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# Sequence variation among populations of sawfishes (Pristiformes: Pristidae) from Indonesia and Australia

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**Abstract.** *Sutarno, Budiharjo A, Setyawan AD, Lymbery AJ. 2017. Sequence variation among populations of sawfishes (Pristiformes: Pristidae) from Indonesia and Australia. Biodiversitas 18: 850-856.* The sawfishes (Pristiformes: Pristidae) are very rare and critically endangered species globally, calling for conservation efforts around the world. This species is taxonomically interesting because molecular research in recent years has led to re-groupings in some species. The aim of this research was to sequence the control region (CR) of mitochondrial DNA of sawfishes from Indonesia and Australia, compare the sequences between samples, and construct phylogenetic trees to know the relationship of those samples. To achieve these aims, dried rostra of samples from both countries are collected, total DNA were purified and amplified using PCR, followed by sequencing reaction. The sequence data were then lined followed by constructing phylogenetic trees. The results of the BLASTN and phylogenetic tree analysis found two species, the all Australian samples belong to *Pristis pristis* (formerly *P. microdon*), one Indonesian sample also belongs to *P. pristis*, while the other Indonesian samples belong to *Anoxypristis cuspidata*.

**Keywords:** *Anoxypristis cuspidata*, mitochondrial DNA, *Pristis pristis* (*P. microdon*), sawfishes

## INTRODUCTION

Sawfishes (Pristiformes: Pristidae) are a small group of elasmobranch large shark-like batoid of the order Pristiformes, and the unique family of Pristidae (Compagno and Last 1999; Sutarno et al. 2012). The taxonomy of sawfishes is confusing (c.f. Sutarno et al. 2012), but refer to current molecular and morphological studies there are five species of sawfishes from two genera, *Pistis* and *Anoxypristis* (Last et al. 2016). Faria et al. (2013) suggest that based on a combination of mitochondrial DNA and morphological characters, the three previously known species (*Pristis pristis, P. microdon* and *P. perotteti*) appear to be one species, *Pristis pristis*.

The all five species of sawfishes are found in the Indo-West Pacific, where *Anoxypristis cuspidata* (Latham, 1794), *Pristis pristis* (Linnaeus, 1758) (formerly known as *Pristis microdon* Latham, 1794), and *Pristis zijsron* (Bleeker, 1851) has widespread distribution; and *Pristis clavata* (Garman 1906) appears to be limited to northern Australia (Compagno and Last 1999; Last and Stevens 2009; Phillips et al 2011); whereas, *Pristis pectinata* (Latham, 1794) reportedly originated from the Red Sea and East Africa to the Philippines (Compagno and Last 1999; Last and Stevens 2009; Phillips et al. 2011). In the East-Pacific Atlantic are found two species, including *P. pristis* and *P. pectinata* (Compagno and Last 1999; Last and Stevens 2009; Faria et al. 2013). In Southeast Asian waters, these five species of sawfishes can be recorded, including *Pristis pristis (P. microdon), P. clavata, P. zijsron, P. pectinata* and *Anoxypristis cuspidata* (Sutarno et al. 2012).

Sawfishes are characterized by a distinctive flattened, greatly elongated rostrum armed on each side with a row of the large transverse teeth, presumably used for hunting and defense (Bigelow and Schroeder 1953; Wueringer et al. 2009; Sutarno et al. 2012). The rostral teeth grow continuously from the base and attach to the rostrum through the alveoli (Slaughter and Springer 1968; Compagno and Last 1999). The peduncle is not expanded and the dentine cap is easily removed (Slaughter and Springer 1968). The maximum total length is around 7 m (Last and Stevens 1994).

Sawfishes usually inhabit shallow inner shelf and coastal habitat, in tropical and subtropical waters including estuarine and freshwater habitats (Nelson 2006; Wueringer et al. 2009; Waters et al. 2014; Hollensead et al. 2015), as well as marine environments to a maximum depth of 122 m (McEachran and de Carvalho 2002; Simpfendorfer 2006). While presenting a wide spectrum of salinity, sawfish prefer coastal marine and estuarine habitats (Peverell 2005; Whitty et al. 2009; Norton et al. 2012). They can migrate from euryhaline, brackish and freshwaters habitats, as a possible behavioral mechanism to cleanse the body of undesirable parasites (Morgan et al. 2010).

