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Evidence of Distinct Genetic Stocks of the Bottlenose Wedgefish (*Rhynchobatus australiae*) in the Indo-West Pacific

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Abstract

Populations of the bottlenose wedgefish (*Rhynchobatus australiae*) in the Indo-West Pacific (IWP) have declined by nearly 80% in recent decades. In response, IWP countries are establishing sanctuaries to provide refuge for the fish. However, little is known about the genetic stock structure of the fish in the region. Hence, this study analysed partial sequences (610 base pairs) of the cytochrome oxidase subunit (COI) gene from eight bottlenose wedgefish populations in the IWP to assess the genetic stock structure of the fishery. The sequences revealed that Western Indian Ocean (WIO) populations are genetically distinct from those in the West Pacific (WP) (F_{CT} = 0.24, p = 0.01) and Australia (F_{CT} = 0.88, p = 0.01). Similarly, WP populations were genetically distinct from Australian populations (F_{CT} = 0.42, p = 0.01). This suggests that the IWP contains three genetically distinct stocks of the bottlenose wedgefish: the WIO, WP, and Australia. The indices of genetic diversity and population size showed that the WIO stock has low genetic diversity and population size when compared to the WP and Australia. This shows that efforts to establish elasmobranch sanctuaries in the IWP should take into account the three identified stocks, with priority given to the WIO.

Keywords: Restricted gene flow, genetic connectivity, elasmobranch sanctuaries, Indo-West Pacific Ocean

Introduction

The bottlenose wedgefish *Rhynchobatus australiae* Whitley, 1939 is a large benthopelagic shark-like batoid found throughout the Indo-West Pacific (IWP), from the Western Indian Ocean (WIO) to the Western Pacific (WP) Ocean (White and Last 2013, Bineesh et al. 2017). The fish is distinguished from other wedgefishes by its bottle-shaped snout, and it can be found in inshore waters from near shore to depths of 60 meters (Kyne et al. 2019). The wedgefish has long been used as a food source for many coastal communities in the IWP (Daly et al. 2021). Yet, they have been fished to alarmingly low levels throughout the IWP due to poor management and high demand for their fins in Asian markets (Clark-Shen et al. 2021). As a result, catch records show that stocks of bottlenose wedgefishes have plummeted by roughly 80% in the Arabian Sea and surrounding waters during the last three decades (Valinassab and Dulvy 2018). Similarly, studies show that catch and abundance of bottlenose wedgefish in the Eastern and Western Indian Ocean have declined by over 65% since 1977 (Faizah and Chodrijah 2020, Daly et al. 2021, Wulandari et al. 2021). Because they grow slowly and produce few young, the decline presents an extremely high risk of extinction (Spaet and Berumen 2015). In response, the fish was

classified by the IUCN Red List of Threatened Species as critically endangered globally (Kyne et al. 2019). Similarly, the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) has acted to regulate international trade in bottlenose wedgefish by listing the fish in Appendix II. This implies that the fish cannot be exported to international markets without a permit issued by the authority of the exporting country confirming that it was caught according to national laws, and that the trade is not harmful for the survival of the species (Cardeñosa et al. 2018). Additionally, some countries in the IWP have acted by banning finning and trade of bottlenose wedgefish and their products. Furthermore, since 2009, one country in the WIO and sixteen countries in the Pacific have designated their Exclusive Economic Zones (EEZ) as shark sanctuaries in order to protect and recover bottlenose wedgefish and other elasmobranch by reducing fishing mortality (Ward-Paige and Worm 2017). These sanctuaries currently cover more than 3% of the global ocean, and more countries are likely to follow suit (Ward-Paige 2017). Despite the recent progress towards the establishment of shark sanctuaries, little is known about the genetic stock structure of bottlenose wedgefish in the IWP. The few available data show significant genetic divergence between the Andaman Sea and Southeast Asia ($\Phi_{ST} = 0.249$, $p < 0.00001$) as well as Southeast Asia and Australia ($\Phi_{ST} = 0.260$, $p < 0.00001$), indicating that the fish in these regions should be managed as separate stocks (Giles et al. 2016). Yet, the pattern of genetic connectivity between bottlenose wedgefish populations in the WIO and other populations in the IWP is largely unknown. Because evidence of significant genetic divergence between the WIO, Eastern Indian Ocean (EIO) and WP have been documented in other marine fauna (Otwoma and Kochzius 2016, Huyghe and Kochzius 2018), distinct stocks of bottlenose wedgefish may also exist in the region. Therefore, there is a need to assess the patterns of genetic connectivity among the bottlenose wedgefish in the IWP to evaluate whether there are distinct stocks which should be managed independently. Generally, delineation of stocks is very crucial for effective management, since implementing conservation policies and fisheries management measures without taking genetic stock structures into account often leads into failed recovery and impede sustainable fisheries management (Kerr et al. 2017). Therefore, the aim of this study was to assess the genetic stock structure of the bottlenose wedgefish in the IWP.

