

## Research Article

**Cite this article:** Vilasboa A, Lamarca F, Solé-Cava AM, Vianna M (2022). Genetic evidence for cryptic species in the vulnerable spiny butterfly ray *Gymnura altavela* (Rajiformes: Gymnuridae). *Journal of the Marine Biological Association of the United Kingdom* **102**, 345–349. <https://doi.org/10.1017/S002531542200056X>

Received: 7 May 2021

Revised: 30 May 2022

Accepted: 21 June 2022

First published online: 30 August 2022

### Key words:





Barcode; conservation; cytochrome oxidase I; Eastern Atlantic; Elasmobranch; Mediterranean; Western Atlantic

### Author for correspondence:

Anderson Vilasboa,

E-mail: [anderson.vasconcellos@uerj.br](mailto:anderson.vasconcellos@uerj.br)

# Genetic evidence for cryptic species in the vulnerable spiny butterfly ray *Gymnura altavela* (Rajiformes: Gymnuridae)

Anderson Vilasboa<sup>1</sup> , Felipe Lamarca<sup>1,2</sup> , Antônio M. Solé-Cava<sup>3</sup>   
and Marcelo Vianna<sup>2,4</sup> 

<sup>1</sup>Department of Genetics, Laboratório de Genética Pesqueira e da Conservação, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, RJ, Brazil; <sup>2</sup>Department of Marine Biology, Laboratório de Biologia e Tecnologia Pesqueira, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil; <sup>3</sup>Department of Genetics, Centro Nacional para a Identificação Molecular do Pescado, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil and <sup>4</sup>Rio de Janeiro Marine Aquarium Research Center (IMAM, AquaRio), Rio de Janeiro, Brazil

## Abstract

The spiny butterfly ray *Gymnura altavela* (Linnaeus 1758) is classified as Endangered by the International Union for Nature Conservation (IUCN) and considered Critically Endangered in the Mediterranean Sea and South-west Atlantic. Despite its status, few studies on the population biology and genetics for the species are available, undermining conservation efforts. In the present study, we evaluated the taxonomic status of *Gymnura altavela* along both sides of the Atlantic using COI sequences. Our genetic data support the existence of two genetically differentiated *G. altavela* lineages (West and East Atlantic) and further studies are required to evaluate the hypothesis that these lineages could, in fact, be cryptic species within this endangered batoid's genus.

## Introduction

Butterfly rays, *Gymnura* van Hasselt, 1823, are marine and demersal batoids displaying a worldwide geographic distribution, occurring in shallow waters with sandy and muddy bottoms in tropical and temperate climates (Muktha *et al.*, 2016). Although *Gymnura* genus rays are easily distinguished from other ray species, significant morphological similarities are noted among congeneric species, making their taxonomy complex. Three nominal species have been described in the Atlantic, by 2017, namely *G. altavela* (Linnaeus 1758), *G. natalensis* (Gilchrist & Thompson 1911) and *G. micrura* (Bloch & Schneider 1801). Yokota & de Carvalho (2017) presented an extensive taxonomic revision of *Gymnura micrura* based on external and internal morphological features and considering specimens from the entire Atlantic Ocean geographic distribution. These authors redescribed *G. micrura* and two newly described species (*G. lessae* and *G. sereti*) previously included in *G. micrura*, highlighting the possibility of the occurrence of cryptic species within the genus. The possible occurrence of cryptic diversity is especially worrisome in species exploited by fisheries with a marked reduction in population size, as in the case of various *Gymnura* species.

The spiny butterfly ray, *Gymnura altavela* (Linnaeus 1758), is considered as presenting an amphiatlantic distribution, at depths ranging from 5–100 metres, targeted by intense commercial fisheries along its distribution (Yokota *et al.*, 2016; ICMBio, 2018). In the western Atlantic Ocean, the species is distributed from New England (USA) to Argentina (Alkusaury *et al.*, 2014) (Figure 1). In Brazil, it is classified as a resident breeder and its presence is confirmed only for the south–south-east region. Furthermore, the only currently known nursery area for the species on the Brazilian coast is Guanabara Bay (Rio de Janeiro) (Gonçalves-Silva & Vianna, 2018a, 2018b). In the eastern Atlantic, this species has been recorded from Portugal to Angola (ICMBio, 2018) (Figure 1), and in the Mediterranean Sea, spread from Gibraltar to Lebanon (McEachran & Capapé, 1984). Spiny butterfly rays are economically important where they occur (Alkusaury *et al.*, 2014), and landings of the species in the Mediterranean have fluctuated spatially and in time (Capapé *et al.*, 1992).

