platinum *Taq* polymerase (Perkin-Elmer, Foster City, CA). In the case of DNA extracted from Whatman's FTA cards (see above), each purified sample was added to 25 μl of PCR master mix and this protocol followed. Some samples amplified weakly using standard PCR. In those instances, 0.75 μl of Bovine Serum Albumin (BSA) was added to each reaction tube prior to adding the DNA template to decrease inhibition of the PCR. The temperature profile was an initial denaturation at 94 °C for 3 min, followed by 35 cycles at 94 °C for 30 s, Ta (Supplementary Table S1) for 45 s, 72 °C for 60 s. A final extension at 72 °C for 3 min concluded each thermal profile. To control for contamination due to handling, a PCR negative control was run in all PCRs. PCR products were separated by electrophoresis in a 1.6% agarose gel stained with ethidium bromide (0.5 μg μl^{-1}) and photographed digitally. Free nucleotides and primers were removed from PCR products using Shrimp Alkaline Phosphatase and Exonuclease I (USB) (Werle et al. 1994), and the products were directly sequenced in one or both directions using the standard protocols of BigDye terminator sequencing chemistry on an ABI PRISM 3100 Genetic Analyser (Perkin-Elmer, Foster City, CA) automated capillary sequencer. Unincorporated dye-labeled nucleotides were removed using the CleanSEQ (Agencourt Bioscience Corporation, Beverly, MA) magnetic bead protocol under recommended conditions. To improve the confidence and accuracy of the results, PCR products were sequenced in both forward and reverse directions when sequenced fragments were shorter than expected and/or of poor quality (Phred quality scores < 20). Sequences with Phred score values between 20 and 40 (a probability between 1/100 and 1/10 000 of being incorrectly called) were edited manually, and sequences with Phred score values ≥ 40 were checked by eye to confirm variable sites. Final sequences all had Phred quality scores >30. All 176 *D. brevicaudata* DNA sequences were aligned using Sequencher version 4.7 (Gene Codes Corporation, Ann Arbor, MI) and alignment results confirmed by eye. Sequences were grouped into haplotypes and exported into MEGA 4.0 (Tamura et al. 2007).

Sequence Analyses

Intraspecific relationships of mtDNA haplotypes were inferred using a statistical parsimony network using the software TCS 1.2.1 (Clement et al. 2000). Summary statistics (number of haplotypes, haplotype frequencies, number of polymorphic sites, number of transitions and transversions, and nucleotide composition) were calculated with DnaSP (Rozas et al. 2003) and MEGA 4.0. For regional analyses, individuals were binned into 4 groups based on proximity of collection sites: Melbourne, Victoria (VIC), Tasmania (Tas), and Gascoyne Seamount (GS) samples in eastern Australia ($n = 8$); all New Zealand samples in New Zealand ($n = 152$); eastern Cape (EC) and western Cape (WCA) in South Africa ($n = 10$); and western Australia ($n = 6$) (Table 1). Haplotype (h), and nucleotide (π) diversities within each region and overall were calculated with DnaSP.

Genetic differentiation within and among all sampling regions was estimated with an analysis of molecular variance (AMOVA, Excoffier et al. 1992 in Arlequin 3.11 [Excoffier et al. 2005]). The degree of genetic heterogeneity between geographic areas was described using Wright's F statistics (F_{ST}) and their molecular analogue Φ_{ST}. For AMOVA analyses, the Tamura and Nei (1993) model of sequence evolution was used. Sequence divergence (d) between *D. brevicaudata* populations was calculated using MEGA 4.0. In the absence of a known mtDNA CR molecular clock for dasyatids, divergence times between *D. brevicaudata* populations were calculated using rates of mtDNA CR evolution published for shark and batoid populations. Values were chosen to encompass the known range of divergence rates between pairs of lineages (0.8% My^{-1} in the scalloped hammerhead shark, *S. lewini* [Duncan et al. 2006], and 0.4% My^{-1} in the shovelnose guitarfish, *Rhinobatos productus* [Sandoval-Castillo et al. 2004]).

Haplotype frequencies were calculated for Australia (EA and WA) and South Africa, plotted as pie charts and overlaid on a map. The same was done for haplotype frequencies within New Zealand. To test the level of gene flow between the breeding site at the Poor Knights Islands (PKI) and nearby coastal locations, an AMOVA was undertaken. Given the small sample sizes from some locations, additional pooling of adjacent samples (not significantly different) was undertaken to increase the power of these tests.