Materials and Methods

Study area

This study was conducted in the IWP, which extends from the tropical waters of the WIO to the WP (Figure 1). The region has a very rich diversity of important marine fauna including the bottlenose wedgefish (Rumisha et al. 2015, Kyne et al. 2020). The region is characterized by oceanographic geographies like deep water trenches, very heavy currents and continental land mass that may limit genetic connectivity of fish including the bottlenose wedgefish (Dudgeon et al. 2009). The wedgefish fishery in the region is predominantly artisanal but the fish are also intentionally or incidentally caught by commercial fishers. Due to high exploitation, the bottlenose wedgefish populations have declined throughout the IWP and the fish is increasingly becoming rare in the catch. The IWP contain seventeen shark sanctuaries that were established to provide refuge to the threatened bottlenose wedgefish, one of which is found in the WIO (Figure 1A).

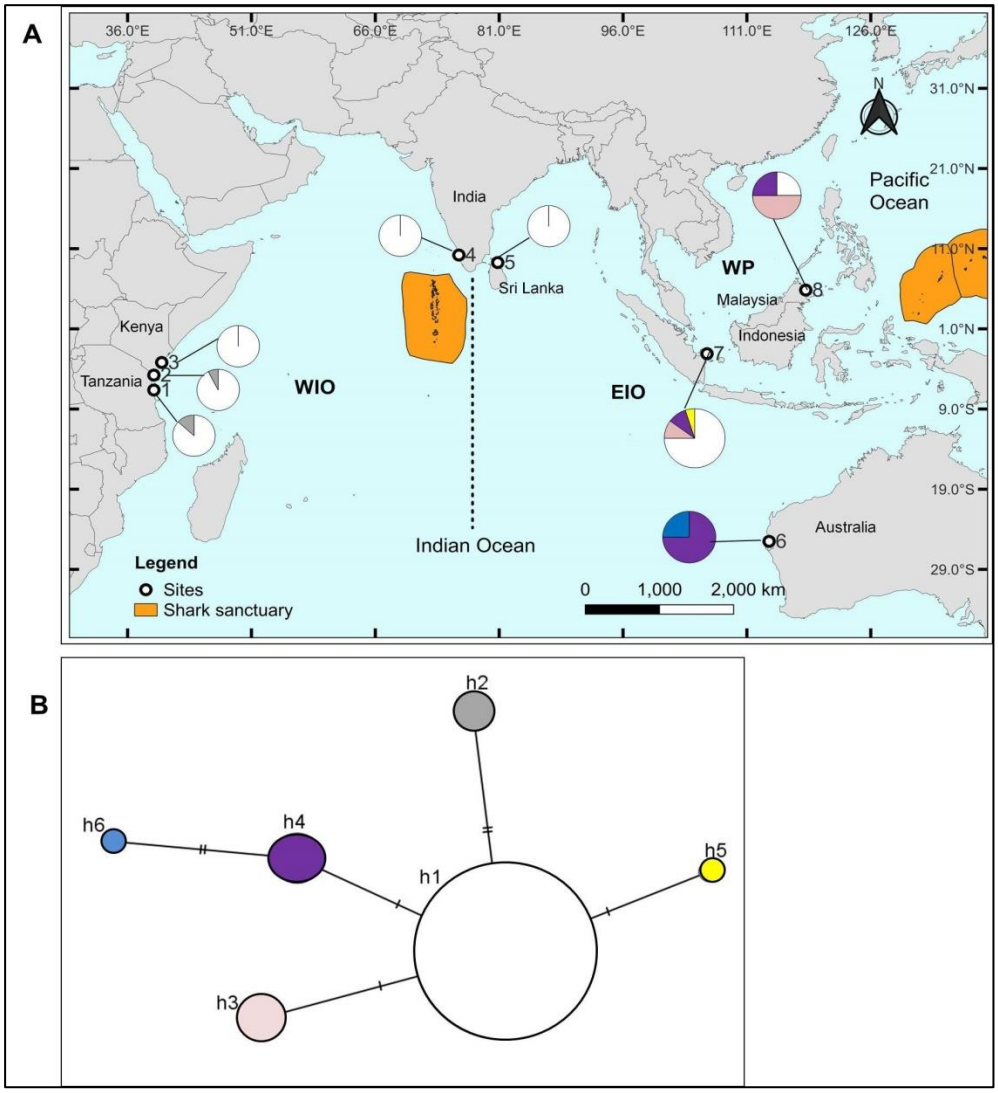

Figure 1 A: Map of the Indo-West Pacific (IWP) showing the sample sites. Pie charts represent the proportion of each haplotype at each site. WIO = Western Indian Ocean, EIO = Eastern Indian Ocean, WP = Western Pacific. **B:** Minimum spanning haplotype network showing the relationship among the partial cytochrome oxidase subunit haplotypes of the bottlenose wedgefish from the IWP. Each circle represents a haplotype (h). Size of each circle is proportional to the number of individuals carrying each haplotype. The central haplotype represents 88 sequences. Hatch marks = number of mutations. For sample sites, see Table 1.

Sampling and DNA extraction

A total of 101 bottlenose wedgefish were sampled from local fishermen at three landing sites in the WIO between January 2020 and June 2022 (Table 1). Because the bottlenose wedgefish are becoming increasingly rare in the catch, sampling was carried out at each site for at least six months and every wedgefish landed was sampled. About 5 g of the muscle tissue was dissected from the pelvic fin of each wedgefish using a sterile surgical blade and preserved in 2 ml sampling tubes containing 99.9% ethanol. The samples were then transported to the

