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Molecular phylogenetic inference of White-Spotted Guitarfish (*Rhynchobatus australiae*) collected from local Malaysian fish markets

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Abstract. *Md-Zain BM, Abdul-Mutalib SA, Aifat NR, Masstor NH, Mohd-Yusof NS, Mohd-Hashim A, Abdul-Latiff MAB, Yaakop S, Samat A. 2018. Molecular phylogenetic inference of White-Spotted Guitarfish (*Rhynchobatus australiae*) collected from local Malaysian fish markets. Biodiversitas 19: 1382-1386.* The white-spotted guitarfish (*Rhynchobatus australiae*) is in high demand at local Malaysian fish markets because its fins are a valuable food source. To date, few molecular studies have characterized their genetic identity. We have conducted a molecular study to infer the phylogenetic relationships of white-spotted guitarfish, which portray a similar morphology to sharks and rays. The main objective of this study was to determine the phylogenetic position of *R. australiae* using cytochrome oxidase I (COI) sequences of mitochondrial DNA based on fish samples collected from local Malaysian fish markets. This study included nine genetic samples of *R. australiae* and fourteen samples from other members of the shark and ray families, including *Sphyrna lewini* (Sphyrnidae), *Rhizoprionodon oligolinx* and *Carcharhinus sorrah* (Carcharhinidae), *Dasyatis zugei*, *Himantura walga*, *Himantura gerradi*, *Himantura jenkinsii* and *Neotrygon kuhlii* (Dasyatidae). *Chimaera fulva*, a member of the Chimaera family, was used as the outgroup. Sequences in size of ~701 base pairs were successfully obtained from all fish samples. The phylogenetic tree topology was reconstructed using distance-based (neighbor-joining) and character-based (maximum parsimony) methods using MEGA and PAUP software. Results indicated that *R. australiae* formed monophyletic clade and is closely related to sharks (Sphyrnidae and Carcharhinidae). This conclusion was also supported by genetic distance analysis which indicated that Rhynchobatidae and sharks (Carcharhinidae and Sphyrnidae) were closer to each other than to rays (Dasyatidae). This study has proven the efficiency of the COI mitochondrial locus in revealing the phylogenetic position of *R. australiae*. Research findings from this study have increased our understanding of the phylogenetic relationships among guitarfish, sharks, and rays, and their respective taxonomic positions are given their shared morphological characters. This will benefit us in identifying these fish species before consumption from local fish markets.

Keywords: Phylogenetic position, ray, *Rhyncobatus australiae*, shark, White-Spotted Guitarfish

INTRODUCTION

The white-spotted guitarfish (*Rhynchobatus australiae*), also known as the bottlenose wedgefish or white-spotted wedgefish, is found in the western Pacific area including Indonesia, Thailand, the Philippines, and Australia (Compagno and Last 2010). In Malaysia, white-spotted guitarfish are commonly found in Sabah and Sarawak in Borneo (White and McAuley 2003; Giles et al. 2016) while its presence in the Malaysian Peninsula has not been scientifically proven. *Rhynchobatus australiae* is frequently captured in artisanal and commercial fisheries either as a target species or as bycatch (White and McAuley 2003). This fish is highly desired because its dorsal fin and tail have a high value in the global shark fin trade (Giles et al. 2016). In Asia, white-spotted guitarfish meat is considered tasty, and their fins are extremely valuable, especially in the Chinese fin trade (Wong et al. 2009; Last et al. 2010; Dulvy et al. 2014). In South Africa and Australia, guitarfish are hunted as a game due to their large size (Compagno and Last 2010). The IUCN has categorized white-spotted guitarfish as vulnerable in the IUCN Red List due to their declining population size, their high catch rate, and their exploitation throughout Southeast Asia (Compagno and Last 2010; Last et al. 2010; Giles et al. 2016).

In the past, white-spotted guitarfish have been referred to as *Rhynchobatus djiddensis* (Compagno and Last 1999). *Rhynchobatus djiddensis* is found in the Western Indian Ocean from the Eastern Cape Province, South Africa, to the Red Sea. The white-spotted guitarfish is also confused with the smoothnose wedgefish, *Rhynchobatus laevis*, which also occurs in Australia (Compagno 1999). Although *R. australiae* can be distinguished from other species in the genus based on vertebral centra morphological characteristics, the lack of external diagnostic features makes species classification more difficult (Compagno and Last 2010; Giles et al. 2016). Furthermore, genetic identification based on *R. australiae* tissue samples is often difficult due to a lack of correct species reference DNA sequences in Genbank (Giles et al. 2016). To date,

phylogenetic studies on *R. australiae* are still limited, and few studies clarify the phylogenetic relationships of this species regarding morphology or genetics. In addition, the white-spotted guitarfish is thought to be one of the ray species that resemble sharks (Figure 1; Giles et al. 2016).