Correlation between geographical distance and genetic differentiation ($\Phi_{ST}/(1-\Phi_{ST})$) was performed for each *D. brevicaudata* "population" using a Mantel test with the software zt (Bonnet and Van de Peer 2002). The normalized Mantel statistic, r (which takes values from -1 to $+1$), was computed and tested for significance by performing 100 000 random permutations of populations in one of the distance matrices, under the null hypothesis that both matrices are not linearly related. Geographic distances between regions sampled ($n = 4$), and between New Zealand locations ($n = 5$) and the 3 other southern hemisphere regions sampled were calculated by using the distance calculator tool in Google Earth (Google). Regional geographic distances were based on the shortest straight-line distances. New Zealand geographical distances were measured as the shortest coastal distances between locations as it is assumed stingrays predominantly move in shallow coastal waters. In addition to correlation

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Table 1 Geographic distribution of *Dasyatis brevicaudata* mtDNA CR haplotypes including number of individuals (*N*) sampled

Grouping	Sample location	N	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	H13	H14	H15	H16	H17	H18	*h* ± SD, π ± SD
	Bay of Islands (BOI)	3	—	2	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	
	Bay of Plenty (BOP)	7	—	1	—	—	3	1	2	—	—	—	—	—	—	—	—	—	—	—	
EC	Hauraki Gulf (HG)	16	—	9	—	—	5	1	1	—	—	—	—	—	—	—	—	—	—	—	
	Whangarei (WH)	3	—	1	—	—	—	—	2	—	—	—	—	—	—	—	—	—	—	—	
	Mokohinau Islands (MOK)	13	—	5	—	1	2	3	2	—	—	—	—	—	—	—	—	—	—	—	0.73 ± 0.03, 0.06 ± 0.003
PKI	Poor Knights Islands	93	—	44	6	—	17	7	15	4	—	—	—	—	—	—	—	—	—	—	
MS	Marlborough Sounds	3	—	3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
SB	Spirits Bay	5	—	—	—	—	1	1	3	—	—	—	—	—	—	—	—	—	—	—	
WC	West Coast	9	3	3	—	—	2	1	—	—	—	—	—	—	—	—	—	—	—	—	
	Victoria (VIC)	6	—	2	—	—	—	—	—	—	—	1	—	—	—	1	2	—	—	—	0.93 ± 0.08, 0.19 ± 0.03
EA	Tasmania (Tas)	1	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	
	Gascoyne Seamount (GS)	1	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	
WA	Western Australia	6	—	—	—	—	—	1	—	—	1	1	1	1	1	—	—	—	—	—	1.00 ± 0.09, 0.11 ± 0.02
SA	Eastern Cape (EC)	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	0.58 ± 0.16, 0.03 ± 0.01
	Western Cape (WCA)	9	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	2	6	1	
	N	176	3	70	6	1	31	16	26	4	1	2	1	1	1	1	2	2	7	1	0.78 ± 0.02 0.09 ± 0.01

Regional haplotype (*h*) and % nucleotide (π) estimates and standard deviations (SD) are listed in the right column.

between pairwise Φ_{ST} values and geographic distances that only consider isolation by distance (IBD), the relationships among regional groupings, also based on the Φ_{ST} values, were visualized using a multidimensional scaling analysis (MDS). MDS analysis uses pairwise distances between objects (pairwise population Φ_{ST} values in the present approach) and approximates those distances in a reduced number of dimensions through an iterative fitting procedure. A 2D plot fitted to pairwise Φ_{ST} values between sites was created in PRIMER 6 (Clarke and Gorley 2006). The goodness of fit between the fitted and observed distances was measured by a stress test, with a zero stress value indicating a perfect fit between the fitted and observed distances.

Results

Genetic Diversity

The mitochondrial CR from 176 *D. brevicaudata* comprised 1928 nucleotides and consisted of 14.5% guanine, 32.5% adenine, 30.8% thymine, and 22.2% cytosine (36.7% GC content). This length is considerably greater than the usual vertebrate CR length of around 1 kb. Fourteen variable nucleotide positions consisting of 12 transitions, 2 transversions, and 1 indel were found, producing 18 haplotypes (Tables 1 and 2). The 24 *D. brevicaudata* sequences from eastern Australia, western Australia, and South Africa possessed unique haplotypes that differed from the New Zealand sequences by a minimum of 2 substitutions (Table 2). Overall, genetic diversity for *D. brevicaudata* sequences were $h = 0.78 \pm 0.02$ standard deviation (SD) and $\pi = 0.09\% \pm 0.01$ SD (Table 1). Regional haplotype diversities were variable, with Australia and South Africa having the highest and lowest genetic diversities, respectively (Table 1). Eight New Zealand haplotypes were determined for 152 sequences (Tables 1 and 2). These were defined by 5 polymorphic sites (4 transitions and 1 transversion) (Table 2). Haplotype diversity was high ($h = 0.73 \pm 0.03$ SD), whereas nucleotide diversity was low ($\pi = 0.06\% \pm 0.003\%$ SD) (Table 1).

