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Molecular evidence supporting the expansion of the geographical distribution of the Brazilian cownose ray *Rhinoptera brasiliensis* (Myliobatiformes: Rhinopteridae) in the western Atlantic

PAOLA PALACIOS-BARRETO^{1, 2}, VANESSA P. CRUZ³, FAUSTO FORESTI³, BIANCA DE S. RANGEL⁴, MANUEL URIBE-ALCOCER⁵ & PÍNDARO DÍAZ-JAIMES^{5, 6}

¹Posgrado en Ciencias del Mar y Limnología/Laboratorio de Genética de Organismos Acuáticos, Instituto de Ciencias del Mar y Limnología, Universidad Nacional Autónoma de México, Postal 70–305 Ciudad Universitaria, 04510 Mexico City, Mexico.

²Fundación Colombiana para la Investigación y Conservación de Tiburones y Rayas, SQUALUS, Cali, Colombia.

E-mail: ppalacios@squalus.org

³Laboratório de Biologia e Genética de Peixes, Universidade Estadual Paulista, Botucatu, São Paulo, Brazil.

E-mail: cruzvp@outlook.com

⁴Departamento de Fisiologia, Instituto de Biociências, Universidade de São Paulo, São Paulo, Brazil.

E-mail: biarangel.sea@gmail.com

⁵Unidad Académica de Ecología y Biodiversidad Acuática, Instituto de Ciencias del Mar y Limnología, Universidad Nacional Autónoma de México, 04510 Mexico City, Mexico. E-mail: pindaro@cmarl.unam.mx

⁶Corresponding author.

Abstract

The genus *Rhinoptera* is composed of eight species widely distributed in tropical, subtropical and temperate coastal waters, which inhabit bays, estuaries and river mouths. Cownose ray *Rhinoptera bonasus* has been reported to inhabit the Western Atlantic including the Gulf of Mexico and the Caribbean, whereas the Brazilian cownose ray *R. brasiliensis* has been considered endemic to the coast of Brazil. Recent reports of *R. brasiliensis* in the Gulf of Mexico bring about the question of whether the species has a wider range than previously reported. Here, the mitochondrial genes COI, Cytb, NADH2 and the nuclear gene *RAG1* were used to distinguish among species and to confirm the presence of *R. brasiliensis* in the Gulf of Mexico. *R. brasiliensis* specimens collected along the southern Gulf of Mexico showed a remarkable genetic and morphological affinity when compared with *R. brasiliensis* specimens from Brazil, supporting the presence of the species in Mexico (from Veracruz through Campeche) and providing evidence that its distribution ranges from Brazil to the Caribbean Sea and the Gulf of Mexico. Both species overlap geographically to a large degree, leading to a reassessment of their conservation status. Our results also show that *R. bonasus* distribution in the Gulf of Mexico may be restricted to the northern portion, in US waters.

Key words: Batoids, cownose rays, range expansion, gene divergence

Introduction

Cownose rays (subfamily Rhinopterinae) are represented by eight living species in a single genus, *Rhinoptera* (Nelson *et al.* 2016). These are mostly benthopelagic organisms inhabiting both continental shelves and the vicinity of offshore islands, but they can also be found in bays and estuaries. They are frequent in the lower reaches of coastal rivers, with a circumglobal distribution in tropical and warm temperate seas (Smith & Merriner, 1987; McEachran & de Carvalho, 2002; Collins *et al.*, 2008). Cownose species are so morphologically similar that it is often difficult to distinguish among them. Key features such as tooth shape and number of plates are variable even within species (Naylor *et al.*, 2012); as a result, the taxonomy of this genus remains relatively poorly known.

Cownose ray *Rhinoptera bonasus* and Brazilian cownose ray *R. brasiliensis* are distributed in the Western Atlantic and can only be distinguished by the number of rows of tooth plates: *R. bonasus* has 5–13 rows (usually 7) in each jaw, whereas *R. brasiliensis* typically has series of nine rows (varying from 7–15) in each jaw (Bigelow &

Schroeder, 1953; Last *et al.*, 2016). According to data listed by Bigelow & Schroeder (1953), *R. brasiliensis* has a broader mouth and greater distances between nostrils and spiracles than *R. bonasus*. In fact, tooth plate conformation corresponds to the main difference between both species, *R. brasiliensis* having three central rows of broad teeth and *R. bonasus* only one row.

