Speciation of two stingrays with antitropical distributions: low levels of divergence in mitochondrial DNA and morphological characters suggest recent evolution

A. Le Port[1,2,*], M. D. M. Pawley[3], S. D. Lavery[1,2]

[1]Leigh Marine Laboratory, University of Auckland, PO Box 349, Warkworth 0941, New Zealand
[2]School of Biological Sciences, University of Auckland, Private Bag 92019, Auckland, New Zealand
[3]Institute of Information and Mathematical Sciences, Massey University, Auckland 0745, New Zealand

ABSTRACT: Disjunct geographic distributions across the equator (antitropicality) occur in many marine and terrestrial taxa. However, their origin and role in forming new species is still poorly understood. Here we examine the phylogenetic relationships and levels of genetic and morphological divergence in 2 stingray species, the Southern Hemisphere *Dasyatis brevicaudata* and the Northern Hemisphere *D. matsubarai*, with antitropical distributions and suspected conserved morphological traits. Analyses of concatenated mtDNA sequences (3160 bp dataset: CO1, cytochrome *b*, control region) suggest *D. brevicaudata* and *D. matsubarai* are closely related sister lineages, with low levels of sequence divergence more akin to intra- rather than interspecific comparisons. Multivariate analyses of morphological traits are largely consistent with the genetic data in showing small but discrete differences in morphometric characteristics, allowing Northern and Southern Hemisphere individuals to be separated into 2 groups. We suggest that *D. brevicaudata* and *D. matsubarai* are closely related sister taxa that may represent an early stage in the process of allopatric speciation.

KEY WORDS: Antitropical distribution · Stingray · Allopatric speciation · *Dasyatis* · Sister taxa

INTRODUCTION

The occurrence of the same organisms to the north and south of the tropical zone but not within the tropics has been coined an antitropical (or amphitropical) distribution (Hubbs 1952). Disjunct distributions of taxa across the tropics are observed for a variety of organisms, both terrestrial and aquatic, at various taxonomic, geographic, and presumably historic scales (Randall 1981, Burridge 2002). Over time, they are likely to result in allopatric speciation as genetic and phenotypic differences accumulate between populations, leading to reproductive isolation. The origins of such distributions are intriguing: How did taxa apparently intolerant to tropical conditions (i.e.

temperate species) become represented in both Northern and Southern Hemispheres (Briggs 2003)? Several mechanisms have been proposed for the development of antitropical distributions, from dispersal and vicariance events (Burridge 2002), to the 'tropical extinction hypothesis' (Briggs 2003). The majority of examples of antitropicality in the literature deal with fish species with long-lived larval stages and/or mobile pelagic adults (Graves 1998, Burridge 2002), for which dispersal is often strongly influenced by currents, and long-distance deep water crossings are not an obstacle. However, little attention has been given to the dispersal patterns and resulting antitropical distributions of more shallow-water or coastal live-bearing species such as elasmo-

branchs (sharks and rays), which rely mostly on the active dispersal of adults and juveniles, and are mostly limited to the continental shelf in their movements. There are a few documented examples of elasmobranchs with such life histories with antitropical distributions either side of the equator, e.g. spiny dogfish *Squalus acanthias* and bronze whaler shark *Carcharhinus brachyurus* (Compagno et al. 2005). Despite the commercial importance of some of these taxa, very little work has been done describing the potential dispersal mechanisms leading to their antitropical distributions (Ward et al. 2008b, Veríssimo et al. 2010, Benavides et al. 2011). Recent molecular work on the evolutionary dispersal of coastal shark species with disjunct distributions has proposed a stepping-stone model of dispersal for these species (Duncan et al. 2006, Keeney & Heist 2006, Benavides et al. 2011).

Although phylogenetic relationships can be reconstructed from a variety of characters, including morphological features, molecular data are particularly applicable for taxa that have diverged recently and exhibit limited variation in morphological traits (Avise 1994). Morphological phylogenetic studies can be hindered in the resolution of relationships between taxa by the difficulty in defining the polarity of morphological character states. This is often due to a very poor fossil record, many instances of parallel evolution and a lack of understanding of the character states of plesiomorphic outgroup taxa (Martin 1995). Furthermore, elasmobranchs and more specifically batoids (skates and rays), appear to be subject to a high level of morphological conservatism (McEachran & Dunn 1998). Molecular phylogenetic analysis may be the only means to confidently infer systematic relationships of chondrichthyian fishes (Martin 1995), and mitochondrial-encoded genes have proven valuable as a source of characters for inferring phylogenetic relationships in elasmobranchs (Kitamura et al. 1996, Douady et al. 2003, Dunn et al. 2003, Human et al. 2006, Rocco et al. 2007, Pasolini et al. 2011). Mitochondrial DNA (mtDNA) offers a particularly rich source of markers for the study of closely related taxa largely because of the reduced effective population size and relatively fast rate of sequence evolution (Chow & Kishino 1995). To account for issues like stochastic lineage sorting in single loci, the addition of nuclear DNA data is preferable, when possible, although this has proved difficult for elasmobranchs.

Stingrays (family Dasyatidae) are most diverse and abundant in coastal tropical and warm temperate waters worldwide. This family currently comprises at least 9 genera (*Dasyatis, Taeniura, Pteroplatytrygon, Makarararja, Neotrygon, Himantura, Urogymnus, Pastinachus,* and *Taeniurops*) and about 79 currently recognised species, most of which are found in the Indo-West Pacific (Last & Stevens 2009; W. White pers. comm.). The short-tailed stingray *Dasyatis brevicaudata* (Hutton 1875) has a disjunct Southern Hemisphere range with centres of distribution in the southwest Pacific (New Zealand/eastern Australia), eastern Indian Ocean (western Australia), and western Indian Ocean/southeast Atlantic (South Africa) (Compagno et al. 1989, Last & Stevens 2009). Recent molecular work using the rapidly evolving mtDNA control region has confirmed that *D. brevicaudata* populations from across the Southern Hemisphere belong to a single species, but has also shown considerable isolation between these regions (Le Port & Lavery 2012). Interestingly, this ray is morphologically very similar and shares several unique key morphological characters with the pitted stingray *D. matsubarai* (Miyosi 1939), which is a Northern Hemisphere species found in the warm temperate to subtropical waters of the northwest Pacific and Sea of Japan (Japan, Korea) (Nishida & Nakaya 1990, Lee & Joo 1996). Preliminary phylogenetic analyses of 11 *Dasyatis* species using mitochondrial 16S, cytochrome *c* oxidase subunit 1 (CO1) and cytochrome *b* (cytb) sequences suggest that *D. brevicaudata* and *D. matsubarai* are closely related (Le Port 2009). With no apparent overlap in their distributions across the tropics, it is an open question as to whether *D. matsubarai* and *D. brevicaudata* are antitropical species (or subspecies) resulting from the dispersal and/or vicariant event of an ancestral, perhaps tropical species, or whether they are phenotypically similar but distinct species due to the parallel or convergent evolution of separate ancestral species.