*Gymnura altavela* is currently classified as globally Endangered (criteria A2d) by the International Union for Nature Conservation (IUCN) (Dulvy *et al.*, 2021) and locally considered Critically Endangered in both the Mediterranean Sea (Walls *et al.*, 2016) and in Brazil (ICMBio, 2018).

On a time scale of only three generations (33 years, from 1982–2015), estimates based on landing data indicate that *G. altavela* populations have declined 42.5% in Morocco, 54% in Senegal and 98.8% in southern Brazil, with an overall population decline estimated as 50–79% during this time frame (Dulvy *et al.*, 2021). This dramatic reduction in population size is due to strong fisheries pressure, both as targeted and as bycatch due to the use of multiple types of fishing gear.

Despite its status, few studies on the species population biology and genetics are available, undermining conservation efforts, although it has increasingly attracted attention in the last



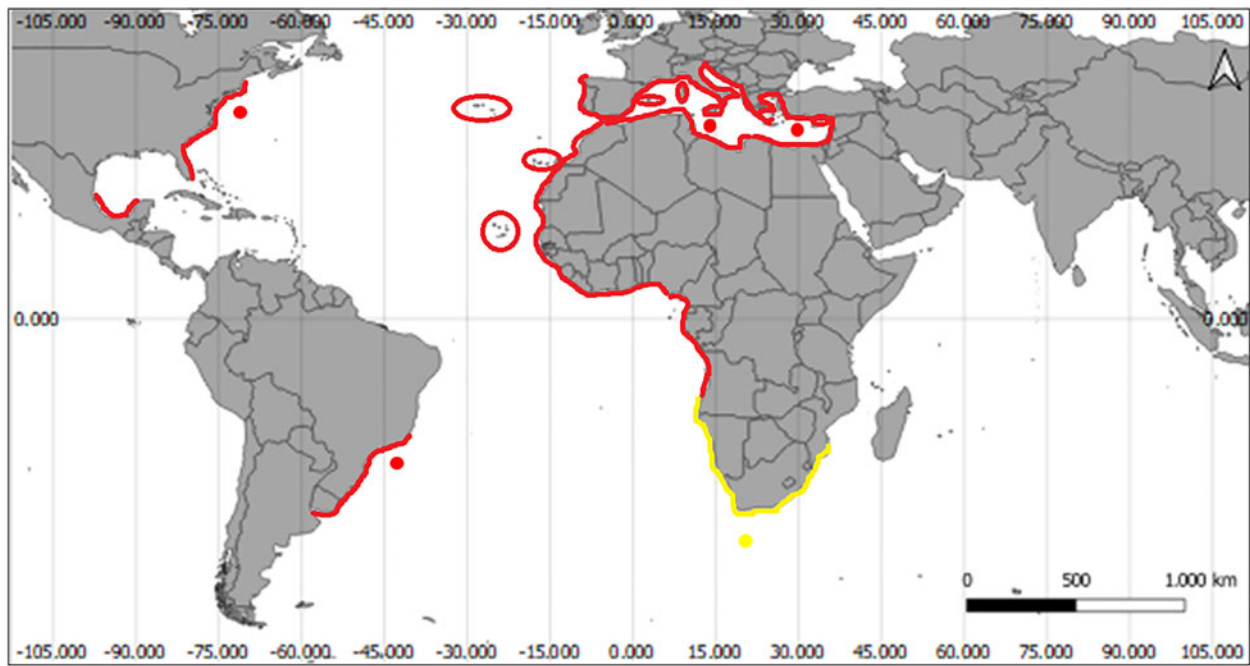


Fig. 1. Distribution for *Gymnura altavela* (black) and *G. natalensis* (grey) and *G. altavela* (black circle) and *G. natalensis* (grey circle) sampling sites.