In 2013, about 250 dead cownose rays were found stranded on Chachalacas beach in Veracruz, Mexico. These were reported to be *R. bonasus*, a species commonly described as being present throughout the western Atlantic from southern Massachusetts to Florida, and from the Gulf of Mexico to southern Brazil (Blaylock, 1993; Neer & Thompson, 2005). Upon examination of tooth plate configuration, it was noted that they matched the description of *R. brasiliensis*; the row count for dental plates in the upper and lower jaws equaled nine, resembling that of *R. brasiliensis*, which had been considered to be endemic to the Brazilian coasts between Rio de Janeiro and Rio Grande do Sul (Bigelow & Schroeder, 1953; Menni & Stehmann, 2000). This native range was proposed based on the number of series and/or rows of dental plates, and in the past there had been few reports of *R. brasiliensis* occurring in other waters considering this single evidence. There have been reports of the presence of *R. brasiliensis* off Colombian coasts on Isla Salamanca, Magdalena (Acero & Garzón, 1982), but also in the southern Gulf of Mexico along the Mexican coast off Tuxpan, Veracruz (González-Isais & Montes-Domínguez, 1996), in North Carolina in the Northwestern Atlantic (McEachran & de Carvalho, 2002), and off Venezuelan coasts, on the Orinoco River delta (Lasso *et al.*, 2004). All these reports were seemingly rare occurrences, with only a small number of individuals found in each case.

Recurrent captures of specimens showing dental characteristics similar to those of *R. brasiliensis* off Mexican coasts, in Veracruz, Campeche and Quintana Roo (Palacios-Barreto, 2016) bring about the question of whether the presence of *R. brasiliensis* in the Gulf of Mexico corresponds to the species' range expansion. The schooling nature and inshore habitat of cownose rays makes the species vulnerable to capture, and this together with relatively late maturity and very low fecundity increases susceptibility to overexploitation and would limit their ability to recover from a population decline. For this reason the Shark Specialist Group of the International Union for the Conservation of Nature (IUCN) has considered *R. brasiliensis* as "critically endangered" (Vooren & Lamónaca, 2004) whereas *R. bonasus* is currently classified as "Near Threatened" (Barker, 2006). Distinguishing between both species is difficult, as the only way to correctly differentiate between them is by dental morphology. Hence, this peculiar feature can prove to be problematic due to the variations reported concerning dental counts in the field.

Taxonomic identification at the species level constitutes a major issue, not only as a contribution to group systematics but also for appropriate fishery management (Fisher *et al.*, 2013; Rangel *et al.*, 2017). Molecular techniques are now commonly employed as diagnostic tools in species identification that are often applied when traditional morphological and meristic data have proven to be insufficient or unsatisfactory (de Franco *et al.* 2010), as it seems to be the case of cownose rays. Here, the mitochondrial genes COI, Cytb, NADH2 and the nuclear gene *RAG1* have been used to confirm the presence of *R. brasiliensis* in the Gulf of Mexico. If these two species overlap geographically to a much greater extent than was previously thought, such information will be critical in management plans and should lead to the reassessment of their conservation statuses.

Material and methods

We obtained tissue samples of bycaught specimens from artisanal fishery for both species from six coastal localities in Brazil (Bertioga, São Paulo n=20), Colombia (Manaure, La Guajira n=15), and in the Southern Gulf of Mexico, including Tamiahua (n=5), Tecolutla (n=8) and Chachalacas (n=27) in Veracruz and Seybaplaya in Campeche (n=28) (Table 1). The specimens were identified as *R. brasiliensis* based on tooth series counts (McEachran & de Carvalho, 2002). A total of four jaws of individuals from Veracruz, Campeche and Quintana Roo were collected and deposited in the National Fish collection of the Instituto de Biología, Universidad Nacional Autónoma de México (CNPE-IBUNAM; catalog numbers: CNPE-IBUNAM 22282, 22283, 22284 and 22285). Tissue samples from each specimen were preserved in 70 % ethanol for molecular analyses.