The aims of this research were to clarify the phylogenetic evolutionary relationships between *D. brevicaudata* and the morphologically similar *D. matsubarai*. To address these aims, previous analyses of *D. brevicaudata* mtDNA control region sequences (Le Port & Lavery 2012) were combined with new data: control region sequences from *D. matsubarai*; sequence data from both species for 2 additional mtDNA markers (CO1, cytb); and comprehensive morphometric and meristic data from both species. More specifically we asked: (1) Do molecular and morphological data allow us to unambiguously discriminate *D. brevicaudata* and *D. matsubarai*?; (2) Does this data support *D. brevicaudata* and *D. matsubarai* as sister species?; and (3) What biogeographic scenario might best explain their current distributions?

MATERIALS AND METHODS

Molecular data

Taxon sampling

Dasyatis brevicaudata samples were collected from several locations throughout the species' range, while *D. matsubarai* samples were collected from 2 locations in Japan (JPN), 1000 km apart (Fig. 1, Table S1 in the Supplement at www.int-res.com/articles/suppl/b019p153_supp.pdf). Samples were collected as skin/muscle tissue from the wild using a biopsy dart fixed on a modified biopsy pole while on SCUBA, caught on rod and reel, or from research collection trips. For animals kept in captivity, a small section of the ventral tail fold was cut with a scalpel, or blood samples were taken and preserved on Whatman FTA cards. Tissue samples were stored in 95% ethanol at −20°C. Details of the individuals sequenced in this study are provided in the Supplement (Table S1), including details of all related outgroups used (*D. thetidis, D. lata*, *D. sabina*).

DNA extraction, amplification, and sequencing

Total genomic DNA extraction, amplification, and sequencing procedures follow Le Port & Lavery (2012). Amplification of partial fragments of mitochondrial genes was performed by PCR (Saiki et al. 1988). The following primers were used for amplification and sequencing: for CO1: Fish-F2 (5′-TCG ACT AAT CAT AAA GAT ATC GGC AC-3′) and Fish-R2 (5′-ACT TCA GGG TGA CCG AAG AAT CAG AA-3′) (Ward et al. 2005); cytb: CB2 (5′-CCC TCA GAA TGA TAT TTG TCC TCA-3′), CB3 (5′-GGC AAA TAG GAA RTA TCA TTC-3′), and GLUDG (5′-TGA CTT GAA RAA CCA YCG TTG-3′) (Palumbi 1996); and the entire control region (dloop): Pro_dasyatis1 (5′-GTC TTG TAA ACC GAA GAG CAG AG-3′) /CR_Middle_R (5′-TAT GCC TTA CAA GGG AAC TAG ACC-3′) pair and CR_Middle_F (5′- GGT TGG CAC TTT ATC TGG TCT AGT-3′) /Phe_dasyatis1 (5′- TAA TAC CAG GTT ATG GAG GCG TGT -3′) pair (Le Port & Lavery 2012). Annealing temperatures for CO1, cytb and dloop were 54, 57 and 60°C, respectively. Two *Dasyatis matsubarai* samples were sequenced for all 3 loci. *D. brevicaudata* sample sizes varied for each mtDNA locus. A total of 176 previously published *D. brevicaudata* samples (New Zealand, NZ, n = 152; Australia, AUS, n = 14; and South Africa, SA, n = 10) were used in the analyses of control region sequences (Le Port & Lavery 2012). A representative selection of these samples were sequenced for cytb and CO1. For cytb, 28 samples (NZ = 12, AUS = 10 and SA = 6) were sequenced. A total of 32 *D. brevicaudata* sequences (NZ = 14, AUS = 12 and SA = 6) were used in the separate CO1 analyses. The dataset comprising the concatenation of the sequences from these 3 mtDNA loci (CO1-cytb-dloop) is hereafter referred to as the 3160 bp dataset and consisted of 30 sequences in total: *D. brevicaudata*, n = 28 (NZ = 12, AUS = 10 and SA = 6 and *D. matsubarai* (n = 2). After considerable efforts, we had no success in amplifying nuclear genes from these species.

Sequence alignment

All sequences were aligned using Sequencher v.4.7 (Gene Codes) and alignment results confirmed by eye. Species represented by multiple specimens were manually examined for variable sites in order to control for intraspecific variation. Identical sequences were collapsed into a single operational taxonomic unit (OTU) for phylogenetic analyses. Final consensus sequences from each gene were aligned using the ClustalW program of MEGA v. 4.0 (Tamura et al. 2007). After final trimming so that all sequences within each dataset were of
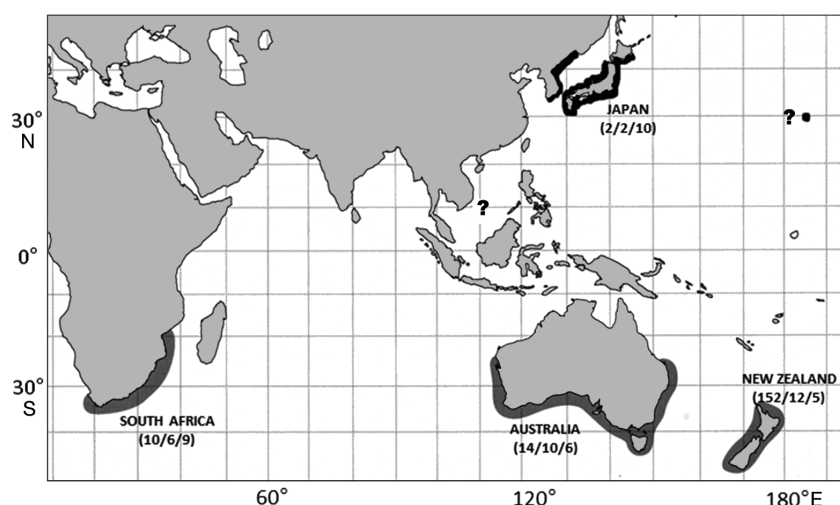

Fig. 1. Geographic distribution and sampled populations of *Dasyatis brevicaudata* (grey) and *D. matsubarai* (black). Numbers in brackets represent the control region (dloop) dataset sample size/3160 bp dataset sample size/morphometric dataset sample size. '?' indicate locations where *D. matsubarai* have been reported but not confirmed

equal length, the cytb, CO1 and dloop partitions contained 558, 623 and 1979 characters, respectively.

