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Genetic diversity in two threatened species of guitarfish (Elasmobranchii: Rhinobatidae) from the Brazilian and Argentinian coasts: an alert for conservation

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The guitarfishes *Pseudobatos horkelii* and *Pseudobatos percellens* meet the criteria for threatened status as Critically Endangered (CR) and Endangered (EN), respectively. Both species occur in the Southern Atlantic Ocean. Considering the lack of data on the genetic structure of these species, the present study evaluated the genetic variability and population structure of the *P. horkelii* and *P. percellens* in the southern region of Brazil and the northern coast of Argentina, based on sequences of mitochondrial DNA, Control Region (D-loop). Samples of *P. horkelii* (n = 135) were analyzed in six localities situated in Northern Argentina, along the Brazilian states' coast. The mean of nucleotide diversity was 0.0053, the Φ_{ST} was 0.4277 and demographic analysis of *P. horkelii* suggests the existence of stability of the populations, with D = 0.9929, F_S = 2.0155, SSD = 0.0817, R = 0.2153. In *P. percellens* (n = 101) were analyzed from six Brazilian localities along the coast of Santa Catarina, Paraná, and São Paulo. The mean nucleotide diversity was 0.0014 and Φ_{ST} value of 0.2921, the demographic analysis indicates a high migration rate of *P. percellens* among the localities evaluated, with D = 0.5222, F_S = 0.3528, SSD = 0.01785, R = 0.3890.

Keywords: D-loop, Endangered species, Populations, *Pseudobatos horkelii*, *Pseudobatos percellens*.

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As raias violas *Pseudobatos horkelii* e *Pseudobatos percellens*, são listados como "Criticamente em Perigo" (CR) e "Em Perigo" (EN), respectivamente. Ambas as espécies ocorrem no Sul do Oceano Atlântico. Considerando a falta de dados sobre a estrutura genética dessas espécies, o presente estudo avaliou a variabilidade genética e a estrutura populacional de *P. horkelii* e *P. percellens* na região sudeste do Brasil e litoral norte da Argentina, com base em sequências de DNA mitocondrial, região de controle (D-loop). Amostras de 135 indivíduos de *P. horkelii* analisados em seis localidades, situadas no norte da Argentina e ao longo da costa dos estados brasileiros. A média da diversidade nucleotídica foi de 0.0053, o índice Φ_{ST} foi de 0.4277 e a análise demográfica de *P. horkelii*, indicou a existência de estabilidade das populações, com D = 0.9929, Fus = 2.0155, SSD = 0.0817, R = 0.2153. Em 101 exemplares de *P. percellens*, foram analisados em seis localidades brasileiras ao longo do litoral de Santa Catarina, Paraná e São Paulo. A diversidade nucleotídica média foi de 0.0014 e o valor Φ_{ST} de 0.2921, a análise demográfica indicou uma alta taxa de migração de *P. percellens* entre as localidades analisadas, com D = 0.5222, F_S = 0.3528, SSD = 0.01785, R = 0.3890.

Palavras-chave: D-loop, Espécies ameaçadas, Populações, *Pseudobatos horkelii, Pseudobatos percellens*.

INTRODUCTION

The rapid and crescent expansion of human activities in the world have resulted in progressive and compromising effects for most natural environments, including marine ecosystems (Marchese, 2015; Quiros *et al*., 2017; Vázquez-Rowe *et al*., 2020) in form of pollution, destruction of natural habitats, climate change, and overfishing, among others (Andersen *et al*., 2017; Clarke *et al*., 2021). Unsustainable fishing pressure has resulted in a considerable number of documented cases of collapse in the natural stocks of many elasmobranchs (sharks and rays), in addition to many other fish taxa (Dulvy *et al*., 2014; Lessa *et al*., 2016; Dulvy *et al*., 2017; MacKeracher *et al*., 2019; Santana *et al*., 2020).

A quarter of all Elasmobranch species are thought to be threatened by overfishing, either as fishery targets or as bycatch, according to the Red List of International Union for Conservation of Nature (IUCN) (Dulvy *et al*., 2014). In general, elasmobranchs have a complex life history characterized by low rates of survival and population growth, which reinforces their sensitivity to mortality (Worm *et al*., 2013; Dulvy *et al*., 2014; Pardo *et al*., 2016). The crucial life history characteristics include slow growth, late maturity, relatively long life expectancy, and low fecundity and reproductive frequency (Cortés, 1998). It is considered that the reduction of elasmobranch populations may have a wide range of negative consequences for both ecological and economic systems (Stevens *et al*., 2000; Dulvy *et al*., 2014; Lessa *et al*., 2016).