Sawfishes have been important as a source of food and medicines as well as religious and cultural symbols (Gonzalez MMB. 2005; Robillard and Séret 2006; Clarke et al. 2007). The pressure from commercial and recreational fishing, the loss of near-shore habitat due to development, and low reproductive potential lead to a decrease in population (Thorson 1982; Simpfendorfer 2000; Seitz and Poulakis 2006; Carlson and Simpfendorfer

2015). Nowadays, sawfishes are among the most threatened marine fishes, with declining numbers and reduced distributions worldwide (Wueringer et al. 2009; Simpfendorfer 2005; Faria et al. 2013). All five species face a very high risk of global extinction (Dulvy et al. 2014). The decline has been observed in all parts of their distribution range (Leeney and Poncelet 2013; Moore 2014; Fernandez-Carvalho et al. 2014). Sawfishes are highly sensitive to exploitation and habitat destruction due to their large size and their coastal and riverine habitats (Robillard and Séret 2006).

All five sawfishes species have now been listed as *endangered* or *critically endangered* species by the International Union for Conservation of Nature (Carlson et al. 2013, D'Anastasi et al. 2013; Kyne et al. 2013a, 2013b; Simpfendorfer 2013; IUCN 2017). They have also been listed in Appendix I of CITES, which prohibits their international trade (CITES 2007). In Indonesia, populations of sawfishes have declined severely, with recent surveys of several sites where the species has historically been recorded, including Lake Sentani and the Mahakam River, finding no individuals. Legislation has recently been enacted to protect all member of *Pistis* genus (as well as *Anoxypristis*) in Indonesia, where overfishing and habitat destruction has severely reduced population numbers (PP 7/1999). Population declines in northern Australia have not been as severe, although the species is still listed as *vulnerable* under the Australian Environmental Protection and Biodiversity Conservation Act 1999 (EPBC 1999).

Genetic studies are essential for the effective conservation of sawfishes, because (i) there is confusion over the taxonomic status of sawfishes (Faria et al. 2013; Sutarno et al. 2012), and (ii) population genetic data can be used provide information on current and historical population sizes and migration rates, which determine how the species should be managed. Molecular genetic studies using mitochondrial DNA sequencing analysis was used in the research. Skin tissue from dried rosta of sawfishes was obtained from collections and purchased from individuals and markets in Indonesia. This means that genetic data were obtained using previously collected samples, without the need to obtain further specimens of this highly endangered species to determine the relationship and taxonomic status of Australian and Indonesian populations of sawfishes.

The aim of this research was to sequence the control region (CR) of mitochondrial DNA of sawfishes from Indonesia and Australia, compare the sequences between samples, and construct phylogenetic trees to know the relationship of those samples.

## MATERIALS AND METHODS

*Sampling*

Tissue samples were obtained from dried rostra sourced from the museum, university and private collections in Indonesia and Australia, and purchased from markets throughout Indonesia (from a five-year survey, 2011-2015). Fifteen samples from Australian collection (DNA material stored in the Fish Health Unit, Murdoch University, Australia) and seven samples from Indonesian collection (rostra stored at the Department of Biology, Universitas Sebelas Maret, Surakarta, Indonesia; this is part of 24 samples collected over five years of field research) were used in this study. A total of 22 samples were assessed. Sampling from dried rostra obviates the need to catch and obtain tissue samples of these rare and threatened fishes.

*DNA extraction*

Total genomic DNA will be extracted from tissue obtained from dried rostra using a Masterpure™ (Epicentre Technologies, Sydney) DNA extraction kit, and following the procedures of Phillips (2006) and Phillips et al. (2009). To minimize the risk of contamination with non-target DNA, all genetic work involving dry rostra samples will be carried out referring to the protocols for working with ancient DNA set out by Mulligan (2005). The quantity and quality of the extracted DNA will be assessed through the appearance of a 2 µL aliquot of the extract on a 2% agarose gel subject to electrophoresis for 25 min at 46 mAmps, stained with ethidium bromide, and illuminated with UV light.

*mtDNA sequencing*

Polymerase chain reaction (PCR) will be used to amplify a 353-351-bp portion of the control region of the mtDNA of sawfishes using the forward primer (CRF: 5'-ACGTATCCGTAATACTCAT) and reverse primer (CRR: 5'-ATGCAAATATTATGTCGAGGGTAG), as described by Phillips et al. (2008). The PCR amplification will be carried out in a reaction mixture containing about 10 ng of DNA template, 10 mM TAQ buffer with 1.5 mM $MgCl_2$ (Roche), 0.1 mM of each of the dNTPs (Promega), 0.5 U of Taq polymerase (Roche), 20 µmol of each primer, and adjusted to the final volume of 50 µL with PCR-grade water. The amplification conditions will consist of an initial 5 min denaturation phase at 94°C, followed by 35 cycles, each cycle consisting of 30 sec of denaturation at 94°C, 30 sec of annealing at 59°C, and 30 sec of extension at 72°C; followed by a final 7 min extension phase at 72°C.

