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Mitochondrial Haplotypes Indicate Parapatric-like Phylogeographic Structure in Blue-Spotted Maskray (*Neotrygon kuhlii*) from the Coral Triangle Region

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Phylogeographic structure was investigated in the blue-spotted maskray, *Neotrygon kuhlii*, focusing on the Coral Triangle region. We used as genetic marker a 519-bp fragment of the cytochrome c-oxidase subunit I (*COI*) gene, sequenced in a total of 147 individuals from 26 sampling locations. The parsimony network of *COI* haplotypes was split into seven distinct clades within the Coral Triangle region. Different clades had exclusive but contiguous geographic distributions, indicating parapatric-like phylogeographic structure. Strong genetic differences were also inferred between local populations within a clade, where reciprocal monophyly between geographically adjacent samples was observed on several instances. Nearly 25% of the total molecular variance could be ascribed to differences between geographic samples within a clade, whereas interclade variation accounted for >65% of the total variance. The strong phylogeographic structure observed within a clade can be explained by either sedentarity or female philopatry. We interpret the parapatric distribution of clades as the joint result of 1) expansion from refuge populations at times of low sea level, and 2) possible enhanced competition between individuals from different clades, or assortative mating, or hybrid zones, along lines of secondary contact. The parapatric-like structure uncovered in the present study parallels regional differences at nuclear marker loci, thus pointing to incipient speciation within Coral Triangle *N. kuhlii*.

Key words: *Myliobatoidei, genetic differentiation, speciation*

The population genetics of stingrays (Myliobatoidei: Aschliman et al. 2012) are among the most poorly understood among vertebrates (Beheregaray 2008). Studying patterns and processes of genetic differentiation in stingrays is fundamental to understanding speciation in this group. Previous genetic surveys in marine stingrays have revealed generally high levels of genetic differentiation between populations. These include the phylogeography and systematics of the white-spotted eagle ray, *Aetobatus narinari* (Richards et al. 2009; Schluessel et al. 2010); the population genetic structure of the round stingray, *Urobatis halleri* (Plank et al. 2010), and the Pacific cownose ray, *Rhinoptera steindachneri* (Sandoval-Castillo and Rocha-Olivares 2011); the phylogeography of the short-tailed stingray, *Dasyatis brevicaudata* (Le Port and Lavery 2012); and nuclear genetic variation in the white-spotted whipray, *Himantura gerrardi*, the blue-spotted maskray, *Neotrygon kuhlii*, and the blue-spotted ribbontail ray, *Taeniura lymna* (Borsa et al. 2012). Part of the total genetic variation in white-spotted eagle ray and Pacific cownose ray has been ascribed to the occurrence of cryptic species (Richards et al 2009; Sandoval-Castillo and Rocha-Olivares 2011). Low connectivity between populations separated by deep water was suggested as the cause of genetic differentiation in the round stingray, short-tailed stingray, and possibly blue-spotted maskray (Plank et al. 2010; Borsa et al. 2012; Le Port and Lavery 2012).

The fish barcoding project (Ward et al. 2009), based on the systematic sequencing of a portion of the cytochrome

c-oxidase subunit I (*COI*) gene, has brought additional perspectives to the biodiversity and taxonomy of stingrays. In a barcoding study of Australasian Chondrichthyans, Ward et al. (2008) identified a few problematic cases relative to the definition of species boundaries, where substantially divergent haplotypes were observed within a given nominal species. One of these cases was the blue-spotted maskray, *Neotrygon kuhlii* (Müller and Henle 1841). Based on limited geographic sampling, *N. kuhlii* showed unusually high within-species diversity, with distinct lineages characterizing the four populations sampled by Ward et al. (2008), that is, from the Java Sea, Bali, Taiwan, and the Gulf of Carpentaria. Average nucleotide distances at the *COI* locus among and within these four lineages were estimated to be 2.80% and 0.18%, respectively. As large ratios of intra- to inter-haplogroup nucleotide distances may indicate cryptic species, Ward et al. (2008) suggested *N. kuhlii* may require additional taxonomic scrutiny. Denser sampling of populations, across a broader geographic range, would be necessary to further address this issue. The recently published systematic survey of Elasmobranchs by Naylor et al. (2012) using the fast-evolving nicotinamide adenine dinucleotide dehydrogenase subunit 2 gene reached similar conclusions. Thus, the hypothesis that *N. kuhlii* may consist of a complex of multiple species has been raised repeatedly (Last et al. 2010; Naylor et al. 2012; Puckridge et al. 2013), apparently based on the sole observation of relatively high nucleotide distances between mitochondrial haplogroups (Ward et al. 2008; Naylor et al. 2012; Puckridge et al. 2013). Given that geographic sampling in the foregoing studies only represented patches of the geographic range of *N. kuhlii*, these findings might as well merely reflect high intraspecific diversity in a species with low gene flow, but without reproductive barriers between populations. A nuclear marker–based diversity study in three stingray species from the Coral Triangle, including *N. kuhlii*, confirmed the remarkably high degree of genetic differentiation among populations, but failed to provide conclusive evidence as to the occurrence of multiple cryptic species within *N. kuhlii* (Borsa et al. 2012). Although strong allelic frequency differences were observed between *N. kuhlii* populations from adjacent basins in the Coral Triangle, a trend of increasing genetic differences with increasing geographic distance was also uncovered, which leaves open the hypothesis that part of the differences between populations might be explained by isolation by distance (Borsa et al. 2012).