molecular laboratory at Sokoine University of Agriculture (SUA) and stored at -20 °C until further analysis. Genomic DNA was extracted from the sampled tissues using the Quick-DNA™ Mini prep plus kit (Zymo Research Inc, CA, USA) according to the manufacturer's protocol. The quality of the DNA extracts was checked on a 1% agarose gel (Rumisha et al. 2018). Additional 35 cytochrome oxidase subunit (COI) sequences of bottlenose wedgefish from India (JN108018-19, and JN022596), Sri Lanka (MT983930-32), Australia (EU399007-9, and DQ108199), Indonesia (MW509710-29), and Malaysia (MG792125-27, and MG644272) were retrieved from GenBank and included in the analysis (Ward et al. 2008, Bineesh et al. 2014, Peiris et al. 2021) (Table 1).

Table 1: The number of bottlenose wedgefish individuals sampled from the Indo-West Pacific. Dar = Dar es Salaam, WIO = Western Indian Ocean, EIO = Eastern Indian Ocean, WP = Western Pacific, COI* = COI sequences obtained from previous studies (Ward et al. 2008, Bineesh et al. 2014, Peiris et al. 2021).

Site code	Landing site/ Region	Country	Coordinates		Number of samples	COI*
			Latitude	Longitude		
	WIO					
1	Ununio, Dar	Tanzania	-6.62	39.18	46	-
2	Moa, Tanga	Tanzania	-5.05	39.12	26	-
3	Malindi	Kenya	-6.16	39.2	29	-
4	Kochi	India	10.04	75.56	-	4
	EIO					
5	Mullaitivu	Sri Lanka	9.27	80.82	-	3
6	Shark Bay	Australia	-25.5	113.68	-	4
	WP					
7	Bangka Belitunga	Indonesia	-2.09	106.16	-	20
8	Sandakan	Malaysia	5.84	118.12	-	4
Total					**101**	**35**

COI amplification and sequencing

Fragments (610 base pairs) of the COI gene were amplified from each DNA extract in a T100™ Thermal cycler machine (Bio-Lab Inc, GA, USA) using the forward primer FishF1: 5'-TCAACCAACCACAAAGACATTGGCAC-3' and the reverse primer FishR1:5'-TAGACTTCTGGGTGGCCAAAGAATCA-3' (Ward et al. 2005). The reactions were performed in a total volume of 35 µl containing 2 µl template DNA, 5 mg bovine serum albumin, 0.3 µM of forward and reverse primer, and 1 x OneTaq 2X Master mix with standard buffer (New England BioLabs Inc., MA, USA). The following temperature profile was used: 94 °C for 3 min, followed by 35 cycles of 1 min at 94 °C, 1 min at 54 °C and 1 min at 72 °C. Final extension was conducted at 72° C for 10 min. The quality of the PCR products was checked on a 1% agarose gel. Successful amplicons were Sanger dideoxy sequenced using the ABI 3730 DNA Analyzer (Applied Biosystems).

