Aquatic Conservation: Marine and Freshwater Ecosystems

WILEY

ARTICLE OPEN ACCESS

Unwedging the Secrets: Species and Genetic Diversity of Wedgefishes (Rhinidae) in Malaysian Waters

Amy Yee-Hui Then[1,2] | Kean Chong Lim[3] | Amanda Jhu-Xhin Leung[3] | Oscar Puebla[2,4] | Martin Helmkampf[2] | Serena Adam[5]

[1]Faculty of Science, Universiti Malaya, Institute of Biological Sciences, Kuala Lumpur, Malaysia | [2]Leibniz Centre for Tropical Marine Research (ZMT), Bremen, Germany | [3]Institute of Ocean and Earth Sciences, Universiti Malaya, Kuala Lumpur, Malaysia | [4]Institute for Chemistry and Biology of the Marine Environment (ICBM), Carl von Ossietzky Universität Oldenburg, Oldenburg, Germany | [5]World Wide Fund for Nature Malaysia, Petaling Jaya, Selangor, Malaysia

Correspondence: Amy Yee-Hui Then (amy_then@um.edu.my)

Received: 6 August 2024 | **Revised:** 3 February 2025 | **Accepted:** 12 February 2025

Funding: This study was supported by Save Our Seas Foundation Small Grant (IF033-2021/leun_smallgrant2021), WWF-Malaysia fund PV022-2022/WWF-MY/FY22/GEN/019 and Universiti Malaya Research Programme Grant RP018C-16SUSC. Part of AT's time for the manuscript writing was supported by the NAM ZMT Fellowship 2023.

Keywords: conservation | genetic structure | Indo-West Pacific | Rhinopristiformes | *Rhynchobatus*

ABSTRACT

The wedgefishes (Rhinidae) stand out as one of the most endangered marine lineages due to the conjunction of shallow coastal habitats, slow growth, low fecundity and high fishing pressure. The Indo-West Pacific region, including Malaysia, is of particular interest as it hosts a large share of the wedgefish diversity. Here, we shed light on the species and genetic diversity of wedgefishes found in Malaysian waters. A total of 85 *Rhynchobatus* samples were collected at 15 locations over 7 years across Malaysia and were identified using both morphological characters and genetics. We highlight the difficulty in identifying species based on morphological characters alone, and that molecular confirmation was needed for ambiguous specimens. *Rhynchobatus australiae*, broadly distributed across coastal Malaysia, represented a large fraction of the samples (87%), followed by *R. palpebratus* (11%) and a single *R. springeri* individual. The occurrence of *R. palpebratus* is a new record for this species in Malaysia and indicates a wider distribution than previously thought. Population genetic analysis within *R. australiae* revealed fine-scale structuring within the Strait of Malacca, notwithstanding the fact that this is a relatively small and shallow waterway that does not obviously hinder the movement of *R. australiae* along the coast. These results reinforce the importance to manage and protect these species and populations in Malaysian waters.

1 | Introduction

The wedgefishes, a group of medium to large-sized dorsoventrally flattened rays from the family Rhinidae, has been highlighted as an emerging group of conservation urgency, due to their uplisted 'most endangered' status alongside giant guitarfishes (family Glaucostegidae) and sawfishes (family Pristidae). The primary threat to the wedgefishes is overfishing that has resulted in population declines exceeding 80% over the course of the last three generations (Kyne et al. 2020). For this reason, the latest IUCN assessments for wedgefishes has conferred the status of Critically Endangered to all valid wedgefish species, with the exception of *Rhynchobatus palpebratus* due to its primary distribution within Australia where fishing pressure is low and proper fisheries management is implemented (Kyne and Rigby 2019). Given their generally restricted distribution (Koeda et al. 2021), association with shallow warm coastal waters that host intense demersal fishing pressure, and intrinsic

biological characteristics such as slow growth and low fecundity (Kyne et al. 2020), there is a pressing need for research to inform conservation-based management plans for the wedgefishes.

The Indo-Pacific region is known as the centre of diversity of wedgefishes (Giles et al. 2016), and a few of the Indo-Pacific countries, including Malaysia, have been flagged over concerns of intense fishing pressures driven by the high demands for their fins, meat, and increasingly for the gelatinous filling in the snout (Choy et al. 2022). The taxonomic status of wedgefishes has undergone considerable revisions over the past two decades with recent species descriptions in the Indo-Pacific waters, e.g. *R. palpebratus* in 2008 (Compagno and Last 2008), *R. springeri* in 2010 (Compagno and Last 2010), *R. immaculatus* in 2013 (Last, Ho, and Chen 2013), *R. cooki* in 2016 (Last, Kyne, and Compagno 2016), and *R. mononoke* in 2021 (Koeda et al. 2021), and revision of the family placement within the order Rhinopristiformes (Kyne et al. 2020).

Among the three genera within the Rhinidae family, *Rhynchobatus* is the most speciose [eight valid species, Koeda et al. 2021], followed by the monotypic *Rhina* (*R. ancylostomus*) and *Rhynchorhina* (*R. mauritaniensis*). Of the eight *Rhynchobatus* species, three have been reported to occur in Malaysian waters, especially in the Malaysian Borneo states of Sabah and Sarawak, where much of the local research on sharks and rays has been concentrated. *Rhynchobatus australiae* has been reported in Peninsular Malaysia, Sarawak, and Sabah (Last et al. 2010; Md-Zain et al. 2018; Booth et al. 2021), *R. springeri* in Sarawak (Last et al. 2010; Booth et al. 2021), and *R. laevis* in northeastern Sabah and Sarawak (Last et al. 2010; see also supplementary information in Booth et al. 2021). *Rhynchobatus djiddensis* was also recorded as another species in Malaysia based on earlier literature in the mid-1970s (Department of Fisheries Malaysia 2006). This record is however likely to be erroneous since Indo-Pacific wedgefishes were all thought to be a single wide-ranging species (*R. djiddensis*) until the late 1990s (Giles et al. 2016). It is now established that *R. djiddensis* is restricted to the Western Indian Ocean (Last et al. 2016).

The bottlenose wedgefish *R. australiae* is one of the largest and most widespread wedgefish species across the Indo-West Pacific region (Kyne et al. 2019), but clarification of the taxonomic ambiguity in the whitespotted wedgefish species complex, which includes *R. australiae*, will likely result in updated distributional information (Kyne et al. 2019; Groeneveld et al. 2023). In an earlier study conducted across its known range at that time within the Indo-West Pacific region, *R. australiae* was shown to dominate local wedgefish catch composition and exhibited moderate spatial structure at the NADH dehydrogenase subunit 2 (ND2) mitochondrial DNA marker (Giles et al. 2016). The same study showed significant biogeographic barriers for the species across the Andaman Sea, Southeast Asia, and Australia, but was largely missing Malaysian specimens. Similar conclusions were derived from another study using a different marker (COI), revealing three genetically distinct stocks in the Western Indian Ocean, Western Pacific, and Australian regions (Simwanza and Rumisha 2023).

The broadnose wedgefish, *R. springeri*, is also part of the whitespotted wedgefish species complex but has a narrower distribution compared to that of *R. australiae* based on the latest IUCN assessment (Kyne 2019). The Borneo region is known as one of the type localities for *R. springeri* (Last et al. 2010). Booth et al. (2021) did not report exact locations for the 43 records of *R. springeri* mentioned in their study, but they were also likely caught in the vicinity of southern Sarawak. The latest IUCN distributional map of the species includes the broader east and west coasts of Peninsular Malaysia (Kyne 2019). It was noted that this species may be a '*habitat specialist occupying shallow brackish coastal and estuarine waters*' as opposed to other congeners occupying open sea regions (Compagno and Last 2010).

The smoothnose wedgefish, *R. laevis*, had been recorded in northeastern Sabah (Last et al. 2010). This species was originally described from India, although the holotype specimen is considered to be lost (Paepke and Schmidt (1988) in Koeda et al. (2021)). Upon re-assessment, *R. laevis* was shown to be distributed widely across the contiguous coastline of the Arabian Sea and the Bay of Bengal, and off coastal mainland China and Japan in the Western Pacific, but absent from the Southeast Asian region (Last et al. 2016). The latest IUCN assessment highlighted that *R. laevis* is part of the whitespotted wedgefish species complex that includes *R. australiae* and *R. djiddensis* (Kyne and Jabado 2019). The species complex description was likely based on molecular data, but the details are unclear. It was noted that some wedgefishes from Australia that were provisionally identified as *R. laevis* showed morphological variations that require additional investigation, but were nonetheless found to be morphologically distinct from an Indo-West Pacific wedgefish, *R. palpebratus* (Compagno and Last 2008).