The haplotype network showed clear regional groupings (Figure 2). Haplotypes were shared between both eastern and western Australia and New Zealand. South Africa, on the other hand, shared none with either Australia or New Zealand (Figure 2). This was further reflected in the mapping of individual haplotype frequencies from all 4 regions (Figure 3). Four of the *D. brevicaudata* haplotypes (H2, H5, H6, and H7) were shared between New Zealand and Australia. Half of the haplotypes found in eastern Australian locations were also found in New Zealand, whereas only 1 haplotype was shared between western Australia and New Zealand. Eastern Australia (Victoria) shared 1 haplotype with western Australia (H10) (Table 1 and Figures 2 and 3). In New Zealand, 3 CR haplotypes (H2, H5, and H7) were found in 81% of samples analyzed (Figure 3 and Table 1). Haplotype 2 was the most widely distributed throughout the study area, except at Spirits Bay (SB); haplotype 5 was particularly abundant in the Bay of Plenty (BOP) sites but was absent from the Whangarei (WH)

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Table 2 Polymorphic nucleotide positions in 18 mtDNA CR haplotypes for *Dasyatis brevicaudata*

	Nucleotide position														
Haplotype	34	104	223	270	295	317	707	845	1166	1376	1428	1708	1753	1851	1914
H1	C	C	C	C	C	T	A	C	A	T	A	C	—	G	T
H2	.	.	.	.	.	C	.	.	.	.	.	.	—	.	.
H3	T	.	.	.	.	C	.	.	.	.	.	.	—	.	.
H4	T	.	.	T	.	C	.	.	.	.	.	.	—	.	.
H5	.	.	.	T	.	C	.	.	.	.	.	.	—	.	.
H6	.	.	.	.	.	C	.	.	.	A	.	.	—	.	.
H7	.	.	.	T	.	C	.	.	.	A	.	.	—	.	.
H8	.	.	T	T	.	C	.	.	.	A	.	.	—	.	.
H9	.	.	.	.	.	C	.	.	.	A	.	T	T	.	.
H10	.	.	.	.	T	C	.	.	.	A	.	T	T	.	.
H11	.	.	.	T	T	C	.	.	.	A	.	T	T	.	.
H12	.	.	.	T	T	C	.	.	.	A	G	T	T	.	.
H13	.	.	.	T	T	C	G	.	.	A	.	T	T	.	.
H14	.	.	.	T	T	C	G	.	.	A	G	T	T	.	.
H15	.	.	.	T	T	C	G	.	.	A	G	T	T	.	C
H16	.	T	.	.	T	C	.	.	T	.	.	T	T	A	.
H17	.	T	.	.	T	C	.	.	T	.	.	T	T	.	.
H18	.	T	.	.	T	C	.	T	T	.	.	T	T	.	.

Haplotype numbers are listed in the left column and the positions of polymorphic base pairs are listed across the top row. The nucleotide at each position is given for haplotype 1. Only nucleotides different from haplotype 1 are given for all other haplotypes. Nucleotides identical to haplotype 1 are indicated with periods (·) and deletions are indicated with dashes (—). Note that some haplotypes are shared between regions (see Figures 2 and 3) (Genbank accession no. JN400034).

and Bay of Islands (BOI) sites; haplotype 7 was absent from BOI and West Coast (WC) sites (Figure 3). Unique haplotypes were also found. Haplotype 1 was the only haplotype found in high frequency (33%) and was recovered only in the WC region. Haplotypes 3 and 8 were found only at Poor Knights Islands (PKI) and haplotype 4 at the Mokohinau Islands (MOK). When looking at the most distant regions sampled, only haplotypes 5–7 were found in SB samples and only haplotype 2 in Marlborough Sounds (MS) samples (Figure 3 and Table 1).

Southern Hemisphere Regional Population Differentiation Analyses

An AMOVA analysis revealed significant population structure between *D. brevicaudata* from all 4 regions sampled (overall $\Phi_{ST} = 0.67$, $P < 0.001$) (Table 3). Significant pairwise differences were detected between all regions with the exception of eastern Australia and western Australia (Table 3).

A Mantel test indicated a significant IBD pattern ($r = 0.84$, $P = 0.0006$) when New Zealand was split into 5 areas (EC, MS, SB, PKI, and WC), and these data were combined with the 3 remaining geographic regions (EA, WA, and SA) in a plot of pairwise genetic distances ($\Phi_{ST}/(1 - \Phi_{ST})$) versus euclidean geographic distances. Visualization of these data in an MDS plot shows increased separation of regional groupings with increased distance and a clear grouping of all New Zealand locations in agreement with their spatial distribution (Figure 4).

Net sequence divergence between *D. brevicaudata* lineages confirmed that overall the South African sequences were the most genetically divergent, and the sequences from New Zealand/Australia the most similar. Using commonly used rates of CR evolution of 0.8% (Duncan et al. 2006) and 0.4% My^{-1} (Sandoval-Castillo et al. 2004), we estimate that the South African population diverged from New Zealand 112 000 to 225 000 years ago ($d = 0.18\%$) and from the Australian population 75 000 to 150 000 years ago ($d = 0.12\%$), respectively. New Zealand *D. brevicaudata*,