The guitarfish *Pseudobatos horkelii* (Müller & Henle, 1841) and *Pseudobatos percellens* (Walbaum, 1792) are demersal species, usually found on sandy or loamy bottoms of the continental shelf, feeding mainly on small fish, crustaceans, and small invertebrates (Bigelow, Schroeder, 1953; McEachran, Carvalho, 2002; Bornatowski *et al*., 2010).

Pseudobatos horkelii, currently listed by the IUCN as "Critically Endangered" (Pollom *et al*., 2020a), is found between Rio de Janeiro in Brazil and Mar del Plata in Argentina (Miranda, Vooren, 2003) and *Pseudobatos percellens*, currently listed as "Endangered" (Pollom *et al*., 2020b), is distributed from the Gulf of Mexico to Northern Argentina (McEachran, Carvalho, 2002).

The guitarfish species of the family Rhinobatidae have been under fishing pressure mainly because they are bycatch of trawling fisheries such as beach seine, single and double trawling, and gillnets (Garstin *et al*., 2018), primarily off the coast of southern and southeastern Brazil, Uruguay, and Argentina (Martins, Schwingel, 2003; Massa *et al*., 2004; Costa, Chaves, 2006; Bornatowski *et al*., 2010). The stocks of *P. horkelii* were reduced by more than 50% between 1994 and 1999 by fisheries operating in the coastal waters of Argentina and Uruguay (Massa *et al*., 2004). Overfishing in Southern Brazil also led to the near exhaustion of *P. horkelii* stocks in the 1980s. Currently, this species is thought to be at 16% of its original stocks, a level considered far below the maximum sustainable yield (Miranda, Vooren, 2003). In 2004, the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) of the Brazilian Ministry of the Environment banned the fishing and sale of this species through normative instruction MMA 5/2004.

The available studies on *P. horkelli* and *P. percellens* include research mainly on reproductive biology and population dynamics (Lessa *et al*., 1999; Rocha, Gadig, 2013; Pasquino *et al*., 2016; Martins *et al*., 2018), feeding habits, and distribution (Menni, Stehmann, 2000; Bornatowski *et al*., 2010; Carmo *et al*., 2015; Rezende *et al*., 2020). However, no data are available on the genetic diversity, population genetic structure or gene flow of either species. These data are fundamental to the development of regulatory programs for the conservation of natural stocks and recovery of endangered species (Ovenden *et al*., 2015; Carrier *et al*., 2018; Domingues *et al*., 2018). When the stock of a species declines, there may be dramatic effects on evolutionary processes such as inbreeding and genetic drift, highlighting the importance of genetic data to develop conservation initiatives. These possible impacts on the adaptive potential of a population confronted by environmental changes, irrespective of other active factors, contribute to a predictable increase in effective extinction risk (Ovenden *et al*., 2015; Carrillo-Briceño *et al*., 2018).

Since *P. horkelli* and *P. percellens* have been overfished in recent years, and given the lack of data on the genetic structure of their populations, the present study aimed to identify the genetic diversity and population structure of these two species along the area from Southeastern Brazil to Northern Argentina using DNA sequences of the mitochondrial Control Region (D-loop), as well as their population dynamics and demographic history and test the hypothesis of panmixia. This study's outcomes will be of great value to a better understanding of the species and populations' structure and distribution and contribute to establishing appropriate programs of management and conservation for these species.

MATERIAL AND METHODS

Sample collection. Samples of the *P. horkelii* (n = 135) and *P. percellens* (n = 101) were collected by artisanal fishers at different localities along the Atlantic Ocean coast from north of Argentina to Southeastern Brazil (**S1**). Samples of *P. horkelii* were collected in

Mar del Plata, Argentina (AR, n = 12) and in five localities in Brazil, being 41 in Torrinha (RS); 22 in Florianópolis (SC); 16 in Pontal do Paraná (PR); 20 in Santos (SP); and 24 in Rio de Janeiro (RJ). Samples of *P. percellens* were collected in three localities in Brazil, being 19 in Florianópolis (SC); 25 in Pontal do Paraná (PR); and in three points in the State of São Paulo, being 16 in Cananéia (SP); 12 in Mongaguá (SP2); and 29 in Santos (SP).

A small fragment of muscle tissue (< 1 cm^2) was collected from each animal, placed in 2ml sample vials, and preserved in 96% ethanol. All samples were collected in strict accordance with the regulations of the Brazilian Federal Animal Ethics Committee (SISBIO 13843–1), and the analyses followed the International Guidelines for Animal Experiments, as authorized by CEEAA IBB/UNESP, protocol number 556. The tissues were deposited in the collection of the Laboratory of Fish Biology and Genetics – UNESP in Botucatu, São Paulo, Brazil.