decade. For example, several studies on *G. altavela* have been recently published in Brazil, on the following subjects: length-weight relationships (Silva-Junior et al., 2011), embryo descriptions (Paiva et al., 2018), distribution and density in an estuarine zone (Gonçalves-Silva et al., 2018a), reinforcing its diet, reproductive aspects and a probable nursery area (Gonçalves-Silva et al., 2018b), highlighting its fishing importance and molecular and morphometric relationships (Marques et al., 2019), revealing differences in *in situ* and *ex situ* bacterial communities associated with the skin and the stinger areas (Gonçalves-Silva et al., 2020), and quantifying high Persistent Organic Pollutant contamination levels (Rosenfelder et al., 2012; Paiva et al., 2021).

Given the taxonomic uncertainties associated with the species (Yokota & de Carvalho, 2017), the present study aims to assess genetic relationships among *Gymnura altavela* specimens sampled along both sides of the Atlantic and Mediterranean. Comparisons between cytochrome oxidase subunit 1 (COI) gene DNA sequences were performed in a phylogeographic framework. DNA sequence-based approaches are widely employed in studies that aim to inventory taxa diversity in groups affected by extraordinary morphological stasis and ecological traits (Cariani et al., 2017; Crobe et al., 2021), with important contributions to the taxonomy of *Gymnura* species (Smith et al., 2009; Shen et al., 2012).

## Materials and methods

A total of 95 Cytochrome oxidase I (COI) sequences from nine nominal *Gymnura* genus species were obtained from GenBank (Table 1; accession numbers in Table S2). Furthermore, we generated 37 sequences (GenBank numbers MW321984–MW322020) from *G. altavela* samples sampled off Rio de Janeiro, Brazil (Table S2) and identified according to McEachran & Carvalho (2003). All sequences and information about specimens are recorded in the public BOLD Project ‘*Gymnura altavela*\_OTUs’ (project code: GYMNU). DNA extraction, PCR and sequencing procedures followed the methodology described in Marques et al. (2019). Sequences for *Himantura uarnacoides* (N = 1), *Mobula mobular* (N = 2), *Myliobatis chilensis* (N = 1) and *Myliobatis longirostris* (N = 1) were used as outgroups (accession numbers available in Table S2).