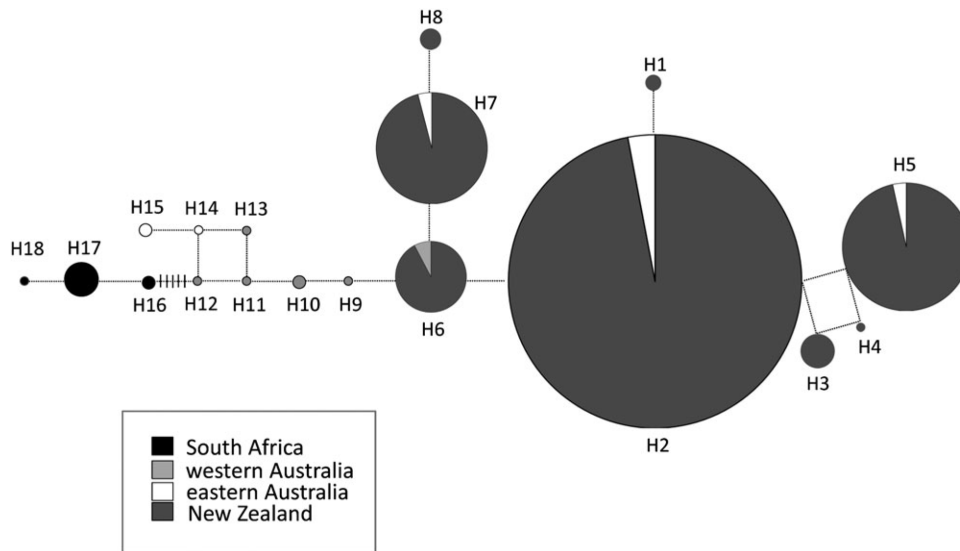

Figure 2. Regional network of mtDNA CR haplotypes and frequencies for *Dasyatis brevicaudata* populations. Haplotypes are indicated by circles color-coded to their collection location with the haplotype number corresponding to Table 2. Relative area of circles reflects sample sizes, connecting lines represent single mutational steps, and uninterrupted lines represent hypothetical missing haplotypes.

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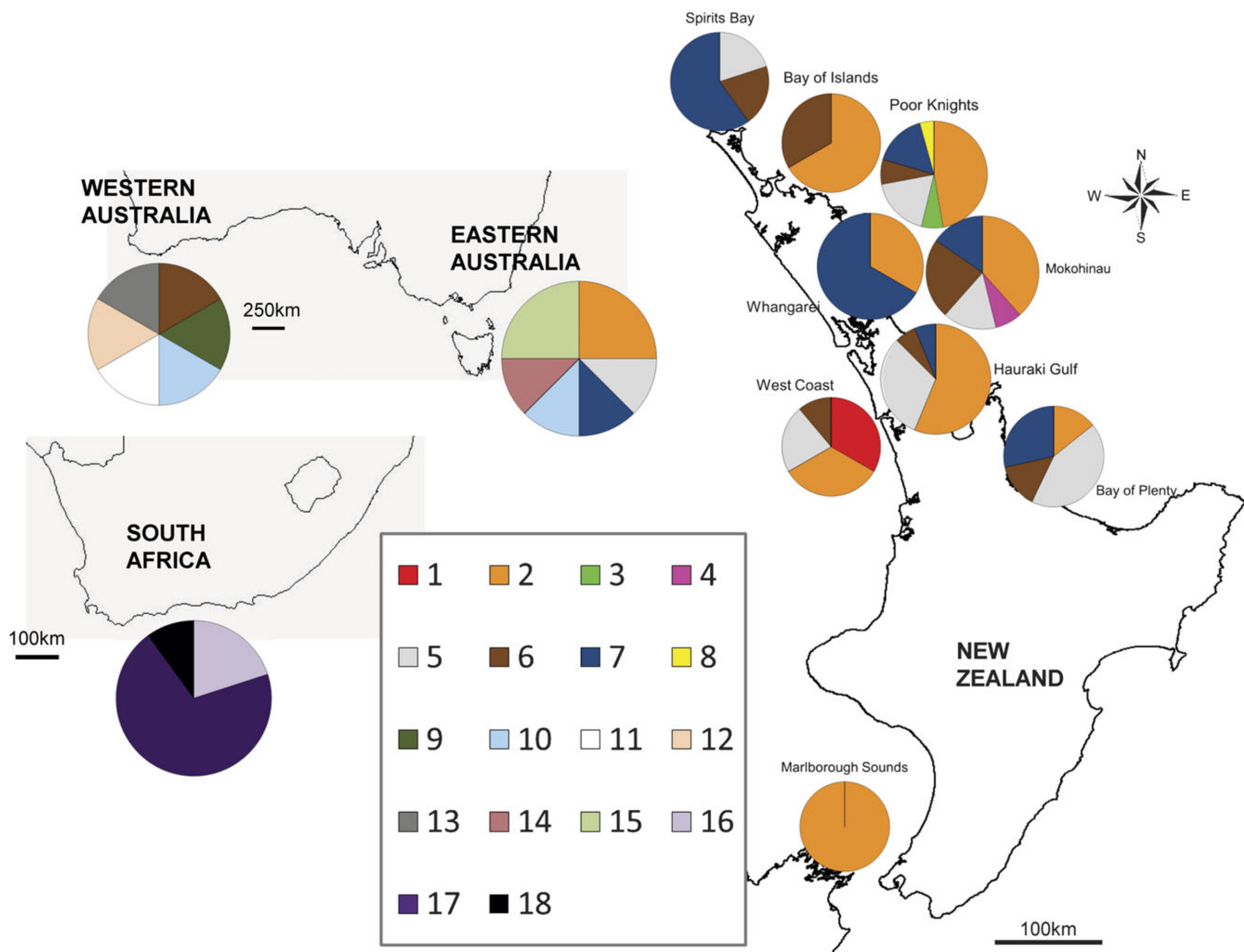

Figure 3. Haplotype frequency map distribution for all *Dasyatis brevicaudata* populations.

on the other hand, diverged from the eastern Australian population 19 000 to 38 000 years ago ($d = 0.03\%$) and from the western Australian population 44 000 to 87 500 years ago ($d = 0.07\%$), respectively.