Much previous research has portrayed relationships between the ray (Batoidea) and sharks (Maisey, 2012). Several hypotheses have been proposed based on morphological data as well as on molecular findings (Shirai 1992, 1996; Winchell et al. 2004). There are many hypotheses about systematic relationships between sharks and rays (Pavan-Kumar et al. 2014). The similarity of overall body morphology requires expertise, primarily in the taxonomic identification of rays (Coulson et al. 2011). Molecular systematic approaches are now commonly used to identify and validate species relationships and avoid the weakness of complex identification keys based on morphological characters (Wong et al. 2009). This has been especially true of the members of the Rhinidae family to which the white-spotted guitarfish belongs. Thus our main objective was to clarify the phylogenetic position of *R. australiae* toward rays and sharks based on DNA sequences from the mitochondrial cytochrome oxidase 1 (COI) gene. All samples were collected from local Malaysian fish markets. Mitochondrial DNA sequences have many advantages in molecular research for species identification and relationships (Ang et al. 2011; Abdul-Latiff et al. 2017) and have proven successful in differentiating species of fish from local markets (Rasmussen and Morrissey 2008; Md-Zain et al. 2018).

MATERIALS AND METHODS

Sample collection, DNA extraction, and polymerase chain reaction (PCR) amplification

A total of 23 fish samples were collected from local fish markets in Sabah (Malaysia Borneo) and Peninsular Malaysia (Table 1). Research methods reported in this manuscript adhered to the legal requirements of Department of Wildlife and National Parks (PERHILITAN) and Malaysian Department of Fisheries. Nine samples were identified as white-spotted guitarfish, five sharks, nine rays and based on morphology described by Last et al. (2010) and Ahmad et al. (2013). A partial tissue sample was taken from each of the individuals for DNA extraction. Total genomic DNA was extracted from 0.02 to 0.05 g of the tissue sample using Invisorb DNA Mini kit (Analytik Jena, Germany), following the manufacturer's protocol.

DNA amplification was performed of the targeted locus, COI (Table 2), in the mitochondrial DNA (mtDNA) (Ward et al. 2005; Ivanova et al. 2007). We used Red Taq Mix (BIOLINE) to conduct the PCR using the following steps: 95 °C initial denaturation for 3 minutes, followed by 30 cycles of denaturation at 95 °C for 1 minute, 52 °C annealing for 15 seconds, and 72 °C extension for 10 minutes. PCR products were viewed using 1.5% agarose gel electrophoresis. The successful PCR products were sent to First Base Laboratories Sdn Bhd (Malaysia) for DNA sequencing (Aifat et al. 2016).

Figure 1. White-Spotted Guitarfish (*Rhynchobatus australiae*)

Table 1. Details of genetic samples

Species/ code	Locality
Rhynchobatus australiae RA1, RA2	Tawau
R. australiae RA4	Malay Peninsular
R. australiae RA5	Sabah
R. australiae RA6, RA7	Tawau
R. australiae RA8, RA9	Johor
R. australiae NC 030254	Thailand
Dasyatis zugei 78, 90	Malay Peninsular
Neotrygon kuhlii 152, 158	Malay Peninsular
Himantura gerrardi 90	Malay Peninsular
H. jenkinsii 433, 434	Malay Peninsular
H. walga 78, 101	Malay Peninsular
Shyrna lewini SL1, SL2	Tawau
Carcharhinus sorrah S3	Sabah
Rhizoprionodon oligolinx S1, S2	Tawau
Chimaera fulva NC014288.1	Genebank

Table 2. Details of primer pairs used in this study

Primer/Reference	Sequence
FISH F2 Ward et al. (2005)	5'TCGACTAATCATAAAGATATCGGCAC3'
FISH R2 Ward et al. (2005)	5'ACTTCAGGGTGACCGAAGAATCAGAA3'
VF2 Ward et al. (2005)	5'TGTAAAACGACGGCCAGTCAACCAACC3'
FR1d Ivanova et al. (2007)	5'CAGGAAACAGCTATGACACCTCAGGGT3'