The DNA was extracted using the NucleoSpin Tissue XS kit (Macherey and Nagel, Dand, Germany). Partial sequences of the control region of the mitochondrial DNA (D-loop) were obtained by polymerase chain reaction (PCR) using the Dloop2F (CAA AGC CWA GAT TTT TAT TAA AC) and Dloop5R (RCW WAT TAA TAG GAC GGT AMT GGA Y), in hypervariable region II, developed specifically for this research. The samples were amplified in reactions of 12.5 µl containing: 10.35 ul of ultrapure water; 0.90 ul buffer (Tris–HCl 20 mM pH 8.4 and KCl 50 mM), 0.75 µl dNTPs (2mM); of 0,1 µl of *Taq* DNA polymerase Taq, 0.2 µl of each primer. The cycling conditions used in PCR reactions (Veriti® 96-well Thermal Cycler, Biosystems TM Applied or Mastercycler® EPGradient, Eppendorf) were performed using the following thermal temperatures: initial denaturation at 94°C for 5 min, followed by 35 cycles including denaturation at 94°C of 30s, annealing at 52°C for 30s, extension at 72°C for 1min, and a final extension at 72°C for 10 min. PCR products were sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) to sequence the samples in an automated ABI 3130xl (Applied Biosystems). The sequences were edited in GENEIOUS 6.0 (Kearse *et al*., 2012) and aligned using the MUSCLE algorithm (Edgar, 2004) run in GENEIOUS 6.0. The haplotype sequences were deposited in GenBank under accession numbers MK809354–MK809366.

Population analysis. The relative nucleotide composition, the number of polymorphic sites, the number and relative frequency of haplotypes, haplotype diversity (H_d), nucleotide diversity (π), and the pairwise nucleotide differences between populations were all calculated in ARLEQUIN 3.5.1.3 (Excoffier, Lischer, 2010). A haplotype network was obtained by the Median-Joining method (Bandelt *et al*., 1999) using the software PopART 1.7 (Leigh, Bryant, 2015).

The pairwise Φ_{ST} diversity index was used to estimate the levels of genetic divergence between the localities for *P. horkelii* and *P. percellens*, that were tested non-parametrically using 1000 bootstrap replicates (Felsenstein, 1985), runned in ARLEQUIN 3.5.1.3 and adjusted for simultaneous pairwise comparisons using the sequential Bonferroni procedure (Rice, 1989). Indeed, genetic divergence was calculated by the Molecular Analysis of Variance, or AMOVA (Excoffier *et al*., 1992), with an a priori criterion to test panmixia among samples, and differentiation was assessed among (Φ_{CT}) and within (Φ_{SC}) regions. For *P. horkelii* we grouped into Northern Argentina and Southern Brazil (AR, RS, SC,

PR), and between Southeastern Brazil (SP3, RJ), whereas for *P. percellens*, we grouped into Southern Brazil (SC, PR) and between Southeastern Brazil (SP1, SP2, SP3).

The demographic parameters Fu's F (Fu, 1996) and Tajima's D test (Tajima, 1989), as implemented in Arlequin, was used to test departures from neutrality due to recent population size expansions, or alternatively because of selection, and were obtained from nucleotide mismatch distributions with the Arlequin software to examine the possibility of demographic expansion, with the Sum of Square Deviation (SSD) and Raggedness index.

We used BAPS v 6.0 (Corander *et al*., 2013) to identify discrete genetic clusters within the dataset, with the most probable number of genetic groups formed by the sequences being inferred by a Bayesian analysis of the population structure.

To test for isolation by distance (IBD) patterns were examined in guitarfish species using Mantel tests in GENEPOP 4 (Raymond, Rousset, 1995; Rousset 2008) to determine the correlation between geographical distances and genetic distances (Mantel, 1967; Slatkin, 1993). Geographical distance was calculated as from sampling points using GPS and were estimated in km from Google Earth Pro (http://earth.google.co.uk).

RESULTS

Pseudobatos horkelii. A total of 135 consensus D-loop sequences of 702 base pairs in length were obtained for *P. horkelii* from localities situated along the northern coast of Argentina (AR), and Southern (RS, SC, PR) and Southeastern Brazil (SP3, RJ). The nucleotide frequencies of these sequences were A = 32.1%; C = 11.2%; G = 23.5% and T = 33.1%. Ten polymorphic sites (S) were detected (Tab. 1), with nucleotide diversity (π) ranging from 0.0013±0.0006 in AR to 0.0043±0.0018 in RS, with a mean of 0.0053. In total, 16 haplotypes (H) were identified as Ph1 to Ph16 (Fig. 1A).

The haplotypes that were present in a great number of specimens were Ph8 (15.5%) and Ph12 (17%) (Tab. 1). The less frequent haplotypes Ph 11 and Ph 12 were found in RJ and RS, respectively with a unique haplotype observed in only one geographic sample. Ten among the 16 haplotypes analyzed with an overall haplotype diversity (H_d) of 0.8992, are composed of specimens from RS which presented the greatest H_d = 0.8902±0.0214 (Tab. 2). The geographic region with the lowest number of haplotypes was PR, with H_d = 0.5333±0.0456.