New Zealand Population Level Differentiation Analyses

An AMOVA analysis revealed significant population structure among all 9 New Zealand locations sampled (overall $\Phi_{ST} = 0.058$, $P = 0.033$). Significant pairwise differences were detected between samples collected at Bay of Plenty (BOP) and West Coast (WC) ($\Phi_{ST} = 0.226$, $P = 0.036$), BOP and Marlborough Sounds (MS) ($\Phi_{ST} = 0.392$, $P = 0.027$), and Spirits Bay (SB) with Hauraki Gulf (HG), MS, Poor Knights Islands (PKI), and WC. Due to some New Zealand locations being represented by relatively small sample sizes (Figure 1), some pooling of adjacent genetically similar samples was undertaken to increase the power of tests of differentiation among New Zealand areas. Significant pairwise differences were taken into account when pooling sampling locations, resulting in 5 groups (EC, MS, SB, PKI, and WC; Table 4). Pairwise comparisons were significant between East Coast (EC), SB, PKI, and WC. The greatest magnitude of genetic differentiation occurred among the most geographically extreme New Zealand locations (i.e., between SB and both MS and WC). PKI was different only from SB and WC (Table 4). Females showed significantly greater population structure around New Zealand ($\Phi_{ST} = 0.08$, $P < 0.05$) compared with males ($\Phi_{ST} = -0.07$, not significant), although sampling was biased toward females. No significant temporal variability was detected among *D. brevicaudata* samples collected at PKI during the 2005, 2006, and 2007 summer seasons (overall years, $\Phi_{ST} = -0.01$, $P = 0.63$).

Discussion

Our study is one of the first to investigate the phylogeography and population structure in a benthopelagic stingray (*D. brevicaudata*). As expected, analyses of the mtDNA CR revealed a strong regional population differentiation and evidence of IBD throughout the sampled range, although some haplotypes were shared between New Zealand and Australia. Significant population differentiation was also

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Table 3 Pairwise regional AMOVA F_{ST} (above diagonal) and Φ_{ST} (below diagonal) values (* = $P < 0.05$, ** = $P < 0.001$) for *Dasyatis brevicaudata*

	Eastern Australia	Western Australia	New Zealand	South Africa
Eastern Australia (8)	—	0.01	0.03	0.29**
Western Australia (6)	0.001	—	0.16*	0.27**
New Zealand (152)	0.44**	0.59**	—	0.34**
South Africa (10)	0.64**	0.75**	0.80**	—

Sample size in brackets. Overall values: F_{ST} = 0.21**, Φ_{ST} = 0.67**

found among coastal New Zealand locations. These results suggest a possible role for low mobility, behavioral philopatry, or both to limit dispersal where physical barriers are absent.

Control region

The CR has proven useful for population studies of sharks and is used for the first time in this study in a stingray species. The specific primers designed were successful for 4 dasyatid species (*D. brevicaudata*, *D. thetidis*, *D. lata*, and *D. matsubarai*) (Le Port A, unpublished data) and amplified the largest mitochondrial CR fragment known in a marine vertebrate (>1900 bp). The largest CR reported to date in a cartilaginous fish was 1143–1332 bp from the whale shark (*R. typus*) (Castro et al. 2007), the majority of other elasmobranchs' CRs being 1030–1050 bp long (Stoner et al. 2003). There is no evidence here of a tandem duplication event being responsible for the long *Dasyatis* CR and it seems this may be the norm in the family Dasyatidae, as the CR in *Neotrygon kuhlii* may be approximately 2000 bp long (Kashiwagi K, personal communication).

Genetic Diversity

Average haplotype diversity indices for *D. brevicaudata* across regions sampled were high (h = 0.78) indicating high levels of genetic diversity. These values are comparable to those found in many elasmobranch species, including, among others, the scalloped hammerhead shark (*S. lewini*, h = 0.80) (Duncan et al. 2006), the shovelnose guitarfish (*R. productus*, h = 0.77) (Sandoval-Castillo et al. 2004), the thorny skate (*Amblyraja radiata*, h = 0.79) (Chevolot et al. 2007), and the longtail stingray (*D. thetidis*, h = 0.58) (Le Port A, unpublished data). Nucleotide sequence diversities, on the other hand, were much lower (π = 0.09%) than those found in the CR of most other elasmobranch species (π = 0.50–1.30%, Sandoval-Castillo et al. 2004; Duncan et al. 2006; Lewallen et al. 2007; Ramirez-Macias et al. 2007), with the exception of those reported for blacktip sharks (*C. limbatus*, π = 0.10%) (Keeney et al. 2003), the sicklefin lemon shark (*N. acutidens*, π = 0.05%) (Schultz et al. 2008), and *D. thetidis* (π = 0.18%) (Le Port A, unpublished data). Although genetic diversity was high overall, genetic diversities within southern hemisphere regions varied