DNA was extracted from tissue samples using the Wizard Genomic DNA Purification kit (Promega) following the manufacturer's instructions. There were amplified by PCR fragments for three mtDNA genes: Cytochrome oxidase 1 (*COI*), Cytochrome b (*Cyt b*) and Nicotinamide adenine dehydrogenase Subunit 2 (*NADH2*). An

additional fragment of the nuclear DNA (nDNA) Recombination activating gene 1 (*RAG1*) was also amplified. PCR amplifications consisted of 15 µl reactions using 10–50 ng of template DNA, 1.5 mM of MgCl₂, 0.2 mM of each dNTP, 10 pm/µl of each primer, and 1 U of Taq DNA polymerase. The universal primers Fish1F and Fish1R reported in Ward *et al.* (2005) were used for amplification of COI, whereas the primers described by McDowell & Fisher (2013), RbCytbF (GGCCTHTTYCTRGCTATACTACTACAC) and RbCytbR (AGGGRTGGAATGGRATTTT), as well as RhinND2F1 (GAACCCYTTAATCCTCTYCATC) and RhinND2R2 (TATRGGGGTTAATGGRAGRAG), were used for amplification of *Cytb* and *NADH2*, respectively. For *RAG1*, the primers used were RhinRAG1F (CACTGAAGTGGGAATAATAGATG) and RhinRAG1R (TTATCGAGCATTGCTTGCC).

TABLE 1. Samples used in the analyses, sampling sites and number of individuals.

Species	Sampling site	Coordinates		N					
		latitude	Longitude	Cyt b	ND2	COI	Cyt b+ND2+COI	RAG	mtDNA+RAG1
<i>R. bonasus</i>	La Guajira, Manaure state, Colombia (15)	11.804389	-72.49138	6	6	8	6	2	2
<i>R. bonasus</i>	Bertioga, Sao Paulo state, Brazil (10)	-46.03412	-23.86057	10	10	10	8	8	8
<i>R. brasiliensis</i>	Chachalacas, Veracruz state, Gulf of Mexico (27)	19.439484	-96.3241	3	1	2	2	1	1
<i>R. brasiliensis</i>	Tecolutla, Veracruz state, Gulf of Mexico (8)	20.481918	-97.00867	6	4	6	3	2	2
<i>R. brasiliensis</i>	Tamiahua, Veracruz state, Gulf of Mexico (5)	21.267888	-97.44387	2	2	2	1	1	1
<i>R. brasiliensis</i>	Seybaplaya, Campeche state, Gulf of Mexico (28)	19.641109	-90.69045	12	7	8	7	7	7
<i>R. brasiliensis</i>	Bertioga, Sao Paulo state, Brazil (10)	-46.03412	-23.86057	10	10	10	10	10	10

Sequences were aligned using the ClustalW facility (Thompson *et al.*, 1994), implemented in Bioedit 7.0.9 (Hall, 1999). We inferred the phylogenetic relationships by using mitochondrial DNA genes concatenated (*Cyt b*+*NADH2*+*COI*) independently from the *RAG1*, but also using the concatenated mtDNA+nDNA data set by including only the sequences available in GenBank for the above four markers (Table 1). A Bayesian inference (BI) for combinations of both concatenated data was conducted for phylogeny reconstruction using MrBayes 3.2.1 (Ronquist *et al.*, 2012). We used a partition by gene for both concatenated data; the HKY+G substitution model was used for *Cyt b*, *NADH2* and *COI*, while TPM2uf was used for *RAG1*, as previously determined using the best fitting nucleotide substitution model assessed through the Bayesian Information Criterion (BIC) approximation in jModeltest 2.1.7 (Darriba *et al.*, 2012). Analysis in MrBayes consisted of four simultaneous runs and four Markov chains using 50,000,000 generations, with a burn-in of 25% of the initial trees. Additionally, a Maximum Likelihood (ML) approach was conducted using RAxML-HPC2 on XSEDE (Stamatakis, 2014) on the CIPRES Science Gateway (Miller *et al.*, 2010) using 1000 bootstrap replicates with the GTR-GAMMA model. Mitochondrial DNA and nuclear sequences for the species *Mobula japonica* (GeneBank accession number JX392983) and *Myliobatis californica* (KM364985) were used as an outgroup. We analyzed the COI sequences using the Automatic barcode gap discovery (ABGD) method, which consists of a program that is optimized for use with the genetic barcode based on data available for the COI gene (Puillandre *et al.*, 2012). This method employs a

genetic distance–based approach to detect a “barcode gap” separating candidate species, based on non–overlapping values of intra– and interspecific genetic distances, and is independent of any tree topology. ABGD analyses were performed using the web interface (<http://wwwabi.snv.jussieu.fr/public/abgd/>, web version ‘May 31 2017’). The analysis was run with default settings [Steps=10, X (relative gap width) =1.5, Nb bins=20], and pairwise differences were estimated with K2P (Kimura, 1980) together with *p*–distance. All assignments for intraspecific divergence with *P*–values between 0.001 and 0.100 divided into 10 partitions were recorded. A parsimony haplotype network for the concatenated mtDNA sequences was generated based on median–joining networks, as implemented in the Network 4.6 software package (Bandelt *et al.*, 1999), in order to distinguish clades in relation to the spatial distribution of haplotypes.