The pairwise Φ_{ST} were estimated based on the control region, the values ranged from 0.0969 between AR and RS, to 0.6427 between AR and SP3 (**S2**), and a higher level of genetic differentiation with statistically significant Φ_{ST} was found between all localities ($P < 0.05$). The molecular variance analysis (AMOVA) resulted in a partition of the genetic variation into Northern of Argentina/Southern of Brazil (AR, RS, SC, PR) to Southeastern (SP3, RJ) of Brazil (Φ_{CT} = 0.2576; P = 0.0000), and significant differences between locations within regions of Φ_{SC} = 0.2664; P = 0.0000 (Tab. 3). The overall Φ_{ST} value was 0.4277 (P = 0.0000), thus rejecting a hypothesis of panmixia.

The demographic analysis considering the locality or all samples as a local population, showed non-significant values of neutrality by Tajima's D and Fu's FS tests (**S3**). Similar results represented by non-significant values were found by the AMOVA neutrality tests when two groups as AR, RS, SC, PR and SP3, RJ were considered. Although the

TABLE 1 | Polymorphisms and frequency found in haplotypes of *Pseudobatos horkelii* and *P. percellens*, based on D-loop. Regions of the North of Argentina coast and South-eastern / Southern regions of Brazil. AR – Argentina, RS – Rio Grande do Sul, SC – Santa Catarina, PR – Paraná, SP1 – Cananéia/SP, SP2 – Mongaguá/SP, SP3 – Santos /SP, RJ – Rio de Janeiro.

Species	Haplotypes	Position										Sampling location						
Pseudobatos horkelii		1	1	2	3	3	3	3	4	5	6	AR	RS	SC	PR	SP3	RJ	Total
		4	9	4	0	5	6	7	1	0	4	(12)	(41)	(22)	(16)	(20)	(24)	(135)
		9	3	0	6	3	6	9	5	7	3							
	Ph 1	A	C	A	C	A	A	G	A	T	G	2	6	0	0	5	0	13
	Ph 2	G	.	.	.	.	.	.	.	.	.	0	2	0	0	0	0	2
	Ph 3	.	.	.	.	G	G	.	.	.	.	0	3	0	8	0	0	11
	Ph 4	G	.	.	.	.	G	.	.	.	.	0	2	0	0	0	0	2
	Ph 5	G	.	.	.	G	.	.	.	.	.	0	3	0	0	0	0	3
	Ph 6	G	.	.	.	G	G	.	.	.	.	2	4	7	0	0	0	13
	Ph 7	.	.	.	.	.	G	.	.	.	.	8	6	0	0	0	1	15
	Ph 8	.	.	T	.	.	G	.	.	.	.	0	5	0	0	0	16	21
	Ph 9	.	.	T	.	.	G	.	T	.	.	0	0	0	0	0	3	3
	Ph 10	.	.	T	.	.	G	C	T	.	.	0	0	0	0	0	3	3
	Ph 11	.	.	.	.	.	G	.	T	.	.	0	0	0	0	0	1	1
	Ph 12	G	T	.	T	.	.	.	.	C	A	0	1	0	0	0	0	1
	Ph 13	G	T	.	T	.	.	.	T	C	A	0	9	0	0	11	3	23
	Ph 14	G	T	.	.	G	G	.	.	C	A	0	0	7	8	0	0	15
	Ph 15	G	T	.	.	G	G	C	T	C	A	0	0	5	0	0	0	5
	Ph 16	G	T	.	.	G	G	.	T	C	A	0	0	3	0	0	0	3
Pseudobatos percellens		7	2	4	4	4	4						SC	PR	SP1	SP2	SP3	Total
		5	7	1	5	5	8						(19)	(25)	(16)	(12)	(29)	(101)
			3	9	8	9	3											
	Pp 1	T	C	T	T	C	T						8	23	7	8	15	61
	Pp 2	.	.	A	.	T	.						8	0	0	0	0	8
	Pp 3	.	.	.	C	.	.						0	2	0	0	0	2
	Pp 4	.	.	.	.	.	A						0	0	0	4	9	13
	Pp 5	G	.	.	.	.	A						0	0	0	0	2	2
	Pp 6	.	T	.	.	.	.						0	0	0	0	2	2
	Pp 7	G	T	.	.	.	.						0	0	2	0	0	2
	Pp 8	G	.	.	.	.	.						3	0	7	0	1	11