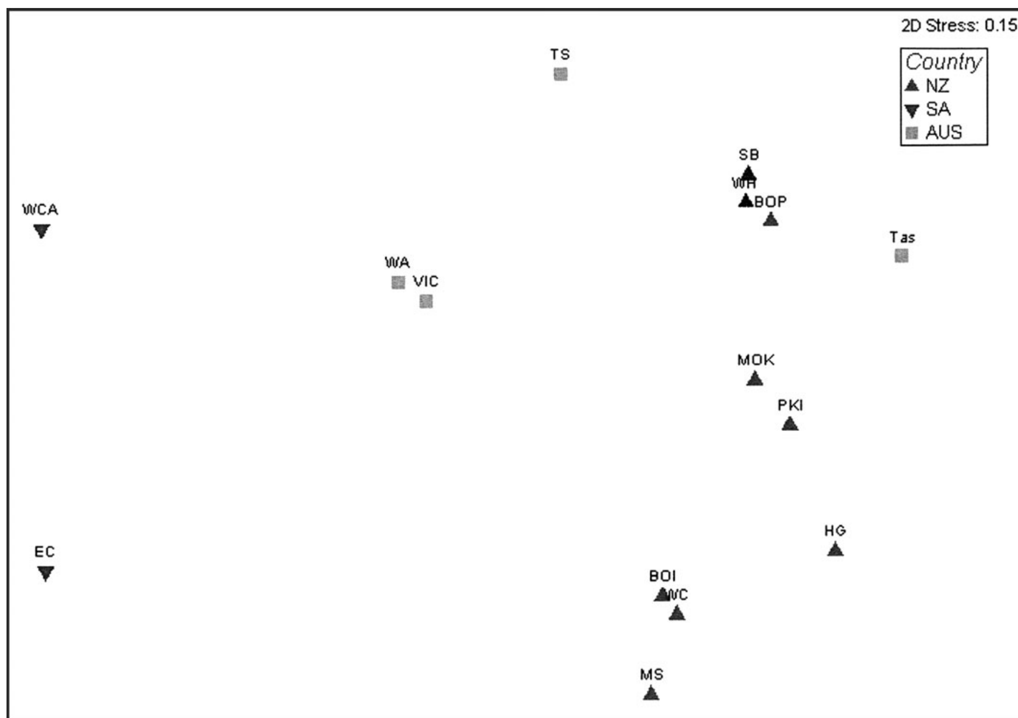

Figure 4. MDS plot of *Dasyatis brevicaudata* for all locations using Φ_{ST} matrix values (for location abbreviations, see Table 1).

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Table 4 Pairwise New Zealand AMOVA F_{ST} (above diagonal) and Φ_{ST} (below diagonal) values (* = $P < 0.05$, ** = $P < 0.001$) for *Dasyatis brevicaudata*

	EC	MS	SB	PKI	WC
East Coast (EC): 42	—	0.117	0.128	0.006	0.032
Marlborough Sounds (MS): 3	0.106	—	0.560*	0.072	0.204
Spirits Bay (SB): 5	0.217*	0.680*	—	0.168	0.185
Poor Knights Islands (PKI): 93	0.011	0.044	0.235**	—	0.051
West Coast (WC): 9	0.111*	0.028	0.431*	0.088*	—

Sample size next to locations. Overall F_{ST} = 0.035*; overall Φ_{ST} = 0.054*

strongly regardless of sample sizes. It could be that the low genetic diversity found in the South African population may be the result of it being a smaller peripheral population, whereas the much higher diversity found in Australia could be indicative of it being a larger and older population, perhaps acting as a center of radiation in the southern hemisphere. The position of the South African haplotypes on the periphery of the haplotype network perhaps supports this contention. However, more intense sampling in those areas is necessary to confirm this hypothesis.

Southern Hemisphere Phylogeography

Although South African *D. brevicaudata* share no haplotypes with Australia and New Zealand, analyses of the mitochondrial CR support the likely existence of a single species with strongly defined regional populations. Recent shark and batoid population studies have estimated divergence times between populations using rates of CR evolution of 0.8% and 0.4% My^{-1} (Sandoval-Castillo et al. 2004; Duncan et al. 2006; Keeney and Heist 2006; Chabot and Allen 2009). Using these 2 divergence rates as a guide, *D. brevicaudata* populations may have diverged very recently, between 19 000 and 38 000 (New Zealand/eastern Australia) and 112 500–225 000 (New Zealand/South Africa) years ago. Accurately dating the isolation of populations is difficult due to the lack of calibrated evolutionary rates of the mitochondrial CR in the ray species studied here, or in any batoid. Furthermore, levels of molecular difference observed in one lineage (i.e., sharks) may not represent the same divergence time as comparable molecular difference in another lineage, at any taxonomic level (Burridge 2002). Therefore, results presented here should be interpreted with caution. However, regardless of how accurate the rates of mitochondrial evolution used here are for stingrays, the apparent lack of major morphological (Le Port 2009), genetic, or ecological differences among populations, as well as the relatively low levels of nucleotide diversity among populations, support isolation occurring in relatively recent times during the glaciations of the Pleistocene (1.8 Ma to 10 000 years ago) (Chappell and Shackleton 1986).