Morphological and meristic data

Taxon sampling

Distinguishing morphological characters for *Dasyatis brevicaudata* from other dasyatids include, but are not restricted to: a row of small white spots at the base of each pectoral fin, a bluntly angular snout, a thick base to a whip-like tail which is the same length or shorter than the body in adult specimens, the presence of an 'omega shaped' coracoid groove on the ventral surface, a small dorsal keel and ventral finfold, and the absence of thorn-like denticles along the dorsal midline of the disc (smooth disc) (Figs. 2a,c,e). *D. matsubarai* possesses *D. brevicaudata*'s typical key characters, namely a row of small white spots at the base of each pectoral fin, and an 'omega shaped' coracoid groove on the ventral surface (Figs. 2b,d,f) (Nishida & Nakaya 1990). Thus, there are at present no clear morphological distinctions between the 2 species' taxonomic descriptions. A total of 43 morphometric parameters were obtained from 30 specimens (*D. brevicaudata* n = 20, *D. matsubarai* n = 10) deposited in the collections of the Te Papa National Museum, Wellington (voucher code: P, DMN); CSIRO Museum, Hobart (H, CA); South African Museum, Cape Town (SAM); South African Institute for Aquatic Biology, Grahamstown (SAIAB); and Hokkaido University Museum, Hakodate, Japan (HUMZ). Meristic counts were recorded for 27 individuals (*D. brevicaudata* n = 17, *D. matsubarai* n = 10). Specimens used for morphological and meristic analyses are listed in Table S2.

Clarification of terms and measurements used in the paper

Methods and terminology of counts and morphometric parameters are adapted from other published work on rays (Bigelow & Schroeder 1953, Compagno & Roberts 1982, Compagno & Roberts 1984, Nishida & Nakaya 1990) (Table S3). Measurements were taken as a straight-line distance. Disc width (DW) is the standard measurement of size in rays, and proportional measurements are expressed as %DW (Table S4). Counts of oral papillae follow Nishida & Nakaya (1990).

Vertebral and radial counts

The morphological affinities of 3 populations of *Dasyatis brevicaudata* with the Japanese ray *D. matsubarai* were evaluated. Counts of radial cartilages and vertebrae were made from radiographs and digital scanning (CT scan), and follow Compagno & Roberts (1982). Radial counts include all radials with terminal branching. Pectoral radial counts include the radials in the anterior-most extension of the propterygium and the posterior-most extension of the metapterygium. Pelvic radial counts include the 'fused' radials in the anterior-most large compound 'radial'. Vertebral counts included in the analyses include: total vertebral segments; first synarcual free centra; intersynarcual centra; second synarcual and free monospondylous centra combined; diplospondylous tail centra; post-sting centra. Sample sizes vary among analyses. This is due in most cases to poor preservation of specimens (strongly curved wings, closed mouths), or preservation of specimens in mediums inadequate for long-term storage of calcified structures (i.e. formalin). For the larger specimens for which several x-ray films had to be used in order to visualise the entire wing and/or tail sections, a dissection pin was placed at the edge of the frame prior to exposure to ensure a consistent point of reference for all counts.