Results

For phylogenetic analyses, a total of 1670 bp were obtained for the three concatenated mitochondrial markers (423 bp for *Cyt b*, 758 bp for *NADH2* and 489 bp for *COI*), whereas the nuclear *RAG1* DNA sequences consisted of a 645 bp fragment. Altogether, mtDNA and nuclear genes produced a 2316 bp fragment. A total of 39 sequences were analyzed for the concatenated mtDNA, 33 for the *RAG1* and 32 for the concatenated mtDNA+nDNA alignments, respectively. Our phylogenetic reconstruction using both ML and BI analyses produced identical well–supported topologies, which grouped the *R. bonasus* and *R. brasiliensis* sequences into two reciprocally monophyletic lineages (Fig. 1).

The ABGD method delimited four to nine groups in both the full (operational taxonomic units, OTUs) and haplotype datasets. Analysis with K2P produced three initial partitions with 4 (*P*=0.0599), 5 (*P*=0.0027) and 9 (*P*=0.0016) OTUs respectively, whereas the use of *p*–distance returned only one initial value of 4 OTUs (*P*=0.0599). We selected the partition that recovered four groups (intraspecific distance *P*=0.001 to *P*=0.059), since this was the most consistent partition coinciding with the phylogenetic analyses (Table 1).

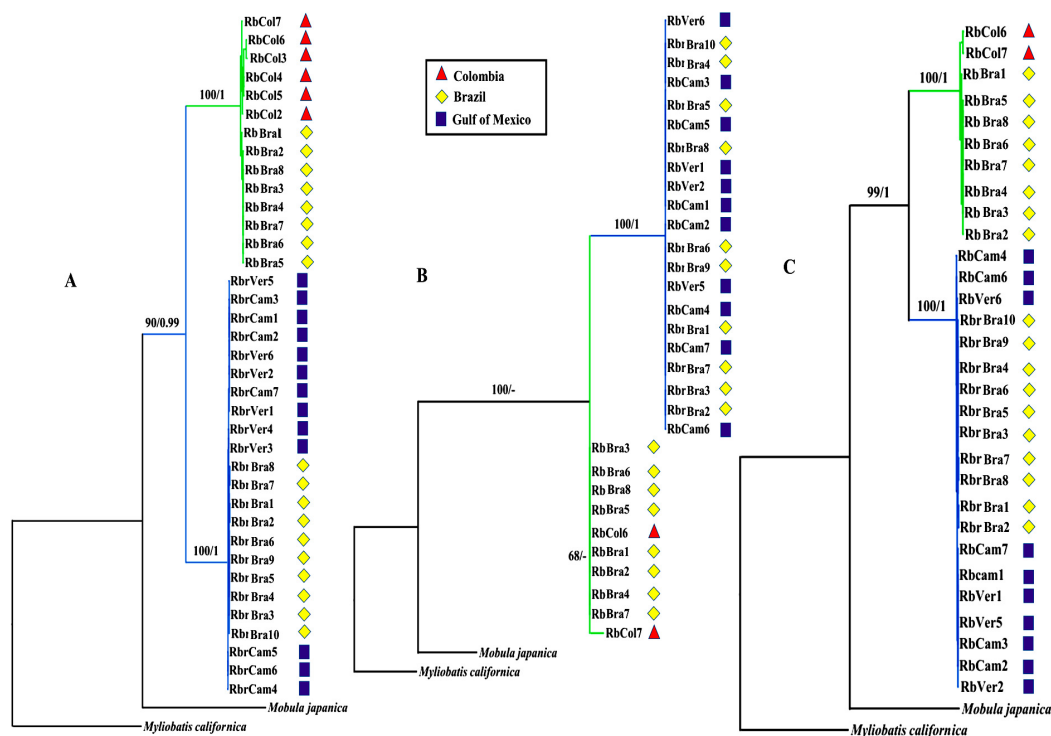


FIGURE 1. (A) Maximum likelihood and Bayesian inference topologies for concatenated data mtDNA (*Cyt b* + *NADH2* + *COI*) (B), nuclear (*RAG1*), and (C) concatenated data mtDNA+ *RAG1*. The numbers on branches are maximum likelihood bootstrap values and posterior probabilities of Bayesian inference, respectively. Lines have been color–coded: (*R. bonasus*) – green; (*R. brasiliensis*) –blue.