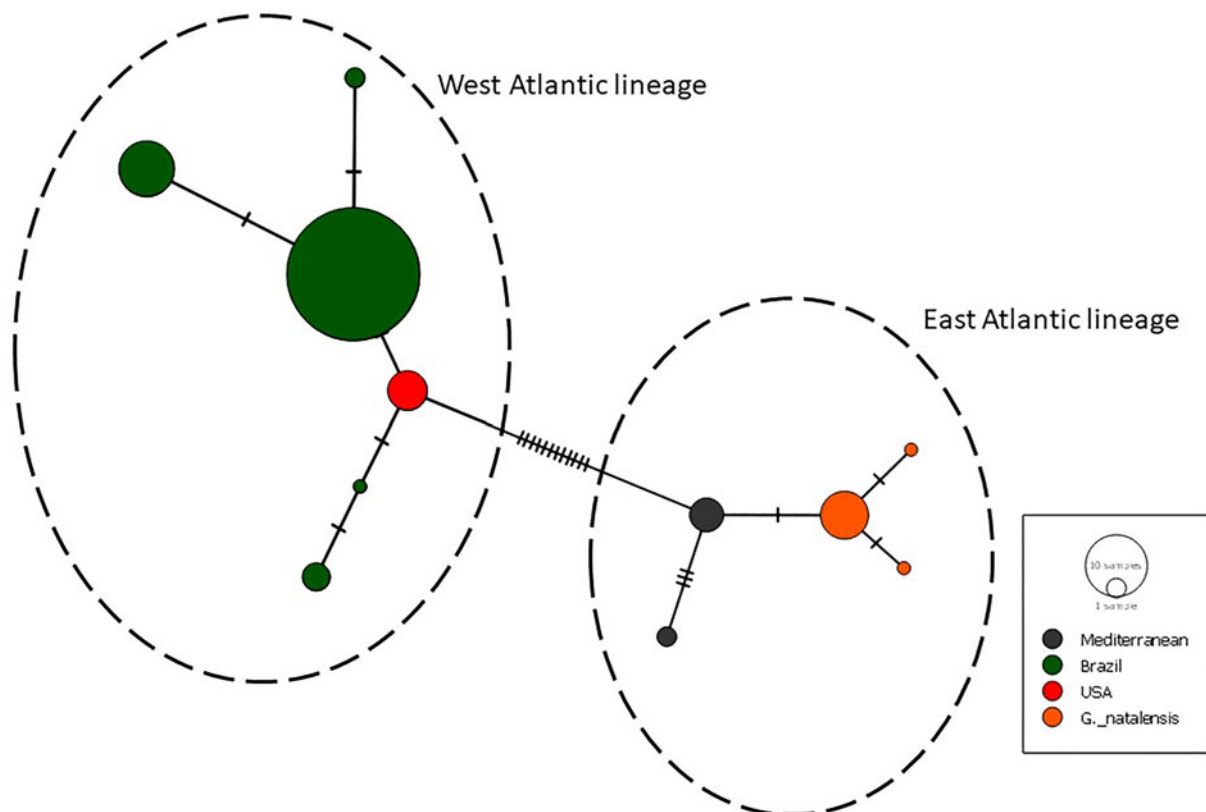
The sequences were aligned using the MEGA 7.0 software (Kumar et al., 2016) through the Clustal W algorithm (Thompson et al., 1994) and carefully checked visually. The HKY + G + I (Hasegawa et al., 1995) nucleotide substitution model was selected using the Bayesian Information criterion as implemented in the aforementioned software (Kumar et al., 2016). Tree topologies were generated using Maximum likelihood (ML) and Neighbour joining (NJ) in the same software with 1000 bootstrap replicates. The MrBayes 3.2.0 (Ronquist et al., 2012) was applied for the Bayesian Inference (BI), as implemented in NGPhylogeny.fr (Lemoine et al., 2019), with two independent analyses of four concomitant Markov Chain Monte Carlo (MCMC) runs for 15 million generations and sampling parameters every 1000 generations. The first 25% of the trees were discarded as burn-in and a 50% majority-rule consensus tree was estimated. Intra- and interspecific pairwise genetic distances employing the Kimura 2-parameter distance model (K2P) (Kimura, 1980) were estimated using the MEGA 7.0 software. We used the Automatic Barcoding

Table 1. Number of individual specimens per species

Taxon	Samples (N)
<i>G. altavela</i> East Atlantic (Mediterranean)	4
<i>G. altavela</i> North-west Atlantic (EUA)	4
<i>G. altavela</i> South-west Atlantic (Brazil)	57
<i>G. natalensis</i>	5
<i>G. australis</i>	9
<i>G. crebipunctata</i>	1
<i>G. japonica</i>	5
<i>G. marmorata</i>	3
<i>G. micrura</i>	4
<i>G. poecilura</i>	38
<i>G. zonura</i>	2
Total	132







**Fig. 3.** Median joining haplotype network from the COI gene, indicating two distinct groups.

from the entire geographic distribution of the species are required to infer species boundaries more securely within the genus.

Another finding highly relevant towards conservation efforts is that *G. natalensis*, to date, considered an endemic Southern Africa species (Figure 1), is phylogenetically closely related to *G. altavela* from the Mediterranean Sea. The possible synonymy of *G. natalensis* and *G. altavela* has already been suggested based on previous morphological analyses (Yokota *et al.*, 2016) and, if future data support this hypothesis, *G. natalensis* should be reclassified as a junior synonym of *G. altavela*, consequently extending its distribution from the Mediterranean to Mozambique and Madagascar.

