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Genetic and phenotypic diversity in the wedgefish *Rhynchobatus australiae*, a threatened ray of high value in the shark fin trade

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ABSTRACT: *Rhynchobatus* spp. (wedgefishes) are large benthopelagic shark-like rays with fins that are highly prized in the international shark fin trade. They are among the most threatened groups of sharks and rays globally. While *Rhynchobatus* spp. are known to be under considerable fishing pressure as a group, taxonomic confusion among species within the genus has compromised species-specific fishery and demographic data that are urgently needed for developing effective management strategies. *Rhynchobatus australiae* (Whitley, 1939) is a large Indo-West Pacific species reaching 2 to 3 m that is classified as Vulnerable on the IUCN Red List. This study combines new empirical data from field surveys with data obtained from verified reference specimens to investigate genetic and phenotypic variation in *R. australiae* and its relative incidence in fisheries. *R. australiae* dominated *Rhynchobatus* catch in fisheries surveys across Southeast Asia, and was the most commonly recorded species of the genus in Australia (94% and 58% of captures respectively, n = 207). Study specimens were consistent with a single species with moderate spatial mtDNA variation (Φ_{ST} = 0.198, p < 0.0001). We show that *R. australiae* can be reliably differentiated from other Indo-Pacific species with *nadh2* (1044bp), and a section of the control region (456bp) short enough to amplify DNA from processed fins in international trade. We document aspects of morphological variability to assist in the description of external characters that differentiate this species. This is the first range-wide intraspecific study on any wedgefish species, and provides the most complete synthesis of mtDNA data to date for identifying *Rhynchobatus* fins in the global shark fin trade.

KEY WORDS: Wedgefishes · Rays · Shark fin trade · Phylogeography · Southeast Asia

INTRODUCTION

The wedgefishes (family Rhynchobatidae) are large shark-like batoids (0.8–3 m total length) comprising a single genus *Rhynchobatus* of 7 described species, 6 of which are endemic to the Indo-West Pacific. The family was recently described as the third most threatened family of all sharks and rays globally on the basis of life-history sensitivity and availability to fisheries (Dulvy et al. 2014). *Rhynchobatus* spp. are caught throughout their range as target and bycatch in demersal trawl, net, and longlining fisheries for their fins and flesh (e.g. White & Dharmadi 2007, White et al. 2013). The fins of *Rhynchobatus* spp. in particular are highly desirable in the international fin trade and fetch some of the highest prices for their size (Chen 1996, Vannuccini 1999, Rose & McLoughlin 2001, Clarke et al. 2006). All

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assessed species are in threatened categories on the IUCN Red List on the basis of their large body size and K-selected life strategy, susceptibility to capture by multiple gears, and use of inshore habitat in some of the world's most heavily fished coastal regions (e.g. Stobutzki et al. 2006, White & Kyne 2010, Lam & Sadovy de Mitcheson 2011). While available fisheries data suggest that populations of *Rhynchobatus* are declining as a whole (White et al. 2013), species-specific assessments have been confounded by difficulties in assigning individuals to species: difficulties that have been compounded by recent taxonomic revisions within the genus.

Until the late 1990s, all Indo-West Pacific *Rhynchobatus* were considered a single wide-ranging species, *R. djiddensis* (Forsskål, 1775) (Compagno & Last 1999). Five more species have since been either reinstated or newly described in these waters. As a result of these revisions and uncertainty associated with species assignments, fishery catch statistics and associated life-history data have only been reliable at the generic level. Fisheries researchers have accommodated this uncertainty by recording *Rhynchobatus* catch as a group, as morphotypes, or as genotypes where comparative DNA reference material is lacking (e.g. Spaet & Berumen 2015). Even dedicated efforts to describe basic age and growth parameters in Australian *Rhynchobatus* have been thwarted by an inability to reliably distinguish among species using external characters (White et al. 2014). In the absence of species-level data, managing the genus as a group risks the overexploitation of rare or spatially restricted species and demographic stocks that may exist.

The 6 described Indo-West Pacific species of *Rhynchobatus* include *R. australiae* (Whitley, 1939) occurring in Southeast Asia and Australia, ranging from Thailand, Taiwan and Indonesia to the Australian sub-tropics (Last et al. 2013) (see Fig. 1); *R. springeri* (Compagno & Last, 2010) in Southeast Asia; *R. immaculatus* (Last et al. 2013), known only from Taiwan; and *R. palpebratus* (Compagno & Last, 2008) in northern Australia, and recorded in Thailand and Taiwan. Following Last et al. (2013), *R. djiddensis* occurs in the western Indian Ocean, including South Africa, Mozambique and the Red Sea, and *R. laevis* (Bloch & Schneider, 1801) occurs in Zanzibar, the Arabian Sea, Oman, Persian Gulf, India, Sri Lanka and Bangladesh. An additional 3 potential new species that await description are *R.* cf. *laevis* in Australia (Naylor et al. 2012), *R.* cf. *djiddensis* from the Arabian Gulf (Moore et al. 2012), and *Rhynchobatus* sp. 1, known from a few specimens from Singapore and Indonesia (Compagno & Last 1999). A single species *R. luebberti* (Ehrenbaum, 1914) occurs in the eastern Atlantic (Compagno & Last 2008).

R. australiae is one of the larger species in the genus, with much of its range in a region of high fishing intensity. The species' 2 subequal dorsal fins and caudal fin reach large sizes and are extremely valuable as a traded product for shark fin soup. Globally, this species has been assessed as Vulnerable on the IUCN Red List on the basis of apparent declines in catch rates for the genus in Indonesia and very high levels of exploitation throughout Southeast Asia (White & McAuley 2003). To determine the extent to which populations of this species are or may be declining, and to monitor and manage these populations, rapid and reliable methods are needed to identify *R. australiae* specimens and fins in fisheries and global trade.

While *R. australiae* can be reliably differentiated from other species of the genus by counts of vertebral centra, the lack of unambiguous external diagnostic features has made species assignment in the field difficult (Compagno & Last 2008, 2010, Last & Stevens 2009, Last et al. 2013). *R. australiae* is frequently confused with other large species, particularly *R. laevis* and *R. djiddensis*. This confusion has been compounded by uncertainty over the relative ranges of these species, and their relative incidence in fisheries, particularly in Southeast Asia and northern Australia. *R. laevis* and *R. djiddensis* have recently been noted as Indian Ocean endemics (Last et al. 2013) and also as widespread throughout Southeast Asia and northern Australia (e.g. Larson et al. 2013, Eschmeyer & Fricke 2015), demonstrating the need for details on the status, range, fixed external characters and demographics of these species. In addition, the status of provisional species *R.* cf. *laevis* as a potential Australian endemic is yet to be resolved.

Mitochondrial identification of *R. australiae* tissue has been hampered by a lack of correctly identified and vouchered reference sequences and the unsettling observation that mitochondrial sequences may not always reliably distinguish among *Rhynchobatus* species. In the survey by Naylor et al. (2012) which included 19 *Rhynchobatus* specimens from Southeast Asia and Australia, they described 1 mtDNA lineage (1044 bp of *nadh2*) that corresponded to *R. australiae* (n = 12), and 1 mtDNA lineage that corresponded to 2 morphologically distinct taxa: *R. palpebratus* (n = 2) and the provisional *R.* cf. *laevis* (n = 5). These findings underscore the importance of first establishing that the intraspecific genetic variation associated

with the chosen molecular marker in *R. australiae* is consistent with a single species. Once established, describing spatial patterns of genetic diversity can contribute to our understanding of demographic connectivity across the species' range.

The Southeast Asian and Australasian archipelagos spanning *R. australiae*'s described range form a network of partial and historically intermittent biogeographic barriers that have diverse impacts on genetic diversification in marine taxa (e.g. Briggs 1999, Rocha et al. 2007, reviewed in Carpenter et al. 2011, Gaither & Rocha 2013), including sharks and rays (e.g. Naylor et al. 2012, Giles et al. 2014). As sharks and rays lack a planktonic larval stage, realized dispersal is driven primarily by adult vagility and habitat use. A range of phylogeographic studies on coastally oriented species have demonstrated shelf-habitat-associated dispersal linked with vagility, and the importance of reproductive habitat use and episodic migration over seascape features which otherwise restrict adult movement (reviewed in Dudgeon et al. 2012). Two major features of this seascape that may act, or have acted, as a partial or total barrier to dispersal in this species, and thus influence mtDNA phylogeographic patterns, are explored in the present study. The first is the contemporary Indonesian Throughflow, a major current that transports water from the Pacific Ocean through Indonesia and into the Indian Ocean, and associated with deep trenches interrupting shelf habitat. The second is the historical Sunda Shelf Barrier, a major Pleistocene land bridge that extended southward from contemporary Thailand and into Indonesia.