Prior to sequencing, the PCR products will be cleaned by using Qiaquick columns (Qiagen), referring to the manufacturer's protocol. The sequencing will be carried out by using the dye terminator cycle sequencing method. Each sequencing reaction will be prepared using approximately 30 ng of clean PCR product, 3.2 pmol of the forward or reverse primer and a Big Dye 3.1 terminator cycle sequencing ready reaction kit following the manufacturer's protocol (Applied Biosystems Inc. 2001). The sequencing products will be electrophoresed, and the raw data chromatograms generated using an Applied Biosystems 3230 DNA Analyzer automated sequencer.

*mtDNA data analysis*

The forward and reverse sequences of the mitochondrial control region will be aligned using GeneTool™ Lite v1.0, the primer sequences removed from both ends and a forward reading consensus sequence generated. Tajima's

(1989) and Fu's (1997) Fs-tests will be applied to test whether the patterns of variation in the control region sequences are selectively neutral. The statistical significance of the tests will be assessed by randomly sampling the data under the assumptions of selective neutrality and population equilibrium. The level of genetic diversity within populations will be described by haplotype diversity (h), nucleotide diversity ($\pi$) and the standardized number of haplotypes (SNH), estimated using ARLEQUIN v3.1. (http://cmpg.unibe.ch/). Appropriate values of the gamma correction for the control region haplotypes will be empirically determined with the software GZ gamma (Gu and Zhang 1997). The number of haplotypes observed in a sample strongly depends on the size of the sample; haplotype numbers will, therefore, be standardized according to the numbers of individuals present in the smallest sample involved in the comparison.The percentage of similarity between CR region of mtDNA of Indonesian and Australian will be compared to the central data of GeneBank using BLASTN (https://blast.ncbi.nlm.nih.gov/).

Analysis of molecular variance, implemented with ARLEQUIN v3.1, will be used to assess how genetic variation is partitioned within and between populations from different geographic localities, with the statistical significance of the variance estimates assessed using a nonparametric permutation approach. Exact tests will be used to ascertain whether the haplotype frequency distributions in selected pairs of samples are significantly different to each other.

Evolutionary relationships among haplotypes will be estimated by constructing a haplotype network using the parsimony method of Templeton et al. (1992) and implemented with the software TCS v1.21 (http://darwin.uvigo.es/software/tcs.html). This method estimates the maximum number of substitutions required to connect any two haplotypes parsimoniously (with 95% confidence) and builds the network by firstly linking sequences with the smallest number of differences.

## RESULTS AND DISCUSSION

### Phylogenetic relationship

The results of the sequencing reaction to 22 samples from Indonesia and Australia have then checked the similarity with BLASTN data, and the results are presented in Table 1.

The similarity percentage between CR region of mtDNA of Indonesian and Australian sawfishes after comparing it to the central data of GeneBank using BLASTN indicated that there are two species of the sawfishes found in Indonesia, *P. pristis* and *A. cuspidata*. The *P. pristis* was found from Samarinda, a region in Kalimantan (Borneo) island, while *A. cuspidata* was found from Merauke in Papua island. Unfortunately, the sample of Samarinda was only found 1 sample from this island due to the species was facing extinction since couple years ago. The sawfishes samples from Australia all belong to *P. pristis*. The sequences resulted were analyzed using the program MEGA 5.10 to construct the phylogenetic tree, and the result is shown in Figure 1.

The phylogenetic trees presented in Figure 1 indicated the relationship between the population of *P. pristis* from Samarinda and Australia. The *Pristis* of Samarinda population is at the same taxonomic group of most populations from Australia, indicating that the Samarinda populations have high sequence similarity compared to most Australian populations. While samples of sawfishes from Merauke in Papua island are all indicating taxonomic different, and it belongs to species of *A. cuspidata*.