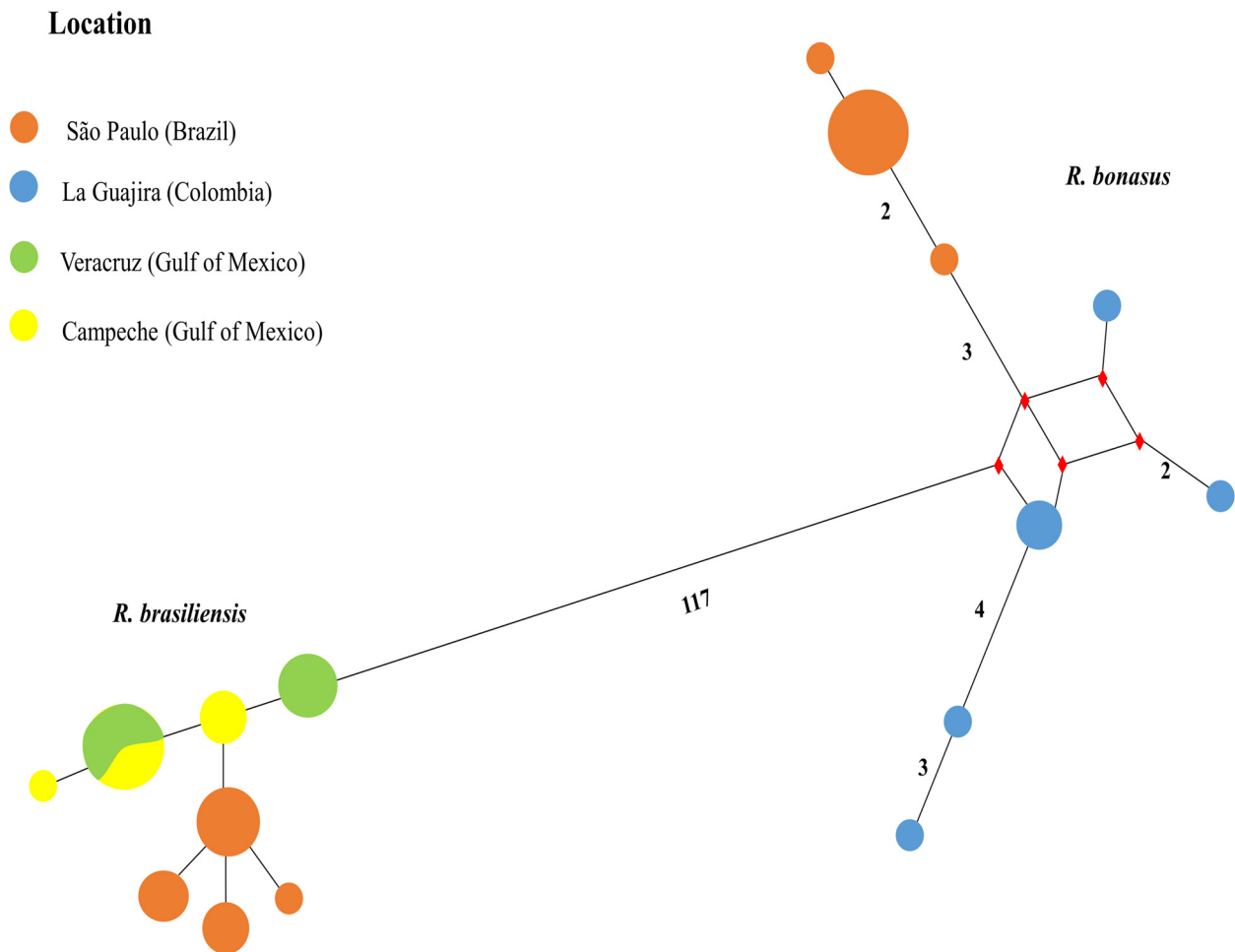


FIGURE 2. Median-joining network illustrating genealogical relationships among haplotypes of concatenated mtDNA (*Cyt b+NADH2+COI*) (1671 bp). The size of each haplotype circle is proportional to its frequency. Small intermediate circles represent median vectors of either unsampled or extinct taxa. Numbers indicate mutational steps between haplotype (one, if not shown). Color-coded by haplotypes between geographical locations.