Data analyses

Dataset construction and combination of loci

The data sets for each locus (CO1, cytb, and dloop) were analysed separately to maximise the number of samples in each analysis (34, 30, and 178, respectively) and to examine whether the data sets converge on different trees. A combined data set (3160 bp dataset), using 30 sequences available, was also constructed by concatenating all 3 mitochondrial loci (CO1, cytb, dloop) to combine the phylogenetic signal from each data set, and maximise the possibility of finding an optimal topology. A partitioning of homogeneity test was first used to evaluate overall congruence in phylogenetic signal among the 3 loci. The test was implemented in a beta version of PAUP (PAUP v.4.0b10, Swofford 2002), using the heuristic search option with 10 random addition sequence replicates and 100 total replicates to generate the null distribution for the test. Tree bisection-reconnection (TBR) was chosen as a branch-swapping algorithm. Since this test indicated no significant overall
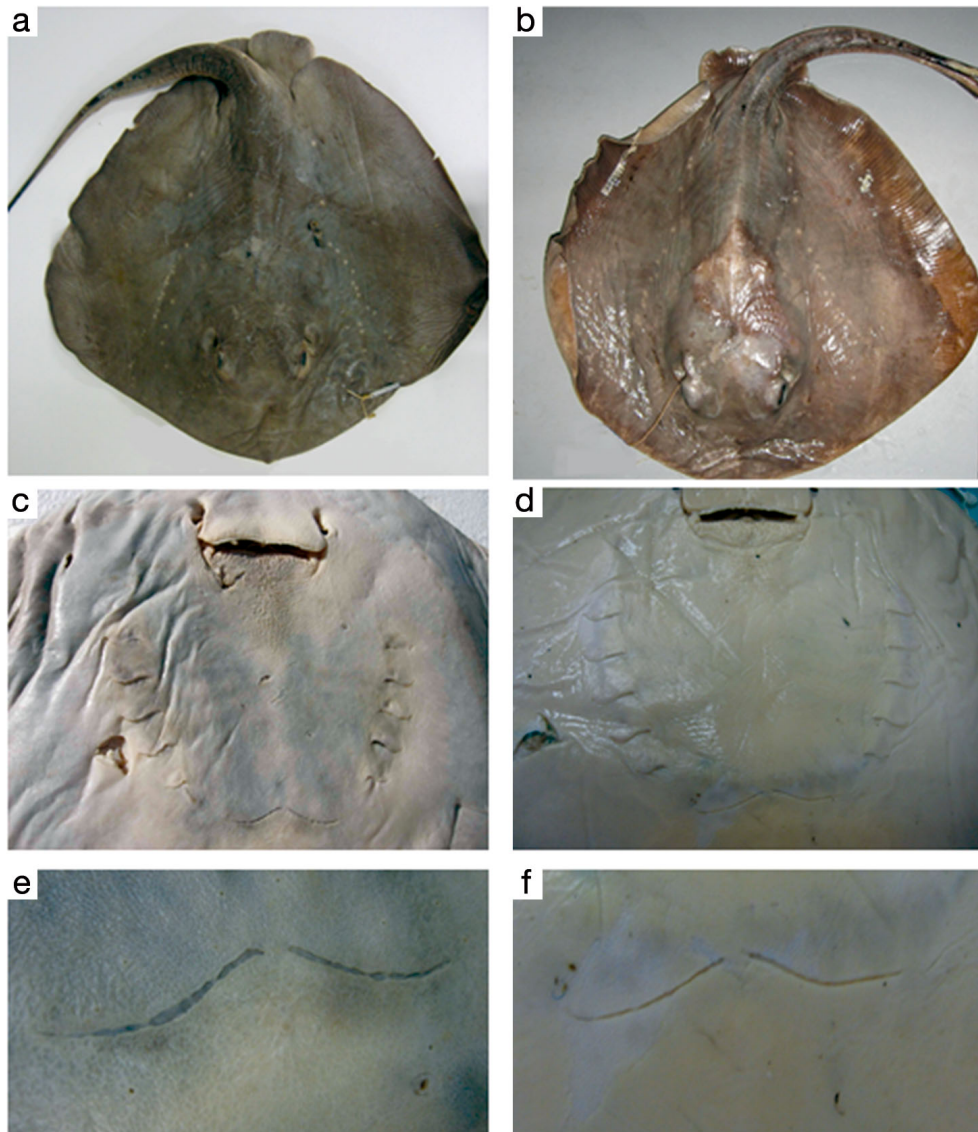
Fig. 2. *Dasyatis brevicaudata* and *D. matsubarai*. Photos comparing key morphological traits in *D. brevicaudata* (left) and *D. matsubarai* (right). (a,b) Dorsal view showing rhomboid disc shape and lateral lines of white spots; (c,d) ventral surface with coracoid groove posterior to 5th gill opening; (e,f) close-up of coracoid groove

conflict (p = 1.00) for the individual partitions (loci), a concatenated dataset was built in MacClade 4.07 (Maddison & Maddison 2005). The concatenated gene order of the combined dataset was 5′-CO1-cytb-dloop-3′ for all analyses.

Phylogenetic analyses

Phylogenetic relationships between the haplotypes found in *Dasyatis brevicaudata* and *D. matsubarai* were inferred using distance-based and model-based (neighbour-joining, NJ; maximum likelihood, ML and Bayesian inference, BA) methods of phylogenetic analyses. The TrN + I + G model was selected as the best model of sequence evolution by the ML test in Modeltest 3.7 (Posada & Crandall 1998) for all datasets. Parameters for the TrN + I + G model were: $-\ln L = 16871.78$; K = 7; A:C:G:T = 0.3232, 0.1946, 0.1948, 0.2873, respectively; I = 0.5132, G = 0.9124. The ML (k = 100 bootstrap replicates), and NJ with bootstrapping (k = 1000 replicates) approaches were based on the described TrN + I + G model parameters using the PHYML extension in Geneious Pro 4.0.2 (Guindon & Gascuel 2003) and MEGA 4 (Tamura et al. 2007) for each dataset (CO1, cytb, dloop, 3160 bp dataset).

To calculate posterior probabilities of clades we used the MrBayes extension in Geneious Pro 4.0.2 (Ronquist & Huelsenbeck 2003). A general time reversible (GTR + G + I) model of substitution with base frequencies was estimated from each dataset for this analysis. To avoid potential problems such as autocorrelation and slow mixing in the Bayesian analysis, it is necessary to perform numerous Markov chain Monte Carlo (MCMC) generations, and in the current study 4 incrementally heated chains were simultaneously run for 1 million MCMC generations for each of the data sets using the program default priors as starting values for the model. Trees were sampled every 1000 generations during the analysis. Bayesian posterior probabilities were obtained from the 50% majority-rule consensus of all trees sampled after trees from the initial 'burn-in' stage had been removed. The first 10% of the collected posterior data were discarded to allow 'burn-in' (Ronquist et al. 2005). The phylogenetic trees were visualized and edited where necessary using the Tree View extension within Geneious Pro 4.0.2. Consensus trees are presented. The net nucleotide sequence divergence of the 3160 bp mitochondrial DNA sequences were calculated using DnaSP v.4.5 (Rozas et al. 2003).

Phenotypic variation among populations

A total of 43 morphological measurements were recorded. Since the sample size (n) was less than the number of variables in the multivariate dataset (p), we used a canonical analysis of principal coordinates (CAP) to try and discriminate between *a priori* defined groups (Anderson & Willis 2003). The CAP was run in PRIMER v.6 (Clarke & Gorley 2006) on the variables without missing values (n = 33, Table S4 in the Supplement) using Euclidean distances in the resemblance matrix. We defined 2 *a priori* groupings and ran separate analyses. A 'Species' analyses was run to determine whether *Dasyatis brevicaudata* (n = 20) and *D. matsubarai* (n = 10) specimens could be reliably distinguished, while the 'Country' grouping allowed us to examine the natural variability in morphological characters (i.e. compare intraspecific vs. interspecific variability) between *D. brevicaudata* populations (NZ, SA, AUS) and *D. matsubarai* (JPN). Here, we used the 'leave-one-out allocation' (cross-validation) measure. This removes a single individual (i.e. ray) from the data, then does the CAP discrimination on the remaining data and tries to predict what group the 'removed ray' would be in.

Comparison of genetic and morphological datasets

In order to compare the levels of divergence in genetic and morphological characters, the pairwise population distances were plotted for both datasets together. For the genetic data, the average sequence divergence between populations was used, while for the morphological data, the patristic distances from the CAP analysis were used as a summary measure across all characters. Datasets could not be compared at the individual level as there was insufficient overlap in the samples with both genetic and morphological data, due to the difficulties of amplifying DNA from formalin-preserved museum specimens.