Linnaeus (1758) first described the spiny butterfly ray from the Mediterranean Sea as *Raja altavela*. The species was later transferred to the *Gymnura* genus. The rays currently identified as *G. altavela* in the South-west Atlantic were originally identified as *Gymnura binotata*, described by Lunel (1879, as *Pteroplatea binotata*) from juvenile samples from Rio de Janeiro, the same area where our samples were collected. Interestingly, the main difference used to discriminate between *G. altavela* and *G. binotata* was ‘two white blotches on dorsal surface’. In Rio de Janeiro, we observed two *G. altavela* morphotypes, one classic (Figure S4A) and the other very rare (Figure S4B and Video S2), with only one specimen captured during 15 years of sampling efforts, with two white spots on the dorsum, which possibly gave rise to the description of *G. binotata* as a distinct species. More recently, we video recorded one individual with spots at Armação dos Búzios, in the state of Rio de Janeiro (22°46′06″S 41°47′26″W) (Video S2). These blotches are probably simply a polymorphism within the species, similar to the one also found in *G. bimaculata* (Norman, 1925). This species was differentiated from *G. japonica* based on the presence of a pair of white ocelli at the posterior part of the spiracle. Half a century later, Isouchi (1977) studied white ocelli pattern variations in *G. bimaculata* and described that one female with ocelli gave birth to pups

with no ocelli, comprising strong evidence that the ocelli are a mere polymorphism. Alternatively, these blotches could be the sign of interspecific hybridization, and, thus, very rare and not detectable by a mitochondrial gene, or the result of reproductive secondary contacts overcoming species boundaries, which has been already recorded in batoids and sharks (Portnoy *et al.*, 2010).

Recently, two new *Gymnura* species were distinguished (Yokota & de Carvalho, 2017) within specimens formerly assigned as *G. micrura*. Alongside the evidence presented here, this underlines the high taxonomic instability of spiny butterfly rays. Therefore, morphological and molecular taxonomic studies are urgently required to help elucidate *Gymnura* species boundaries worldwide.

### Consequences for conservation

Although the findings presented herein should be viewed with caution due to the use of a single mitochondrial gene, they are significant, as they indicate that a vulnerable and overfished species may be even more threatened than previously noted, since global population size estimates may be inflated by aggregating data from two different lineages that could, in fact, comprise distinct species. If confirmed by further morphological and genetic studies, the implications of this study will likely result in the uplisting of *Gymnura altavela* and, furthermore, indicate that the south-western Atlantic taxon is under even greater threat. These preliminary results should result in a greater engagement of the scientific community interested in fish conservation, which may be crucial for conservation efforts towards this important species.

**Supplementary material.** The supplementary material for this article can be found at <https://doi.org/10.1017/S002531542200056X>.

**Data.** Sequences used here can be retrieved from GenBank. Accession numbers are listed in Table S2.

**Acknowledgements.** The authors thank Rebeca A. Marques for aiding in laboratory procedures and two anonymous reviewers for valuable manuscript contributions. The authors thank the National Council for Scientific and Technological Development (CNPq) and Seed grants from IMAM-AquaRio. Thanks are also due to Eduardo Lukezic and the 'Azul Profundo' dive operator for ceding the video reported herein.

**Author contributions.** A.V.: contributed to data acquisition and analysis, interpreting the findings and writing the manuscript. F.L.: contributed to data acquisition and writing the manuscript. A.M.S.C.: contributed providing financial support, helping in data analysis, interpreting the findings and writing the manuscript. M.V.: contributed to formulating research questions, providing financial support, interpreting the findings and writing the manuscript.

**Financial support.** National Council for Scientific and Technological Development (CNPq) and Seed grants from IMAM-AquaRio.