Median-joining network analysis of the 1670 bp based on the concatenated mtDNA alignment revealed 16 haplotypes, eight for *R. bonasus* and eight for *R. brasiliensis*, arranged into two divergent clades separated by 117 mutational steps. Within clades, the specimens of *R. brasiliensis* sampled in the Gulf of Mexico and Brazil showed low divergence consisting of one mutational step (the Gulf of Mexico vs. Sao Paulo, Brazil. ~10,600 km apart). Connections between Colombia and Brazil for *R. bonasus* were also low, ranging from one to three mutational steps (Fig. 2).

Discussion

The phylogenetic tree showed the existence of two clearly separated clades—one including individuals of *R. brasiliensis* and a second composed of individuals of *R. bonasus*—supporting the evidence provided in previous studies separating both species based on morphological and molecular data (Jones *et al.*, 2017; Rangel *et al.*, 2017). The specimens collected in the Gulf of Mexico bear close genetic affinity to those from the Brazilian cownose ray *R. brasiliensis*. Moreover, these specimens clustered away from *R. bonasus* specimens from Colombia. In addition, the highest significant *P* value from the ABGD analysis was consistent with 4 OTUs corresponding to the four species *R. bonasus*, *R. brasiliensis*, *Mobula japonica* and *Myliobatis californica*, as defined by the phylogenetic tree. This method proposes the grouping of the input sequences into a number of possible species by using the pairwise differences from a distance matrix. As any method based on pairwise distances, it relies on divergence and

not on tree genealogy or tree shape. Indeed, the method works well when speciation comes from radiations, bifurcating events or a mixture of both (Puillandre *et al.*, 2012).

The remarkable genetic affinity between cownose ray specimens from the Gulf of Mexico and those of *R. brasiliensis* from Brazil also coincides with the configuration of the number of rows (nine) for the dental plates as defined for *R. brasiliensis* by Bigelow & Schroeder (1953) and Menni & Stehmann (2000). As a consequence, results in this study confirm the presence of *R. brasiliensis* in the southeastern Gulf of Mexico. In light of this, it can be suggested that *R. brasiliensis* should not continue to be considered endemic or restricted to the Brazilian coasts between Rio de Janeiro and Rio Grande do Sul (Bigelow & Schroeder, 1953; Menni & Stehmann, 2000). The current range of *R. brasiliensis* should be extended from southern Brazil to the Gulf of Mexico. It has recently been described how cownose rays may occur sympatrically and syntopically on the coasts of Brazil (Rangel *et al.*, 2017). In other studies, a number of observations indicate that cownose rays forage in groups and selectively prey on highly abundant species. The highly opportunistic and aggregate feeding behavior of the cownose ray (Fisher, 2010) could favor the geographical expansion of *R. brasiliensis*.

There has been no evidence of a second species of *Rhinoptera* in the southeastern Gulf of Mexico based on tooth series counts or molecular data; however, the presence of *R. bonasus* off the coast of Mexico should not be discarded. Castro–Aguirre (1965) reported a cownose ray captured displaying seven tooth rows in the upper jaw that was identified as *R. bonasus*, although this has been the only report for the region. A formal description and range distribution review should be considered as a priority for this species.

Rhinoptera brasiliensis and *R. bonasus* are considered species of concern for conservational purposes (Vooren & Lamónaca, 2004; Barker, 2006). Both species share similar vulnerable life histories, making them equally susceptible to threats from bycatch and targeted fisheries. Monitoring elasmobranch landings (including species-specific catch details) is necessary to provide valuable information on the biology and population status of these rays (Barker, 2006). Due to the transient nature of this schooling ray and the evidence concerning its extended range distribution, coordinated national and international efforts are necessary to adequately assess movements, abundance, and impact of fishing. Further research is required, including both an extensive tracking study to understand their movement patterns, and studies focused on recovering the species' life history data across its range in addition to the characterization of habitat use for the identification of potential nursery areas.

TABLE 2. Results of the Automatic Barcode Gap Discovery (ABGD) analysis sequences (total N = 39). *X*, relative gap width; Simple, *p*-distance and *K2P*, Kimura 2-parameter substitution model.

Subst. model	<i>X</i>	Partition	Prior intraspecific divergence (<i>P</i>)								
			0.0599	0.0359	0.0215	0.0129	0.0077	0.0046	0.0028	0.0017	0.001
p-distance	1.5	Initial	4	4	4	4	4	4	4	4	4
		Recursive									
K2P	1.5	Initial	4	4	4	4	4	4	4	9	9
		Recursive							5		

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