In the present study, we analyzed nucleotide sequence variation at the *COI* locus in *N. kuhlii* from an extended number of samples collected across most of the species range, including the western Indian Ocean and the Coral Triangle region (Figure 1). This did not include blue-spotted maskrays from the Coral Sea, which we consider to belong to a related species, *N. trigonoides*, based on distinctive spotting patterns (de Castelnau 1873) and mitochondrial haplotypes (Borsa et al. 2013). Separate clades were recognized within *N. kuhlii* and their distribution was mapped. Patterns of genetic variation were further analyzed within each clade, to infer processes of genetic differentiation in *N. kuhlii*.

Materials and Methods

Blue-spotted maskray, *N. kuhlii* (Myliobatoidei: Dasyatidae), specimens were collected between January 2008 and February 2012 from Pemba Island in the western Indian Ocean and from the Coral Triangle region, including 21 local fish-landing places throughout Indonesia, one site in Taiwan, two sites in the Philippines, and one site in West Papua (Table 1, Figure 1). Tissue samples of a total $N = 119$ individuals were collected. Voucher specimens were deposited at the ichthyological collection of Lembaga Ilmu Pengetahuan Indonesia (LIPI), Jakarta, in 2009–2010.

Approximately 30 mg of skin or muscle was removed from the pelvic fin, the pectoral fin, or the tail using surgical scissors and preserved in 95% ethanol at ambient temperature and used for the extraction of DNA. DNA extraction was done using either the Viogene tissue genomic DNA extraction protocol (Viogene, Taiwan) or the DNeasy DNA extraction kit of Qiagen GmbH (Hilden, Germany). DNA was stored in 1×, pH 8.0 TE buffer (AppliChem, Darmstadt, Germany). Polymerase chain reaction (PCR) amplification of a fragment of the *COI* gene was done in a T-Gradient thermal cycler (Biometra, Göttingen, Germany) using 20 µL reaction mixture with the following concentrations: 0.05 units/µL Taq DNA polymerase in 2× DFS-Taq Mastermix (Bioron GmbH, Ludwigshafen, Germany), 16 mM $(NH_4)_2SO_4$, 65 mM Tris–HCl, pH 8.8 at 25 °C, 0.01% Tween-20, 2.75 mM $MgCl_2$, 0.8 mM dNTP mix, 0.4 µM of each primer (AITBiotech, Singapore), and 2 µL DNA template. The primers were *FishF1* (5'-TCAACCAACCACAAAGACATTGGCAC-3') and *FishR1* (5'-TAGACTTCTGGGTGGCCAAAGAATCA-3') (Ward et al. 2005). Negative PCR controls were run alongside the assayed samples. PCR parameters were an initial denaturation at 94 °C for 2 min, followed by 35 cycles of heating (94 °C for 1 min), annealing (47 °C for 1 min), and extension (72 °C for 1 min), with a final extension step at 72 °C for 10 min. PCR products were visualized on 1% agarose gels and their size was estimated as approximately 670 bp. All PCR samples were sequenced, except a few individuals that did not produce a single bright band. After purification (by isopropanol precipitation), 1 µL 1/8 diluted PCR product was subjected to sequencing reaction, in both forward and reverse directions, using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA). Cycling conditions were according to the manufacturer's protocol. Sequencing reaction products were cleaned by removing dye terminator (CleanSEQ kit, Beckman Coulter) and loaded onto an ABI Prism 3100 DNA sequencer (Beckman Coulter, Beverly, MA). Sequences were deposited in GenBank and have accession nos. JX304798–JX304915 and KC295416.