RESULTS

Phylogenetic relationships

Phylogenetic analyses

Previous phylogenetic analyses of Dasyatidae (Le Port 2009) using mitochondrial CO1, cytb and 16S sequences revealed that *Dasyatis thetidis* and *D. lata* were the most closely related species to *D. brevicaudata* and *D. matsubarai*, and hence these were used as an outgroup in the current analyses. The 2 *D. matsubarai* samples had the same haplotype for the CO1 and cytb loci, but different haplotypes for the control region. *D. brevicaudata* samples had the same haplotype for CO1, 4 for cytb and 18 for the control region (Fig. 3). Consensus trees for CO1 and the control region agreed in the relationships between the species with *D. brevicaudata* and *D. matsubarai* coming out as sister clades. However, for cytb, both species do not come out as sister clades. Instead, *D. matsubarai* is nested within *D. brevicaudata* populations, although the support for this relationship is low due to poor phylogenetic signal (Fig. 3b). The combined 3160 bp dataset showed the greatest resolution (Fig. 4). In this tree, *D. brevicaudata* and *D. matsubarai* formed a well-supported clade, which was sister to a clade formed by *D. thetidis* from NZ and SA and *D. lata*. *D. sabina* was basal to the other *Dasyatis* species sequenced (Fig. 4).

Genetic diversity and divergence

Rates of nucleotide divergence were similar across all 3 mtDNA regions e.g. maximum values between *Dasyatis brevicaudata* and *D. matsubarai* for CO1
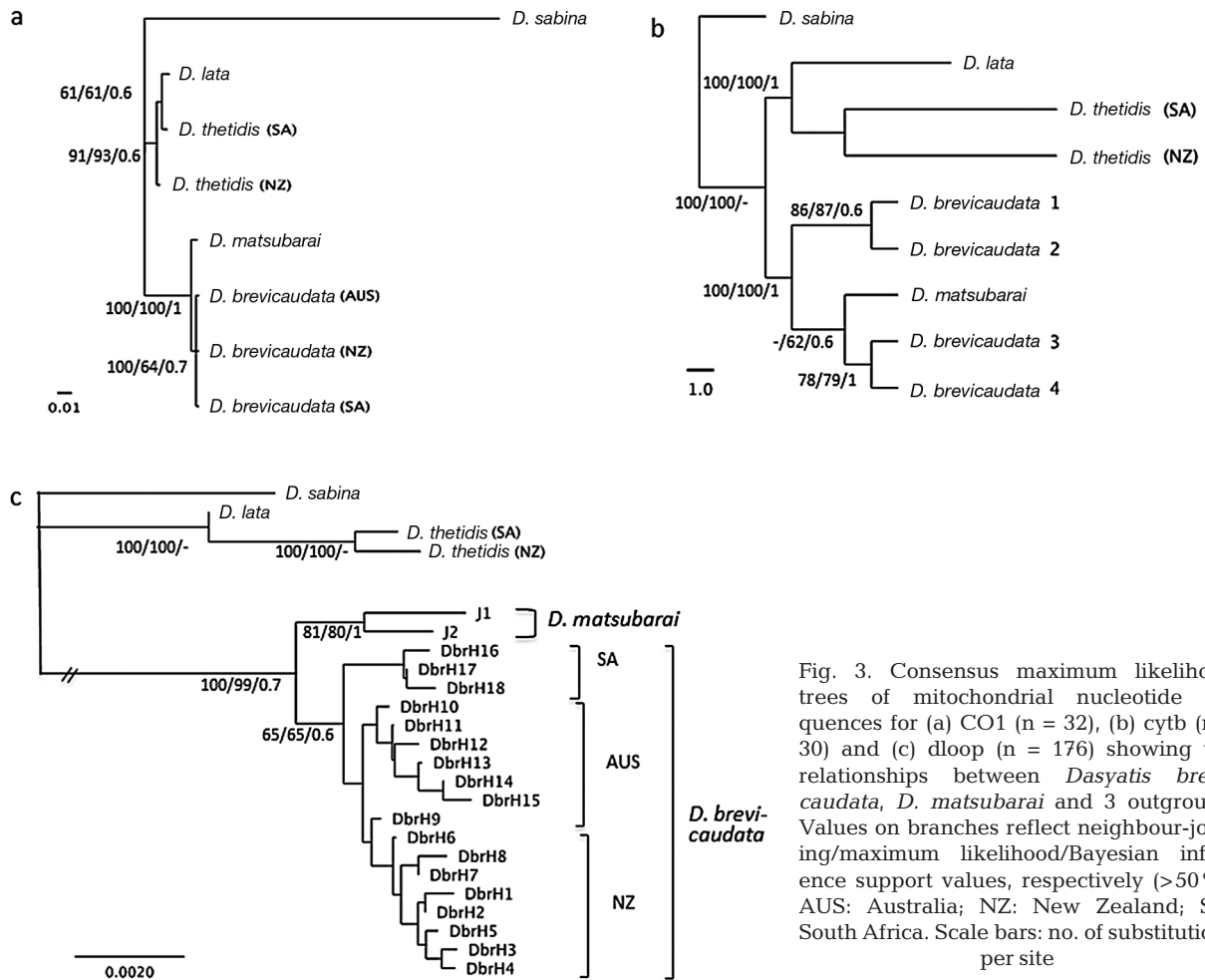
Fig. 3. Consensus maximum likelihood trees of mitochondrial nucleotide sequences for (a) CO1 (n = 32), (b) cytb (n = 30) and (c) dloop (n = 176) showing the relationships between *Dasyatis brevicaudata*, *D. matsubarai* and 3 outgroups. Values on branches reflect neighbour-joining/maximum likelihood/Bayesian inference support values, respectively (>50%). AUS: Australia; NZ: New Zealand; SA: South Africa. Scale bars: no. of substitutions per site

(0.35%), cytb (0.70%), dloop (0.30%). This does not support the common expectation of a faster rate of substitution for the non-coding dloop region. Within the combined 3160 bp *D. brevicaudata/D. matsubarai* dataset, 25 variable nucleotide positions were detected, comprised of 16 transitions, 5 transversions, and 1 indel. This produced 16 *D. brevicaudata* and 2 *D. matsubarai* haplotypes (Fig. 4). Sequence divergence among *D. brevicaudata* populations ranged between 0.1 and 0.2%, while distances between *D. brevicaudata* and *D. matsubarai* ranged between 0.3 and 0.5%. Comparatively, the next species most closely related to *D. brevicaudata* in this dataset, *D. thetidis* (NZ and SA) diverged by 5.2 and 5.6%, respectively, and intraspecies sequence divergence for *D. thetidis* was 0.8% (Table 1).