DNA sequence and phylogenetic analysis

Both forward and reverse DNA sequences were proofread using Bioedit software (Aifat et al. 2016). GenBank BLASTn software confirmed the DNA sequence similarity to that of the target species. DNA sequences were aligned using MEGA 6 (Tamura et al. 2013), and phylogenetic analysis was performed using two methods: the distance-based neighbor-joining (NJ) method, and the character-based maximum parsimony (MP) method. Both analyses were completed using MEGA 6 software (Tamura et al. 2013). The Kimura 2-parameter model was selected for the NJ phylogenetic analysis, while the MP phylogenetic tree was heuristically searched using 1,000 random stepwise additions and a 50% majority rule consensus. The MP tree was then reconstructed using the tree bisection and reconnection algorithm (Abdul-Latiff et al. 2014a, 2014b). Both phylogenetic trees were tested with 1,000 bootstrap replications to obtain the bootstrap confidence level.

RESULTS AND DISCUSSION

Genomic DNA from a total of 24 genetic samples was successfully extracted and amplified. Sequences in size of ~701 bp of COI locus were obtained and subjected to sequence analysis. Of this obtained DNA sequence, 433 (61.77%) bases were conserved, 268 (38.23%) variable and 254 (36.23%) were parsimony informative characters. Genetic distance analysis indicated that white-spotted guitarfish (Rhyncobatidae) are closer to sharks (Carcharhinidae) with a genetic distance of 0.224 than they were to rays (Dasyatidae) where the genetic distance was of 0.233. The highest genetic distance (0.257) was found between the Rhyncobatidae and the outgroup of this study (Chimaeridae). This result indicated that *C. fulva* was correctly chosen as the outgroup.

The NJ (Figure 2) and MP (Figure 3) phylogenetic trees revealed the phylogeny of white-spotted guitarfish. The best MP tree indicated a tree length of 684, a consistency index of 0.588, a homoplasy index of 0.421, a retention index of 0.820 and a rescaled consistency index of 0.482. The NJ and MP tree topology revealed a clear separation between the outgroup, Chimaera, and the in-group, sharks and rays. Two main clades are detected in the NJ tree, with clade A consisting of sharks (*R. oligolinx, C. sorrah* and *S. lewini*) and white-spotted guitarfish, *R. australiae*, and clade B solely composed of members of the ray family (*Himantura* spp., *D. zugei* and *N. kuhlii*). Clade A, consisting of *R. australiae* and sharks (Sphyrnidae and Carcharhinidae), was supported with an 84% bootstrap value.

Furthermore, *R. australiae* formed a monophyletic clade within clade A, supported by a 100% bootstrap value. Two further subclades can be differentiated within *R. australiae*, namely subclade I and II. Subclade I encompassed samples from Sabah and Peninsular Malaysia, while subclade II consisted of samples from Sabah, Peninsular Malaysia, and Thailand (Genbank sequences).

Parallel to the NJ tree, the MP tree portrayed a clear separation between the ingroup (sharks and rays) and the outgroup (Chimaera). Clade formation in the MP tree followed the same topology as the NJ tree, as clade A consisted of sharks and white-spotted guitarfish, while clade B consisted of rays. *R. australiae* formed a monophyletic clade with sharks (Sphyrnidae and Carcharhinidae) supported with a 64% bootstrap value. The same monophyletic clade of *R. australiae* was detected within clade A, thus separating it from sharks. Based on the MP tree, clade B was separated from sharks and guitarfish, supported by 52% bootstrap value.

Results from our phylogenetic analysis revealed a monophyletic clade for rays (Dasyatidae). Sharks (Sphyrnidae and Carcharhinidae) and white-spotted guitarfish (Rynchobatidae) formed a distinct monophyletic clade from each other supported by a 100% bootstrap value. These findings, together with their closer genetic distance to each other, indicated that sharks and guitarfish shared the same common ancestor, while rays were more distantly related. Few phylogenetic studies have been conducted on sharks and batoids, and studies on *R. australiae* are limited. According to Aschliman et al. (2012), rays are a derived group that formed a monophyletic clade, supported by some synapomorphic characters. Thus, classifications of guitarfish as closer to rays (Batoidea) proved to be a contradicting hypothesis. In this study, NJ, and MP phylogenetic tree topology indicated that *R. australiae* formed a monophyletic clade within a clade containing sharks, and this result is concurrent with Maisey (2012).