Similarity analysis using BLASTN between the sequence of CR region of Sawfishes from Indonesia and Australia to the central data of GeneBank indicated that there are variations found in those samples both from Indonesia and Australia. Analysis of phylogenetic trees to find out the relationship between those samples indicated that one of Indonesian *Pristis* (from Samarinda) is closely related to PM1023, PM10220, PM3196, PM1027 and Flyma03; the other samples from Merauke belongs to different species of origin compared to any other samples studied, *A. cuspidata*.

### Conservation effort

The decline and extinction of sawfishes have been reported or found from across the Indonesian archipelago. This is evident from five years of field and market surveys (2011-2015) in various regions of Indonesia, where part of the sample is used in this study. The field research and market surveys cover the western coast of Sumatra and the Mentawai Islands, the northern coast of Sumatra (Aceh), the eastern coast of Sumatra (Jambi), the western coast of Kalimantan and the mouth of Kapuas and Peniti rivers, the southern coast of Kalimantan and the mouth of the Barito River; the Mahakam River estuary in East Kalimantan, the coastal areas of Manado (Sulawesi), the coastal areas of Sorong and Manokwari (west of Papua), the northern coast of Papua and Lake Sentani, and the river estuaries and coastal areas of Merauke, southern Papua, and several Fish Auction Places (TPI) in Java's northern coastal fishing port, especially in Juwana and Pekalongan.

In the field research and market surveys (2011-2015), there are only 24 individual sawfishes were recorded, represented by the rostrum. A total of two samples of *P. pristis* were recorded, i.e. from Lake Sentani, Papua (1 sample) and Mahakam river estuary, East Kalimantan (1 sample was used in this study and the identity was confirmed as *P. pristis*, under the code of Samarinda); besides, 21 *A. cuspidata* was recorded from south coast of Merauke (6 samples were used in this study and the identity was confirmed as *A. cuspidata*, under the code of Merauke, HGM1, HGM2, PMB141, PMB148, PMC143).

**Table 1.** Percentage of similarity between CR region of mtDNA of Indonesian and Australian sawfishes comparing to the central data of GeneBank using BLASTN