The present study combines new empirical data from specimens sampled in fisheries surveys with data from validated reference specimens to examine important fisheries and demographic questions useful for separate assessment and management of catch and trade of *R. australiae*. We investigate the relative catch abundance of this species in fisheries across their described range, mtDNA differentiation from other *Rhynchobatus* species, and genetic and phenotypic variation within the species. The core study area of Southeast Asia and northern Australia spans the most recent published range description for this species after Last et al. (2013). This region is a marine biodiversity hotspot with intense fisheries effort and a major source of elasmobranch fins for global trade. The extensive mtDNA data presented for *R. australiae* and comparative data for other members of the genus contribute the most complete resource to date for identifying *Rhynchobatus* fins in the global shark fin trade.

MATERIALS AND METHODS

Study area and specimen data sources

Tissue samples were collected from 207 *Rhynchobatus* specimens across *Rhynchobatus australiae*'s described range after Last et al. (2013) from the East Andaman Sea (west) across Indonesia to Taiwan (north) and to sub-tropical Australia (south) (collectively, the core study area) and 4 specimens from the wider Indo-Pacific (Fig. 1). In Southeast Asia, all encountered *Rhynchobatus* specimens were sampled from landing site surveys (n = 153), and in Australia opportunistically from fisheries observer programs or research (n = 54). Samples from Kuwait, Saudi Arabia, Mozambique and Fiji were collected for this study or contributed by collaborators from fisheries and research sources (n = 4) (Fig. 1, Table 1). A total of 23 genetic reference sequences were included from verified reference specimens, and morphological data was included from 10 museum specimens. These reference data were either published data relating to museum specimens, or collected for this study from museum specimens (Table S1 in the Supplement at www.int-res.com/articles/suppl/m548p165_supp.pdf). *R. australiae* is defined here on the basis of molecular data in Naylor et al. (2012), corresponding to specimens in the Australian National Fish Collection (Table S1). Tissue was obtained from the *R. australiae* holotype (AMS IA4959; Manning River mouth, NSW, Australia), but amplification was unsuccessful.

Landing site surveys

Tissue samples obtained from landing site surveys were collected from whole sharks landed by artisanal or commercial fishing fleets. Landing sites were chosen where elasmobranch catch was expected or known to be high based on gears, fishing grounds and targeted catch (e.g. surface and bottom set longlines operating in inshore waters). All encountered *Rhynchobatus* specimens were sampled. Specimens were sourced primarily from local in-shore operations at landing sites where fleets operate within a somewhat restricted distance. Observation of fishing gear used, catch composition, and interview data on trip length and fishing grounds were used to assess whether catch was likely to come from within approximately 300 km or a more distant location. Samples collected in Jakarta, Indonesia were determined by interview as originating from more distant

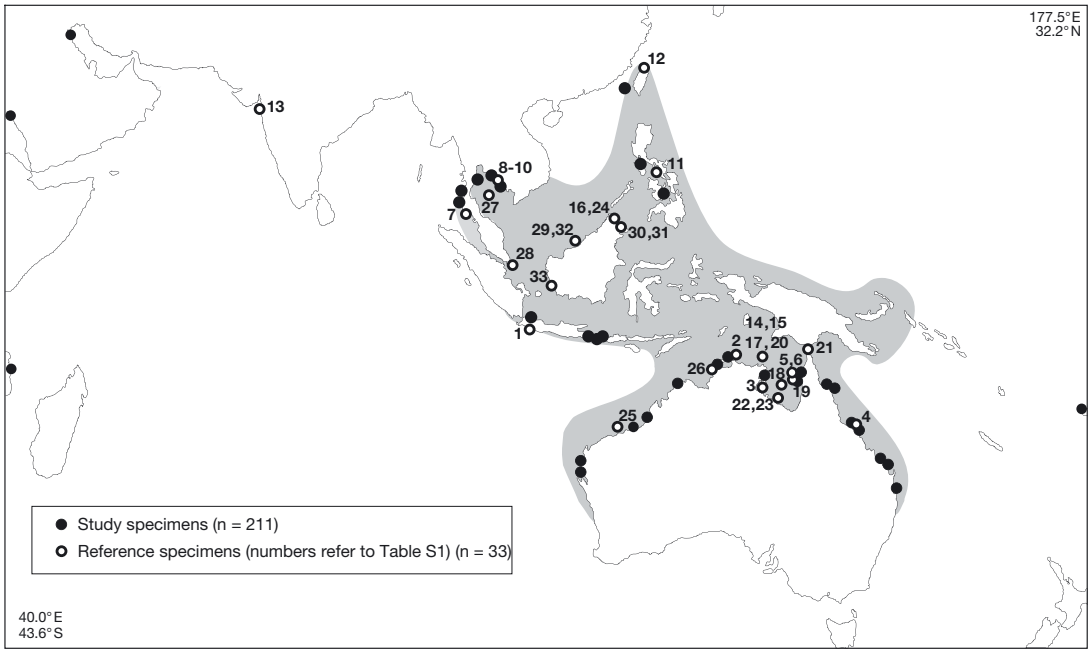


Fig. 1. Source locations of *Rhynchobatus* specimens (n = 244), with the distribution of *R. australiae* (the core study area) marked in dark grey (Last et al. 2013). The 33 reference specimens are numbered (see Table S1 in the Supplement at www.int-res.com/articles/suppl/m548p165_supp.pdf)

Table 1. Sampling locations (geographic midpoints), sample sizes (n), and haplotypes (H) of *Rhynchobatus* study specimens (symbols as in Fig. 2) for the mtDNA control region (CR) and *nadh2*. Sampling type indicated as either landing site (LS), fisheries observer programs (FO), and fisheries research (FR), or additional samples (AD) (no catch or effort data). GoC: Gulf of Carpentaria; N(S) QLD: north (south) Queensland; NT: Northern Territories

Symbol	mtDNA lineage	Location	Type	Midpoints		Total n	CR		*nadh2*	
				Lat.	Long.		n	H	n	H
∇	*R. australiae*	Thailand: Andaman Sea	LS	9.6	98.4	30	30	6	8	3
		Thailand: Gulf	LS	13.1	100.6	10	10	3	2	2
		Indonesia: Jakarta/Kalimantan	LS	–6.1	106.7	28	27	9	10	3
		Indonesia: Bali	LS	–8.7	115.0	7	7	2	3	2
		Indonesia: Lombok	LS	–8.8	116.5	14	14	2	4	1
		Taiwan: PengHu Islands	LS	23.6	119.6	54	45	7	14	4
		Philippines: Palawan	LS	11.6	122.5	2	2	2	1	1
		Australia, west coast: Kimberley	FR	–16.3	124.4	1	1	1	–	–
		Australia, west coast: Shark Bay	AD	–25.6	113.7	5	5	1	4	1
		Australia, north coast: GoC	FR	–10.4	140.5	1	1	1	–	–
		Australia, east coast: N QLD	FO	–16.2	145.5	10	10	3	2	1
		Australia, east coast: S QLD	FR	–26.2	153.1	14	14	4	–	–
		Fiji	AD	–18.2	178.4	1	1	1	1	1
●	*R. palpebratus*/	Australia, east coast	FO, FR	–18.7	147.2	7	7	5	4	3
	R. cf. *laevis*[a]	Australia, north coast: GoC	FO, FR	–12.6	139.9	11	11	4	2	2
		Australia, north coast: west NT	FO, FR	–12.7	130.2	4	4	3	–	–
		Indonesia: Jakarta	LS	–6.1	106.7	5	5	4	2	2
		Australia, west coast	AD	–	–	1	1	1	–	–
▲	*R. immaculatus*	Taiwan: PengHu Islands	LS	23.6	119.6	1	1	1	1	1
■	*R. springeri*	Indonesia: Jakarta	LS	–6.1	106.7	2	2	1	1	1
○	*R.* sp. (Arabian Gulf)	Kuwait	LS	29.4	48.0	1	1	1	–	–
★	*R.* sp. (Red Sea,	Mozambique	AD	–	–	1	1	1	–	–
	Mozambique)	Saudi Arabia: Red Sea	AD	21.4	39.2	1	1	1	1	1

[a]Provisional species after Naylor et al. (2012)

waters in Indonesia. A sample collected in Mindoro, Philippines was determined by interview to originate from Palawan. Tissue samples were preserved in salt saturated 20% DMSO. Total length (TL, measured ventrally), sex, and male outer clasper length (CLO) and clasper calcification were recorded. Specimens were photographed for subsequent recording of phenotypic characters, and linear measurements were taken where possible.