The phylogenetic study of elasmobranch fishes in our study was based on a 701 bp sequence of the COI gene. However, the COI locus of mtDNA was not employed by either Douady et al. (2003) nor Winchell et al. (2004) in their batoid analyses. However, findings in our study, especially of the phylogenetic tree topology, were similar to those of Douady et al. (2003) and Winchell et al. (2004). This evidence points to a separation of batoids from sharks. Winchell et al. (2004), who analyzed the cytochrome *b* sequences of mtDNA, supported the separation of sharks from batoids, while phylogenetic tree reconstructions by Douady et al. (2003) also found evidence that batoids are a separate lineage from sharks. Douady et al. (2003) proposed an evolutionary scenario where sharks and batoids underwent earlier diversification to form distinctive monophyletic clades.

It is irrefutable that rays formed a monophyletic clade as compared to sharks. Rays have many distinctive and unique morphological characteristics, separating them from sharks. These include internal anatomical structures that support disc set like chest cartilage, anteorbital elements, and synarcual cartilage (Last et al. 2016). The external morphology of *R. australiae* resembles that of sharks (especially from the genus Squatinidae, angel sharks). Numerous other external characters differentiate sharks from rays, especially the positions of gills, which in sharks are on the side of the head, while in rays the gills are underneath the head, closer to the mouth (Ahmad et al. 2008).