Homologous sequences available from GenBank (from a total of 28 *N. kuhlii* from India, Ningaloo Reef, the Java Sea, Vietnam, the South China Sea, Bali, Taiwan, Japan, and

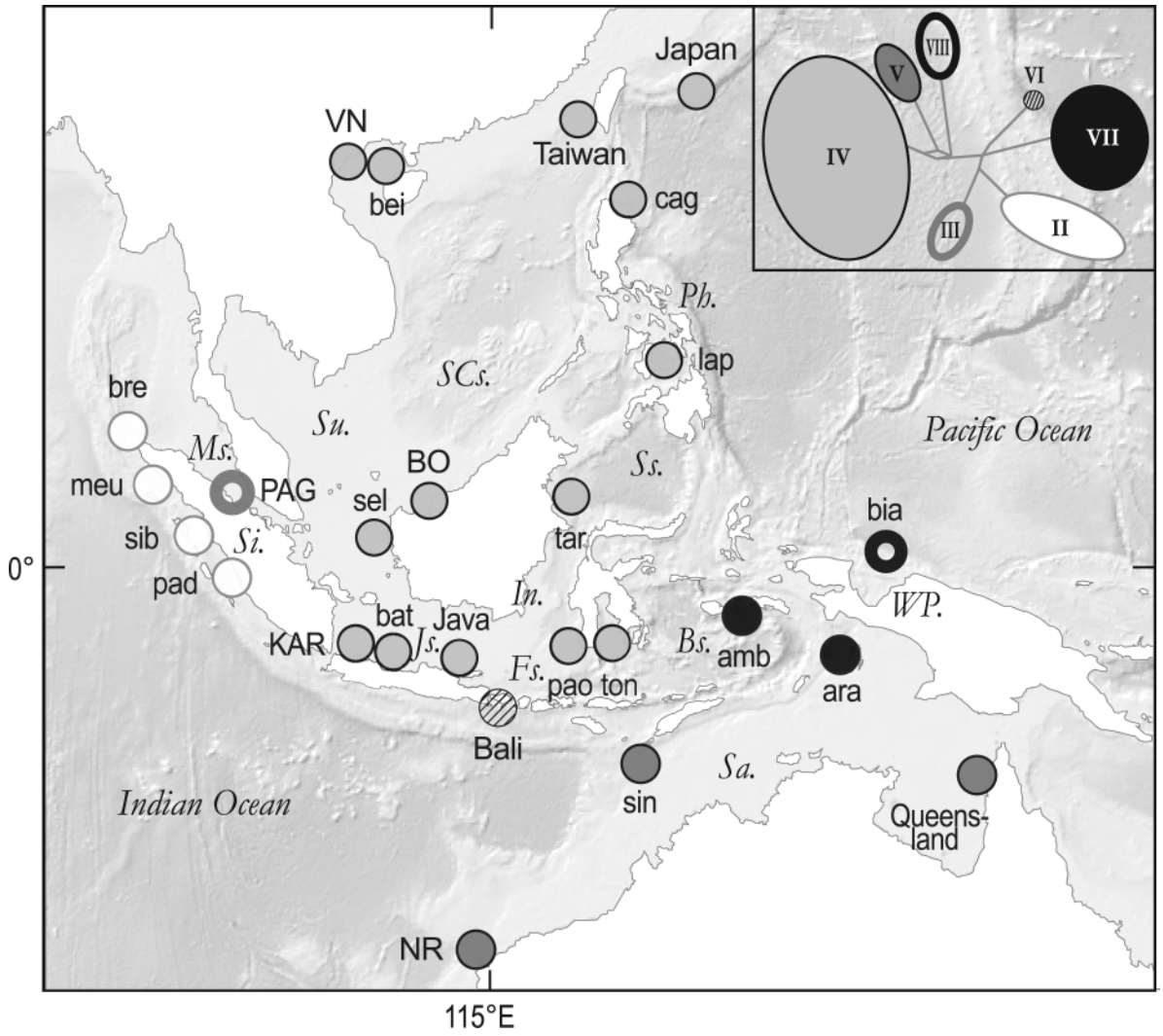

Figure 1. Map of the central Indo-West Pacific region, with sampling sites for blue-spotted maskray, *N. kuhlii*. Abbreviations for samples and sampling details in Table 1. *Circles* indicate sampling localities; *inset* presents a simplified version of the parsimony network of haplotypes (Figure 2); *colour codes* identical to those symbolizing haplotypes of Figure 2 (*white*: Clade II; *open grey*: Clade III; *closed pale grey*: Clade IV; *closed dark grey*: Clade V; *hatchured*: Clade VI; *closed black*: Clade VII; *open black*: Clade VIII). Abbreviations in italics correspond to localities referred to in text: *Ms.* Malacca Strait; *Si.* Sumatra island; *Su.* Sunda shelf; *Js.* Java Sea; *SCs.* South China Sea; *Fs.* Flores Sea; *Ss.* Sulawesi Sea; *Bs.* Banda Sea; *Sa.* Sahul shelf; *In.* Indonesia; *Ph.* Philippines; *WP.* West Papua. Samples from Tanzania and India have not been represented. Background topographic map from GeoMapApp (Ryan et al. 2009).