In addition to the three known valid species records for Malaysia, there is likely another species awaiting description, currently known as *Rhynchobatus* sp. 2 (Compagno and Last 1999) and found in Sarawak, Singapore, and Java. The eyebrow wedgefish *R. palpebratus*, described on the basis of specimens from northern Australia and the Andaman Sea, may also occur more widely within the Indo-Malaya region. These recent species descriptions and re-descriptions are primarily based on external morphological features and counts of vertebral centra, as well as molecular insights in some cases. However, morphological similarities coupled with the lack of definitive diagnostic features among congeners continue to impede accurate species identification in the group (see Compagno and Last 2010; Giles et al. 2016; Kyne et al. 2020).

To date, the information on *Rhynchobatus* species in Malaysia is rather limited, primarily based on short-term surveys of fisheries landings and markets, sometimes complemented with genetic analysis but using poorly informative markers. None of the wedgefish species are currently protected within the country, although it sits at the heart of the wedgefish distribution. The lack of decisive protection for the taxon is in part due to insufficient local research. Considerable research gaps remain regarding species delineation in this taxon and their broader Indo-Pacific distribution. Given the dynamic taxonomic updates and the prevailing species identification challenges for wedgefishes, the objectives of this paper were to (1) clarify species record status and distribution of wedgefishes in Malaysia using both morphological characters and genetic data, (2) investigate genetic variation within and phylogenetic relationships

between species of the genus *Rhynchobatus* represented in this study, and (3) assess the population genetic structure of *R. australiae* within Malaysia. The findings from this paper will serve as important baseline to shape short-term fisheries management interventions and long-term conservation priorities for the endangered wedgefishes.

2 | Material and Methods

2.1 | Sampling

Field surveys were conducted at various landing sites and markets along the coastal area covering all three major water bodies in Malaysia between September 2015 to February 2023 (Figure 1). Encountered *Rhynchobatus* specimens were identified on site to species level (when possible) based on morphological characters following Compagno and Last (2008, 2010), Last et al. (2010), and Jabado (2019). Particular attention was paid to the three *Rhynchobatus* species known to occur in Malaysia, i.e. *R. australiae*, *R. springeri*, and *R. laevis*, as well as other species within the whitespotted wedgefish species complex, especially *R. palpebratus* that had previously been recorded in Australia and Thailand (Compagno and Last 2008). Based on existing descriptions, these species are distinguishable by the following features: *R. australiae*—diagonal row of three equidistant white spots above black pectoral fin markings; *R. laevis*—three white spots surrounding black pectoral fin markings with no black spot around eyes (Last et al. 2010); *R. springeri*—dark marking on and/or behind eyes, white spots covering most of the trunk (extending towards the edge of the pectoral fin) and tail (Compagno and Last 2010); and *R. palpebratus*—dorsal surface of each eyeball with two curved black bars, white spots on dorsal surface sparsely located from post pectoral-fin insertion to approximately the free rear tip of the first dorsal fin, and continuing in the form of pale faint line (Compagno and Last 2008). Dorsal view photographs (whole specimen and snout to trunk) were taken for reference and identification verification, and fin clips were collected and preserved in absolute ethanol for subsequent molecular analysis. External morphological characteristics that were visible from photographs of the animal's dorsal surface, including presence or absence of 'eyebrow'-like marking and black spots around eye region, pattern of white spots around black pectoral fin spot, and number of white spots by section (straight line count) were examined and recorded (Figure 2).

2.2 | Laboratory Procedures

For molecular confirmation of species identity and phylogenetic analysis, a maximum of 10 samples per species were selected, covering multiple locations across Malaysia. DNA was extracted using PrimeWay Genomic DNA extraction kits (Malaysia) following the manufacturer protocol for tissue samples. Two mitochondrial DNA (mtDNA) markers were targeted, namely cytochrome oxidase subunit 1 (COI) using a newly designed primer set of RhyCOI-F 5′ TCA GCC ATC TTA CCT GTG GC 3′ and RhyCOI-R 5′ CCG GAG TAA TAG GCA ACG ACA 3′, and NADH dehydrogenase subunit 2 (ND2) using the primers ILEM 5′ AAG GAG CAG TTT GAT AGA GT 3′ and ASNM 5' AAC GCT TAG CTG TTA ATT AA 3′ (Naylor et al. 2012). Polymerase chain reaction (PCR) amplification was performed using a 20 μL reaction mix containing 10 μL of exTEN 2X PCR Master Mix (Malaysia), 1 μL of 10 pmol primer (both primers), 50 pg to 1.0 μg DNA template, and molecular grade water. The PCR cycles for both markers consisted of 2 min initial denaturation at 95 °C followed by 40 cycles of 25 s at 95 °C, 25 s at 50 °C and 50 s at 72 °C, and subsequently a final extension of 5 min at 72 °C. All PCR products were examined using 1% agarose in TAE buffer prior to Sanger sequencing by Apical Scientific Sendirian Berhad (Selangor, Malaysia).

For the population genetic analysis of *R. australiae*, a maximum of two individuals per boat/landing were sampled to limit over-sampling of sibs that may result in reduction of haplotype

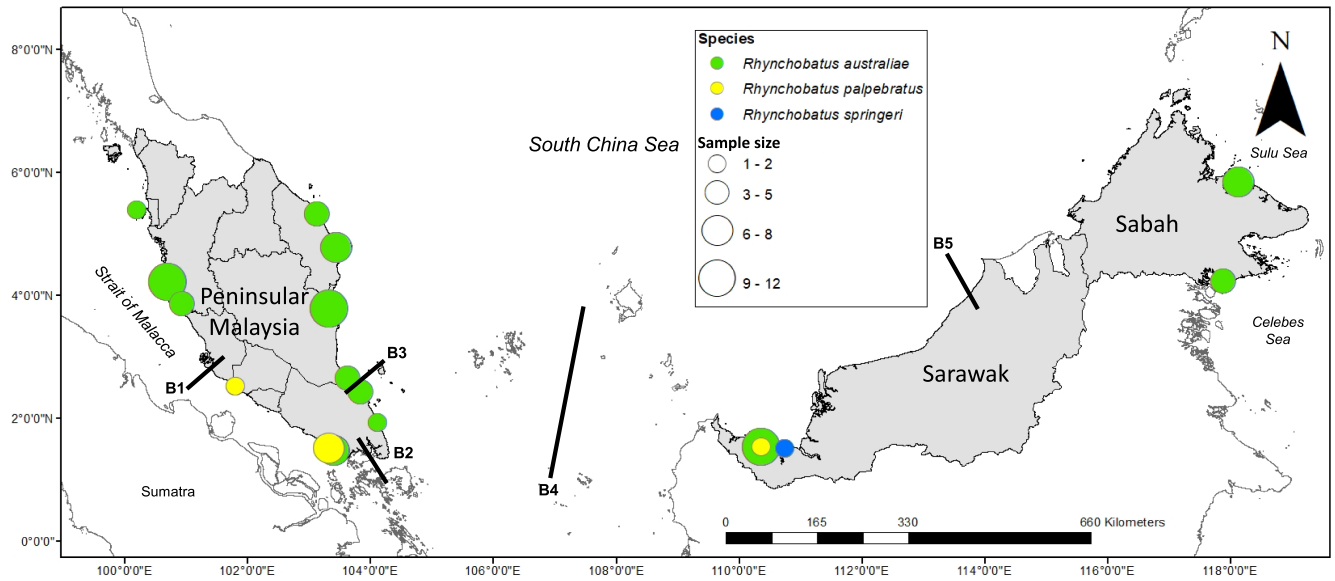

FIGURE 1 | Sampling sites across Malaysia (grey shading) covering northern and southern west (WP) and east (EP) coasts of Peninsular Malaysia, and coastal Sarawak and Sabah.*R* Black lines denote putative biogeographical barriers (B1 to B5) considered in the population genetic analysis.