Data analysis

The obtained COI sequences were edited to trim the ends and aligned using the ClustalW algorithm as implemented in the software MEGA ver. 11 (Tamura et al. 2021). Each sequence was then translated into amino acid sequences using the vertebrate mitochondrial genetic code to identify and remove nuclear pseudogenes and sequencing artifacts from the dataset (Bugota and Rumisha 2023). The aligned COI sequences (610 base pairs) were then submitted to GenBank and given the accession numbers ON678555-ON678608. The FaBox (1.61)

online fasta sequence toolbox was used to collapse the sequences into haplotypes. The indices of genetic diversity such as number of polymorphic sites, number of haplotypes, haplotype diversity and nucleotide diversity were calculated using the program Arlequin ver. 3.5 (Excoffier and Lischer 2010). The same program was used to estimate the indices of genetic differentiation among the studied populations. Similarly, the same software was used to compare populations by computing pairwise F_{ST} values and their corresponding significance levels. The F_{ST} p-values were adjusted using the Holm-Bonferroni sequential procedure. Hierarchical AMOVA was performed to determine if there is a significant genetic differentiation between groups of populations. The relationships between the different haplotypes were assessed using a minimum spanning haplotype network constructed with the PopART ver. 1.7 software (Leigh and Bryant 2015). Bayesian estimates of the effective population size (Θ) and pairwise migration rate (m) were estimated by the program MIGRATE-N ver. 3.6.11 (Beerli and Palczewski 2010). The program was run based on a full migration matrix model and Bayesian inferences (Rumisha and Kochzius 2023).

Results

Genetic stock structure

The Analysis of Molecular Variance (AMOVA) revealed significant genetic differentiation between sites (F_{ST} = 0.33, $p < 0.05$: Φ_{ST} = 0.29, $p < 0.05$). Pairwise population F_{ST} comparison showed that the populations of bottlenose wedgefish in the WP are genetically distinct from the populations in the WIO and Australia (Table 2). Similarly, it showed that the populations in Australia are genetically distinct from populations in the WIO. Hierarchical AMOVA grouping of WIO against Australia was significant (F_{CT} = 0.88, $p < 0.01$). Similarly, hierarchical AMOVA showed significant genetic differentiation between WIO and WP (F_{CT} = 0.24, $p < 0.01$) and between WP and Australia (F_{CT} = 0.42, $p < 0.01$). This shows that there are three distinct stocks of bottlenose wedgefish in the IWP. Evidence of distinct stocks of bottlenose wedgefish in the study area was also revealed by the constructed haplotype network. The network showed that some of the haplotypes are restricted in one region and do not occur in other regions (Figure 1B). While haplotype 2 was only restricted in the WIO, haplotype 3 was only restricted in the WP. Similarly, h6 was only observed in Australia, suggesting that there is restricted genetic connectivity between the WIO, WP, and Australia.

Table 2: Pairwise comparison of F_{ST} and F_{CT} values of the bottlenose wedgefish populations in the Indo-West Pacific. Bolded values are significant after Holm-Bonferroni correction

		1	2	3	4	5	6	7	8
Pairwise	1	0							
F_{ST}	2	-0.03	0						
	3	0.06	0.01	0					
	4	-0.10	-0.14	0.00	0				
	5	-0.16	-0.20	0.00	0.00	0			
	6	**0.77**	**0.85**	**0.94**	0.75	0.71	0		
	7	0.04	0.08	**0.15**	-0.04	-0.09	**0.51**	0	
	8	**0.57**	**0.68**	**0.83**	0.44	0.37	0.18	0.23	0
Pairwise			Australi						
F_{CT}		WIO	a	WP					
	WIO	0							
	Australi								
	a	**0.88**	0						
	WP	**0.24**	**0.42**	0					

Genetic diversity

The bottlenose wedgefish from the IWP showed a total of six haplotypes. The most common haplotype accounted for 83.1% of all individuals and it was found at all sites except Australia (Figure 1B). The WIO and EIO each showed two private substitutions, whereas the WP showed three. The WP population had the highest number of haplotypes and the highest haplotype diversity (Table 3). The WIO, on the other hand, had the lowest haplotype and nucleotide diversity. Similarly, Bayesian estimates of the effective population size revealed that the WIO had the smallest population size, and the WP had the largest (Table 4).