Divergence times

The net evolutionary rate of the whole elasmobranch mtDNA has been estimated at ~0.31% Myr^{-1}

(Dunn et al. 2003) and the cytb third codon silent rate of substitution estimated at ~0.71% Myr^{-1} (Martin 1995). We estimated divergence times between and within species for the 3160 bp dataset by using 2 rates of control region divergence reported for shark lineages (0.8 and 0.67% Myr^{-1}) (Duncan et al. 2006, Schultz et al. 2008). The 3 *Dasyatis brevicaudata* lineages with sequence divergence values ranging between 0.1 and 0.2% may have diverged only 62 500 to 149 000 yr ago. On the other hand, nucleotide divergences ranging from 0.3 and 0.5% between *D. brevicaudata* and *D. matsubarai* suggest these 2 species found either side of the tropics may have evolved in isolation for 187 000 to 373 000 yr.

Morphological differences

The CAP analysis on 33 morphological variables using 'Species' as the grouping variable was able to distinguish *Dasyatis brevicaudata* from *D. matsubarai* specimens with a high degree of accuracy (87%)
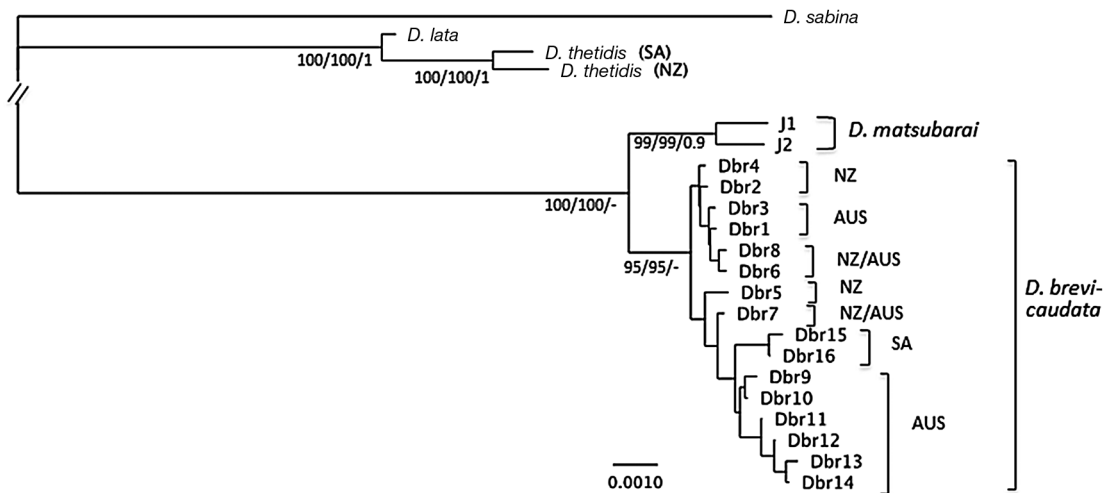
Fig. 4. Consensus maximum likelihood tree of the combined mitochondrial nucleotide sequences (3160 bp dataset: CO1/cytb/dloop) showing relationships between *Dasyatis brevicaudata* (n = 28), *D. matsubarai* (n = 2) and 3 outgroups. Details as in Fig. 3

(Fig. S1 in the Supplement). Only 4 out of 30 individuals were incorrectly classified (3 *D. brevicaudata* and 1 *D. matsubarai*). On the other hand, when the variable 'Country' was used, 19 out of 30 (63%) of individuals were correctly classified (Fig. 5). Interestingly, based on morphological characters, 3 groups could be discriminated in the latter analysis: NZ (*D. brevicaudata*), SA/AUS (*D. brevicaudata*), and JPN (*D. matsubarai*), suggesting that natural phenotypic variations may be very similar intra- and interspecifically. Using a correlation plot, the variables IPD (interpectoral distance) and HED (horizontal eye diameter) were the most useful in distinguishing groups NZ and JPN, while IOW (interorbital width), SPV (prepelvic length), DL2 (disc length including pelvics), INW2 (internasal width–posterior) appear to be the most useful in discriminating between groups NZ and SA/AUS, and PVL (pelvic fin length) and VTL (ventral tailfold length) for groups SA/AUS and JPN (Fig. 5, Table S3 in the Supplement).

Meristic counts were comparable between *Dasyatis brevicaudata* individuals from the 3 *D. brevicaudata* Southern Hemisphere populations and *D. matsubarai* from JPN (Table 2). Total number of vertebral segments ranged between 154 and 175 in *D. brevicaudata* and between 160 and 172 in *D. matsubarai*. Also, the total number of pectoral and pelvic radials did not differ between *D. brevicaudata* and *D. matsubarai* specimens (Table 2).

Genetic and morphological concordance

Overall, there appears to be concordance between the genetic and morphological datasets, in that both can distinguish between the 2 taxa, but that they have not diverged greatly. When the divergences between the various populations are compared across the 2 datasets, some discordance is observed (Fig. 6). The New Zealand population is particularly

Table 1. Pairwise comparisons of genetic distances (% divergence, Tamura-Nei model) for *Dasyatis brevicaudata*, *D. matsubarai* and the outgroups in the 3160 bp dataset. AUS: Australia; NZ: New Zealand; SA: South Africa. Sample size in parentheses