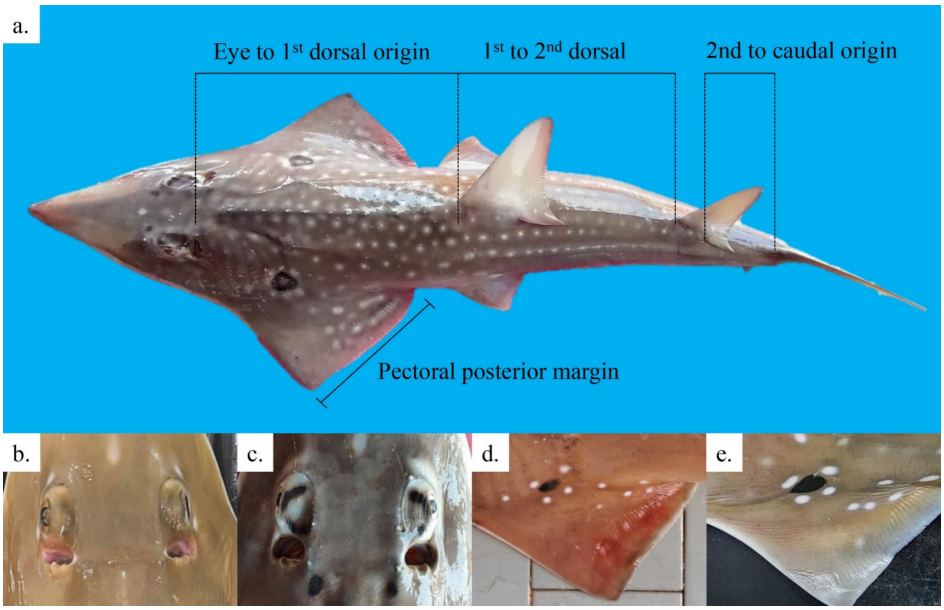

FIGURE 2 | (a) Major regions inspected in fresh *Rhynchobatus* specimens (see also Table 2), with examples of morphological variations in the eye region (b,c) and in the pectoral fin region (d,e). Specifically, the lack of eyebrow marking and black spot in *R. australiae* (b), the presence of both eyebrow marking and one black spot behind each spiracle in *R. palpebratus* (c, also present in *R. springeri*), the diagonal white spots above the black pectoral spot in *R. australiae* (d), and the white spots surrounding the black pectoral spot in *R. palpebratus* (e, also present in *R. springeri*).

diversity (note that several boat/landings were considered per landing site). This also contributed to reducing heterogeneity in sample size among landing sites. The DNA of each sample was extracted using PrimeWay Genomic DNA extraction kit (Malaysia), and the mitochondrial control region (CR) as well as ND2 were amplified. The primer sets used for the targeted regions were newly designed primer set of CR (RS)-F2 5′ TCA AAC TCT CGT CCT TGG CTC 3′ and CR (RS)-R2 5′ GCA TCT TCA GTG CCA TGC TT 3′ for CR, and ILEM 5′ AAG GAG CAG TTT GAT AGA GT 3′ and ASNM 5′ AAC GCT TAG CTG TTA ATT AA 3′ for ND2 (Naylor et al. 2012). Details of the PCR amplification were similar to the above except that an annealing temperature of 56 °C was used for CR.

2.3 | Data Analysis

2.3.1 | Species Identification and Phylogenetic Analyses

Sequences for both COI and ND2 were manually inspected, aligned and edited using ClustalX (Thompson et al. 1997) and BioEdit (Hall 1999). All sequences were submitted to the National Center for Biotechnology Information (NCBI) GenBank database, with the accession numbers provided in Supplemental Table S1. For species identification based on the highest similarity to known records, the sequences were queried against the core nucleotide database at NCBI using blastn.

To confirm species identity, further investigate the genetic variation within and between species, and examine their phylogenetic relationships, we conducted a phylogenetic analysis based on the ND2 sequences of all collected specimens, 11 *Rhynchobatus* entries available in GenBank, and two representatives of *R. ancylostomus* as outgroup. In addition, smaller and less redundant subsets of the data were created by selecting 10 sequences assigned to *R. australiae*, covering the full range of genetic variation among the population sample. These were combined with 11 (ND2) and 31 (COI) sequences of *Rhynchobatus* entries available in GenBank and the Barcode of Life Data System (BOLD), which similarly represented a wide range of genetic variation with limited redundancy. In all cases, two representatives of *R. ancylostomus* served as the outgroup. Accession numbers of all sequences from public databases used in the phylogenetic analyses are found in Supplemental Table S2.

For each dataset—ND2 with all samples, ND2 and COI subsets (just ND2 and COI from here on), and ND2 + COI concatenated—sequences were aligned as described above and subjected to a best model search based on the Akaike information criterion (AIC) and the Bayesian information criterion (BIC) for maximum likelihood (ML) and Bayesian inference (BI) analyses, respectively, using Kakusan v3 (Tanabe 2007). A phylogenetic tree of each dataset was constructed using Treefinder version March 2011 for ML (Jobb, von Haeseler, and Strimmer 2004) and MrBayes version 3.1.2 for BI (Huelsenbeck and Ronquist 2001). The ML analyses were performed by searching for the best ML tree ('reconstruct phylogeny' option), and independently running with 1000 nonparametric bootstrap replicates using the J1 + Gamma (COI), TN93 + Gamma (ND2), TIM + Gamma (ND2 + COI), and TN93 + Gamma (all ND2 sequence dataset) models. The BI analyses were initiated using J1 + Gamma (COI), HKY85 + Gamma (ND2), HKY85 + Gamma (ND2 + COI), and TN93 + Gamma (all ND2 sequence dataset) with a random starting tree and two parallel runs, each of which consisted of four chains of Markov chain Monte Carlo (MCMC) iterations for 2,000,000 generations (sampled every 100th generation for each chain). Likelihood values for post-analysis trees and parameters were evaluated for convergence and burn-in. Five thousand of the sampled trees were discarded as burn-in (25%), and the remaining trees after burn-in were used to calculate 50% majority-rule consensus

trees and the posterior probabilities of the nodes. Due to the high level of agreement between the ML and BI trees, we opted to present the best ML tree for each dataset, annotated with nonparametric bootstrap values and Bayesian posterior probabilities at the congruent nodes. These final trees were rooted with *R. ancylostomus*, and visualized using ggtree v3.8.2 and dependent packages in R v4.3.3, with manual edits performed in PowerPoint. Uncorrected p-distances were calculated using PAUP*40b10 (Swofford 2002) to evaluate the genetic divergence among *Rhynchobatus* species.

2.3.2 | Population Genetics of *Rhynchobatus australiae*

The collected *R. australiae* samples were divided into six major coastal regions (Supplemental Table S3): northern and southern west coast of Peninsular Malaysia (WP north and WP south, respectively), northern and southern east coast of Peninsular Malaysia (EP north and EP south, respectively), Sarawak (Sar) and Sabah (Sb). These regions encompass the three major water bodies in Malaysia, i.e. the Strait of Malacca, the southern South China Sea and the Sulu-Celebes Sea. They were determined with respect to fine-scale biogeographical barriers informed by previous work (Lim et al. 2021): the historical Sunda Shelf Barrier hypothetically positioned around Selangor (B1) and Singapore (B2), depth and distance barriers along EP (B3), South China Sea (B4) and along Malaysian Borneo (B5) (Figure 1).

Sequences of the CR and ND2 markers were inspected manually, aligned and edited using ClustalX (Thompson et al. 1997) and BioEdit (Hall 1999). All haplotype sequences used for the following analyses were submitted to GenBank with accession numbers provided in Supplemental Table S2. Intra- and inter-population genetic diversity were estimated as number of haplotypes (N_{ha}) and polymorphic sites (k), haplotype diversity (ha) and nucleotide diversity (π) for individual and concatenated markers (ND2 + CR) using ARLEQUIN v3.5.2.2 (Excoffier and Lischer 2010). Haplotype or gene diversity is the probability of obtaining two different alleles from random sampling while nucleotide diversity is the average differences in number of nucleotides per site among DNA sequences (Nei 1987). The median-joining haplotype network was then plotted using PopART v 1.7 (Bandelt, Forster, and Röhl 1999) to visualize the distribution of haplotypes among the six regions.