Queensland; Table 1) were added to our 119-sequence dataset. The nucleotide sequences were aligned visually under BioEdit (Hall 1999). A median-joining parsimony analysis was done using Network (Bandelt et al. 1999) on the nucleotide sequence matrix of 147 individual *COI* haplotypes trimmed to a core length of 519 bp. The network mode of representation was favored over phylogenetic trees because it is more relevant to describing relationships between haplotypes within a species, where ancestral haplotypes are a priori expected to persist alongside mutated haplotypes (Posada and Crandall 2001). The robustness of nodes in the network was tested using bootstrap resampling on the trees of haplotypes rooted by *N. trigonoides*, constructed using Mega5 (Tamura et al. 2011).

Hierarchical analysis of molecular variance was run on the same sequence dataset using Arlequin (Excoffier and Lischer 2010), to partition genetic diversity into within- and between-population components. The Kimura-2 parameter (K2P) model of nucleotide substitution with nonuniform evolutionary rates among sites modelled by discrete Gamma distribution (+G) was chosen to estimate genetic distance between haplotypes, as it was the most likely according to the Bayesian information criterion (Arlequin). Significance of *F*-statistics, Φ-statistics (estimating population differentiation based on the K2P+G model), and associated variance components was tested by comparing the actual parameter values to a pseudo-distribution generated by 1000 random permutations (Arlequin).

Table 1 Samples of blue-spotted maskray, *N. kuhlii*, analyzed for nucleotide sequence variation at the *COI* gene locus. Photographs available from corresponding author upon request. Additional information in the Dryad repository (http://datadryad.org): doi:10.5061/dryad.qp85g.

Sampling location	Coordinates	Abbreviation	Sampling date	Sample size, *N*	Collector	Vouchers, references	GenBank accession no.
W Indian Ocean: Pemba I., Tanzania	06°09'S 39°10'E	—	May 2010	1	J.-D. Durand	Photograph	KC295416
W Indian Ocean: Tanga, Tanzania	~05°N ~39°E	—	—	1	—	Puckridge et al. (2013)	KC249906[a]
Laccadive Sea: Kochi, India	~10°N ~76°E	—	—	1	—	NBFGR:CHN 156	HM467799[a]
Bay of Bengal: Vizakhapatnam, India	17°25'N 83°14'E	—	July 2010	1	A. Pavan Kumar	CIFE:VIZ-DK1	JX978329
Andaman Sea: Pulau Breueh, Aceh	05°53'N 95°02'E	*bre*	April 2009	6	I.S. Arlyza	Photographs	JX304798–JX304803
NE Indian Ocean: Meulaboh, Aceh	04°07'N 96°08'E	*meu*	April 2009	2	I.S. Arlyza	MZB 20843; photographs	JX304804–JX304805
NE Indian Ocean: Sibolga, N Sumatera	01°45'N 98°46'E	*sib*	March 2009	10	I.S. Arlyza	Photographs	JX304806–JX304815
Malacca Strait: Perbaungan	03°39'N 98°59'E	*PAG*	December 2008–March 2009	4	I.S. Arlyza	MZB 20847; photographs	JX304816–JX304819
Malacca Strait: Pagurawan	03°26'N 99°16'E	*PAG*	March 2009	8	I.S. Arlyza	Photographs	JX304820–JX304827
NE Indian Ocean: Padang, W Sumatera	00°56'S 100°21'E	*pad*	August 2009	1	I.S. Arlyza	MZB 20845; photographs	JX304828
Java Sea: Karangantu, Banten Bay	06°01'S 106°10'E	*KAR*	May 2009	6	A. Arifin	Photographs	JX304830–JX304835
Java Sea: Pulau Pabelokan	05°27'S 106°29'E	*KAR*	August 2009	1	I.S. Arlyza	MZB 20852; photographs	JX304829
Java Sea: Pulau Pari	05°51'S 106°37'E	*KAR*	December 2008	1	Mumu	MZB 20851; photographs	JX304836
Java Sea: Pulau Peniki	05°46'S 106°38'E	*KAR*	March 2009	8	I.S. Arlyza	MZB 20850; photographs	JX304837–JX304844
South China Sea: Haiphong	20°46'N 106°52'E	*VN*	September 2010	2	—	Cerutti-Pereyra et al. (2012)	JQ765561, JQ765562
South China Sea: Beibu Gulf	19°52'N 108°14'E	*bei*	February–July 2011	1	—	Wang et al. (2012)	JQ681494
Karimata Strait: Selakau, Pontianak	01°09'N 109°01'E	*sel*	August 2009	8	Fahmi; M. Adrim	Photographs	JX304845–JX304852
Java Sea: Batang, Central Java	06°51'S 109°47'E	*bat*	January 2008	7	I.S. Arlyza	—	JX304853–JX304859
Java Sea: unspecified location	—	*Java*	April 2004	5	W.T. White	Ward et al. (2008)	EU398737–EU398741
South China Sea: Tanjung Manis	02°07'N 111°19'E	*BO*	April 2004	2	J. Caira, K. Jensen, C. Healy	BO423, BO424 (Naylor et al. 2012)	KC249903[b,c], JN184065[b,d]
South China Sea: Mukah	02°54'N 112°06'E	*BO*	April 2004	1	J. Caira, K. Jensen	BO473 (Naylor et al. 2012)	KC249905[b,d]
South China Sea: Sarawak	02°49'N 110°53'E	*BO*	April 2004	1	J.N. Caira	BO409 (Naylor et al. 2012)	KC249902[b,e]