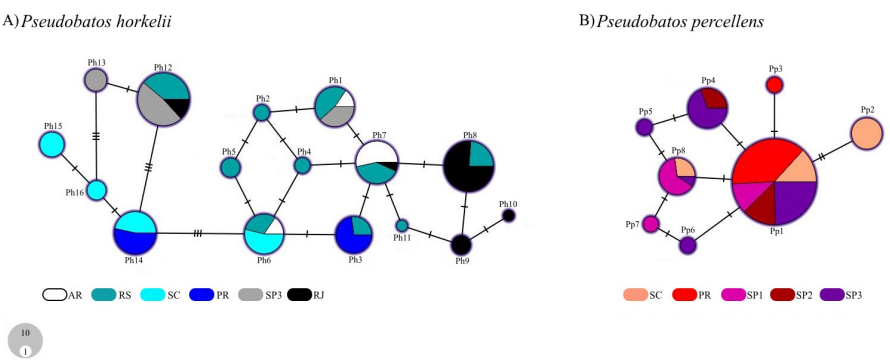

FIGURE 1 | Median-joining network of mtCR haplotypes for **A.** *Pseudobatos horkelii* and **B.** *Pseudobatos percellens*. Haplotypes are represented by circles with size proportional to frequency in the total sample. All hatch marks correspond to one mutation. Samples from northern Argentina (AR), Torrinha/RS (RS), Florianópolis/SC (SC), Pontal do Paraná/PR (PR), Cananéia/ SP (SP1), Mongaguá/SP (SP2), Santos/SP (SP3), Rio de Janeiro/RJ(RJ).

Raggedness and SSD indices are significant ($P > 0.05$) for almost all analysis in different tests by localities or groups, the graph of mismatch showed differences in observed and expected curves, representing a bimodal pattern between all samples (**S4**). The Bayesian analysis generated three groups which did not correspond to the geographic localities (Fig. 2A). Mantel test results a positive value, withal not significant for *P. horkelii* (r = 0.059; $P = 0.4210$).

Pseudobatos percellens. D-loop sequences of 646 bps were obtained from 101 specimens of *P. percellens*, from southern (SC, PR) and southeastern (SP1, SP2, SP3) regions in Brazil, with an overall nucleotide composition of A = 34.9%, C = 24.1%, G = 9.6% and T = 31.4%. Only six polymorphic sites were detected, representing eight haplotypes identified as Pp1 to Pp8 (Tab. 1, Fig. 1B). The mean nucleotide diversity (π) found was 0.0014, ranging from 0.0002±0.0000 in PR to 0.0020±0.0009 in SC. The Pp1 haplotype was found in 60.3% of the samples analyzed comprising the largest number among carrier specimens. Four among the eight haplotypes detected are present in the genome of only two specimens, being the haplotype Pp3 from PR, the haplotypes Pp5 and Pp6 from SP3, and the haplotype Pp7 from SP1.

The mean diversity haplotype found was $H_d = 0.6049$, calculated among samples of the five regions analyzed, and SP3 presented the largest number of haplotypes (five), with $H_d = 0.6578$+/–0.0663; other two regions presented two haplotypes, being PR with $H_d = 0.1533$+/– 0.0915 and SP2 with $H_d = 0.4848$+/– 0.1059 (Tab. 2).

Analysis of pairwise ranged from values of $\Phi_{ST} = 0.0320$ between SP2 and SP3, to 0.3760 between PR and SP1 (**S5**). A high level of genetic differentiation with statistically significant Φ_{ST} was found between almost all localities ($P < 0.05$), excepting the result between SP2 and SP3 that was not significant ($\Phi_{ST} = 0.0320$, $P > 0.05$). Likewise, the results of AMOVA reveal significant differences in the population structuring simulations (Tab. 3), with an overall Φ_{ST} value of 0.29218 ($P = 0.0000$), what rejects a hypothesis of panmixia. The AMOVA analysis also resulted in a partition of the genetic variation into southern (SC, PR) to southeastern (SP1, SP2, SP3) regions of Brazil ($\Phi_{CT} = 0.2011$; $P = 0.0000$),

TABLE 2 | Population statistics of *Pseudobatos horkelii* and *P. percellens*, based on D-loop. Number of individuals (n), polymorphic sites (S), number of haplotypes (H), haplotype diversity (H_d), nucleotide diversity (π), mean number of pairwise differences (K).