An AMOVA with pairwise genetic distances was conducted in ARLEQUIN v3.5.2.2 to identify hierarchical partitioning of genetic variation under the influence of various combinations of the selected biogeographical barriers (B1-B5). Pairwise Φ_{ST} values among regions were estimated as well as their statistical significance after correction for false discovery rate (FDR). Historical demographic expansion was examined using Fu's Fs (Fu 1997), Tajima's D tests of neutrality (Tajima 1989), and mismatch distribution analysis on individual and combined markers in DnaSP v6.10.03 (Nei 1987).

3 | Results

A total of 85 fin clips of *Rhynchobatus* specimens were collected from landing sites and local fish markets in 15 locations across Malaysia and successfully sequenced (Supplemental Table S3); six of the fin clips were taken from processed specimens and

TABLE 1 | Occurrence of markings and counts of spots for the *Rhynchobatus* specimens examined in this study (n = sample size). *If present, black spot occur either individually or in pairs. Round brackets indicate mean values.

Body region and associated morphological features	*R. australiae* $n = 62$	*R. palpebratus* $n = 10$	*R. springeri* $n = 1$
Total length (cm)	23.4–288.0 (83.0)	61.0–157.0 (95.2)	167.0
Pre-spiracle			
Eyebrow-like markings	Absent	Present	Present
Black spot(s)* in anterior/between eyes	25 Present/37 absent	1 Present/9 Absent	Absent
Black spot(s)* posterior to eyes	4 Present/58 absent	3 Present/7 Absent	Absent
White spots between spiracle to origin of first dorsal			
Three diagonal spots above black pectoral spot	59 Present/1 absent	Absent (10)	Absent (1)
Pectoral fin posterior edge	1–16 (5.7)	1–5 (2.6)	10
Eye to 1st dorsal spot (1st from midline)	0–2 (0.2)	0–11 (2.2)	10
Eye to 1st dorsal spot (2nd from midline)	0–4 (1.7)	0–8 (3.1)	10
White spots between origin of first dorsal fin to caudal origin			
1st to 2nd dorsal fin origin (1st from midline)	0–23 (2.9)	0–12 (4.3)	11
1st to 2nd dorsal (2nd from midline)	2–17 (6.2)	3–10 (5.1)	10
Faint line along white spots on tail	3 Present/56 absent	Present	Absent
2nd dorsal fin insertion to caudal fin origin	0–10 (1.0)	0	6

subsequent morphological measurements could not be carried out. Initial on-site identification for 79 whole specimens based on morphological characters yielded 68 *R. australiae*, seven *R. springeri*, and four *R. palpebratus*. When complemented with genetic analysis (details below), species assignment using both approaches were found to be incongruent for 16% of all specimens. Molecular identification based on 99% sequence identity with reference sequences in NCBI's core nucleotide database and the ND2 phylogeny including all samples revealed that *R. australiae* made up 87% of the species composition of all 85 samples, followed by *R. palpebratus* (12%) and one individual of *R. springeri* (Supplemental Table S4, Figure S2). All three species were found in Malaysian Borneo, specifically in Sarawak, but only two (*R. australiae* and *R. palpebratus*) were recorded in Peninsular Malaysia. Mean intraspecific uncorrected p-distances ranged from 0.08 to 0.77% for COI, from 0 to 4.0% for ND2, and from 0.17 to 0.33% for both markers combined (Supplemental Table S5). In contrast, mean interspecific distances ranged from 0.6 to 6.42% for COI, from 1.38 to 5.83% for ND2, and from 2.03 to 4.44% for both markers combined. After accounting for outdated (i.e. preceding new species descriptions) and likely erroneous identifications, intraspecific genetic distances were substantially lower than interspecific genetic distances, validating our approach for the genetic identification of species.

3.1 | Identification Based on Morphological Characters

Variation in the examined marking and spot patterns of the three *Rhynchobatus* species genetically identified in the present study, based on the subset of photographs that were sufficiently clear, are presented in Table 1 and Figure 3. Characteristics that were useful to distinguish these species include the eyebrow marking (*R. palpebratus*, *R. springeri*), three diagonal white spots above the black pectoral spot (*R. australiae*), and a faint white line along the white spots on the tail (*R. australiae*, *R. palpebratus*). Other examined characteristics were less useful to delineate species: The occurrence of black spots around the eye region was relatively low (35.6% and 9.6% of *Rhynchobatus* species showed black spots between and behind the eyes, respectively), and the count of white spots on the body region was found to vary widely across

FIGURE 3 | Dorsal view of the head region of selected *Rhynchobatus* specimens collected for this study. The code for each specimen used in the molecular analyses is shown in the top left corner of each photograph. [A]*R. australiae*, [P]*R. palpebratus*, [S]*R. springeri*.

individuals. *Rhynchobatus australiae* was distinguishable by the absence of eyebrow-like dark markings typically seen in *R. palpebratus*, and the presence of three diagonal spots above the black pectoral spot. Apart from the shared characteristics of eyebrow marking and the black pectoral spot surrounded by white spots, *R. palpebratus* differs from *R. springeri* by having the posterior edge of the pectoral fin partially covered with white spots (fully covered in *R. springeri*), a faint line along the white spots on the tail (absent in *R. springeri*), and the absence of white spots between the second dorsal fin insertion and caudal fin origin (present in *R. springeri*).

3.2 | Phylogenetic Relationships

The single-gene (1191 bp for ND2, 645 bp for COI and additional 1191 bp for ND2 including all specimens of *R. australiae* shown in Figures 4, 5, and Supplemental Figure S1, respectively) and concatenated phylogenetic trees (a total of 1836 bp for ND2 + COI shown in Supplemental Figure S2) were largely congruent in terms of topology. All *Rhynchobatus* species were consistently recovered as monophyletic with strong support, with the exception of *R. palpebratus*, which emerged as