**Conflict of interest.** The authors declare no conflict of interest.

## References

- Alkusaury H, Ali M, Saad A, Reynaud C and Capapé C (2014) Maturity, reproductive cycle, and fecundity of spiny butterfly ray, *Gymnura altavela* (Elasmobranchii: Rajiformes: Gymnuridae), from the coast of Syria (Eastern Mediterranean). *Acta Ichthyologica et Piscatoria* **44**, 229–240.
- Bandelt H, Forster P and Röhl A (1999) Median-Joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution* **16**, 37–48.
- Capapé C, Zaouali J, Tomasini JA and Bouchereau JL (1992) Reproductive biology of the spiny butterfly ray, *Gymnura altavela* (Linnaeus, 1758) (Pisces: Gymnuridae) from off the Tunisian coasts. *Scientia Marina* **56**, 347–355.
- Cariani A, Messinetti S, Ferrari A, Arculeo M, Bonello JJ, Bonnici L, Cannas R, Carbonara P, Cau A, Charilaou C, El Ouamari N, Fiorentino F, Follsea MC, Garofalo G, Golani D, Guarniero I, Hanner R, Hemida F, Kada O, Lo Brutto S, Mancusi C, Morey G, Schembri PJ, Serena F, Sion L, Stagioni M, Tursi A, Vrgoc N, Steinke D and Tinti F (2017) Improving the conservation of Mediterranean Chondrichthyan: the ELASMOMED DNA barcode reference library. *PLoS One* **12**, e0170244. doi: 10.1371/journal.pone.0170244
- Crobe V, Ferrari A, Hanner R, Leslie RW, Steinke D, Tinti F and Cariani A (2021) Molecular taxonomy and diversification of Atlantic skates (Chondrichthyes, Rajiformes): adding more pieces to the puzzle of their evolutionary history. *Life* **11**, 596.
- Dulvy N, Charvet P, Carlson J, Badji L, Blanco-Parra M, Chartrain E, De Bruyne G, Derrick D, Dia M, Doherty P, Dossa J, Ducrocq M, Leurs G, Notarbartolo di Sciara G, Pérez Jiménez J, Pires J, Seidu I, Serena F, Soares A, Tamo A, Vacchi M, Walls R and Williams A (2021) *Gymnura altavela*. In *The IUCN Red List of Threatened Species 2021*: e.T63153A3123409.
- Gonçalves-Silva F, Dos Santos H, de Assis Leite D, Lutfi D, Vianna M and Rosado A (2020) Skin and stinger bacterial communities in two critically endangered rays from the South Atlantic in natural and aquarium settings. *Microbiology Open* **9**, e1141.
- Gonçalves-Silva F and Vianna M (2018a) Use of a species-rich and degraded tropical estuary by elasmobranchs. *Brazilian Journal of Oceanography* **66**, 339–346.
- Gonçalves-Silva F and Vianna M (2018b) Diet and reproductive aspects on the endangered butterfly ray *Gymnura altavela* raising the discussion of a possible nursery area in a highly impacted environment. *Brazilian Journal of Oceanography* **66**, 315–324.
- Hasegawa M, Kishino H and Yano T (1995) Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *Journal of Molecular Evolution* **22**, 160–174.
- ICMBio (2018) Livro vermelho da fauna Brasileira ameaçada de extinção: Volume VI Peixes. Ministério do Meio Ambiente: Brasília, Brasil: ICMBio.
- Isouchi T (1977) Butterfly ray *Gymnura bimaculata*, a junior synonym of *G. japonica*. *Japanese Journal of Ichthyology* **23**, 242–244.
- Kimura K (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* **16**, 111–120.
- Kumar S, Stecher G and Tamura K (2016) MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution* **33**, 1870–1874.
- Leigh J and Bryant D (2015) PopART: full-feature software for haplotype network construction. *Methods in Ecology and Evolution* **6**, 1110–1116.
- Lemoine F, Correia D, Lefort V, Doppelt-Azeroual O, Mareuil F, Cohen-Boulakia S and Gascuel O (2019) NGPhylogeny.fr: new generation phylogenetic services for non-specialists. *Nucleic Acids Research* **47**, 260–265.
- Linnaeus L (1758) *Systema Naturae per regna tria naturae, secundum classes, ordines, genera, species, cum characteribus, differentiis, synonymis, locis. Editio decima, reformata [10th revised edition], vol. 1. Laurentius Salvius: Holmiae*, 824 pp.