DNA sequencing and alignment

DNA was extracted using a 20% Chelex solution (Bio-Rad) after Walsh et al. (1991) for a total of 211 study specimens and 2 reference specimens. For 203 specimens, 456 bp of the mitochondrial control region 203 (CR) was amplified using the forward primer GwF (Pardini et al. 2001) and reverse primer 470R2 (Giles et al. 2014). For 60 specimens, 1044 bp of *nadh2* was amplified using forward and reverse primers ILEMFWD and ASNMREV, respectively (Naylor et al. 2012). Amplicons were purified with New England Biolab enzymes Exonuclease I and Antarctic Shrimp Phosphatase at 1 unit of each per microlitre of template. Purified amplicons were sequenced either in-house and visualised on an ABI 3130 Genetic Analyser, or outsourced to Macrogen, South Korea, or Beckman-Coulter, USA. Forward and reverse primers used for PCR were also used for sequencing. Sequence data were aligned and ambiguities resolved manually using CodonCodeAligner 3.7.11 (CodonCode Corporation). The resulting dataset consisted of 211 study specimens and 22 reference sequences, each amplified for either the mtDNA control region or *nadh2*, or both (Table 1, Table S1 in the Supplement).

Phylogenetic trees

Phylogenies were estimated under Bayesian and Maximum Likelihood criteria in MrBayes 3.2 (Huelsenbeck & Ronquist 2001, Ronquist et al. 2012) and RAxML (Randomized Axelerated Maximum Likelihood) (Stamatakis et al. 2008), respectively. In MrBayes, trees were constructed under a general time reversible (GTR) substitution model with gamma-distributed rate variation across sites and a proportion of invariable sites estimated from the data, with 4 discrete gamma categories, uniform alpha and a relative burn-in of 25%. This model was selected using ModelTest 3.4 (Posada & Crandall 1998) in conjunction with PAUP* v4.0b10 (Swofford 2002). The Mr. Bayes search was run for 5 million generations on the University of Queensland high performance computing cluster with 4 metropolis-coupled chains. Convergence across chains was assessed by examining estimated sample size, potential scale reduction factor and the average standard deviation of split frequencies. RAxML's online black box facility (http://embnet.vital-it.ch/raxml-bb/) was used to generate the best fit Maximum Likelihood tree for 100 bootstrap replicates. Five sets of unique haplotypes were analysed for each tree-building method ('taxon sets')—A: most restrictive (concatenated sequences for samples with both mtDNA regions); B: CR only; C: *nadh2* only; D: least restrictive (either CR or *nadh2* or both); and E: most restrictive (concatenated study specimens only, i.e. taxon set A) plus all reference material sequences (for either *nadh2* or CR). Consensus trees from MrBayes and best fit trees from RAxML were plotted in the ape package 3.0-7 (Paradis et al. 2004) in R (R Core Team 2013) using RStudio (RStudio Team 2013). Lineage identities were assigned based on the identity of verified reference material (Table S1 in the Supplement). No verified DNA reference material was available for *R. luebberti, R. djiddensis*, *R. laevis*, or provisional species *Rhynchobatus* sp. 1. *nadh2* reference material is available in GenBank for a museum reference specimen from Malaysia labelled as *R. laevis*; however, this species was recently noted as an Indian Ocean endemic by Last et al. (2013). The Australian National Fish Collection confirmed that the specimen from which this sequence was obtained is the newly described *R. springeri* (P. Last pers. comm.), and we have used that species identity here.

Genetic diversity and spatial differentiation in *R. australiae*

Intraspecific analyses were conducted using mtDNA control region data. Genetic variation in each collection location with $n \geq 5$ specimens is given as number of haplotypes (H), haplotype diversity (h) and nucleotide diversity (π) (Nei 1987). Tajima's D (Tajima 1989) was estimated to test whether variation in each sampled population was consistent with expectations under a hypothesis of constant population size and neutral mutations. A haplotype network diagram was constructed under parsimony criteria for a 95% connection limit in TCS 1.21 (Clement et al. 2000), and related to sampling locations.

Using analysis of molecular variance (AMOVA) (Excoffier et al. 1992), we tested for population sub-

division in *R. australiae* by pooling sampling locations into regions on either side of 2 *a priori* biogeographic features: the contemporary Indonesian Throughflow current, at the major outlet site of the Timor Passage/Ombai Strait, and the historical Sunda Shelf Barrier, at the contemporary Ithsmus of Kra separating the Andaman Sea from the Gulf of Thailand. The Timor Passage/Ombai Strait accounts for approximately 83% of combined current flow and is associated with trenches of around 160 km and 35 km wide reaching over 2000 m deep in parts, dividing Indonesia from the adjacent Australian continental shelf (Gordon et al. 2010, Rosenfield et al. 2010). Although the Lombok Strait (~38 km wide and ~350 m deep) has been used in marine studies as a site to test divergence associated with the Indonesian Throughflow, owing to its concordance with the terrestrial Wallace Line, the Timor Passage/Ombai Strait is a much more substantial seascape feature and physical interruption in shelf habitat. This is therefore likely to be a more reasonable site to test for impact on adult dispersal between Australia and Southeast Asia for a reasonably vagile shelf-associated elasmobranch, as demonstrated for the spot-tail shark *Carcharhinus sorrah* in Giles et al. (2014). Specimens collected in Jakarta were understood to originate from Indonesian waters and therefore were included with other Southeast Asian locations on the northern side of the Timor Passage and east of the Isthmus of Kra. The AMOVA was conducted under the Tamura & Nei (1993) model in Arlequin 3.5 (Excoffier & Lischer 2010). Significance was tested using 1-tailed p-values with Holm-Bonferroni sequential correction (Holm 1979).

Phenotypic variation in *R. australiae*

Phenotypic descriptors were selected from those used in previous descriptions (Compagno & Last 1999, 2008, 2010, Last & Stevens 2009). Nine simple categorical dorsal features were recorded to describe patterning, snout shape and first dorsal fin position (n = 106 specimens), along with 18 linear measurements (relative to total length) to describe basic body plan features (n = 57 specimens) (Fig. S1 in the Supplement at www.int-res.com/articles/suppl/m548p165_supp.pdf). Outer clasper length and degree of calcification relative to total length were plotted as a proxy for male maturity. A non-metric multidimensional scaling (NMDS) ordination was performed to observe the relationship between linear measurements of body plans among *R. australiae* specimens, in the context of other sampled species. A dissimilarity matrix was calculated for the linear measurements using Gower's coefficient in the cluster package 1.14.3 (Maechler et al. 2013) in R. Gower's coefficient was selected as an appropriate measure for data that include both continuous and categorical variables. The matrix was analysed by NMDS in the MASS package 7.3.22 (Venables & Ripley 2002). Corresponding symbols and colouring were used to match these data to mtDNA lineages and categorical variables. A principal component analysis (PCA) of the 17 linear measurements was then constructed in the ade4 package 1.4-17 (Dray & Dufour 2007) in R. The relative contribution of measurements to differentiation among specimens of *R. australiae* was examined.