Specie	Locality	Code	S	H	H_d	ϖ	K
P. horkelii	Mar del Plata, Argentina	AR	3	3	0.5455+/-0.1436	0.0013+/-0.0006	0.9090+/-0.6760
	Torrinha, Rio Grande do Sul, Brazil	RS	9	10	0.8902+/-0.0214	0.0043+/-0.0018	3.0756+/-1.6323
	Florianópolis, Santa Catarina, Brazil	SC	5	4	0.7619+/-0.0401	0.0031+/-0.0015	2.2164+/-1.2710
	Pontal do Paraná, Brazil	PR	4	2	0.5333+/-0.0456	0.0030+/-0.0015	2.1333+/-1.2514
	Santos, São Paulo, Brazil	SP3	6	3	0.6263+/-0.0787	0.0032+/-0.0009	2.3105+/-1.3195
	Angra dos Reis, Rio de Janeiro, Brazil	RJ	9	5	0.5540 +/-0.1104	0.0029+/-0.0010	2.0326+/-1.1830
P. percellens	Florianópolis, Santa Catarina, Brazil	SC	3	3	0.6550 +/- 0.0566	0.0020+/-0.0009	1.3099 +/-0.8546
	Paraná, Brazil	PR	1	2	0.1533 +/- 0.0915	0.0002+/-0.0000	0.1533 +/-0.2217
	Cananéia, São Paulo, Brazil	SP1	2	3	0.6417 +/- 0.0670	0.0011+/-0.0004	0.7583 +/-0.5874
	Mongaguá, São Paulo, Brazil	SP2	1	2	0.4848 +/- 0.1059	0.0007+/-0.0000	0.4848 +/-0.4475
	Santos, São Paulo, Brazil	SP3	3	5	0.6578 +/- 0.0663	0.0012+/-0.0005	0.8128 +/-0.6014

and revealed significant differences between locations within regions (Φ_{SC} = 0.01972; P = 0.0000) (Tab. 3).

The indices of demographic analysis using the neutrality index showed non-significant values of neutrality by Tajima's D and Fu's FS tests (**S3**). Despite of the Raggedness and SSD indices are significant (P > 0.05) for almost all analysis in different tests, the graph of mismatch showed unimodal pattern between all samples (**S4**). The Bayesian analysis generated two groups which did not correspond to the geographic localities (Fig. 2A). Mantel test results a negative correlation for *P. percellens* (r = –0.3160, P = 0.3780).

TABLE 3 | Hierarchical AMOVA for the control region of *Pseudobatos horkelii* and *P. percellens*. Samples of *P. horkelii* were grouped into northern Argentina (AR), southern Brazil (BRA: RS, SC, PR) and southeastern Brazil (BRA: SP3, RJ). Whereas for *P. percellens*, were grouped into southern Brazil (BRA: SC, PR) and southeastern Brazil (BRA: SP1, SP2, SP3).

Species		Source of variation	Degrees of freedom	Sum of squares	Variance component	Percentage of variation	Fixation index	*P* value
P. horkelii	All sampling areas	Among groups	5	101.083	0.87547	42.77	0.4277	0.0000±0.0000
		Within groups	129	151.110	1.1713	57.23	-	
	northern AR + southern BRA *vs* southeastern BRA	Among groups	1	3.605	0.0056	1.18	0.2576	0.0000±0.0000
		Among populations within groups	4	11.641	0.1210	25.46	0.2664	0.0000±0.0000
		Within population	129	45.005	0.3488	73.36	0.0118	0.3167±0.0153
P. percellens	All sampling areas	Among groups	4	12.716	0.1434	29.22	0.29218	0.0000±0.0000
		Within groups	96	33.363	0.3475	70.78	-	
	southern BRA *vs* southeastern BRA	Among groups	1	1.587	-0.0015	-0.48	0.2011	0.0000±0.0000
		Among populations within groups	3	4.348	0.0636	20.21	0.1972	0.0000±0.0000
		Within populations	96	24.283	0.2529	80.27	-0.0048	0.4975±0.0157