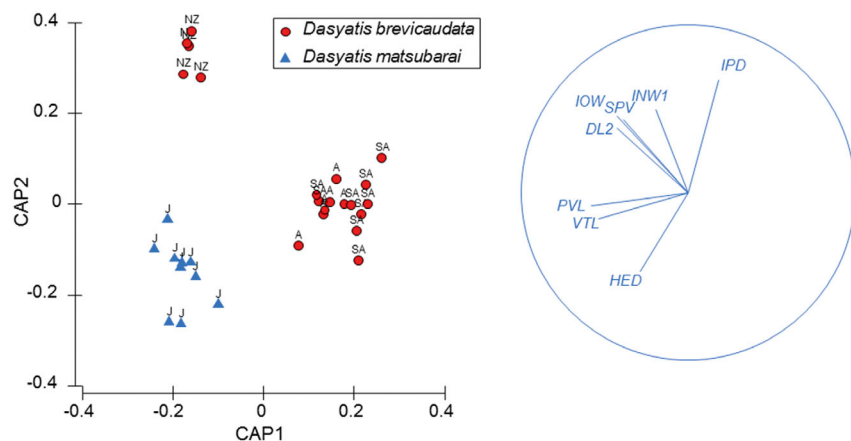| | | *D. brevicaudata* | | | *D. matsubarai* | *D. thetidis* | | *D. lata* | *D. sabina* |
		AUS (10)	NZ (12)	SA (6)	(2)	NZ (2)	SA (2)	(1)	(1)
D. brevicaudata	AUS	–							
	NZ	0.07	–						
	SA	0.09	0.18	–					
D. matsubarai		0.35	0.37	0.49	–				
D. thetidis	NZ	5.60	5.53	5.60	5.76	–			
	SA	5.29	5.22	5.33	5.46	0.85	–		
D. lata		5.47	5.43	5.56	5.60	1.53	1.60	–	
D. sabina		9.56	9.56	9.54	9.60	11.43	11.37	11.63	–

Fig. 5. Relative positions in plots of first 2 canonical functions of specimens (*Dasyatis brevicaudata* [n = 20] and *D. matsubarai* [n = 10]) subjected to canonical analysis of principal coordinates (CAP) on 33 morphological characters using the variable 'Country'. A: Australia; J: Japan; NZ: New Zealand; SA: South Africa. The correlations (r > 0.50) of the 4 groupings with the 2 CAP axes are presented to the right. See Table S3 in the Supplement for character abbreviations

interesting, in that it appears to have diverged morphologically to a similar degree as *Dasyatis matsubarai*, while maintaining relatively close genetic similarity to the other *D. brevicaudata* populations.

DISCUSSION

We have confirmed here that *Dasyatis brevicaudata* and *D. matsubarai* are distinguishable both genetically and morphologically. We have also confirmed that, as suspected, these are sister taxa. How-

ever, the levels of inter-specific divergence seen here are much smaller than expected, suggesting that the antitropical species *D. brevicaudata* and *D. matsubarai* are in the beginning stages of diverging into allopatric lineages.

Divergence between *Dasyatis brevicaudata* and *D. matsubarai*

Molecular data are particularly applicable for taxa that have diverged recently and exhibit limited variation in morphological traits (Avise 1994). It has been suggested in cetaceans that at least 2 lines of evidence indicating irreversible divergence are needed to distinguish species (e.g. genetic and morphological) (Reeves et al. 2004). In the present study, evidence from mtDNA (CO1, cytb, dloop), as well as morphometric data strongly support that the 2 described stingray species are distinct, but very closely related. However, several aspects of these results are worth discussing further.

Mitochondrial CO1 barcodes have been used successfully to discriminate elasmobranch species (Ward et al. 2008a). CO1 results from this study revealed a detectable divergence between *Dasyatis matsubarai* and *D. brevicaudata* (minimum number of fixed base differences of 2 [0.4 %]) compared to no divergence between all *D. brevicaudata* populations. However,

Table 2. *Dasyatis brevicaudata* and *D. matsubarai*. Vertebral counts. Information on size range (disc width in mm), sex and total numbers of individuals examined are provided below population headings. AUS: Australia; NZ: New Zealand; JPN: Japan; SA: South Africa; M: male; F: female; na: not applicable

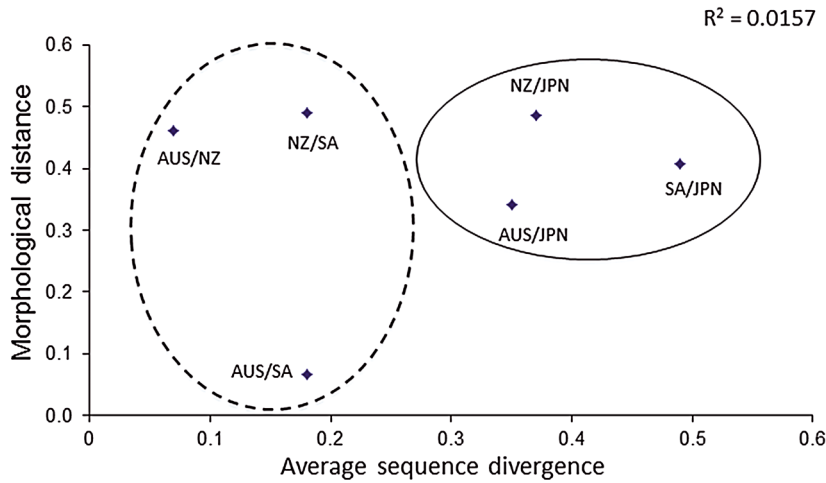| Counts	*D. brevicaudata* (NZ) 290.0–540.0 (4F)	*D. brevicaudata* (AUS) 325.0–673.0 (1M, 3F)	*D. brevicaudata* (SA) 390.0–750.0 (5M, 4F)	*D. matsubarai* (JPN) 367.0–470.0 (4M, 6F)
Oral papillae	na	7 (n = 1)	5 (n = 2)	5–11 (n = 9)
1st synarcual free centra	1–2	0–2	2–3	
Intersynarcual centra	6–7	5–6	5–7	3–6
2nd synarcual + monosp.	34–36	31–39	27–39	31–36
Pre-sting diplospondylous vertebrae	71–84	83–88	72–88	81–85
Post-sting centra	38–56	34–41	40–50	41–47
Total diplospondylous	109–140	117–129	116–138	122–132
Total vertebral segments	164–169	156–174	154–175	160–172
Propterygial radials (left)	52–56	54–56	46–56	54–57
Mesopterygial radials (left)	15–16	12–16	11–18	15–17
Metapterygial radials (left)	54–56	50–55	48–56	54–56
Total pectoral radials (left)	121–126	120–124	123–125	123–127
Pelvic radials (left)	25–27	22–26	22–25	24–27
Pelvic radials (right)	24	22–26	23–27	23–26