RESULTS

Phylogenetic trees

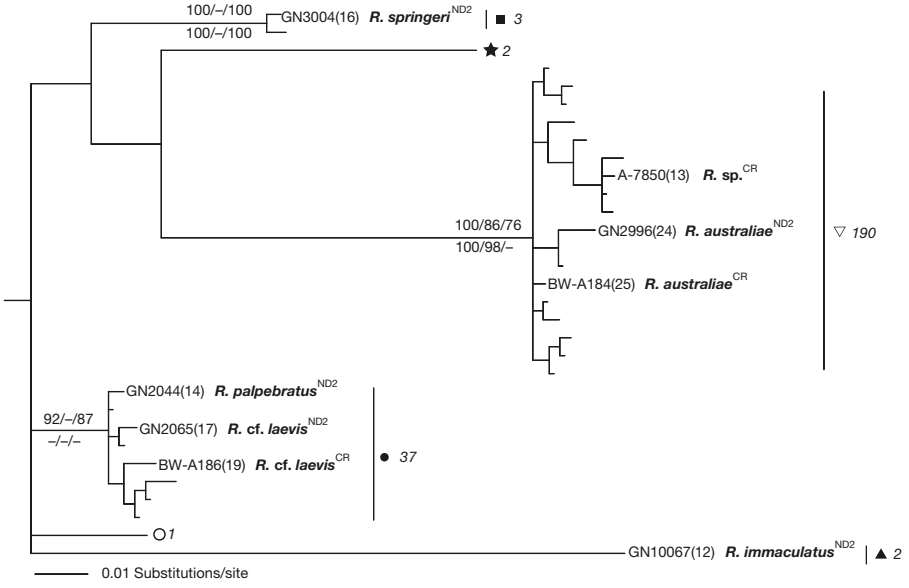
Taxon sets A–E resulted in 65, 31, 28, 25 and 31 unique haplotypes, respectively (E included 6 reference specimen haplotypes). Of the 10 resulting phylogenies, the topology of the Bayesian output for taxon set E is given (Fig. 2), with the results of all other analyses over 80% posterior probability (Bayesian) or bootstrap support (ML) reported at nodes. *nadh2* and CR datasets agreed on topology in isolation and combined. Observed genetic variation was consistent with 6 evolutionarily significant units (ESUs), with 1 ESU comprising 2 described species (Fig. 2). Novel haplotypes were submitted to GenBank with accession numbers KT879758 to KT879786.

Description and spatial distribution of sampled mtDNA lineages

In total, 94% of specimens sampled in the study area formed a single well-supported lineage, including *Rhynchobatus australiae* reference specimens GN2996/ANFC H 6221-01 (Naylor et al. 2012) and BW-A184 (Ward et al. 2005) and distinct from other species (open inverted triangle, Fig. 2). Sequence data from museum specimen MNHN A-7850 from India was also included in this lineage, last recorded as *R. djiddensis* following Séret & McEachran (1986) prior to the revisions of Compagno & Last (1999). The second most abundant lineage included reference specimens from 2 species, *R. palpebratus* (GN2044)

and *R.* cf. *laevis* (GN2065, BW-186) (Ward et al. 2005, Naylor et al. 2012) (closed circle, Fig. 2). A third well supported lineage included reference specimen *R. springeri* from Malaysia (GN3004/ANFC H 6221-02) and 2 specimens from Jakarta (solid square, Fig. 2). A single specimen from Taiwan was recorded from a species which was undescribed at the time of collection. This specimenaligned with sequence data from the newly described *R. immaculatus* (GN10067) (solid triangle, Fig. 2) known only from Taiwan. The specimen collected here was male and mature/maturing (claspers not fully calcified to calcified); at 1146 mm TL (CLO 10.73%), this is the largest specimen of this species reported to date.

Two remaining lineages were well differentiated from other species but did not align to any reference specimens; here, we refer to these taxa as *Rhynchobatus* sp. (Red Sea, Mozambique, symbolized by a solid star in Fig. 2), and *Rhynchobatus* sp. (Arabian Gulf, symbolized by a solid circle in Fig. 2). The *Rhynchobatus* sp. (Arabian Gulf) lineage was a single male specimen that was maturing (claspers not calcified to partially calcified) at 935 mm TL (CLO 7.17%), indicating a moderately sized to large species. The specimen from the Red Sea was male, 1400 mm TL (no maturity data available). These specimens seem likely to represent 2 individuals of *R. djiddensis, R. laevis* after Compagno & Last (2008), and/or *R.* cf. *djiddensis* after Moore et al. (2012). However, verified DNA reference material to identify these forms is currently lacking.



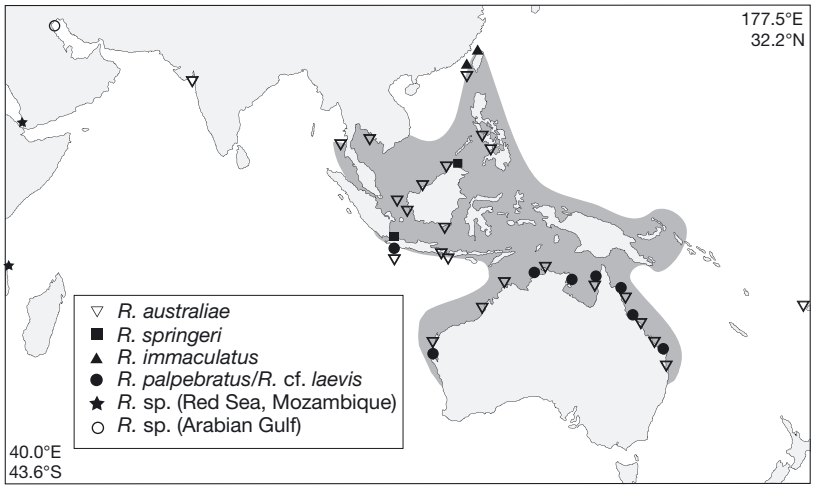


Fig. 2. Top panel: mtDNA phylogeny of unique haplotypes for 1500 bp of concatenated control region (CR, 456 bp) and *nadh2* (ND2, 1044 bp) for *Rhynchobatus* study specimens and reference sequences from vouchered specimens (taxon set E). Support for the major nodes are given for concatenated data/CR/ND2 datasets, repectively, for >80% Bayesian posterior probability (above the line), and >80% bootstrap support for ML (below the line). Symbols are used to identify genetic lineages in bottom panel. Sample sizes are given next to each lineage. Reference sequences are labeled as follows: specimenID (record number in Table S1 in the Supplement) **species** gene region (superscripted). Bottom panel: observed spatial distribution of mtDNA lineages

R. australiae was widespread (and the most abundant species recorded) throughout the core study area, from the Thai Andaman Sea to Indonesia and Taiwan and to the sub-tropics in Australia (Fig. 2). This species was recorded in all landing site surveys in the core area where any species of *Rhynchobatus* was present. In Southeast Asian surveys (excluding Jakarta), *R. australiae* accounted for 98% of specimens (n = 125); in Jakarta it accounted for 81% of specimens (n = 29), and in Australia 58% of specimens (n = 32). The lineage included samples from Fiji and India. MtDNA lineage *R. palpebratus/R.* cf. *laevis* was recorded in Jakarta (14%, n = 5) and throughout the waters of northern Australia (42%, n = 23). Relative contributions and spatial distributions of the 2 taxa represented by this lineage could not be calculated from this dataset.