| Sequence | Query length | The most sequence having similarity | Identity | Access no. |
|---|---|---|---|---|
| **Australia** | | | | |
| 1121 (Australia) | 397 | *Pristismicrodon* haplotype 1 control region, partial sequence; mitochondrial | 99% | GQ980006.1 |
| | | *Pristismicrodon* haplotype 2 control region, partial sequence; mitochondrial | 99% | GQ980007.1 |
| 7879 (Australia) | 401 | *Pristismicrodon* haplotype 1 control region, partial sequence; mitochondrial | 99% | GQ980006.1 |
| | | *Pristismicrodon* haplotype 2 control region, partial sequence; mitochondrial | 99% | GQ980007.1 |
| 7881 (Australia) | 402 | *Pristismicrodon* haplotype 1 control region, partial sequence; mitochondrial | 99% | GQ980006.1 |
| | | *Pristismicrodon* haplotype 2 control region, partial sequence; mitochondrial | 99% | GQ980007.1 |
| 7883 (Australia) | 401 | *Pristismicrodon* haplotype 1 control region, partial sequence; mitochondrial | 99% | GQ980006.1 |
| | | *Pristismicrodon* haplotype 2 control region, partial sequence; mitochondrial | 99% | GQ980007.1 |
| 7885 (Australia) | 399 | *Pristismicrodon* haplotype 1 control region, partial sequence; mitochondrial | 99% | GQ980006.1 |
| | | *Pristismicrodon* haplotype 2 control region, partial sequence; mitochondrial | 99% | GQ980007.1 |
| 7891 (Australia) | 401 | *Pristismicrodon* haplotype 1 control region, partial sequence; mitochondrial | 99% | GQ980006.1 |
| | | *Pristismicrodon* haplotype 2 control region, partial sequence; mitochondrial | 98% | GQ980007.1 |
| 7899 (Australia) | 401 | *Pristismicrodon* haplotype 1 control region, partial sequence; mitochondrial | 99% | GQ980006.1 |
| | | *Pristismicrodon* haplotype 2 control region, partial sequence; mitochondrial | 99% | GQ980007.1 |
| 7901 (Australia) | 329 | *Pristismicrodon* haplotype 1 control region, partial sequence; mitochondrial | 93% | GQ980006.1 |
| | | *Pristismicrodon* haplotype 2 control region, partial sequence; mitochondrial | 93% | GQ980007.1 |
| Flyma02 (Australia) | 404 | *Pristismicrodon* haplotype 1 control region, partial sequence; mitochondrial | 99% | GQ980006.1 |
| | | *Pristismicrodon* haplotype 2 control region, partial sequence; mitochondrial | 99% | GQ980007.1 |
| Flyma03 (Australia) | 410 | *Pristismicrodon* haplotype 1 control region, partial sequence; mitochondrial | 99% | GQ980006.1 |
| | | *Pristismicrodon* haplotype 2 control region, partial sequence; mitochondrial | 99% | GQ980007.1 |
| PM10220 (Australia) | 370 | *Pristismicrodon* haplotype 1 control region, partial sequence; mitochondrial | 89% | GQ980006.1 |
| | | *Pristismicrodon* haplotype 2 control region, partial sequence; mitochondrial | 89% | GQ980007.1 |
| PM10231 (Australia) | 395 | *Pristismicrodon* haplotype 1 control region, partial sequence; mitochondrial | 99% | GQ980006.1 |
| | | *Pristismicrodon* haplotype 2 control region, partial sequence; mitochondrial | 99% | GQ980007.1 |
| PM1027 (Australia) | 407 | *Pristismicrodon* haplotype 1 control region, partial sequence; mitochondrial | 99% | GQ980006.1 |
| | | *Pristismicrodon* haplotype 2 control region, partial sequence; mitochondrial | 99% | GQ980007.1 |
| PM1028 (Australia) | 425 | *Pristismicrodon* haplotype 1 control region, partial sequence; mitochondrial | 99% | GQ980006.1 |
| | | *Pristismicrodon* haplotype 2 control region, partial sequence; mitochondrial | 99% | GQ980007.1 |
| PM3196 (Australia) | 411 | *Pristismicrodon* haplotype 1 control region, partial sequence; mitochondrial | 99% | GQ980006.1 |
| | | *Pristismicrodon* haplotype 2 control region, partial sequence; mitochondrial | 99% | GQ980007.1 |
| **Indonesia** | | | | |
| Merauke | 364 | *Anoxypristis cuspidata* haplotype 2 D-loop, partial sequence; mitochondrial | 93% | JQ026199.1 |
| HGM1 (Merauke) | 405 | *Anoxypristis cuspidata* haplotype 2 D-loop, partial sequence; mitochondrial | 99% | JQ026199.1 |
| HGM2 (Merauke) | 404 | *Anoxypristiscuspidata* haplotype 2 D-loop, partial sequence; mitochondrial | 99% | JQ026199.1 |
| PMB141 (Merauke) | 402 | *Anoxypristiscuspidata* haplotype 6 D-loop, partial sequence; mitochondrial | 98 % | JQ026203.1 |
| PMB148 (Merauke) | 342 | *Anoxypristiscuspidata* haplotype 5 D-loop, partial sequence; mitochondrial | 92 % | JQ026202.1 |
| PMC143 (Merauke) | 368 | *Anoxypristiscuspidata* haplotype 5 D-loop, partial sequence; mitochondrial | 99% | JQ026202.1 |
| Samarinda | 395 | *Pristismicrodon* haplotype 1 control region, partial sequence; mitochondrial | 99% | GQ980006.1 |
| | | *Pristismicrodon* haplotype 2 control region, partial sequence; mitochondrial | 99% | GQ980007.1 |

Note: Refer to Faria et al. (2013), *Pristis microdon* (Latham, 1794) should be included in *Pristis pristis* (Linnaeus, 1758)