Sampling location	Coordinates	Abbreviation	Sampling date	Sample size, *N*	Collector	Vouchers, references	GenBank accession no.
Ningaloo Reef	~22°S ~114°E	*NR*	August–September 2010	2	O. O'Shea	Cerutti-Pereyra et al. (2012)	JQ765536, JQ765537
Bali	08°45'S 115°10'E	*Bali*	August 2002–March 2005	6	W.T. White	Ward et al. (2008)	EF609342, EU398736, EU398742–EU398745[f]
Bali: Kedonganan	08°44'S 115°11'E	*Bali*	January 2008	1	P. Borsa	Photographs	JX304860
Sulawesi Sea: Tarakan, E Kalimantan	03°38'S 117°44'E	*tar*	October 2009	5	Fahmi; M. Adrim	—	JX304863–JX304867
Flores Sea: Paotere, Makassar Strait	05°03'S 119°27'E	*pao*	September 2009	2	I.S. Arlyza	Photographs	JX304861–JX304862
Taiwan: Penghu	~23°37'N ~119°36'E	*Taiwan*	May 2005	3	—	Ward et al. (2008)	EU398733–EU398735[e]
Taiwan: west coast	—	*Taiwan*	October 2009	1	H.-C. Ho	—	JX304868
Flores Sea: Tonra, Bone Bay	04°44'S 120°19'E	*ton*	September 2009	5	I.S. Arlyza	Photographs	JX304869–JX304873
Timor Sea: off Rote island	—	*sin*	September 2009	2	I.S. Arlyza	Photographs	JX304874–JX304875
Philippines: Cagayan, N Luzon	18°17'N 121°53'E	*cag*	February 2012	12	K.-N. Shen	—	JX304876–JX304887
Philippines: Lapu-Lapu, Cebu Strait	10°17'N 124°00'E	*lap*	May 2011	4	A.S. Alino	Photographs	JX304888–JX304891
Japan: Ishigaki-shima	~24°18'N ~124°10'E	*Japan*	—	1	—	NSMT P-91858; Yagishita et al. (2009)	AB485685[g]
Banda Sea: Ambon	03°40'S 128°11'E	*amb*	October–December 2008	6	I.S. Arlyza; La Pay	MZB 20864; photographs	JX304892–JX304897
Arafura Sea: Kei islands	~07°37'S ~135°20'E	*ara*	March–May 2009	8	A. Kusnadi	MZB 20866; photographs	JX304898–JX304905
West Papua: Biak Island	00°58'S 136°16'E	*bia*	May–June 2009	10	Alvi	MZB 20867; photograph	JX304906–JX304915
Northern Australia: Gulf of Carpentaria	12°28'S 141°29'E	*Queensland*	March 1995	1	—	Ward et al. (2008)	DQ108184[b]

[a] "Clade 8" of Puckridge et al. (2013).

[b] Clade "Neotrygon kuhlii 1" of Naylor et al. (2012).

[c] "Clade 3" of Puckridge et al. (2013).

[d] "Clade 2" of Puckridge et al. (2013).

[e] "Clade 1" of Puckridge et al. (2013).

[f] "Clade 6" of Puckridge et al. (2013).

[g] "Clade 4" of Puckridge et al. (2013).

[h] "Clade 5" of Puckridge et al. (2013).

Nucleotide distances (K2P+G model) among haplotypes and clades were estimated using Mega5.

Results

The median-joining parsimony network connecting all 147 *COI* haplotypes (519 bp) is presented in Figure 2. Haplotypes were clustered as seven separate lineages or clades, which were arbitrarily assigned roman numbers II–VIII for convenience. In addition, a loose haplogroup formed by the haplotypes from India and Tanzania (sampling locations not shown on Figure 1) was observed, and it is referred to hereafter as "Haplogroup I." The color codes that distinguish the different clades on Figure 2 were the same as those used on Figure 1. A strong geographic component to clade composition was thus revealed. Clade II was sampled exclusively along northwestern Sumatra Island. Clade III comprised all haplotypes sampled in the Malacca Strait, and only these. Clade IV haplotypes had a wide geographic distribution, encompassing the South China Sea, the Java Sea, the western Sulawesi Sea, the northern Flores Sea, the Philippines, and the East China Sea (Taiwan); we also included the haplotype sampled from southern Japan into Clade IV, the one which it was most closely related to, despite substantial sequence divergence (Figure 2). Clade V exclusively consisted of haplotypes sampled on the Sahul shelf. Clade VI exclusively consisted of haplotypes sampled in southern Bali. Clade VII