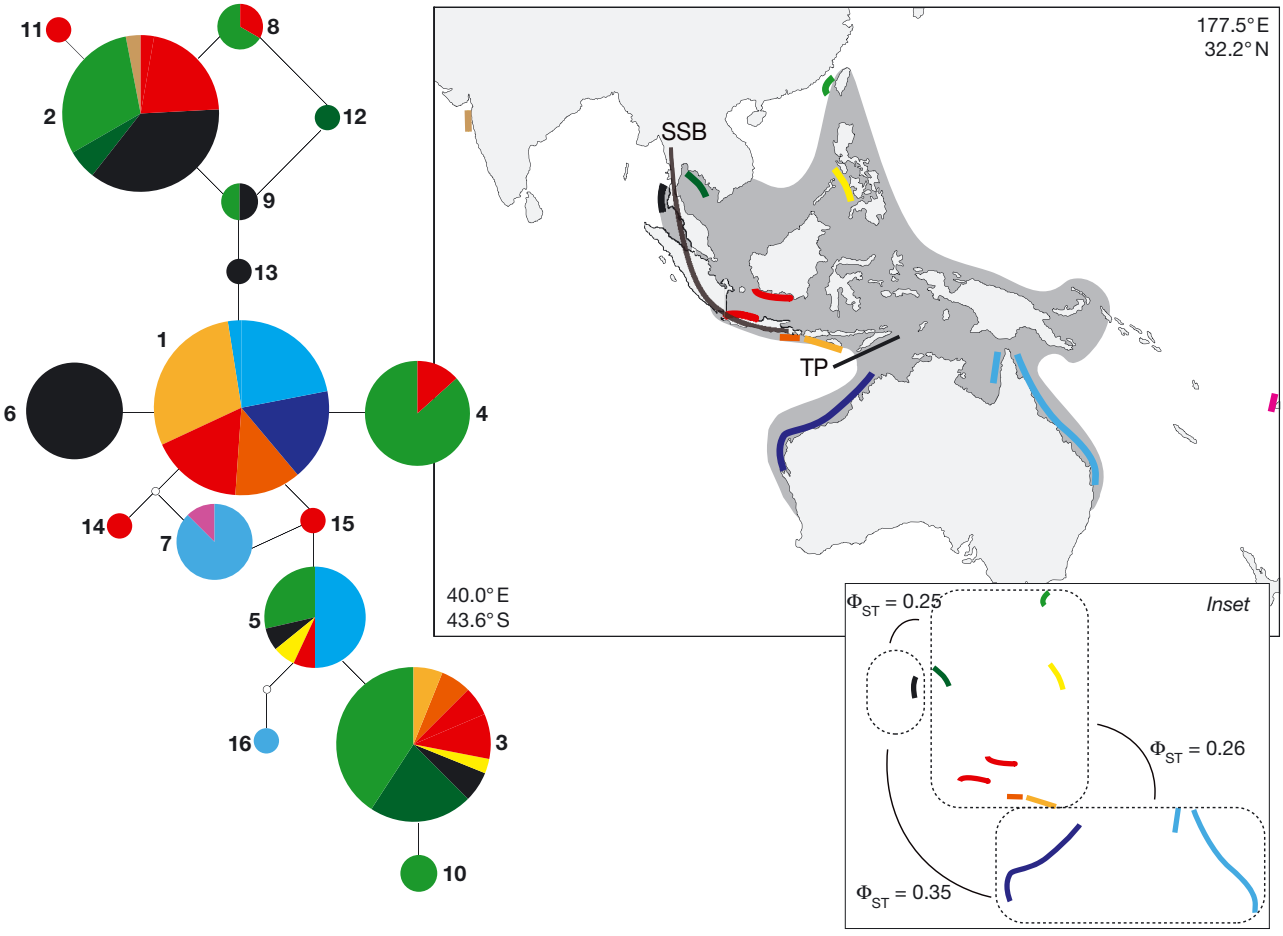


Fig. 3. Geographic distribution of control region haplotypes for *Rhynchobatus australiae* specimens (n = 169). Left: a parsimony network diagram indicates relationships among unique haplotypes. Each haplotype is represented by a circle proportional in size to the number of specimens with that haplotype (ID no. is given next to circle): the most common (haplotype 1), n = 41; and the least common (e.g. haplotype 16), n = 1. Connecting lines represent one base pair difference. Each circle is coloured to indicate the relative occurrence of each haplotype at sampling locations (map, upper right). The most recent published distribution of *R. australiae* is given in dark grey (Last et al. 2013). *A priori* biogeographic barriers used as a basis for testing genetic differentiation are given: Sunda Shelf Barrier (SSB) and the Indonesian Throughflow at the Timor Passage/Ombai Strait (TP, drawn extending from Timor Passage). Inset (lower right) gives AMOVA results for regions divided by these barriers (significant at $p < 0.00001$ after Holm-Bonferroni correction)

Genetic diversity and spatial differentiation in *R. australiae*

Sixteen control region haplotypes were identified for the 169 specimens (maximum sequence divergence of 2%), with moderate spatial structure (study-wide $\Phi_{ST} = 0.198$, $p < 0.0001$) (Fig. 3, Table 2). Haplotype 1, the most common haplotype recorded, was only encountered in Indonesia and Australia (n = 41) and included reference specimen BW-A184 (#25 in Table S1 in the Supplement, Fig. 1). Australian samples (n = 32) were restricted to 4 haplotypes (1, 5, 7, 16), and haplotype 1 was the only haplotype encountered in Australia west of the Torres Strait (n = 8). Haplotype 2 included specimens from the northwest portion of the sampled area—the Andaman Sea and Gulf of Thailand, Taiwan and Jakarta—and included reference specimen MNHN A-7850 from India (#13 in Table S1, Fig. 1). Haplotype 3 included specimens from across Southeast Asia, as well as the Andaman Sea. Haplotype 5 was encountered among specimens in Australia, Southeast Asia and the Andaman Sea. Haplotypes 6 and 13 were recorded only from the Andaman Sea. Haplotype 7 included specimens from the east coast of Australia and the single sample from Fiji. AMOVAs conducted to test *a priori* biogeographic barriers among pooled sampled populations divided by the Indonesian Throughflow at the Timor

Table 2. Summary metrics by (a) sampling locations and (b) biogeographic regions for control region mtDNA lineage *Rhynchobatus australiae*; number of specimens (n), number of haplotypes (H), haplotype diversity (*h*, mean ± SE) and nucleotide diversity (π, mean ± SE). Regions in (b) are divided by major *a priori* biogeographic barriers. –: sample sizes were insufficient to calculate these metrics

(a) Sampling location		n	H	*h*	π
Thailand: Andaman		30	6	0.667 ± 0.055	0.0056 ± 0.0035
Thailand: Gulf		10	3	0.511 ± 0.164	0.0057 ± 0.0037
Indonesia: Bali		7	2	0.476 ± 0.171	0.0031 ± 0.0025
Indonesia: Lombok		14	2	0.264 ± 0.136	0.0017 ± 0.0015
Indonesia: Jakarta		15	7	0.781 ± 0.102	0.0049 ± 0.0032
Indonesia: Kalimantan		12	4	0.636 ± 0.128	0.0063 ± 0.0040
Taiwan		45	7	0.789 ± 0.029	0.0071 ± 0.0041
Philippines		2	2	–	–
Australia: west/north coast		7	1	–	–
Australia: east coast		24	4	0.717 ± 0.039	0.0034 ± 0.0024
Fiji		1	1	–	–
(b) Biogeographic region	Description	n	H	*h*	π
Thailand: Andaman	West of Sunda Shelf Barrier	30	6	0.667 ± 0.055	0.0056 ± 0.0035
Southeast Asia	East of Sunda Shelf Barrier, northwest of Timor Passage	105	12	0.812 ± 0.017	0.0063 ± 0.0037
Australia	Southeast of Timor Passage	32	4	0.641 ± 0.062	0.0031 ± 0.0021

Passage/Ombai Strait and Sunda Shelf barrier (Table 2) indicated significant genetic differentiation across these features (Andaman Sea–Southeast Asia Φ_{ST} = 0.249, p < 0.00001; Southeast Asia–Australia Φ_{ST} = 0.260, p < 0.00001) (Fig. 3). Haplotype and nucleotide diversity were moderate both overall (*h* = 0.848 ± 0.012, π = 0.0061 ± 0.0036; mean ± SE) and for each biogeographic region (Table 2). Lower values in individual sites are likely to be biased by low sample sizes at this scale. In the most extreme case, only 1 haplotype was recorded in the 6 specimens from Western Australia. Tajima's *D* values did not deviate significantly from expectations under neutral equilibrium conditions.

Phenotypic variation in *R. australiae*

An NMDS of linear measurements showed differentiation of *R. australiae* from other species across all size categories. The spread of specimens in the plot was consistent with growth allometry (Fig. 4). The maximum length recorded in males was considerably smaller (1663 mm TL) than for females (2428 mm TL). The

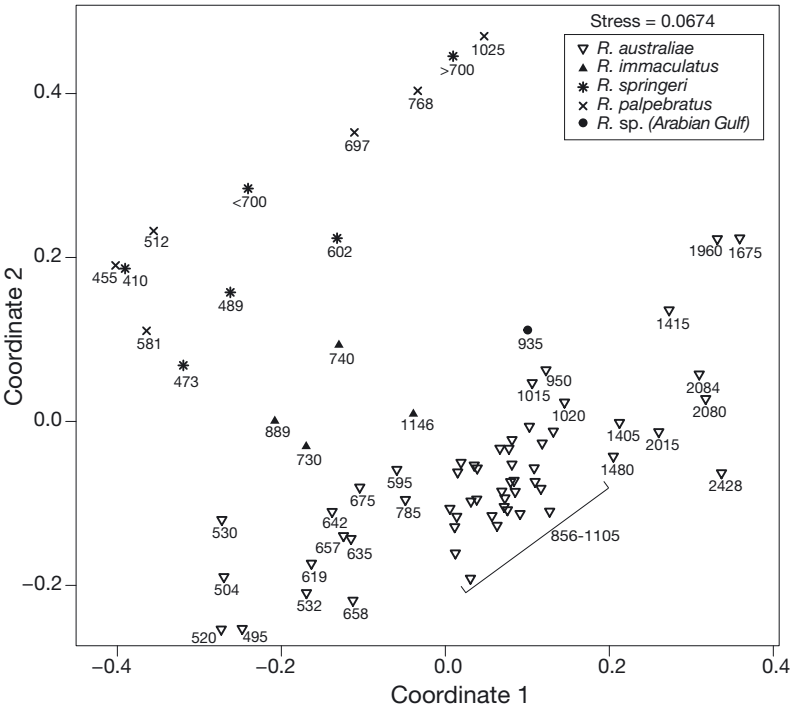
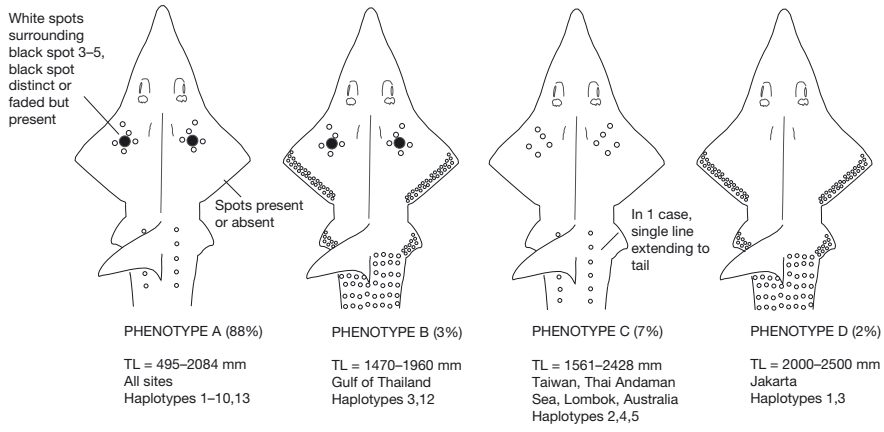