Table 3: Indices of genetic diversity among the bottlenose wedgefish (*Rhynchobatus australiae*) from the Indian Ocean and the Pacific Ocean. n = number of COI sequences analysed, nh = number of haplotypes, nps = number of polymorphic sites, h = haplotype diversity and π = nucleotide diversity.

Site	n	nh	nps	Genetic diversity	
				h	π (%)
WIO		**2**	**2**	**0.0778**	**0.0398**
Dar es Salaam, Tanzania	22	2	2	0.1732	0. 0568
Tanga, Tanzania	23	2	2	0.0870	0. 0285
Malindi, Kenya	26	1	-	-	-
India	4	1	-	-	-
EIO		**2**	**2**	**0.5000**	**0.1639**
Sri Lanka	3	1	-	-	-
Australia	4	2	2	0.5	0.1623
WP		**4**	**3**	**0.5326**	**0.0986**
Indonesia	20	4	3	0.4368	0.0778
Malaysia	4	3	2	0.8333	0.1894

Table 4: Bayesian estimates of the effective population size (Θ) and pairwise migration rate (m) among the Indo-West pacific populations of bottlenose wedgefish *Rhynchobatus australiae*. Θ = mutation-scaled effective population size, *m* = mutation-scaled migration rate, WP = Western Pacific, WIO = Western Indian Ocean, Au = Australia.

Region	Θ		*m*		
	Mean	(2.5%, 97.5%)	Direction	Mean	(2.5%, 97.5%)
WIO	0.00057	0.00014, 0.00106	WP → WIO	130	0, 350.7
WP	0.00096	0.00027, 0.00183	Au → WIO	96.5	0, 282.7
Au	0.00071	0.00003, 0.00156	WIO → WP	126.5	0, 351.3
			Au → WP	173.4	0, 409.3
			WIO → Au	99.4	0, 289.3
			WP → Au	104.9	0, 305.3

Discussion

Genetic stock structure

The findings of this study revealed three distinct stocks of the bottlenose wedgefish in the IWP, implying that there is restricted gene flow in the region. Restricted gene flow has also been observed between the bottlenose wedgefish in Australia, WP, and the Andaman Sea (Giles et al. 2016). Similarly, restricted gene flow has also been observed in the IWP spot-tail shark *Carcharhinus sorrah* between Australia and Indonesia (Ovenden et al. 2009) and between Australia, WP and the northern WIO (Giles et al. 2014). Restricted gene flow among most populations of meroplanktons in the IWP has been attributed to sea surface currents and geographical isolation (Huyghe and Kochzius

2018). But because the bottlenose wedgefish do not produce planktonic larvae, the observed population structures can be explained by the importance of the habitat use and oceanographic geographies like deep water trenches. Studies show that some elasmobranch exhibit localized dispersal pattern in the mid and across the shore waters on the continental shelf, with limited evidence of migration across deep water dividing the continental shelf (Giles et al. 2016). Because the bottlenose wedgefish occur in inshore waters less than 60 m deep and exhibit site fidelity (Flowers et al. 2016), the observed genetic separation of the WIO from Australia and WP populations could be attributed to the deep ocean that separates the continental shelf in these regions. The deep ocean between Australia and WIO is probably creating barriers that prevent gene flow, leading to the evolution of genetically distinct populations between the two regions. The genetic separation of the WIO from other IWP population has previously been reported in skunk clownfish (Huyghe and Kochzius 2018), giant tiger prawns (Duda Jr and Palumbi 1999, You et al. 2008), and the starfish *Linkia laevigata* (Otwoma and Kochzius 2016). The observed genetic differentiation between WP and Australia could be attributed to historical vicariance or to contemporary restricted gene flow caused by deep water trenches between the two regions. Deep waters in the Sunda (Java) trench could act as a barrier to gene flow, leading to the observed population subdivision between Australia and the WP. The trench extends from the Sunda Islands past Java, along the southern coast of Sumatra, and on to the Andaman Islands, forming a barrier to gene flow between Western Australia and Indonesia (Chin et al. 2017). Deep sea trenches between Australia and Indonesia have also been linked to genetic subdivision in the spot-tail shark *Carcharhinus sorrah* (Ovenden et al. 2009, Giles et al. 2014), and other elasmobranch (Dudgeon et al. 2009). Historical vicariance due to the Sunda-Sahul land bridge during the lowest sea levels of the Pleistocene could have also restricted gene flow, leading to the observed genetic differentiation between the WP and other populations in the Indian Ocean (Dudgeon et al. 2009, 2012). However, the fact that the most common haplotype was found in both the WIO and WP (Figure 1) suggests that the WIO was colonized by a single recent radiation event that started from the WP, as previously suggested by other researchers (Fratini et al. 2010, Huyghe and Kochzius 2017).