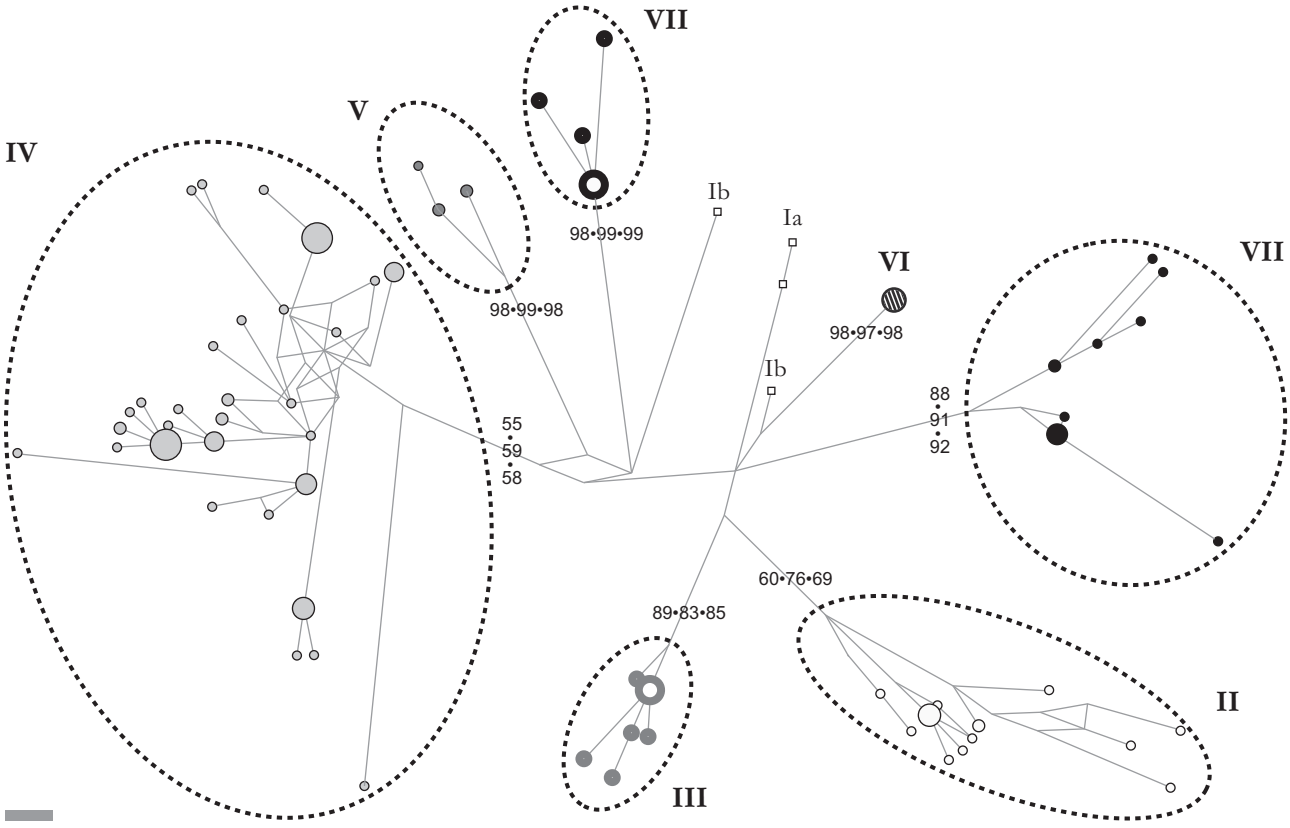

Figure 2. Blue-spotted maskray, *N. kuhlii*. Median-joining parsimony network (Network; Bandelt et al. 1999) of the haplotypes (as determined from 519-bp nucleotide sequences at the *COI* locus) of 147 individuals (sampling details in Table 1). Clades (*II–VIII*) are delineated by ellipses. Branch length is proportional to the number of mutational steps; *circles* represent individual haplotypes, their area being proportional to their frequency in the total sample and their color according with Figure 1; *squares* represent haplotypes from Tanzania and India. Numbers at the origin of each clade are bootstrap scores (1000 bootstraps) from, respectively, maximum parsimony (Mega5; Tamura et al. 2011)/neighbor joining (Kimura-2 parameter distance; Mega5)/maximum likelihood (HKY+G mutation model; Mega5) trees rooted by *N. trigonoides* (GenBank JQ765533–JQ765535, JX263420, JX304916, and JX304917) (Figure S1; see Supplementary Material online). Haplogroups *Ia* and *Ib* include haplotypes from Tanzania and India, respectively. Clades encompass haplotypes from different samples (abbreviations from Table 1) as following: *II*: bre, meu, sib, pad; *III*: PAG; *IV*: KAR, VN, bei, sel, bat, Java, BO, tar, pao, Taiwan, ton, cag, lap, Japan; *V*: NR, sin, Queensland; *VI*: Bali; *VII*: amb, ara; *VIII*: bia. Haplogroups *Ia* and *Ib* (present work) are included in "Clade 8" of Puckridge et al. (2013); Clade *IV* is "Neotrygon kuhlii 1" of Naylor et al. (2012) and includes "Clades 1–4" of Puckridge et al. (2013); Clades *V* and *VI* are, respectively, "Clade 5" and "Clade 6" in Puckridge et al. (2013). Scale bar: 1 mutational step.

Table 2 Hierarchical analysis of molecular variance (Arlequin; Excoffier and Lischer 2010) among population samples of *N. kuhlii* in the Coral Triangle, based on partial sequences (519 bp) of the *COI* gene. Population samples were grouped by clade (see Figure 2). Fixation indices: Φ_{CT}: 0.655; Φ_{SC}: 0.715; Φ_{ST}: 0.901 ($P < 0.001$ for all three)

Source of variation	d.f.	Sum of squares	Variance components	% Variation	*P*-value
Among clades	6	880.3	*Va* = 7.61	65.5	<0.001
Among populations within clades	16	214.7	*Vb* = 2.87	24.7	<0.001
Within populations	120	104.9	*Vc* = 1.14	9.8	<0.001
Total	142	1274.0	11.62		

exclusively consisted of haplotypes sampled on the margins of the Banda Sea, with one subclade from Ambon, the other subclade from Kei islands. Clade VIII exclusively consisted of haplotypes sampled in Biak. The relationship of the clades reported in the present study to the clades mentioned by Naylor et al. (2012) and Puckridge et al. (2013) could not be fully established (see footnote to Table 1).