Fig. 4. Non-metric multi-dimensional scaling ordination of linear measurements of body plan in study specimens of *Rhynchobatus australiae* (n = 57), *R. immaculatus* (n = 1) and *Rhynchobatus* sp. (Arabian Gulf) (n = 1), and corresponding measurements for the holotype and 2 paratypes of *R. immaculatus* (Last et al. 2013), the holotype and 5 paratypes of *R. palpebratus* (Compagno & Last 2008), and the holotype and means of 20 paratypes <700 mm TL and 5 paratypes >700 mm TL of *R. springeri* (Compagno & Last 2010), and 3 additional *R. springeri* paratypes measured for this study. Total length (TL, in mm) is given for each specimen



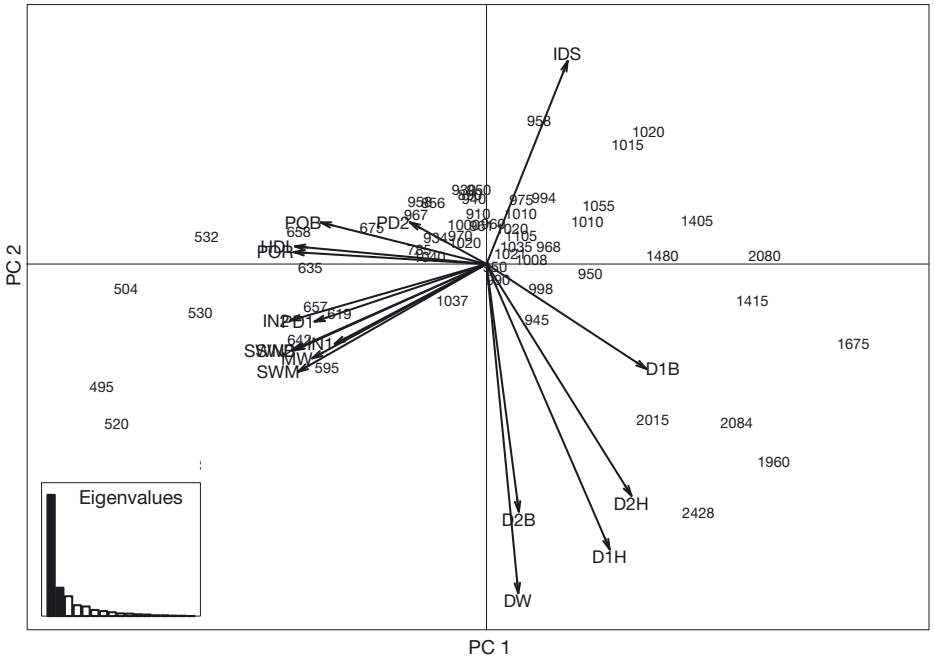


Fig. 5. Phenotypic variation in *Rhynchobatus australiae* (top): observed combinations of categorical phenotypic traits in study specimens (n = 100) overlaid on 1 standard idealised body shape. Principal component analysis biplot (bottom) of 17 linear measurements (n = 57), each specimen represented by their total length in mm. Phenotypic variables are defined in Fig. S1 (see the Supplement at www.int-res.com/articles/suppl/m548p165_supp.pdf). Plotted eigenvalues (inset) show relative proportion of variance explained by axes PC 1 and PC 2, respectively (black bars)

largest specimens recorded in this study had their dorsal and caudal fins removed at sea, suggesting larger maximum lengths estimated at ~1900 mm TL (largest male) and >2500 mm TL (largest female).

A PCA biplot of PC1 vs PC2 indicated an allometric shift in the relationships of the linear measurements (i.e. body shape) with increasing size (Fig. 5, Fig. S1). The loadings of the various input measurements on the 2 main principal components were assessed to determine their relative influence. Head length (HDL), pre-oral length (POR), pre-orbital length (POB), and oronasal features (SWB, MW, IN_P) expressed as relative lengths (% of total length) decreased with increasing total length (TL), while relative disc width (DW) and dorsal fin heights (D1H, D2H) and base lengths (D1B, D2B) increased with increasing total length (Figs. 5 & 6a). Allometric scaling trends reflected power function relationships as expected by biological theory (Zelditch et al. 2012); however, equations were not fitted owing to insufficient sampling of large specimens. All relative measurements of the head, snout and predorsal length decreased until maturity (as inferred) (e.g. pre-oral length, Fig. 6a). Relative dorsal fin base lengths and heights followed curvilinear to linear relationships with increasing total length (e.g. first dorsal fin base, Fig. 6b), and relative disc width was relatively stable until maturity, then increased steadily (Fig. 6c). The interdorsal space (IDS, distance between first dorsal fin insertion and second dorsal fin origin) showed no obvious trend with size. Most male specimens recorded in this study were juvenile or maturing (Fig. 6d). Outer clasper lengths plotted against total lengths indicated males were mature in *R. australiae* by approximately 1400 mm TL (Fig. 6d), but too few data were available for larger males to fit a predictive logistic equation.