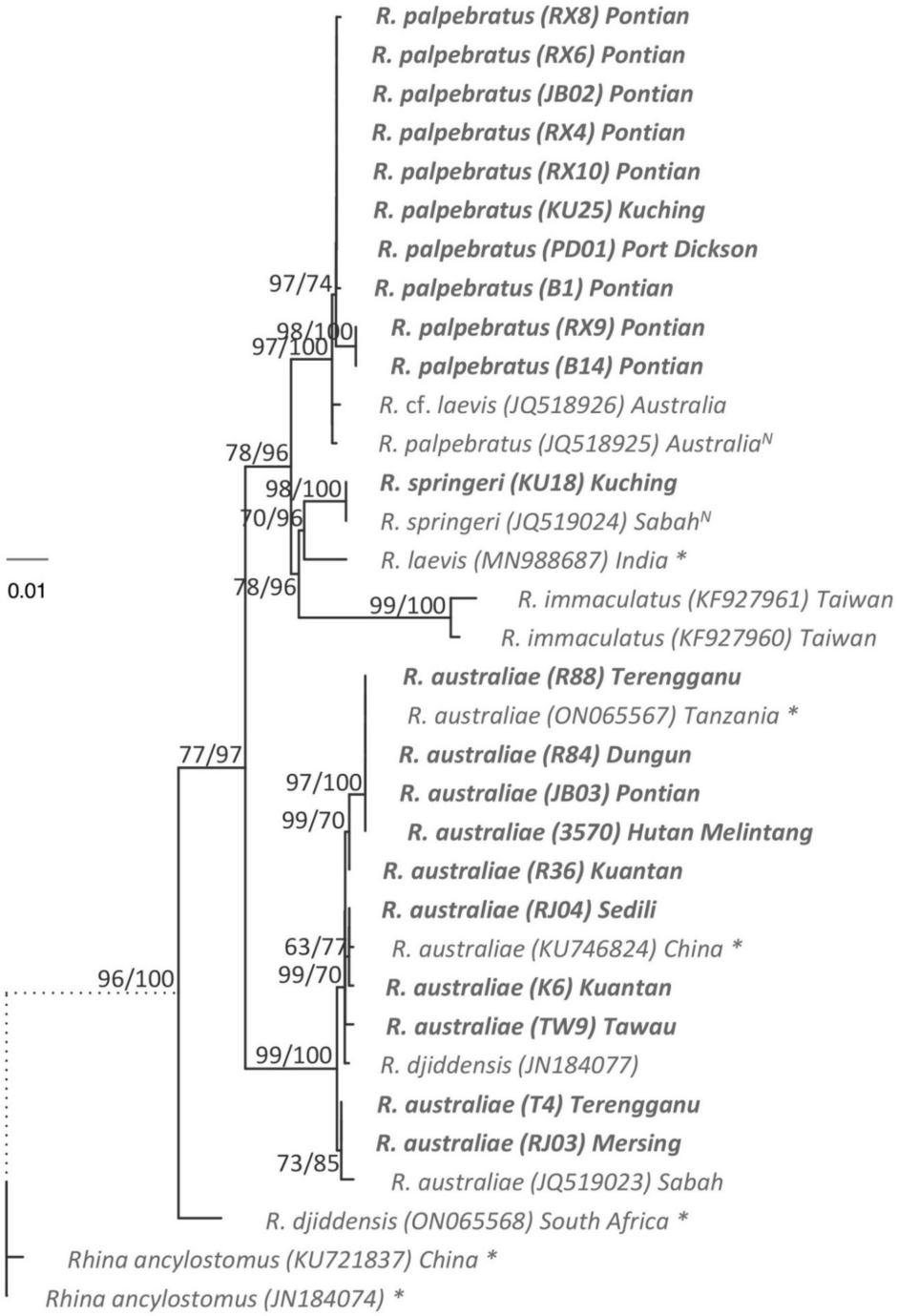

FIGURE 4 | Best maximum likelihood (ML) phylogenetic tree of *Rhynchobatus* species based on the ND2 gene, with ML bootstrap values and BI posterior probabilities shown on the internal branches. Bold = samples collected in this study, *complete genome reference sequences, [N]reference sequences from Naylor et al. 2012. Notes: JQ519024 is identified as *R. laevis* in GenBank but was the specimen used by Peter Last to describe *R. springeri* (Giles et al. 2016). Sample JN184077 identified as *R. djiddensis* was sequenced in the USA but the exact sampling location is not known.

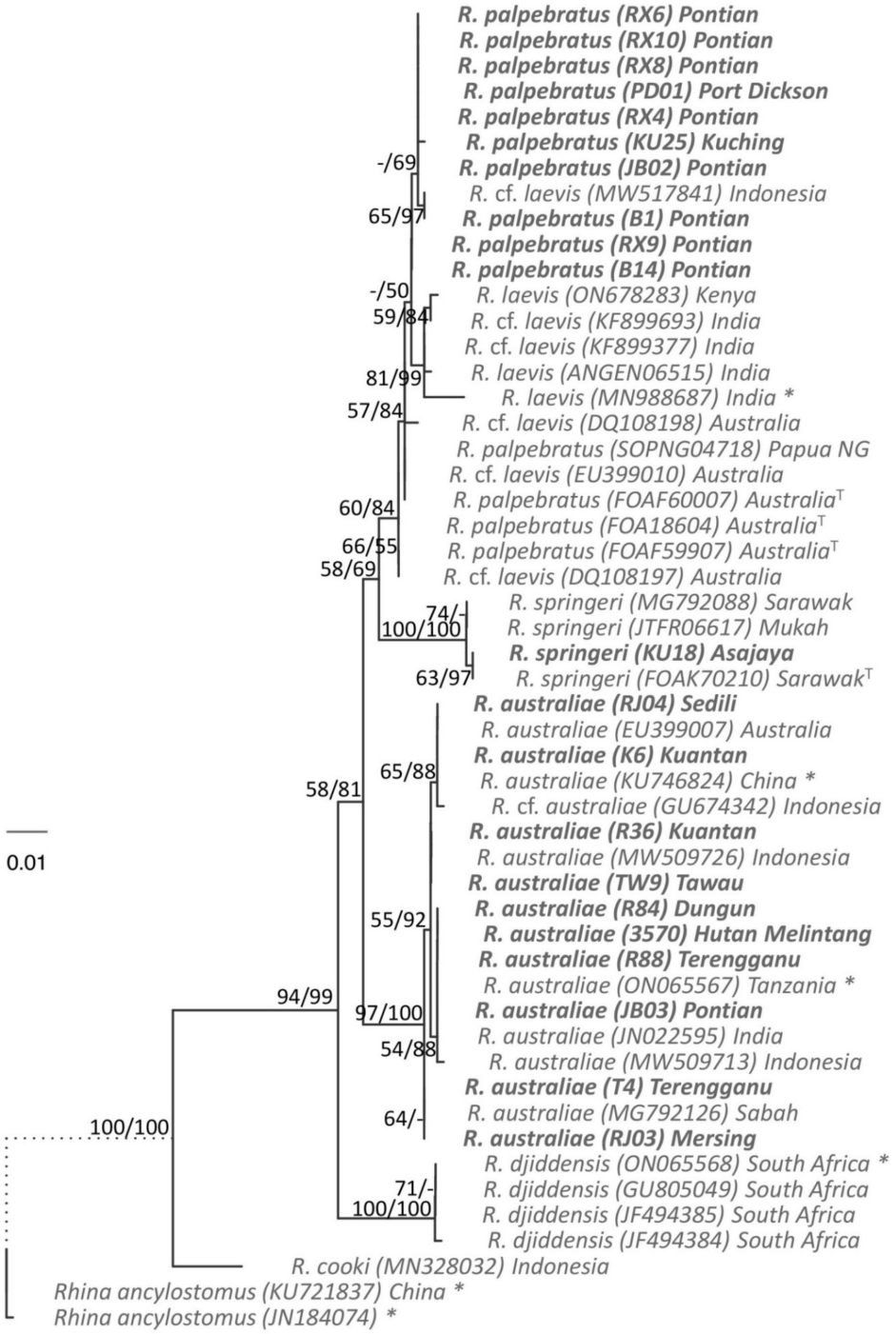

FIGURE 5 | Best maximum likelihood (ML) phylogenetic tree of *Rhynchobatus* species based on the COI gene, with ML bootstrap values and BI posterior probabilities shown on the internal branches. Bold = samples collected in this study, *sequences extracted from complete genome, [T]reference sequences from type specimens. Papua NG, Papua New Guinea.

paraphyletic with respect to *R. laevis* from the Indian Ocean in the COI trees. In addition, *R.* cf. *laevis* from Australia and the Indo-West Pacific was found nested within *R. palpebratus* in the single-gene trees. The single *R. djiddensis* specimen (JN184077) positioned within the *R. australiae* cluster in the ND2 trees has most likely been mis-identified, thus preserving the monophyly of *R. australiae*.

Between-species relationships generally showed weaker support, but *R. australiae* emerged as sister group to the remaining species in all trees with strong support. *Rhynchobatus palpebratus* was placed in a sister-group relationship to *R. springeri* and *R. laevis* (Indian Ocean) in the ND2 and concatenated trees. *Rhynchobatus immaculatus* and *R. cooki* could only be included in the single-gene analyses due to the lack of suitable reference sequences for the concatenated analysis. The former was found in a well-supported sister-group relationship with *R. springeri* and *R. laevis* (Indian Ocean) in the ND2 trees, while the latter was recovered as sister to all remaining ingroup species according to COI.

3.3 | Population Genetics of *Rhynchobatus australiae*

A total of 2284 bp (1191 bp for ND2 and 1093 bp for CR) were successfully amplified for all 74 *R. australiae* samples. From these 29 haplotypes and 30 polymorphic sites were identified, 14 of which in ND2 and 16 in CR (Supplemental Table S6). Most of the haplotypes (89.7%) were represented by less than five individuals, and half of them (58.6%) by a single individual. Haplotype Hap2 was the most common haplotype, represented by 24 individuals from across Peninsular Malaysia and Sarawak. In terms of genetic diversity, ND2 showed lower values (haplotype diversity 0.23–0.90, nucleotide diversity 0.0005–0.0028) compared to CR (haplotype diversity 0.50–0.96, nucleotide diversity 0.0012–0.0042, Table 2). The haplotype (0.58–0.98) and nucleotide (0.0008–0.0033) diversity varied slightly when the two markers were concatenated.