Fig. 6. Pairwise comparisons of the levels of divergence in genetic and morphological characters between *Dasyatis brevicaudata* lineages and *D. matsubarai*. For the genetic data, the average sequence divergence between populations was used, while for the morphological data, we used the average patristic distances between groups from the CAP analysis. Intra-specific (dashed line) and inter-specific (continuous line) groupings are highlighted

these values are much more similar to intra-species divergences (average 0.37%) rather than inter-species differences (average among congeners of 7.5%) reported in over 200 shark and ray species (Ward et al. 2008a, Cerutti-Pereyra et al. 2012). Similarly, the combined 3160 bp mtDNA dataset suggests that *D. matsubarai* is not greatly more divergent from *D. brevicaudata* than are Southern Hemisphere regional populations of *D. brevicaudata* from each other. A comparison among these 2 species and other dasyatids examined in this study or among closely related elasmobranchs in other published studies, shows that the divergence between *D. brevicaudata* and *D. matsubarai* (0.3 to 0.5%) does not fall within the range expected between different species. It is also much lower than mitochondrial control region divergences reported for closely related and morphologically similar batoids (e.g. thornback ray *Raja clavata* vs. biscuit skate *R. straeleni*, 1.8%, Pasolini et al. 2011, and Gulf of California vs. Pacific shovelnose guitarfish *Rhinobatos productus*, 2.5%, Sandoval-Castillo et al. 2004). Therefore, genetic evidence from 3 mtDNA markers showing such low levels of inter-specific divergence suggests that *D. matsubarai* and *D. brevicaudata* have likely diverged very recently, and are in the early stages of allopatric speciation.

Despite considerable sampling efforts, we were able to obtain DNA sequences from only 2 *Dasyatis matsubarai* specimens (from widely separated locations in the Japanese range, ca. 1000 km apart), as all individuals of this species used in the morphological analyses were formalin-preserved. This clearly prevents us from making an accurate estimate of the full genetic diversity within the taxon. However, given the close similarity of these sequences (<0.1% divergence), their considerably greater divergence from all *Dasyatis brevicaudata* sequences (0.3 to 0.5%),

and the very strong support for the reciprocal monophyletic relationship between the taxa, we are very confident of our major conclusions about the close sister relationship between these taxa.

Another line of evidence (morphological and meristic data) was used in this study to assess the status of *Dasyatis matsubarai* and *D. brevicaudata*. Multivariate analyses of 33 morphological characters were useful in discriminating between the 2 species, although once again, the inter-specific variability in morphological characters was similar to that between *D. brevicaudata* populations. Interestingly, no differences in meristic counts between species were found. These subtle phenotypic changes are all the more interesting given that we estimate that these species diverged between 187 000 and 373 000 yr ago, suggesting that these rays could be evolving very slowly. Morphological stasis has been reported in marine organisms (Rocha-Olivares et al. 2001), and may be common in rays and skates (McEachran & Dunn 1998, Pasolini et al. 2011).

The comparison of genetic and morphological data in our study reveals that, not unexpectedly, the geographically separated populations of these taxa have not diverged at the same rate in genetic and morphological characters. The New Zealand population has apparently diverged morphologically more than expected. Mayr (1954) described geographical speciation as a process leading over time to a continuum of stages of increasing divergence. *D. brevicaudata* and *D. matsubarai* may be only part-way through this process.

Migration and distribution patterns: evolutionary history

Our data from the combination of 3 mitochondrial loci suggest that *Dasyatis brevicaudata* and *D. mat-*

subarai diverged during the Pleistocene (187 000 to 373 000 yr ago). However, it is unclear what evolutionary process led to this divergence, and ultimately to their antitropical distribution. Our genetic and morphological data permit us to reject some evolutionary scenarios, but not all. Firstly, the genetic data reject the possibility that there is any ongoing connection between the 2 taxa, as there are no shared or closely related haplotypes, and they are clearly reciprocally monophyletic lineages. We can also reject the scenario that there has been very recent colonization of one hemisphere, as there is no evidence of greatly reduced genetic diversity within either lineage, or of paraphyly, which would be expected in such a scenario.

Two other major scenarios have been proposed to explain antitropical distributions. Older transequatorial dispersal during periods of glaciation has been widely proposed as a mechanism for the formation of antitropical distributions in fishes (Randall 1981, Graves 1998, Benzie 1999, Burridge 2002, Liu et al. 2006). However, fish have high larval dispersal potentials (Graves 1998), while stingrays (Myliobatiformes), on the other hand, as coastal bentho–pelagic elasmobranchs, have comparatively low dispersal capabilities. An alternative scenario that has been invoked for these transequatorial divergences is the vicariant isolation of ancestral populations occupying the tropics during the warming of interglacial phases (the 'tropical extinction' hypothesis, Briggs 2003). Unfortunately, these alternative scenarios are not easily distinguished by molecular data, as both have similar genetic outcomes. There is no evidence for long-distance migrations or deepwater crossings in *Dasyatis brevicaudata* (Le Port et al. 2008, Last & Stevens 2009). However, the occurrence of *D. brevicaudata* at remote offshore locations (e.g. West Norfolk Ridge, Gascoyne Seamount) with deep bottom depths (≥1000 m) (Clark 1988, W. White pers. comm.) suggests the use of these areas as stepping-stones in past or current migrations. We suggest that the current disjunct distribution of *D. brevicaudata* and *D. matsubarai* may be the result of rare long-distance migrations across deep tropical waters during cooler periods, when this species' distribution would likely have extended much further into tropical waters, and lowered sea levels would have provided many more shallow stepping-stones. Additional *D. matsubarai* samples, and mitochondrial and nuclear DNA analyses would be required to further test these scenarios.

Regardless of the exact mechanism, it is clear that *Dasyatis brevicaudata* and *D. matsubarai* are now completely reproductively isolated. They have diverged little in either molecular or morphological characters, but it appears we have caught them in the beginning stages of diverging into allopatric lineages. It is possible that a similar process of allopatric divergence has driven the speciation process in other *Dasyatis* lineages.

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