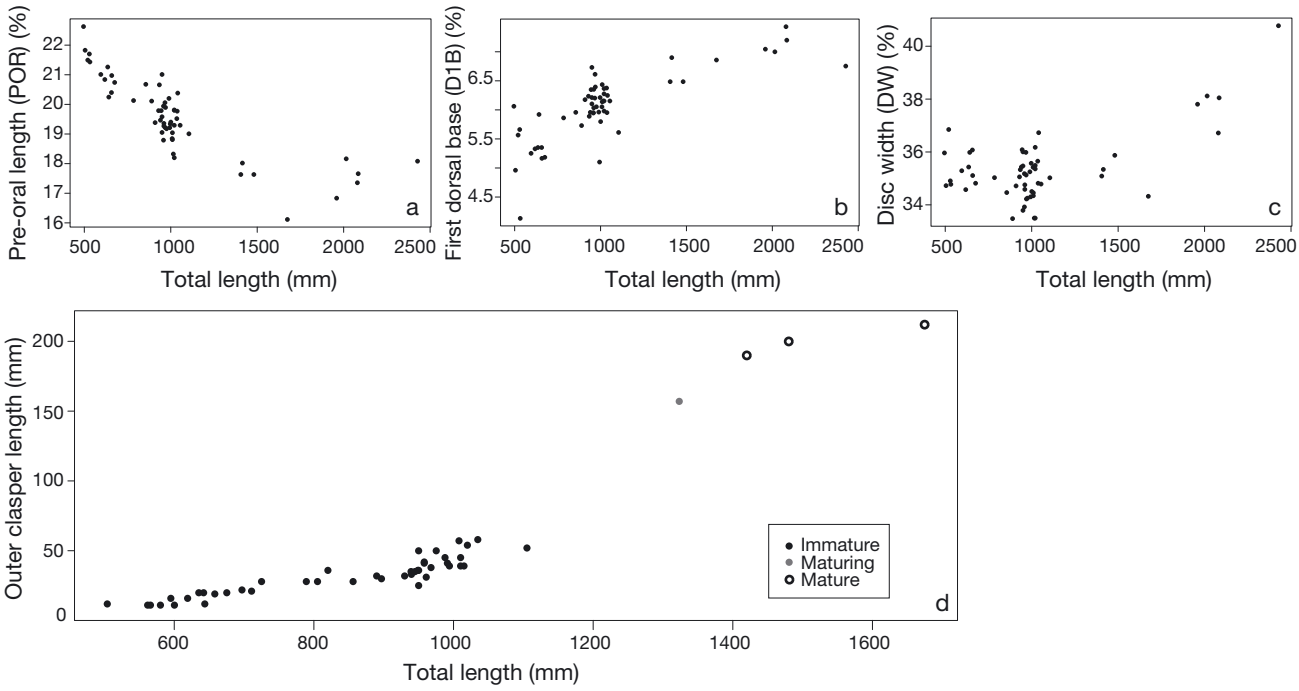


Fig. 6. (a,b,c) Major allometric trends in linear measurements for *Rhynchobatus australiae* from Southeast Asian sites (n = 57): (a) pre-oral length (POR), (b) first dorsal fin base length (D1B), (c) disc width (DW). Each measurement is represented as a percentage of total length (TL) (*y*-axis) versus TL (*x*-axis). (d) Outer clasper length versus TL in male specimens of *R. australiae* from Southeast Asian sites (n = 48), with claspers not calcified (immature), not fully calcified (maturing) and fully calcified (mature)

All specimens in the *R. australiae* lineage for which morphological information were available had a bottle-shaped snout (plain to weakly bicolour), with the first dorsal fin originating approximately over the pelvic fin origins (n = 100). The remaining 5 categorical traits describing dorsal patterning were variable, and are summarised as phenotypes A–D (Fig. 5). A well demarcated black pectoral spot was present in all specimens up to 1405 mm TL; above this size the spot was either faded (up to 2080 mm TL), or absent/not visible (1561–2500 mm TL). Between 3 and 5 (usually 4) white pectoral spots (4 spots in 63% of specimens) surrounded the black pectoral spot or its usual position. A line of 3 white pectoral spots adjacent to the black spot was observed in all but 2 specimens >2000 mm (phenotype D).

The posterior dorsal surface toward the caudal fin was typically plain, with a row of well-demarcated white spots along the mid-body to just behind the free rear tip of the first dorsal fin. In specimens <1500 mm TL, a single faint line of smaller spots sometimes extended down the posterior dorsal surface to the caudal fin. Pectoral and pelvic margins ranged from plain to with many rows of spots. In specimens >1500 mm TL, many had numerous rows of white spots on the posterior surface from the first dorsal fin to the caudal fin, co-occurring with many rows of spots along the pectoral fin margins, and typically along the pelvic fin posterior margins. Large individuals were also observed with a plain posterior dorsal surface and plain pectoral and pelvic margins, and with a single row of well-demarcated spots extending to the tail. There was substantial variation among individuals in additional small spots on the dorsal surface of the head and forward of the first dorsal fin. Dorsal colouration ranged from yellow-brown and dark brown to grey (examples are shown in Fig. 7).

DISCUSSION

By undertaking a large-scale survey of *Rhynchobatus* landings across the central Indo-West Pacific and reconciling species identity against verified reference specimens for the genus following the most recent nomenclature, this study provides insight into a number of areas that have hindered species-specific conservation assessment and management in this threatened group of shark-like rays. Firstly, we show that *Rhynchobatus australiae* is the most commonly caught *Rhynchobatus* species in Southeast Asia, and one of the most, if not the most, commonly caught species in the genus throughout north-

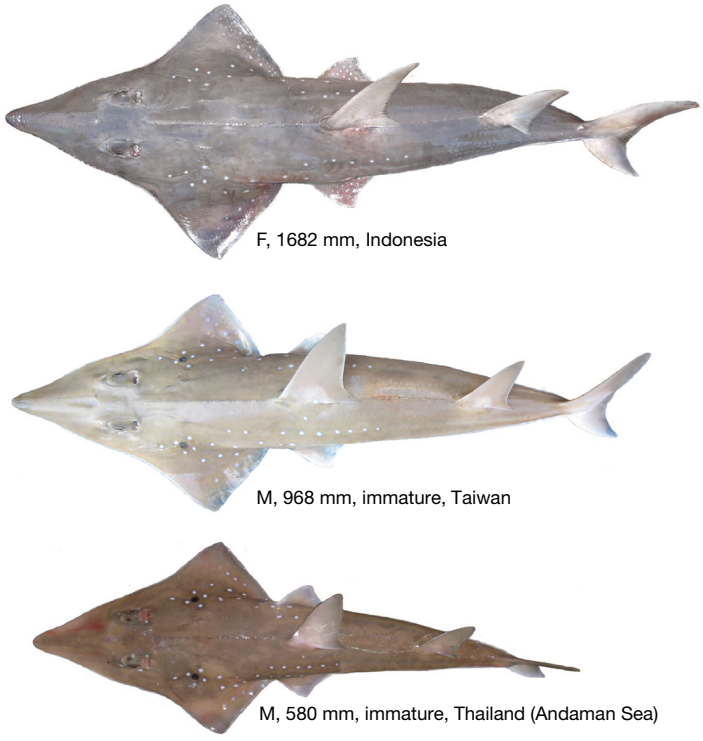


Fig. 7. Examples of colour variation in *Rhynchobatus australiae* for the most common combination of phenotypic variables in 3 specimens of increasing size (phenotype A, bottom and middle; phenotype C, top)

ern Australia. Secondly, in correlating study specimens with reference data using a number of markers, we provide the most current and validated resource for species identification of *Rhynchobatus* tissue using mitochondrial sequences. For *R. australiae*, we contribute detailed information on genetic differentiation, to assist in understanding demographic connectivity, and on phenotypic variation, to assist in identifying fixed external characters that differentiate this species from others. Below, we discuss our findings on *R. australiae* and the implications and utility of this study for fisheries management and the monitoring of fins in trade.

Spatial distribution and relative abundance of *R. australiae*

Our data suggest that *R. australiae* is the only widespread *Rhynchobatus* species throughout the central Indo-West Pacific and evidently occurs more widely in the Indo-West Pacific than described elsewhere. *R. australiae* dominated recorded *Rhynchobatus* catch in Southeast Asian surveys (94%), and accounted for all but one specimen (*R. immaculatus*) recorded north of the Indo-Malay archipelago. In Australian waters, *R. australiae* accounted for approximately half of the total specimens recorded (58%), recorded in sympatry alongside the mtDNA lineage *R. palpebratus*/ *R.* cf. *laevis* to the sub-tropics on both east and west coasts (Fig. 2).

Although the core study area spans the species' range as last reported (Last et al. 2013) from the Thai Andaman Sea (west) and Taiwan (north) through Indonesia to Australia (south), additional samples in this study from as far east as Fiji (Table 1), and as far west as India (#13 in Table S1 in the Supplement) were also identified as *R. australiae*. Specimens recently recorded in fisheries landings from the Arabian Sea (Bineesh et al. 2014) (*R. australiae* e.g. GenBank accession number JN108018) and the Red Sea (Spaet & Berumen 2015) (*Rhynchobatus* sp. 2 GenBank accession number KM396927) were both consistent with *R. australiae* in this study based on *coi* and *nadh2* reference data, respectively (Table S1). These findings suggest that *R. australiae* distribution extends into the west Pacific and well into the western Indian Ocean. The distributional limits of this species and regions of sympatry with other large *Rhynchobatus* species in the Indian Ocean are in need of further investigation.

Genetic diversity and spatial differentiation in *R. australiae*

Moderate mtDNA differentiation was observed across *R. australiae*'s range (study-wide Φ_{ST} = 0.198, $p < 0.0001$), and patterns of differentiation were not suggestive of range-wide connectivity among maternal stocks (Fig. 3). Significant subdivision was observed over the 2 *a priori* biogeographic barriers tested, indicating contemporary or historical restriction of gene flow at these sites. The degree of observed matrilineal subdivision over the Timor Passage/Ombai Strait and adjacent deep waters however suggests that this feature constitutes less of a barrier to dispersal than for some other elasmobranch species with strong shelf-associated behaviour (reviewed in Dudgeon et al. 2012, e.g. Giles et al. 2014). This is somewhat surprising given that large *Rhynchobatus* species in Australia are known to have benthopelagic habits in shallow inshore waters, not exceeding approximately 60 m depth (Last & Stevens 2009). A passive acoustic study of *Rhynchobatus* spp. in a northern Australian embayment reported fre-

quent but variable and apparently size-independent use of very shallow inshore habitat (<10 m deep), not consistent with synchronous philopatric behaviour (White et al. 2014b). Little is known of movement and total habitat use in this genus, and our result for *R. australiae* may reflect episodic migration between Australia and Indonesia (e.g. Whitney et al. 2012).