- Marques R, Guimarães J, Sole-Cava A and Vianna M (2019) A new strategy proposal to monitor ray fins landings in south-east Brazil. *Aquatic Conservation: Marine and Freshwater Ecosystems* **30**, 68–85.
- McEachran JD and Capapé C (1984) Gymnuridae. In Whitehead P, Bauchot M, Hureau J, Nielsen J and Tortonese E (eds), *Fishes of the North-Eastern Atlantic and the Mediterranean*. Paris: UNESCO, pp. 203–204.
- McEachran JD and Carvalho MR (2003) Gymnuridae. Butterfly rays. In Carpenter K (ed.), *FAO species Identification Guide for Fishery Purposes. The Living Marine Resources of the Western Central Atlantic. Vol. 1: Introduction, molluscs, crustaceans, hagfishes, sharks, batoid fishes, and chimaeras*. Rome: FAO, pp. 575–577.
- Muktha M, Akhilesh KV, Sandhya S, Jasmin F, Jishnudev M and Kizhakudan S (2016) Re-description of the longtail butterfly ray, *Gymnura poecilura* (Shaw, 1804) (Gymnuridae: Myliobatiformes) from Bay of Bengal with a neotype designation. *Marine Biodiversity* **48**, 1085–1096.
- Paiva L, Julio T, Marques R and Vianna M (2018) First description of the embryos of the stingray *Gymnura altavela* (Linnaeus, 1758) (Myliobatiformes: Gymnuridae), a species at risk of extinction. *Journal of Applied Ichthyology* **34**, 984–987.
- Paiva L, Vannuci-Silva M, Correa B, Santos-Neto E, Vianna M and Lailson-Brito J (2021) Additional pressure to a threatened species: high persistent organic pollutant concentrations in the tropical estuarine batoid *Gymnura altavela*. *Bulletin of Environmental Contamination and Toxicology* **107**, 37–44.
- Portnoy D, McDowell J, Heist E, Musick J and Graves J (2010) World phylogeography and male-mediated gene flow in the sandbar shark, *Carcharhinus plumbeus*. *Molecular Ecology* **19**, 1994–2010.
- Puillandre N, Lambert A, Brouillet S and Achaz G (2012) ABGD, automatic barcode gap discovery for primary species delimitation. *Molecular Ecology* **21**, 1864–1877.
- Ronquist F, Teslenko M, van der Mark P, Ayres D, Darling A, Höhna S, Larget B, Liu L, Suchard M and Huelsenbeck J (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology* **61**, 539–542.
- Rosenfelder N, Lehnert K, Kaffarnik S, Torres J, Vianna M and Vetter W (2012) Thorough analysis of polyhalogenated compounds in ray liver samples off the coast of Rio de Janeiro, Brazil. *Environmental Science and Pollution Research* **19**, 379–389.
- Shen A, Ma C, Ni Y, Xu Z and Ma L (2012) The taxonomic status of *Gymnura bimaculata* and *G. japonica*: evidence from mitochondrial DNA sequences. *Journal of Life Sciences* **6**, 9–13.
- Silva-Junior L, Andrade A and Vianna M (2011) Technical contribution length-weight relationships for elasmobranchs from southeastern Brazil. *Journal of Applied Ichthyology* **27**, 1408–1410.
- Smith W, Bizzarro J, Richards V, Nielsen J, Márquez-Flarías F and Shivji M (2009) Morphometric convergence and molecular divergence: the taxonomic status and evolutionary history of *Gymnura crebripunctata* and *Gymnura marmorata* in the eastern Pacific Ocean. *Journal of Fish Biology* **75**, 761–783.
- Thompson J, Higgins D and Gibson T (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix. *Nucleic Acids Research* **22**, 4673–4680.
- Walls RHL, Vacchi M, Notarbartolo di Sciara G, Serena F and Dulvy NK (2016) *Gymnura altavela*. The IUCN Red List of Threatened Species 2016: e.T63153A16527909. Accessed on 22 August 2022.
- Yokota L and de Carvalho M (2017) Taxonomic and morphological revision of butterfly rays of the *Gymnura micrura* (Bloch & Schneider 1801) species complex, with description of two new species (Myliobatiformes: Gymnuridae). *Zootaxa* **4332**, 1–74.
- Yokota L, White WT and de Carvalho M (2016) Butterfly rays, family Gymnuridae. In Last P, White W, de Carvalho, Séret B, Stehmann M and Naylor G (eds) *Rays of the World*, 5th edn. Melbourne: CSIRO Publishing, pp. 11–521.