All Tajima's D tests were not significantly different from zero (except for WP North combined markers), which suggests no departure from neutrality (Table 2). Similarly, Fu's Fs, which is more sensitive to recent population expansion than D tests (Fu 1997), suggested weak evidence for population expansion based on the non-significant negative values for both individual and combined markers (WP North, EP North and EP South in ND2; Sarawak and Sabah in CR; EP North, Sarawak and Sabah in combined ND2 + CR).

The haplotype network showed three haplotype clusters separated by five or more mutations, with one to four mutations separating neighbouring haplotypes within each cluster (Figure 6). Haplotypes were largely shared among the six regions, but most of the specimens from WP (83.3%) were found within the cluster of 10 haplotypes on the left of the figure. The cluster on the right (excluding Hap9 and Hap19) included 17 haplotypes whose positioning within the network mirrored the spatial adjacency

TABLE 2 | Genetic diversity of *Rhynchobatus australiae* according to area and markers (individual ND2 and CR markers and concatenated ND2 + CR markers). WP and EP, west and east coasts of Peninsular Malaysia, respectively. N = number of samples, k = number of polymorphic sites, N_{ha} = number of haplotypes, ha = haplotype diversity, π = nucleotide diversity, D = Tajima's D test statistic, F_S = Fu's F_S test statistic. * Represent significant difference at $p < 0.05$.

Area	N	k	N_{ha}	ha	π	D	F_S
ND2							
All	74	14	14	0.69 ± 0.05	0.2244 ± 0.1304	0.27	−1.87
WP North	17	4	3	0.23 ± 0.13	0.0005 ± 0.0005	−1.58*	−0.08
WP South	7	5	2	0.57 ± 0.12	0.0024 ± 0.0016	1.98	4.27
EP North	23	12	10	0.73 ± 0.10	0.0027 ± 0.0016	0	−1.93
EP South	7	9	5	0.90 ± 0.10	0.0026 ± 0.0017	−0.91	−0.67
Sarawak	10	8	4	0.73 ± 0.10	0.0028 ± 0.0018	0.70	1.78
Sabah	10	7	5	0.80 ± 0.10	0.0028 ± 0.0018	1.39	0.54
CR							
All	74	16	20	0.85 ± 0.03	0.2662 ± 0.1479	0.87	−4.23
WP North	17	8	4	0.50 ± 0.14	0.0012 ± 0.0009	−1.56	0.46
WP South	7	8	5	0.90 ± 0.10	0.0042 ± 0.0027	2.10	0.12
EP North	23	12	7	0.75 ± 0.08	0.0040 ± 0.0023	1.18	1.56
EP South	7	10	4	0.81 ± 0.13	0.0035 ± 0.0023	−0.36	1.14
Sarawak	10	10	8	0.96 ± 0.06	0.0039 ± 0.0024	0.92	−2.44
Sabah	10	12	8	0.93 ± 0.08	0.0035 ± 0.0022	−0.45	−2.77
ND2 + CR							
All	74	30	29	0.88 ± 0.03	0.2467 ± 0.1293	0.64	−6.48
WP North	17	12	5	0.58 ± 0.13	0.0008 ± 0.0006	−1.75*	0.28
WP South	7	13	5	0.90 ± 0.10	0.0033 ± 0.0020	2.19	1.07
EP North	23	24	13	0.82 ± 0.08	0.0033 ± 0.0018	0.63	−1.15
EP South	7	19	5	0.90 ± 0.10	0.0030 ± 0.0018	−0.65	0.91
Sarawak	10	18	8	0.96 ± 0.06	0.0033 ± 0.0019	0.88	−0.94
Sabah	10	19	9	0.98 ± 0.05	0.0031 ± 0.0018	0.28	−2.51

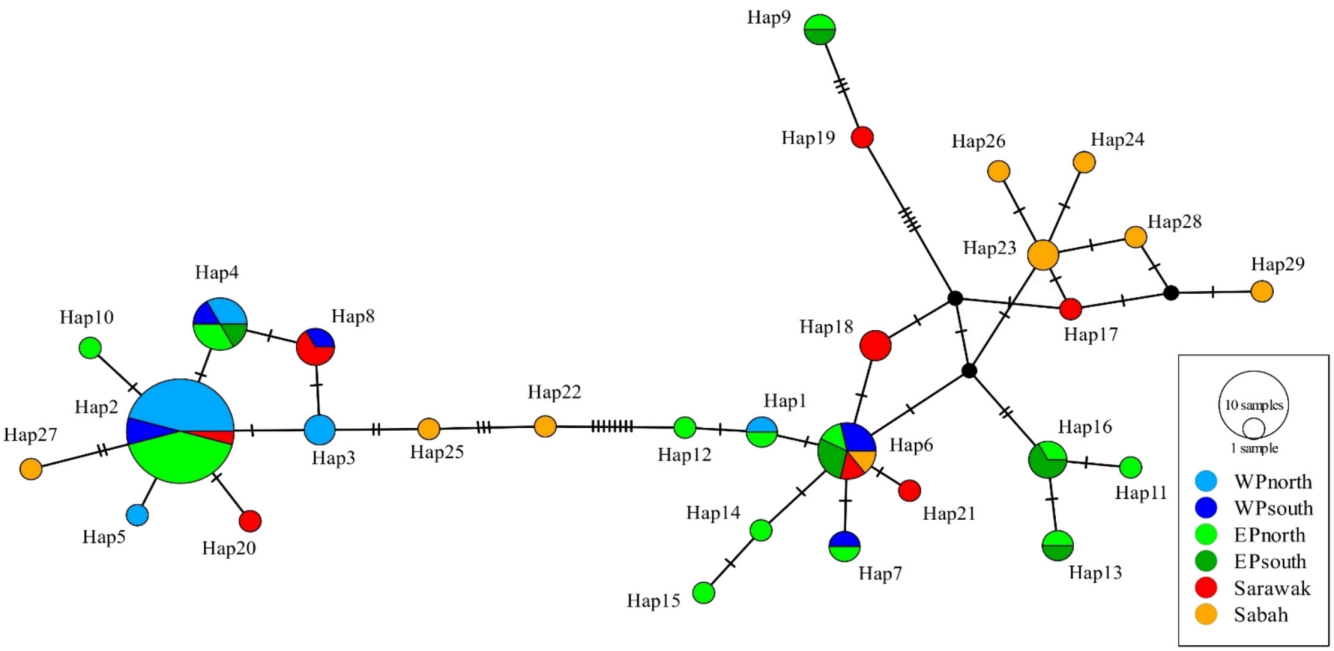

FIGURE 6 | Haplotype network for *Rhynchobatus australiae* ($n = 74$) based on combined ND2 and CR markers. Circle size is proportional to the number of samples sharing the haplotypes. Bars along the lines represent number of mutations between the connected haplotypes.

of the sampling areas (i.e. EP, Sarawak and Sabah). Hap9 and Hap19 formed the smallest cluster involving specimens from EP and Sarawak, with three mutations between the two areas. A similar haplotype network structure was observed for the individual markers, but with fewer mutations between haplotypes, separated by a maximum of three and four mutations for ND2 and CR, respectively (Supplemental Figure S3).

Results from the hierarchical AMOVA showed moderate and highly significant genetic structure, with a Φ_{ST} value of 0.312 ($p < 0.001$) among the six regions (i.e. 31.2% of the genetic variation was due to differences among regions) considering both markers combined (Table 3). Testing for genetic partitioning with various region groupings (Supplemental Tables S7–S8) indicated that a large share of this genetic structure was due to the difference between WP north and the other regions ($\Phi_{ST} = 0.229$, $p < 0.05$). Under this scenario, 22.9% of the genetic variation was partitioned among these two groups, 23.2% within each group, and 40.8% within each region. No significant partitioning of genetic variation was observed in any of the hierarchical AMOVA tests for the CR marker (Supplemental Table S9). Pairwise comparisons using individual and combined markers indicated that WP north was significantly different from the other five regions (p value <0.05) (Table 4). Other significant pairs include EP north-EP south in ND2 and EP north-Sabah in both individual and combined markers ($p < 0.05$).