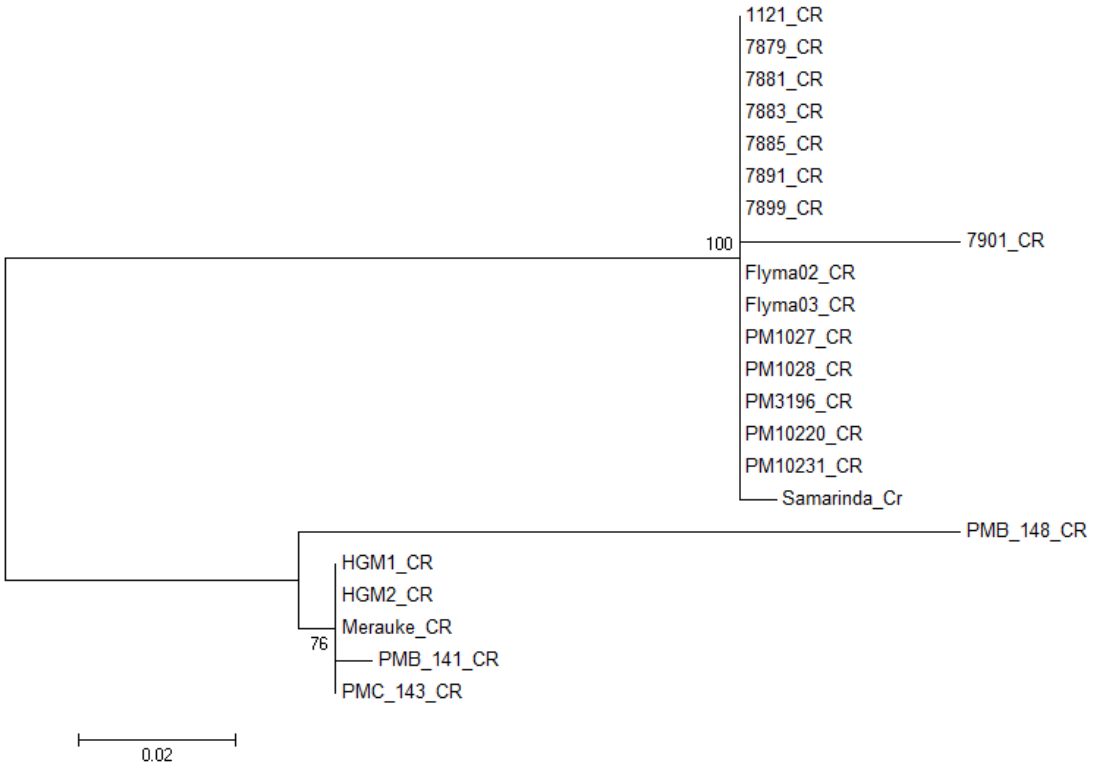


**Figure 1.** Phylogenetic trees of Indonesian and Australian sawfishes based on the CR gene sequences analyzed using MEGA 5.10.

In the previous 20-25 years, fishermen in the surveyed places still catch and sell sawfishes, but at the time of the study (2011-2015), fishermen could no longer catch sawfishes, except on the coast of Merauke. The intensive research at Merauke in 2014 shows that sawfishes rostrum are sold freely as souvenirs in stores around the harbor, but to bring out the island special permits are required. Some fishermen said still to catch and sell sawfishes and offer to provide fresh (or alive) sawfishes caught on the demersal coast or river mouths of the southern coast of Merauke, between Yos Sudarso Island and Wasur National Park. The lack of public awareness to keep the animals protected by law (PP 7/1999) and inadequate law enforcement causing hunting, trade, and consumption of protected animals is still common in this region, not only for sawfishes but also rays, sharks, deer, kangaroos, birds, etc. The pressure of development and conversion of natural habitats is likely to immediately reduce the protected animals if the environmental law enforcement and public awareness to participate does not arise. In PP 7/1999 it is said that the protected sawfishes are *Pistis* spp., but the *Anoxypristis cuspidata* should also be treated equally because this is only a taxonomic case.

In Australia, all sawfishes species have decreased significantly, although most are unquantified. In places, viable populations exist, representing some of the last surviving populations in the Indo-West Pacific, with Australia being one of a number of global strongholds for sawfishes (Stevens et al. 2005), they can find in the Gulf of Carpentaria, Western Australia, Northern Territory and Queensland coastlines, with the distribution of each species and gender is not always equitable (Phillips et al 2011).

The rate of decline in the Genus *Pristis* is generally higher than that of *A. cuspidata*, because the latter species have smaller body sizes that require less food and space, and have a high reproductive capacity. *P. pristis* and *A. cuspidata* are the most distributed species, but in places where some sawfishes are found, *A. cuspidata* is the easiest to be found (Thorburn et al. 2007; Peverell 2009). Although A. cuspidata is relatively common throughout their global distribution, it is relatively declined throughout its range. The accurate information about the decline of sawfishes population is difficult because baseline data is not species-specific (Peverell 2009; D'Anastasi 2010; Harry et al. 2011), misidentification, weak reporting and not appropriate for the observation program. In general, the decline of sawfishes population occurred since the 1960s and in the last 20 years, the remaining population tends to be 20% from the 1960s (D'Anastasi et al. 2013). The appropriate regulation, law enforcement, accurate databases and the growth of environmental awareness are expected to prevent the extinction of sawfishes populations, although unavoidable global climate change can affect the population.

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