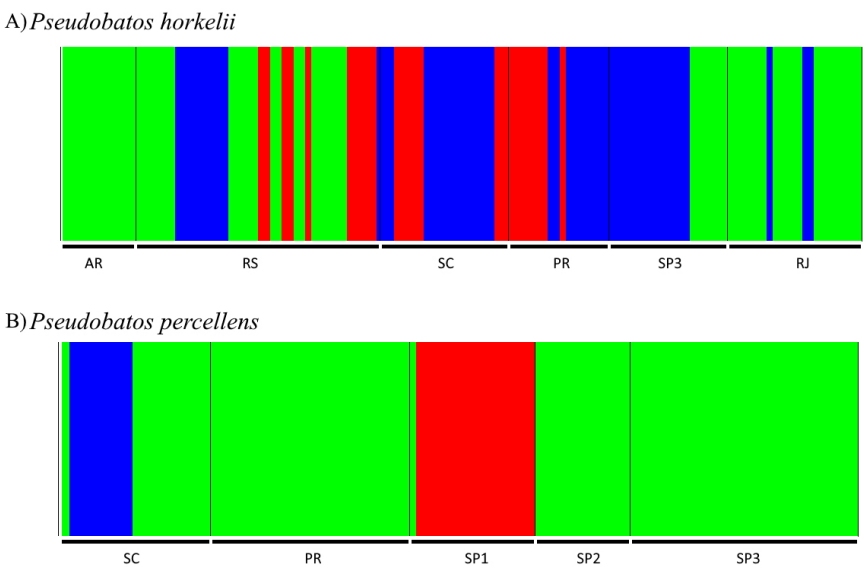

FIGURE 2 | Graph of the Bayesian analysis of population structure of mtCR for **A.** *Pseudobatos horkelii* and **B.** *Pseudobatos percellens*. Samples from northern Argentina (AR), Torrinha/RS (RS), Florianópolis/SC (SC), Pontal do Paraná/PR (PR), Cananéia/ SP (SP1), Mongaguá/SP (SP2), Santos/SP (SP3), Rio de Janeiro/RJ(RJ).

DISCUSSION

The results of the present study provide interesting insights concerning the genetic diversity of two endangered guitarfish species *Pseudobatos horkelii* and *P. percellens* occurring in the Southeastern region of the Atlantic Ocean. The D-loop mitochondrial marker was used to obtain information regarding connectivity, genetic diversity and population structure of the species along the coast of Northern Argentina, Southern and Southeastern Brazil. These indexes indicate that both species present population structure in the different regions analyzed. Our data revealed that populations of *P. horkelii* showed significant differences among all locations sampled in Mar del Plata in Argentina and in five localities along the coast of the states of Rio Grande do Sul, Santa Catarina, Paraná, São Paulo and Rio de Janeiro in Brazil. Similar results were obtained in the analysis of *P. percellens*, which showed significant differences among samples obtained along the coast of the states of Santa Catarina, Paraná, and São Paulo, from which individuals from Cananéia, Mongaguá, and Santos were assessed.

The available genetic studies conducted on *Pseudobatos* species have so far been related to molecular identification of species and fishery products using the DNA barcode methodology as a molecular marker (Franco *et al*., 2012; Souza *et al*., 2018), and refer mainly to illegal trade of threatened species in different regions of the Brazilian coast, including locations in the states of Rio de Janeiro, São Paulo, Paraná, Santa Catarina and Rio Grande do Sul. Interestingly, even though guitarfish are listed as endangered species, our study's data indicate considerable genetic diversity levels. *Pseudobatos horkelii*, which is currently a "Critically Endangered" (CR) species, showed indices of haplotypic diversity of H_d = 0.6518 and nucleotide diversity of π = 0.0029 as a result of ten mutations of the 702 bps of D-loop region sequenced, that resulted in 16 haplotypes identified in 135 individuals sampled. Alternatively, in *P. percellens* currently listed as "Endangered" (EN), the average genetic diversity indices are slightly smaller, with H_d = 0.5185 and π = 0.0010, and only six mutations were found, distributed in 646 bps sequenced, with eight haplotypes.

The same level of diversity have also been found in other species of skates of the family Rajidae, in *Raja straeleni* Poll, 1951 classified as "Deficient Data" (DD) (Smale, 2009), the values found for haplotypic diversity were H_d = 0.67, and for nucleotide diversity π = 0.0025; and in *Raja clavata* Linnaeus, 1758 classified as "Near Threatened" (NT) (Ellis, 2016), the value of H_d was 0.55 and π was 0.0023 (Pasolini *et al*., 2011). In the family Pristidae, the sawfish *Pristis pristis* (Linnaeus, 1758) classified as "Critically Endangered" (CR) (Kyne *et al*., 2013), H_d was 0.39 and π was 0.0011 (Feutry *et al*., 2015). In this context, the real situation presented by the levels of genetic diversity in rays requires a most focused attention concerning conservation of wild stocks of the species in dangered situation of disappearance in this group of organisms. The values of H_d and π found for *Raja straeleni*, a species with deficient data of dangerousness, points to a situation similar as *P. horkelii* currently listed as "Critically Endangered" (CR). According to Domingues *et al.* (2018), despite an increase in the number of genetics studies in the last decade, only ~ 10% of shark and ray species have been investigated in terms of their population genetic structure, genetic diversity and demographic history, theses information's genetics, could help the determine applications for conservation (Hoban *et al*., 2013a,b).