Genetic diversity

The haplotype and nucleotide diversity among the IWP populations of bottlenose wedgefish ranged between 0.077 and 0.83, and 0.028 and 0.18%, respectively. These values are comparable with the levels of haplotype and nucleotide diversity reported in scalloped hammerhead sharks (*Sphyrna lewini*) from the IWP (Hadi et al. 2020). However, the population in the WP showed high haplotype and nucleotide diversity compared to populations in the WIO (Table 3). High genetic diversity in the WP compared to the WIO has also been reported in giant tiger prawns (You et al. 2008), skunk clownfish (Huyghe and Kochzius 2017) and scalloped hammerhead sharks (Hadi et al. 2020). The high genetic diversity in the WP reinforces the hypothesis that the WP is a centre for marine species origins and that populations in the WIO may have resulted from colonization by a recent radiation event that started from the WP. The observed high genetic diversity in the WP suggests that populations in the region have high effective population size compared to the WIO (Hague and Routman 2016). This explanation is supported by the calculated Bayesian estimates of the effective population size which showed that the WIO stock has a low Θ compared to the WP. The low genetic diversity and Θ in the WIO may suggest that the WIO stock is exposed to heavy fishing pressure and that it has been severely exploited compared to the WP. This explanation is consistent with the reported number of shark sanctuaries in the WP and WIO. Since 2009, sixteen shark sanctuaries have been established in the WP and only one in the WIO (Ward-Paige and Worm 2017).

Therefore, high genetic diversity and Θ in the WP is probably due to increased protection resulting from the region's high number of shark sanctuaries, which reduce fishing mortality by prohibiting commercial elasmobranch fishing and the export of elasmobranch products (Ward-Paige 2017).

Because illegal, unreported, and unregulated (IUU) fishing is known to occur in the WIO with wedgefish specifically targeted off the East Africa coast (Kyne et al. 2019), the observed low genetic diversity and Θ in the region is alarming and it suggests that the region should be given priority in future conservation efforts. This is crucial because further reduction in population size could increase genetic drift, thereby increasing the chance of localized extinctions (Hague and Routman 2016). Furthermore, because the bottlenose wedgefish showed limited genetic connectivity between WP and WIO, the WIO stock cannot be replenished by populations from the WP. This implies that increasing the number of elasmobranch sanctuaries in the WP is probably not going to benefit the declining WIO stock. Therefore, there is a need to strengthen management of the bottlenose wedgefish in the WIO to ensure stock recovery. Because the fishery showed high genetic connectivity among sites in the WIO, establishing more elasmobranch sanctuaries and stepping up enforcement of regional and local regulations could benefit the entire WIO stock.

Conclusion

This study revealed significant genetic differentiation among the bottlenose wedgefish populations in the WP, Australia, and the WIO, implying that these regions have limited genetic connectivity and that each stock in each of these regions should be managed separately. Furthermore, it was revealed that the WIO stock has low genetic diversity and Θ compared to the WP. However, since the marker used has a low resolution due to its uniparental inheritance and populations in the WP and Australia were represented by a small number of individuals, the observed patterns need to be verified using hypervariable nuclear markers and more samples from the aforementioned regions. Nonetheless, the fact that significant genetic divergence was detected between the WP and WIO suggests that, despite having high genetic diversity, WP populations cannot replenish the WIO stock. Hence, any conservation efforts in the WP cannot help the WIO stock to recover. Therefore, the ongoing initiatives to establish elasmobranch sanctuaries in the IWP should take into account the three identified stocks, with priority given to the WIO. Because the fishery demonstrated high genetic connectivity among WIO sites, establishing more elasmobranch sanctuaries and strengthening regional and local regulations could benefit the entire WIO stock. Because the Maldives is the only WIO country to have declared over 90,000 square kilometres of its marine waters as a shark sanctuary (Ward-Paige 2017), more WIO countries should follow suit and declare their EEZs as elasmobranch sanctuaries. Studies show that the sanctuaries reduce fishing mortality and could enable the declining bottlenose wedgefish populations to recover (Ward-Paige and Worm 2017).

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