During glaciations, sea levels fell giving access to the extensive continental shelves for nonpelagic species, permitting greater dispersal. Decreases in sea level have facilitated many species to cross barriers such as oceans (Burridge 2002). Changes in conditions during glaciations may have allowed ancestral *D. brevicaudata* to cross the tropics along the continental shelf in an easterly or westerly direction. The end of the glaciation with its rise in sea level and sea temperature would have later isolated South African populations from New Zealand/Australian populations. The complete lack of shared haplotypes between South Africa and Australia/New Zealand supports a longer isolation time for this western population.

Australia and New Zealand are separated by a continental slope and the deep Tasman Sea, which has been shown to be a partial barrier to gene flow in several coastal marine species (Grewe et al. 1994; Ward and Elliott 2001). Given the maximum depths (200–250 m) and limited distances (<100 km) that have been recently reported for *D. brevicaudata* using popup satellite archival (PSAT) tags (Le Port et al. 2008), it is likely that the approximately 1850 km expanse of deep water (up to ~2000 m deep) encountered when crossing the Tasman Sea would be a substantial barrier to the dispersal of this species. This supports the current regional isolation and thus strong population structure between New Zealand and Australian populations. Studies of coastal elasmobranch species have highlighted the importance of species-specific behavioral characteristics (i.e., coastal nursery areas) (Duncan et al. 2006), as well as physical barriers (i.e., depth) (Gaida 1997) in the structuring of populations. The existence of 4 shared haplotypes, as well as distinct, but closely related haplotypes between New Zealand and Australia suggests these populations may have remained in contact for a longer period. Consequently, there may have been insufficient time for lineage sorting to occur due to these populations having diverged relatively recently (<100 000 years ago). During the last glaciation, Australia and New Zealand would have been connected by shallower seas, facilitating their dispersal across the Tasman Sea. *Dasyatis brevicaudata* have been caught from remote areas such as Wanganella Bank in the West Norfolk Ridge, which lies approximately 600 km northwest of New Zealand and is surrounded by waters 1000 m and deeper (Clark 1988). Also, one of the samples used in this study was collected on the Gascoyne Seamount approximately 600 km east of the Australian coast and surrounded by depths of thousands of meters (White W, personal communication), thus making it currently potentially inaccessible to other *D. brevicaudata* from either Australia or New Zealand. Interestingly, this sample's haplotype (H7) was one commonly found on the east coast of New Zealand. Certainly, the presence of *D. brevicaudata* in such remote offshore locations suggests the use of these areas as stepping-stones in past or current trans-Tasman migrations. Thus, this species must be capable of rare long-distance migrations across deep waters, and these migrations would have been facilitated in times of greatly lowered sea levels by the presence of many more shallow stepping-stones in the Tasman, particularly along the Norfolk Ridge and Lord Howe Rise.

New Zealand Population Structure

The CR displayed sufficient variation to detect significant genetic heterogeneity among *D. brevicaudata* sampled at

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locations in New Zealand suggesting somewhat limited dispersal along the New Zealand coast and a potential role for behavioral philopatry. Although there was no evidence of genetic isolation of the breeding ground at the Poor Knights Islands, individuals sampled from the West Coast and Spirits Bay may be isolated from East Coast locations. This is supported by the occurrence in West Coast samples of a high frequency of a unique haplotype (H1). H1 differed from the most common haplotype (H2), which also occurred in high frequency at the West Coast location, by only 1 nucleotide substitution. Spirits Bay, on the other hand, was the only location sampled where the most common haplotype H2 was absent. These results highlight the potential for dispersal barriers along the New Zealand coast in these stingrays. The west coast of New Zealand is only accessible to east coast locations from either the northern or southern tips of the North Island, thus involving relatively large distances (≥400 km) traveled, potentially limiting its accessibility to dispersing individuals. Yet, not only has no genetic heterogeneity been detected among East Coast locations spanning distances >600 km, but there are no known physical barriers to dispersal between the East and West Coasts. The presence of East Coast haplotypes on the West Coast suggests some gene flow may occur, but may not be large enough to result in genetic homogeneity. Other strong restrictions to successful dispersal along the coastline may exist, such as specific habitat requirements and/or site fidelity to mating, pupping, or feeding sites. The lower genetic diversity in *D. brevicaudata* from Marlborough Sounds (the southern limit of its range) may simply be an artifact of a small sample size, but it may also reflect a truly lower diversity of a somewhat isolated population at the extreme of the species' distribution. Stronger conclusions are hampered by the limited knowledge of movement patterns, locations of breeding sites from throughout the range of the species and low sample sizes from critical areas (i.e., Spirits Bay, West Coast, and Marlborough Sounds), where they are much less common.