Population structure and demography. Levels of genetic differentiation among guitarfishes revealed patterns of population structure along the Southeastern Atlantic Ocean area for *P. horkelii* and *P. percellens*. A hypothesis to explain the genetic structure may be the resident behavior of the species, which performs seasonal migrations only among depth zones between 50 and 150 m during the reproductive period that occurs in the winter and remains together up to 20 m depth (Vooren, 1997). Similar findings of population structure in other resident batoids species have been reported with *Hypanus americanus* (Hildebrand & Schroeder, 1928) (Richards *et al*., 2019). The analysis of 267 individuals of this species sampled from Eastern USA and Caribbean using the D-loop marker revealed a high level of genetic partitioning among localities (Φ_{ST} = 0.49; P < 0.0000) that permitted differentiate the samples into three populations. Furthermore, Le Port *et al.* (2011) also observed a high level of genetic structuring in samples of *Bathytoshia brevicaudata* (Hutton, 1875) among South Africa, Australia, and New Zealand populations, with an overall Φ_{ST} = 0.67, P < 0.001, this structuring may be result of that the deep oceanic basins (and tropical waters) act as a major barrier to stingray dispersal.

In addition, other fact that may have influenced the structure of these populations may involve fishing activities. Few abundance estimates are available for the Brazilian guitarfish throughout its range, *P. horkelii* was highly explored in the Rio Grande do Sul region in the years 1980s and 1990s, with the mean biomass of annual catches ranging from 600 to 1,800 tons (Miranda, Vooren, 2003), and the according the authors, believe that the effects of overfishing, may cause a population decline. However, for *P. percellens* there are no regular records of fishing exploitation in the region the available data indicate occurrence of capture by bycatch, that is, as accompanying fauna in fishing of other commercially interesting species (Lessa, Vooren, 2007). Such activity also should determine strong interferences in the structure of populations.

In this case, the tests of neutrality (the Tajima D and Fu FS statistics) were used to check the excess of rare mutations. Non-significant results showed evidence of recent population expansion for *P. horkelii.* The demographic analysis for the *P. horkelii* based on D-loop pairwise nucleotide differences showed a bimodal mismatch distribution model and a high Harpending's raggedness index in values ranging from 0.0183 to 0.7866, with significant P values. The bimodal curve indicates the existence of a population demographically stable (Slatkin, Hudson, 1991; Rogers, Harpending, 1992) that has suffered a sudden size reduction, a population bottleneck and should be in a recent population expansion. In this case, the tests of neutrality (the Tajima D and Fu FS statistics) were used to check the excess of rare mutations. Non-significant results showed evidence of recent population expansion for *P. horkelii*. This could suggest that the bimodal curve characterizes the existence of stable populations, despite the great fishing exploitation that the species has suffered in the last decades.

The mismatch distribution analysis showed unimodal graphic for *P. percellens*, which could indicate the occurrence of population expansion events or events of expansion of the geographic distribution area, with a high migration rates among close populations (Rogers, Harpending, 1992). If this information is analyzed together with the non-significant data from the neutrality tests, the results could be interpreted as a strong indication that in *P. percellens* it is possible that a high migration rate is occurring between the analyzed sites.

In the present study, the results of the Bayesian analysis for *P. horkelli* and *P. percellens*

indicated K = 3 clusters. For *P. horkelii*, Rio Grande do Sul was the only locality showed the three clusters, Santa Catarina and Paraná showed the same clusters (blue and vermelho), while Argentina showed a single cluster (green). In *P. percellens*, presented for Paraná and Santos a single cluster (green), while individuals from Santa Catarina and Cananéia showed different clusters, blue and green (SC) and red and green (SP1), respectively.

Even though *P. horkelii* has suffered a severe population decline of more than 80% due to overfishing (Lessa, Vooren, 2007), its populations revealed high indices of genetic diversity, suggesting the existence of stable populations of this species, with highest haplotypic diversity occurring mostly in the region of Rio Grande do Sul, where the prohibition on the exploitation of this species should be most effective. Even considering the lack of population or capture data available for *P. percellens*, this species is captured by intense fishing practices similar to those observed for *P. horkelii*, in which different types of trawls, gillnets, and seines are used, which has also led to a significant decline in populations of this species in recent years. In this context, it must be considered that the proper management of coastal habitats inhabited by these species, as well as an efficient management and control of fisheries at the regional and national levels should be a priority in planning conservation programs.

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AUTHOR'S CONTRIBUTION

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Pablo H. Oliveira: Methodology, Software.

Giovana S. Ribeiro: Methodology, Software.

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Alexandre S. F. Rodrigues: Data curation.

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Claudio Oliveira: Resources, Supervision, Validation, Writing-original draft, Writing-review and editing.

Rosangela P. Lessa: Conceptualization, Writing-review and editing.

Fausto Foresti: Conceptualization, Resources, Supervision, Writing-original draft, Writing-review and editing.

ETHICAL STATEMENT

All samples were collected in strict accordance with the regulations of the Brazilian Federal Animal Ethics Committee (SISBIO 13843-1), and the analyses followed the International Guidelines for Animal Experiments, as authorized by CEEAA IBB/UNESP, protocol number 556.

COMPETING INTERESTS

The authors declare no competing interests.

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