Ignoring the haplotypes from Tanzania and India, the geographic distribution of the seven clades underlined several phylogeographic discontinuities in Coral Triangle *N. kuhlii*. Such discontinuities were apparent between the western coast of Sumatra Island and the Malacca Strait, between the Malacca Strait and the South China Sea, between the Indian Ocean coast of Bali and the Flores Sea north of it, between the eastern Flores Sea and the Banda Sea, between the Banda Sea and the Sahul shelf, and somewhere between the Banda Sea and the Pacific Ocean coast of West Papua (Figure 1). Pairwise *Fst* estimates between populations from different clades ranged from 0.067 to 1 (average 0.471 ± 0.294) (Table S1; see Supplementary Material online). Homologous pairwise Φ*st* estimates ranged from 0.651 to 1 (average 0.910 ± 0.081) (Table S2; see Supplementary Material online).

In the Coral Triangle, pairwise net nucleotide divergence between clades was 0.014–0.027, whereas mean nucleotide distance within a clade was 0.006. The mean nucleotide distance within a local population was 0.004 ± 0.005 (Table S3; see Supplementary Material online). About 65% of the total molecular variance could be ascribed to the partition of Coral Triangle *N. kuhlii* populations into seven clades (Table 2); nearly 25% of the variance was explained by differences between geographic samples within a clade; and the remainder (<10%) represented interindividual variation. In several instances, haplotypes from geographically adjacent samples within a clade formed sister sub-clades: *meu* versus *sib* within Clade II; *lap* versus *cag* versus *Taiwan* within Clade IV; and *amb* versus *ara* within Clade VII (see above). Elsewhere, samples within a clade generally harbored a high proportion of private haplotypes (Table S3; see Supplementary Material online).

Discussion

The present results provide novel insights into patterns of genetic differentiation in the blue-spotted maskray, *N. kuhlii*, shedding new light on the evolutionary biology of stingrays.

Deeply divergent lineages in *N. kuhlii* correspond to populations that have been geographically isolated for a long period. Using the rate of nucleotide substitution for the *COI* gene in angel sharks, *Squatina* spp. [$2.4 \pm 0.7 \times 10^{-3}$ substitution.site^{-1}per million years (MY); Stelbrink et al. 2010], leads to placing the initial divergence between *N. kuhlii* clades in the Coral Triangle at 0.2–0.8 MY ago. This is compatible with the series of episodes of low sea level through the second half of the Pleistocene (starting 2.4 MY ago; Hewitt 2000). Using the mutation rate proposed for combined cytochrome *b* and *COI* genes in the spotted eagle ray, *A. narinari*, from either side of the Panama isthmus (0.53% or 0.90%; Richards et al. 2009) yields a substantially more ancient initial divergence at 0.8–2.5 MY ago.