4 | Discussion

Through extensive surveys across Malaysia spanning 15 locations over a period of more than 7 years, especially in the Peninsula, we determined that the dominant *Rhynchobatus* species in terms of relative abundance is the bottlenose wedgefish *R. australiae*, which is congruent with reports by Giles et al. (2016) and Booth et al. (2021). The proportions of *Rhynchobatus* species may nonetheless vary, particularly in Malaysian Borneo. Using molecular genetics, we established the presence of two other look-alike congeners, *R. palpebratus* and *R. springeri*, both seemingly showing relatively restricted spatial distribution within Malaysia.

A considerably high percentage (ca. 16%) of our on-site identifications of *Rhynchobatus* specimens based on external morphological features did not match the genetic identification. In particular, a number of *R. australiae* specimens were initially mis-identified as *R. springeri* due to the presence of two black spots behind the eyes. This trait is associated with *R. springeri* (Jabado 2019) but had not been highlighted for *R. australiae* in studies that examined phenotypic characteristics of the species (e.g. Giles et al. 2016; Jabado 2019). Giles et al. (2016) indicated that some of the diagnostic markings for *R. australiae* are obvious in smaller sized individuals but fade in larger ones—examination and description of the external morphological markings need therefore to consider size-related variation. *Rhynchobatus palpebratus* specimens were also initially mis-identified as both *R. australiae* and *R. springeri*. The morphological differences among *Rhynchobatus* species are not trivial and species identification require careful consideration, particularly where congeners occur sympatrically, e.g. in waters of southern Sarawak (all *Rhynchobatus* congeners) and along the southwest coast of Peninsular Malaysia (*R. australiae* and *R. palpebratus*).

The use of phylogenetic trees based on a combination of COI and ND2 markers was instrumental to confirm species identification among *Rhynchobatus* congeners. The identification of the single *R. springeri* from Sarawak could be confirmed unambiguously with the COI sequence of the type specimen for this species (FOAK70210). The sole ND2 sequence for *R. springeri*

TABLE 3 | Results of the AMOVA analyses with lowest p values comparing genetic variation within and among groups for concatenated ND2 + CR markers and for individual ND2 and CR markers. WP and EP, west and east coasts of Peninsular Malaysia, respectively; Sar, Sarawak; SB, Sabah; DF, degree of freedom; SS, sum of squares; VC, variance components; PV, percentage of variation. * and ** represent statistical significance at $p < 0.05$ and $p < 0.001$, respectively.

Source of variation	DF	SS	VC	PV	
ND2 + CR					
All grouped					
Among regions	13	113.168	1.186**	31.19	$\Phi_{ST} = 0.312$
Within regions	60	156.967	2.616	68.81	
Total	73	270.135	3.802		
2 groups (WP north vs WP south EP Sar SB)					
Among groups	1	36.081	1.013*	22.93	$\Phi_{CT} = 0.229$
Among regions within groups	12	77.087	0.789*	17.85	$\Phi_{SC} = 0.232$
Within regions	60	156.967	2.616**	59.22	$\Phi_{ST} = 0.408$
Total	73	270.135	4.417		
3 groups (WP north vs WP south EP Sar vs SB)					
Among groups	2	47.001	0.801	19.36	$\Phi_{CT} = 0.194$
Among regions within groups	11	66.167	0.719*	17.39	$\Phi_{SC} = 0.216$
Within regions	60	156.967	2.616**	63.25	$\Phi_{ST} = 0.368$
Total	73	270.135	4.136		
ND2					
All grouped					
Among regions	13	46.508	0.475**	29.50	$\Phi_{ST} = 0.295$
Within regions	60	68.167	1.136	70.50	
Total	73	114.676	1.612		
2 groups (WP north vs WP south EP Sar SB)					
Among groups	1	15.491	0.447	23.75	$\Phi_{CT} = 0.237$
Among regions within groups	12	31.017	0.300*	15.93	$\Phi_{SC} = 0.209$
Within regions	60	68.167	1.136**	60.32	$\Phi_{ST} = 0.397$
Total	73	114.676			
3 groups (WP north vs WP south EP Sar vs SB)					
Among groups	2	20.139	0.358*	20.33	$\Phi_{CT} = 0.203$
Among regions within groups	11	26.370	0.267*	15.15	$\Phi_{SC} = 0.190$
Within regions	60	68.167	1.136**	64.52	$\Phi_{ST} = 0.355$
Total	73	114.676	1.761		
CR					
All grouped					
Among regions	13	66.660	0.710**	32.43	$\Phi_{ST} = 0.324$
Within regions	60	88.799	1.480	67.57	
Total	73	155.459	2.190		
2 group (WP north vs WP south EP Sar SB)					

(Continues)

TABLE 3 | (Continued)

Source of variation	DF	SS	VC	PV	
Among groups	1	20.591	0.565	22.31	$\Phi_{CT}=0.223$
Among regions within groups	12	46.070	0.489**	19.28	$\Phi_{SC}=0.248$
Within regions	60	88.799	1.480**	58.41	$\Phi_{ST}=0.416$
Total	73	155.459	2.534		
3 group (WP north vs WP south EP Sar vs SB)					
Among groups	2	26.863	0.443	18.64	$\Phi_{CT}=0.186$
Among regions within groups	11	39.798	0.452*	19.05	$\Phi_{SC}=0.234$
Within regions	60	88.799	1.480**	62.31	$\Phi_{ST}=0.377$
Total	73	155.459	2.375		

(JQ519024) was originally identified as *R. laevis* (GN3004/ANFC H 6221-02) in GenBank—this specimen had been used by Peter Last to describe *R. springeri* (Giles et al. 2016). The species confirmation process for *R. palpebratus* was less straightforward. The COI-based phylogenetic tree suggested a *R. laevis*–cf. *laevis*–*palpebratus* species complex that included reference sequences from *R. palpebratus* type specimens and *R. laevis* from India, which is the type locality for *R. laevis*. The ND2 tree contained fewer sequences but also showed a *R. palpebratus*–cf. *laevis* species complex; however, the same *R. laevis* specimen from India was not recovered as a close relative of the *R. palpebratus* or *R.* cf. *laevis*. This latter topology exhibited much higher support, and was not contradicted by COI due to its lack of resolution, suggesting that *R. laevis* from the Indian Ocean and *R.* cf. *laevis* from the Indo-West Pacific should not be regarded as the same species. It also reveals that COI alone is less informative than ND2 in resolving the *R. palpebratus*–*laevis* species complex. This is supported by Groeneveld et al. (2023), who found that ND2 was the most informative mitochondrial marker based on mitogenomes of *R. djiddensis* and *R. australiae* from South Africa. The COI tree however remains useful, despite overall lower support values, due to the more comprehensive sampling of the genus. To conclusively resolve the phylogenetic relationships among wedgefishes, larger nuclear datasets are likely required, e.g. based on multiple nuclear genes, or genome-wide SNPs derived from RAD-seq or whole genome resequencing.

We did not find any *R. laevis* specimens in Sarawak as reported in Booth et al. (2021)—this is likely in part due to our low survey effort in the state. It is also possible that the specimens identified as *R. laevis* in Booth et al. (2021) correspond to the species that we identified as *R. palpebratus*. Last et al. (2016) noted that these two species are very similar but are distinct genetically and do not overlap range-wise. Our study supports that *R. laevis* (sole sequence MN988687 from India) and *R. palpebratus* (from our study area and also from type specimens from Australia) represent distinct ND2 lineages, i.e. separate species. Also, given the revised geographical delineation of *R. laevis* that excluded Malaysia and wider Southeast Asia by Last et al. (2016), previous records of *R. laevis* in Malaysia, including the one reported from the state of Perak (Abd Haris Hilmi, Ahmad, and Kissol 2017), are likely to be *R. palpebratus*. The new species record of *R. palpebratus* in Malaysia indicates that the species distribution range may extend more widely across the Indo-West Pacific region beyond Australia, and a re-assessment of the near-threatened status of *R. palpebratus* (currently the only *Rhynchobatus* species that is not listed as critically endangered; see Kyne and Rigby 2019) may be warranted.