Differences in patterns of genetic variation between males and females have provided evidence for sex-specific philopatry in species that include white sharks (Pardini et al. 2001), lemon sharks (Feldheim et al. 2002), and blacktip sharks (Keeney et al. 2003; Keeney et al. 2005). Although our data are consistent with female philopatry, as reported in studies of coastal elasmobranchs, additional samples and nuclear data are needed to more conclusively ascertain whether sex-biased dispersal is occurring in *D. brevicaudata*.

Comparisons with Other Elasmobranch Species

Regardless of reproductive mode (egg-laying vs. live bearing), climatic preferences (tropical vs. temperate distributions), and now clearly phylogenetic lineage (sharks vs. rays), a recurrent pattern is emerging in the more coastal elasmobranchs, which involves limited or absent gene flow between ocean basins (oceanic divergence), but less genetic differentiation along continuous coastal distributions (coastal connectivity) (Chevolot et al. 2006; Duncan et al. 2006; Keeney and Heist 2006; Stow et al. 2006; Schultz et al. 2008; Chabot and Allen 2009; Ovenden et al. 2009; Plank et al. 2010). Only those elasmobranchs that are strictly pelagic (e.g., whale shark, *R. typus*) (Castro et al. 2007) or range to great depths (e.g., thorny skate, *A. radiata*) (Chevolot et al. 2007) appear to maintain connectivity over ocean basins. Several shark studies have investigated the level of mitochondrial divergence among populations with similar distributions to *D. brevicaudata* (i.e., South Africa/western Australia [Pardini et al. 2001; Stow et al. 2006; Chabot and Allen 2009], western Australia/eastern Australia/New Zealand [Ward and Elliott 2001; Stow et al. 2006]). Most show greater divergence of South African populations but lower mitochondrial differentiation across western Australia/eastern Australia/New Zealand (Stow et al. 2006). However, *D. brevicaudata* exhibits a higher level of divergence across these regions than any other elasmobranch examined previously. Similarly, an even greater level of genetic differentiation has been found in the conspecific *D. thetidis* over the same spatial scale with a CR sequence divergence value of 1.1% between South African ($n = 4$) and New Zealand ($n = 57$) populations compared with 0.2% for *D. brevicaudata* (Le Port A, unpublished data). These results suggest that deep oceanic waters are a greater barrier to dispersal in coastal stingrays than in many coastal sharks, probably due to the benthic habits of stingrays.

Forces Driving Population Structure in Coastal Stingrays

Our study largely reveals that stingrays conform to the emerging pattern found in coastal sharks, of high divergence across oceanic basins and lower differentiation along continuous coastal habitats. It is clear that genetic differences among sites are not explained entirely by distance alone. First, it seems apparent that the deep oceanic basins (and tropical waters) act as a major barrier to stingray dispersal, even greater than that for coastal sharks. Second, a continuous coastal connection appears to promote gene flow. The geographic distance between eastern and western Australia (a distance of over 3000 km) is similar to that between eastern Australia and New Zealand. However, the genetic divergence in *D. brevicaudata* between eastern and western Australia, which are linked by a continuous coastline, is nonsignificant and far less than that between eastern Australia and New Zealand. We have also shown that the breeding site at the Poor Knights Islands shows no evidence of genetic isolation, thus there is no evidence of year-round site fidelity to this location. However, there is still a surprising level of genetic differentiation around New Zealand in this species, indicating that its strong swimming capabilities and potential for dispersal around this continuous coastline may be interrupted by other behavioral barriers. We suggest that fine-scale habitat preferences driven by factors such as reproductive requirements and behavioral traits (such as mating or pupping aggregations) may play a role in these restrictions to gene flow. A better understanding of individual movement patterns and suitable breeding and nursery areas through tagging and tracking data, as well as the use of microsatellite data to get a better insight into the fine-scale structure of *D. brevicaudata* in New Zealand will facilitate more refined hypothesis testing.

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Supplementary Material

Supplementary material can be found at http://www.jhered.oxfordjournals.org/.

Funding

This work was supported by the University of Auckland.

Acknowledgments

All procedures were conducted under the animal care protocols approved by the University of Auckland Animal Ethics Committee (AEC Permit R240), and research within the Poor Knights Islands Marine Reserve was conducted under Department of Conservation Permit no. NO-14234-RES. Many thanks to the skippers of R/V Hawere, B. Doak and M. Birch, and to D. Egli and N. Hannam for their invaluable help in the field. Thanks also go to M. Smale, L. Singh, C. Duffy, P. Last, W. White, A. Graham, D. Phillips, and D. Chapman for the contribution of overseas samples and to Vince Richards for initial help with stingray CR primers.

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Received May 11, 2011; Revised October 11, 2011; Accepted October 19, 2011

Corresponding Editor: C. Scott Baker

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