The observed differentiation between the Andaman Sea and Southeast Asian specimens may represent either a lingering signature of historical vicariance over the Sunda Shelf or reduced contemporary connectivity between peninsular Malaysia and the Indonesian islands dividing Southeast Asia and the northern Indian Ocean. Additional nuclear DNA evidence and spatial habitat use and life-history studies would aid demographic interpretation of the observed maternal phylogeographic patterns in *R. australiae*. Overall and within-region genetic diversity was moderate when considered within the range reported for elasmobranch species (see Hoelzel et al. 2006). Levels of nucleotide diversity in Australia (n = 32) were lower than in other sampled regions, and comparable with those reported for *Pristis zijsron* across an analogous study area in these waters (n = 49) (Phillips et al. 2011), and *Carcharias taurus* in South Africa (n = 26) (Stow et al. 2006).

Phenotypic variation in *R. australiae*

Intraspecific variability in dorsal patterning and body shape was documented to assist ongoing efforts to describe fixed external characters that differentiate *R. australiae* from other species. Females were recorded to reach at least 2428 mm, and males at least 1663 mm in length (TL). The majority of observed variation in body plan (Fig. 4) and dorsal patterning (Fig. 5) was explained by specimen size and showed no correlation to spatial mtDNA differentiation (Figs. 3 & 6). As specimens matured there was relative shortening of longitudinal and lateral head features and a relative increase in dorsal fin bases and heights. Mature specimens had a comparatively shorter head and snout, narrower snout, wider body and taller dorsal fins than immature specimens. Among mature specimens, larger animals were increasingly wide for their length and had increasingly tall fins for their base lengths (Figs. 5 & 6).

A line of 3 white spots was consistently located adjacent to the black pectoral spot (or its usual position if absent), as described for *R. australiae* in Compagno & Last (2010), and was observed in all specimens except 2 large females. These 2 specimens were not freshly captured, and it is possible that this feature was present in life but no longer visible. All specimens under 1500 mm TL had a single black pectoral spot surrounded by 3–5 (usually 4) white spots, a short line of well-demarcated white spots on the mid dorsal surface terminating slightly behind the first dorsal fin free rear tip, no spots on the tail (plain), and pectoral and pelvic fin margins typically plain (phenotype A, examples in Fig. 7). This phenotype was found in all study sites for which morphological data were available and was the most common phenotype, reflecting that most surveyed specimens were small. In specimens larger than 1500 mm TL, the black pectoral spot was often faded or absent, and/or with many rows of white spots on the dorsal surface toward the tail and the margins of the pectoral and pelvic fins. This variation in dorsal patterning with size may reflect differentiated habitat use with developmental stage. Large *Rhynchobatus* specimens are known to occur over sandy bottoms (e.g. White et al. 2014b), and low contrast may offer a camouflage advantage. A greater number of white spots on the tail of large adults compared to smaller individuals has also been observed in *R. springeri* (Compagno & Last 2010).

Implications for fisheries management

We find that *R. australiae* is likely to comprise the largest component of *Rhynchobatus* catch originating from the central Indo-West Pacific. In addition, recent fisheries records contributing mtDNA sequences from India and the Red Sea suggest that this species is also a component of the high intensity coastal fisheries in the western Indian Ocean (Bineesh et al. 2014, Spaet & Berumen 2015). As patterns of genetic differentiation in *R. australiae* did not provide evidence for substantial demographic connectivity among Australia, Southeast Asia and the Andaman Sea, separate conservation assessment and management of the species in each of these regions may be appropriate. In light of the high levels of capture occurring in Southeast Asia and the northern Indian Ocean, separate assessment of Southeast Asian/Andaman stocks and research into the extent and relative catch abundance of populations west of the currently described range of this species are recommended priorities.

In Southeast Asia, other *Rhynchobatus* species are apparently rare in landings and have more restricted and possibly fragmented spatial distributions. Species-specific assessment and management may be urgently

needed to ensure that populations of these endemic species remain viable. Our findings suggest that in Southeast Asian landings, *R. australiae* may be differentiated from these other species on the basis of simple external features, most simply the bottle-shaped snout (plain to weakly bicolour in juveniles). Although the presence of other rare or undescribed species cannot be ruled out, *R. australiae*'s bottle-shaped snout differentiates this species from smaller sympatric species known from these waters — *R. springeri*, *R. immaculatus* and *R. palpebratus* — which each have a bicolour wedge-shaped snout (broad to narrowly pointed).

In Australian waters, uncertainty over the status of provisional *R.* cf. *laevis*, its external differentiation from *R. australiae,* its molecular differentiation from *R. palpebratus,* and the relative abundance of each species has hindered species identification and subsequent assessment of the demographic impact of current catch rates on the different sympatric species (e.g. White et al. 2013, 2014). Unfortunately, owing to the lack of accompanying images for Australian samples, our study offers just a small clarification to this issue. Our results show substantial catch of *R. australiae*, and unknown relative contributions from *R. palpebratus* and provisional *R.* cf. *laevis.* The samples obtained for this study are also just a snapshot of Australian fishery compositions, and further fishery composition, life history and ecological studies at the species level across northern Australian waters are much needed.

Implications for fin trade monitoring

Given its prevalence in *Rhynchobatus* catch in the active fishing region of the central Indo-Pacific, demonstrated extension into western Indian Ocean fisheries, and large attainable fin sizes, *R. australiae* may well account for a substantial component of fins from this genus in global trade. While intact dorsals and caudal fins are readily recognisable as *Rhynchobatus* on the basis of morphology (e.g. Marshall 2011), fixed species-diagnostic morphological traits are yet to be established, largely because of a lack of genus-wide reference material. As subsequent processing of fins for consumer markets obscures morphological characters, a molecular approach is valuable for discriminating among *Rhynchobatus* species in shark fin trade consignments. This study establishes the viability of using partial control region sequences to differentiate *R. australiae* fins from those of all other described and provisional species for which DNA reference material is available to date (456 bp). These include the 4 described and 1 provisional species from the central Indo-West Pacific, and 2 ESUs from the Indian Ocean likely to account for 2 individuals of *R. laevis*, *R. djiddensis* or an undescribed species. The marker is short enough to amplify material from processed fins under trade conditions, and a single primer pair amplified all tested *Rhynchobatus* species.

We provide the largest synthesis so far of sequence data for this genus, and our study is the first to bring together and standardise available reference material from multiple sources. However, as verified DNA reference sequences are not yet available for all global species in the genus, context-specific caution should be used in the use of currently described mtDNA markers as a diagnostic tool for species-level identification. We recommend that in forensic applications, *Rhynchobatus* tissue be assigned to species level only where the available scope of comparative material can be justified for that specific location and application, or where morphological data are available to narrow the diagnostic question. As in prior studies using *coi* and *nadh2*, *R. palpebratus* and provisional species *R.* cf. *laevis* could not be differentiated using the control region, and identifications of *R. palpebratus* using these markers should be treated with caution until this taxonomic question has been resolved. It may be that whole mtDNA genome sequencing can detect regions of differentiation between these taxa that are not captured by the markers described to date (e.g. Feutry et al. 2014). Determining the incidence of *Rhynchobatus* spp. in shark fin imports, exports and domestic seizures will provide a valuable perspective for assessing the suitability of trade regulation in this threatened group of rays, and insight on capture independent of specific fisheries.

Lastly, *Rhynchobatus* species are among a number of threatened rays with dorsal and caudal fins that fetch high prices in the global shark fin trade, including the Endangered and Critically Endangered sawfishes, the only elasmobranchs regulated in international trade under CITES Appendix I. In some jurisdictions, however, recent shark fin trade regulations exclude rays in the taxonomic definition of shark fin, and therefore do not apply to the fins originating from these species. In the interest of accurately representing the taxonomic scope of the fin trade and those species impacted by it, definitions of the trade commodity shark fin should include the dorsal and caudal fins of shark-like batoids in families Rhynchobatidae, Pristidae, Rhinidae and Rhinobatidae.

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