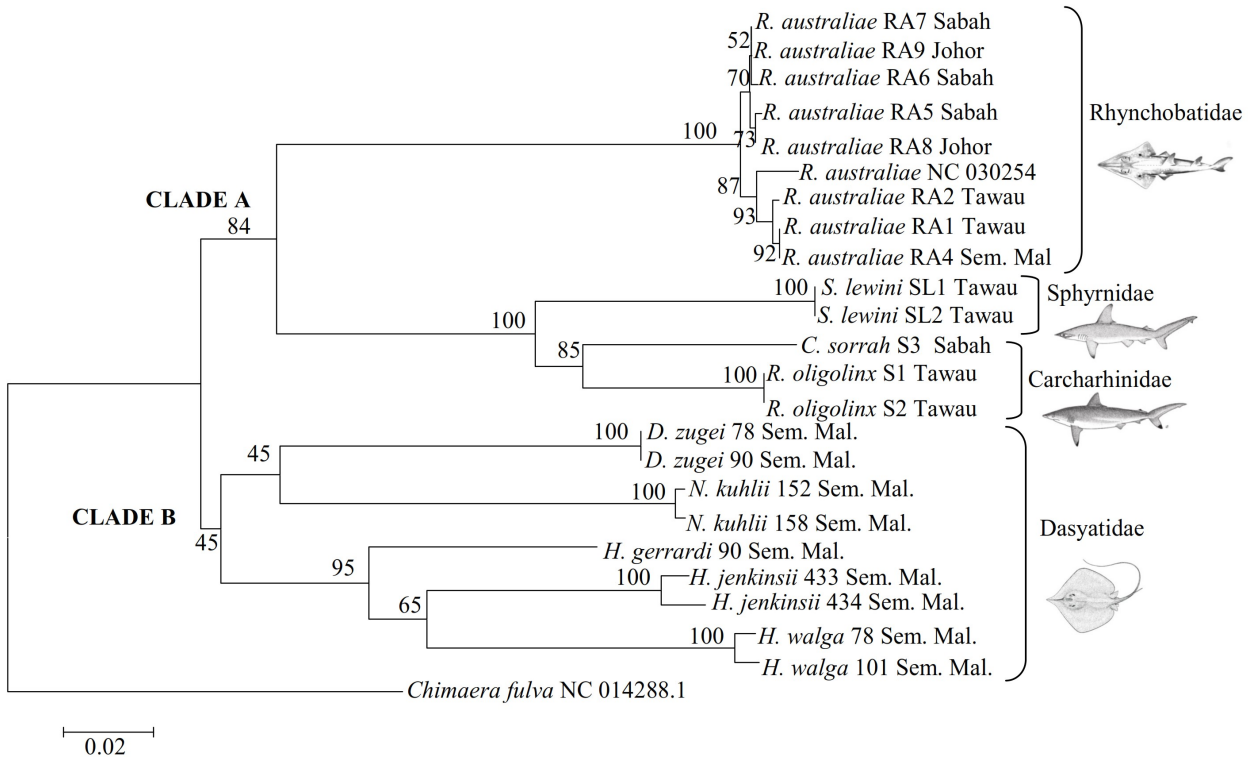

Figure 2. The Neighbor-Joining (NJ) phylogenetic tree of COI gene

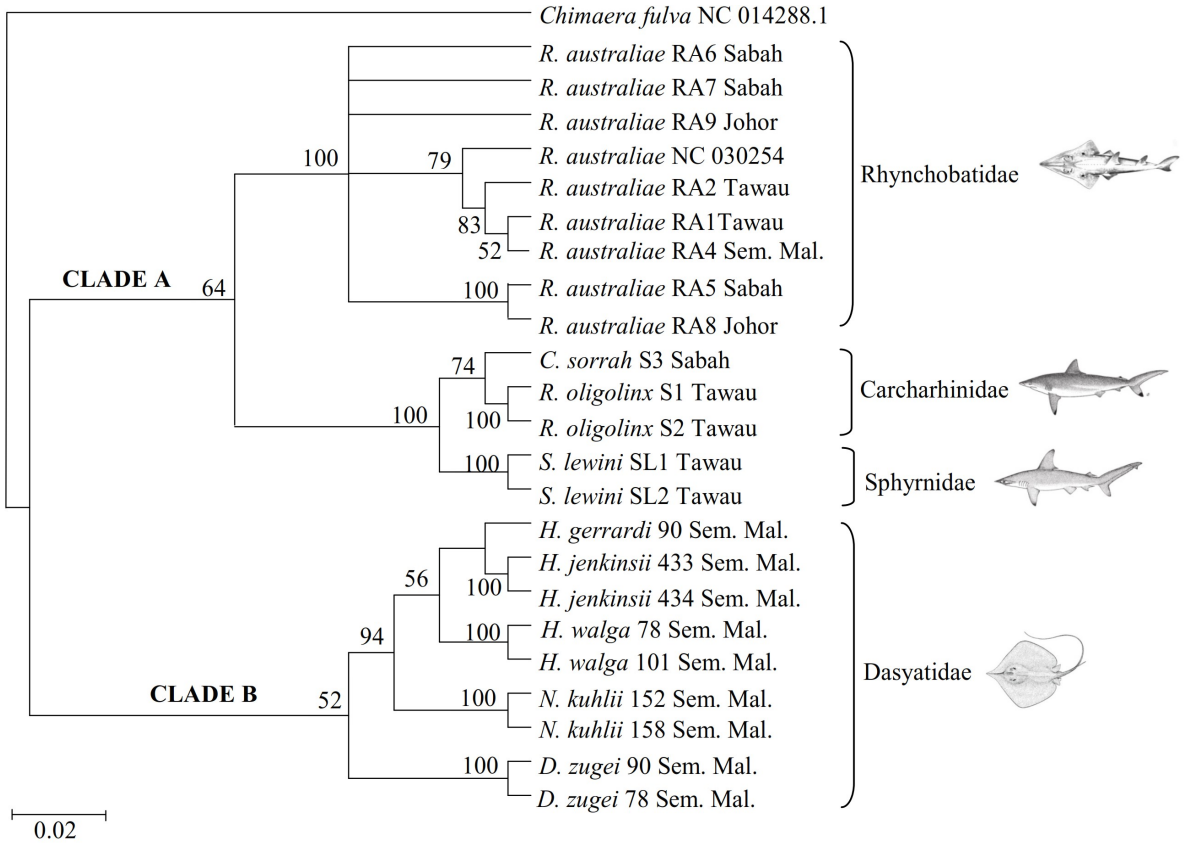

Figure 3. The Maximum Parsimony (MP) phylogenetic tree of COI gene

Findings in this study have proven that the COI locus can determine the phylogenetic and taxonomic position of *R. australiae* and differentiate it from sharks and rays. However, subclade diversifications within *R. australiae* in both NJ and MP tree topologies do not represent geographical patterns of population distribution in Malaysia, which will require alternative markers for better phylogenetic resolution. This implies that the COI locus is suitable for determining the phylogenetic position of guitarfish, but cannot be applied to a biogeographical population-level study. According to Troast et al. (2016), applications of the COI locus alone failed to postulate population structures within species accurately. Thus, we propose that future studies at the population level should exploit other mtDNA gene sequences such as cytochrome *b* and D-loop regions that have been proven to analyze taxa at population levels (Rosli et al. 2011; Ang et al. 2012).

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