The genetic differentiation estimate at the mitochondrial locus (average *Fst* estimate = 0.471 ± 0.294 between clades) should be compared to its nuclear analogues. Although no sample was common to this study and Borsa et al.'s (2012) nuclear DNA study, the geographic scale was similar, hence allowing such a comparison. A high level of nuclear differentiation was effectively reported between *N. kuhlii* populations from different sea basins, with average *Fst* estimates over four intron loci ranging from 0.176 to 0.557 (Borsa et al. 2012). Two of the sea basins (the adjacent Flores Sea and Banda Sea) were sampled in both studies: the *Fst* estimates between populations from these two basins were 0.290 (introns: samples *Flores Sea* versus *Banda Sea*; Borsa et al. 2012) and 0.499 (mitochondrial DNA: samples *ton* versus *amb*; present results). Thus, both mitochondrial and nuclear markers indicate a high level of genetic differentiation between sea basins. The ratio of nuclear to mitochondrial *Fst* was, on the average, less than two, which suggests similarly restricted male and female gene exchange. A slightly greater contrast was observed at the local scale between *Fst* estimates at intron loci (*Fst* within sea basin = 0.105, 0.153; Borsa et al. 2012) and mitochondrial loci (*Fst* within clade = 0.389 ± 0.334; present study), which under a scenario of low migration may be explained by unachieved equilibrium between genetic drift and migration (Larsson et al. 2009).

The high proportion of mitochondrial haplotypes private to a sample within a clade indicates advanced lineage sorting at the local geographic scale. The first possible cause for this observation may be the sedentarity of individuals in *N. kuhlii*. A tagging study has reported that 16.1% of tagged blue-spotted maskray were recaptured at the very site of tagging up to 1081 days after tagging (Pierce et al. 2009), which the

authors interpreted as support to this hypothesis. The second hypothesis is that of a high degree of philopatry in *N. kuhlii*. In this case, eventual dispersal is offset at the time of reproduction when adult females return to their birthplace for parturition, or both adult males and adult females return to their birthplace for mating and the females further stay there until parturition. The use of geographically defined areas for mating, parturition, and juvenile growth has been documented in sharks (Carrier and Pratt 1998; Feldheim et al. 2002; Hueter et al. 2005; Whitney et al. 2012) and hypothesized in stingrays (Capapé 1993; Capapé and Zaouali 1995; Dale et al. 2011).

The main phylogeographic discontinuities uncovered in Coral Triangle *N. kuhlii* were apparently determined by the shallow boundaries between sea basins (Figure 1). For instance, a single clade was represented at the periphery of the South China Sea, including the Sunda shelf east of the Malay Peninsula, whereas a different clade was sampled in the Malacca Strait west of it. Likewise, a single clade was represented all over the Sahul shelf, whereas another clade was sampled at the periphery of the adjacent Banda Sea. *Neotrygon kuhlii* is a shallow water benthic species; it is commonly encountered from the intertidal zone to 90 m (Last and Compagno 1999). Given these habitat preferences, populations from different sea basins would have been likely, repeatedly isolated through the Pleistocene, retreating toward the deeper interior of the sea basins during periods of low sea level. During this time, habitat suitable to blue-spotted maskray was considerably restricted (Voris 2000), and it is possible that small populations would have persisted only in a limited number of refuges. When the sea level rose again, the formerly isolated refuge populations would have been able to expand their range, mostly on the Sunda and Sahul shelves where increasingly large surfaces of suitable habitat became available once again (Voris 2000).

Parapatry is the term used to describe a situation where pairs of taxa present separate but contiguous geographic distributions (Bull 1991). The geographic distributions of the seven clades observed over the Coral Triangle were mutually exclusive and it is sensible to describe them as contiguous at the geographic scale considered here, thus parapatric. Given the context of relative sedentarity or philopatry, we have to explain why the different clades of *N. kuhlii* are geographically distributed in a parapatric fashion. For this we hypothesize that a clade expanded as far as to colonize suitable habitat still unoccupied by conspecifics from another population. When secondary contact occurred, the expansion was stopped, leading to the current distribution. The persistence of parapatry, thus, may be explained either by the occurrence of a hybrid zone or by competition between individuals from genetically differentiated populations, or by assortative mating. In all cases, the parapatric-like population structure uncovered in this study points to incipient speciation, where some degree of reproductive isolation has been achieved but ecological compatibility has not yet been reached (Bull 1991). Investigating the fine-grain genetic structure of *N. kuhlii* populations at the boundaries between clades will be necessary to assess whether genetically differentiated populations interbreed and, if so, how narrow the hybrid zone is.

Supplementary Material

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

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