Unlike the larger-sized *R. australiae*, which is widely distributed across coastal Malaysia, the other two congeners appear to exhibit narrower ranges. To date, *R. palpebratus* is recorded only from the southern Strait of Malacca, the west coast of Peninsular Malaysia and from Kuching, Malaysian Borneo. We did not find *R. springeri* from either coasts of Peninsular Malaysia, contrary to the distributional map of the species (Last et al. 2016; Kyne 2019). It is evident that the abundance of *R. springeri* is relatively low compared to the other two congeners, and current evidence, including the new data presented in this study, suggest a restricted distribution of *R. springeri* around the waters of Sarawak, Malaysian Borneo. Our findings also suggest that the non-contiguous distributional records of *R. springeri* and *R. palpebratus* could reflect a combination of specialized habitat use (Compagno and Last 2010) and/or reduced range from high fishing pressure.

We found moderate and highly significant genetic structure within *R. australiae*, and a large share of this structure was due to the difference between the northern Strait of Malacca and the other regions. This structure was even apparent within the Strait of Malacca itself, i.e. between the northern and southern parts of the strait, which we refer to as fine-scale genetic structure. The pattern was observed even though haplotypes were largely shared among the six regions, implying that it is due to differences in the frequencies of the different haplotypes in the six regions. Although significant genetic differentiation had been detected for *R. australiae* over wider geographical areas (see Giles et al. 2016; Simwanza and Rumisha 2023; Tapilatu et al. 2023; Groeneveld et al. 2024), it is rather surprising that such fine-scale genetic differentiation is found within a relatively small and shallow waterway with no obvious physical barriers that would hinder coast-wide movement of a large-sized vagile batoid like *R. australiae*. To date very limited studies had been conducted on movement of *R. australiae* and *Rhynchobatus* in general. Lear et al. (2024) reported that tagged adult females of *R. australiae* showed strong fidelity to a relatively small, shallow area (< 2 m in depth) in northwestern Australia, and Jordaan

TABLE 4 | Pairwise F_{ST} (lower diagonal) among sampling areas and respective *p* values (upper diagonal) for concatenated ND2 + CR markers and individual ND2 and CR markers. WP and EP, west and east coasts of Peninsular Malaysia respectively, * and ** represent statistical significance at $p < 0.05$ and $p < 0.001$, respectively.

	WP north	WP south	EP north	EP south	Sarawak	Sabah
ND2 + CR						
WP north	—	*	*	**	**	**
WP south	0.303	—	n.s.	n.s.	n.s.	n.s.
EP north	0.178	−0.079	—	n.s.	n.s.	*
EP south	0.664	0.121	0.161	—	n.s.	n.s
Sarawak	0.431	−0.075	0.014	0.046	—	n.s.
Sabah	0.595	0.099	0.166	0.050	0.048	—
ND2						
WP north	—	*	*	**	**	**
WP south	0.323	—	n.s.	n.s.	n.s.	n.s.
EP north	0.193	−0.087	—	*	n.s.	*
EP south	0.713	0.178	0.190	—	n.s.	n.s
Sarawak	0.452	−0.078	−0.008	0.042	—	n.s.
Sabah	0.588	0.100	0.154	0.111	0.015	—
CR						
WP north	—	*	*	**	*	**
WP south	0.292	—	n.s.	n.s.	n.s.	n.s.
EP north	0.168	−0.074	—	n.s.	n.s.	*
EP south	0.624	0.075	0.137	—	n.s.	n.s
Sarawak	0.417	−0.073	0.029	0.049	—	n.s.
Sabah	0.600	0.098	0.175	−0.008	0.072	—

et al. (2021) noted that almost half of tagged *R. djiddensis* were recaptured within 5 km of tag-and-release site in southern Africa; however another study reported that *R. laevis* was capable of moving up to 712 km (Bruns et al. 2024). Collectively, the biological evidence suggests that wedgefishes are capable of long migratory distances but may show preference for limited movement restricted to shallow habitats (Lear et al. 2024), with possible species- and sex-specific patterns that require further investigations.

The Malay Peninsular has been recognized as a potential barrier to gene flow for a number of marine species, including horseshoe crabs (Adibah, Ng, and Tan 2015), paddle crab (Suppapan et al. 2017), and various mangrove plant species (Mantiquilla et al. 2021). Within the narrow and shallow Strait, very limited genetic studies on sharks and rays or other marine organisms have been conducted. However fine-scale genetic structuring within the Strait similar to that of *R. australiae* is seen in another benthopelagic species, the longtail butterfly ray, *Gymnura poecilura* (Leung et al. unpublished data), and the green tiger prawn, *Penaeus semisulcatus* (Halim et al. 2021). These congruent genetic signatures may reflect historical vicariance over the Sunda Shelf region, which are also likely shaped by possible contemporary geographical barriers for these demersal elasmobranchs that lack a larval-mediated dispersal strategy within the narrow Strait.

The moderate levels of genetic diversity detected in *R. australiae* compared to other sharks and rays (Domingues, Hilsdorf, and Gadig 2017) suggest some capacity for adaptation. However, the low population numbers of this species and the lack of protective measures hinder its ability to persist with ongoing threats such as overfishing and habitat degradation. Additionally, sharks, and by extension rays, have been shown to exhibit the lowest rate of mutations among vertebrates, indicating very slow evolution and possibly a reduced ability to survive population bottlenecks (Sendell-Price et al. 2023). The different genetic populations of *R. australiae* should be considered as distinct stocks from a fisheries perspective and conserved separately to preserve genetic diversity of the species. Similar work to examine genetic diversity and population structure for *R. palpebratus* would provide additional insights to inform future IUCN assessments on the status of this species.

High fishing pressure and strong demand for their fins, meat and gelatinous snout will continue to exert pressure on these highly threatened wedgefishes unless specific interventions are introduced. Although none of the wedgefish species are presently protected in Malaysia, conservation action to include them as priority species within the National Plan of Action Shark (NPOA-Shark Plan), and to list them as marine endangered species (Control of Endangered Species of Fish Regulations)

under the national Fisheries Act 1985 is warranted. The protection should be conferred on all the look-alike *Rhynchobatus* congeners collectively, given difficulties in distinguishing species using only external characteristics. Site-specific protection conferred to wedgefish may be beneficial given their fragmented habitat range, and species-specific movement studies are warranted due to unique life history and ecology (White et al. 2014). The recent recognition of the West Tioman Important Shark and Ray Area (ISRA) in the south EP region in supporting undefined aggregations of *R. australiae* (IUCN SSC Shark Specialist Group 2024; Jabado et al. 2024) is a positive step towards evidence-based spatial conservation of the wedgefishes. Our study reveals the importance of Sarawak waters in hosting all three *Rhynchobatus* congeners, which should serve as focal area for further investigations. Future work should focus on clarifying genetic diversity, habitat use, and distributional extent of the two less common *Rhynchobatus* congeners to ensure that their populations can be sufficiently protected.

Author Contributions

AT, AL and SA conceived the study design and obtained funding. AL managed fieldwork and data collection. AL and LKC performed data analyses, with supervision by AT and MH. All authors contributed to the writing and editing of the manuscript and have approved the final version.

Acknowledgements

This manuscript is output for Save Our Seas Foundation grant awarded to AL and WWF Malaysia grant awarded to AT. We thank Department of Fisheries Sarawak, especially Mr Goh and Mr Jabbo, for help to procure samples, and the two anonymous reviewers for their useful feedback. Open Access funding enabled and organized by Projekt DEAL.

Ethics Statement

All tissue samples collected in this study were derived from dead animals originating from fisheries catches. As such, there were no relevant animal welfare implications. All wedgefish species are not legally protected in Malaysia. Collection permits and sampling protocol for samples in Sabah was approved by the Sabah Biodiversity Council [Access Licence Reference No.: JKM/MBS.1000-2/2 JLD.9 (21–24) and Transfer Licence Reference No.: JKM/MBS. 1000-2/3 JLD.4 (18)]. Permission to collect specimens from Sarawak waters was granted by the Marine Fisheries Department of Sarawak. Research permits for samples collected in Peninsular Malaysia were not needed at the time of collection (up till year 2021); permission to collect samples after year 2021 was obtained from relevant state Department of Fisheries.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The genetic sequences generated during the current study have been made publicly available on the NCBI GenBank (see Supplemental Table S1).

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.