

# Integrative taxonomy identifies a new stingray species of the genus *Hypanus* Rafinesque, 1818 (Dasyatidae, Myliobatiformes), from the Tropical Southwestern Atlantic

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## Abstract

An integrative approach by the congruence of genetics, morphology and ecological niche modelling (ENM) was used to delimit a new species of *Hypanus* (Rafinesque, 1818), a recently resurrected genus of marine stingrays comprising eight species, five of which occur in the western Atlantic. The species with the widest distribution, *Hypanus americanus* (Hildebrand and Schroeder, 1928), from the northeastern coast of the United States to southeastern Brazil, was demonstrated to be paraphyletic based on protein-coding mitochondrial genome analyses. This data set also indicates that the genetic distance between the new species *Hypanus berthalutzae* sp. nov. and its three closely related species (*H. americanus*, *H. longus* and *H. rудis*) varies from 0.82% to 3.14%. In addition, Bayesian Analysis of Population Similarity using the mitochondrial gene *mt-nd2* supports the separation of *H. berthalutzae* sp. nov. (southwestern Atlantic) from its sister species *H. rудis* (eastern Atlantic). Similarly, morphological and morphometric analyses corroborated four morphotypes within the *H. americanus* species group and indicated the ventral caudal fold height and length and interspiracular and interorbital lengths as useful measurements to distinguish among them. Claspers of adult males also exhibit morphological differences among species. The ENM agreed with molecular and morphological analyses and delimits the distribution of *H. berthalutzae* sp. nov. to shallow areas close to shore along the Brazilian coast, from the mouth of the Amazon River to São Paulo State, including the northeastern oceanic islands, suggesting that the great outflow of fresh water and sediments and the Mid-Atlantic Ridge might act as barriers. The integration of these data to describe a new species provides information relevant to their conservation status, because all species of the *H. americanus* species group are under the “data-deficient” status.

## KEY WORDS

Brazil, ecological niche modelling, mitogenome, morphology, systematics

## 1 | INTRODUCTION

It has long been known that stingrays in the genus *Dasyatis* Rafinesque (1810) are not monophyletic based on both morphological (Rosenberger, 2001) and genetic (Naylor et al., 2012) data. Nonetheless, it was only recently that the family Dasyatidae was revised (Last, Naylor, & Manjaji-Matsumoto, 2016) and that *Dasyatis* was split into eight genera, one of which was the resurrected genus *Hypanus* Rafinesque (1818), whose delimitation was based on molecular data, without diagnostic morphological characters (Last, Naylor, & Manjaji-Matsumoto, 2016). Distributed in the eastern and western coasts of the Americas and Western Africa, it consists of eight valid species, five of which occur in the western Atlantic Ocean. Nonetheless, samples of *Hypanus marianae* (Gomes et al., 2000) and *Hypanus rufus* (Günther, 1870) were not available to Last, Naylor, and Manjaji-Matsumoto (2016), and so their inclusion in the genus was provisional. *Hypanus* still lacks a morphological diagnosis, and species are grouped based on molecular clusters and morphological similarities (Last, Naylor, & Manjaji-Matsumoto, 2016).

Bigelow and Schroeder (1953) provided a thorough description of these stingrays occurring in the north Atlantic: *Hypanus americanus* (Hildebrand & Schroeder, 1928), *Hypanus guttatus* (Bloch & Schneider, 1801), *Hypanus sabinus* (Lesueur, 1824) and *Hypanus say* (Lesueur, 1817). Nonetheless, other species were described after this revision: *H. marianae* from the northeastern coast of Brazil, *Hypanus longus* (Garman, 1880) and *Hypanus dipterurus* (Jordan & Gilbert, 1880) from the Pacific coast of America and *H. rufus* from Western Africa. An updated review of the whole genus, based on both molecular (Last, Naylor, & Manjaji-Matsumoto, 2016) and morphological data, is now warranted.

The species with the widest distribution is *H. americanus*, extending from New Jersey (United States) to São Paulo (Brazil) in the western Atlantic. Its most closely related congener, *H. longus*, ranges from Baja California to Colombia and the Galapagos Islands [Eastern Pacific (EP)] (Last, Naylor, Séret, et al., 2016). Nonetheless, this assessment of relatedness was not based on comprehensive sampling throughout the range of *H. americanus* but rather was based on sampling that was restricted to the northwestern Atlantic (NWA) and Gulf of Mexico (Last, Naylor, & Manjaji-Matsumoto, 2016). Because most stingrays are benthic coastal species with specific habitat requirements (Le Port & Lavery, 2012), their species distributions could be constrained by external influences (Pyron & Burbrink, 2010) such as freshwater incursions, temperature and marine currents (Last, Naylor, Séret, et al., 2016).

A recent molecular identification of rays traded on the Brazilian Amazon coast has revealed that there appear to be two distinct lineages of *H. americanus* (Rodrigues Filho et al., 2020). These two lineages correspond to samples from the NWA and southwestern Atlantic (SWA) – regions that were previously thought to be homogeneous with respect to the distribution of *H. americanus* (Rodrigues Filho et al., 2020). Interestingly, the Amazon River outflow, a known geographic barrier for many marine species (Rocha, 2003), discharges into the Atlantic Ocean and may be responsible for driving the divergences within *Hypanus* genus.

The currently valid species *H. americanus* is reported as “data deficient” by the Red List of IUCN (Grubbs et al., 2016). In Brazil, stingrays are targeted both for food and as by-catch (Gadig et al., 2000). Nonetheless, a population of *H. americanus* has been identified on a conservation unit in Fernando de Noronha (Pernambuco, Brazil), an oceanic archipelago 250 km from the coast in northeastern Brazil (Aguiar et al., 2009).

In an effort to better understand the distribution and divergence between the two identified lineages of *H. americanus*, a study based on genetics, morphology, and ecology was undertaken to delimit the species as an evolutionary unit based on associations of many taxonomic features (Padial et al., 2010). The authors sequenced DNA data from the mitochondrial (mt) protein-coding region and the gene NADH dehydrogenase 2, mitochondrial (mt-nd2) (Naylor et al., 2012); examined morphological and morphometric data; and applied an ecological niche modelling (ENM) approach (Gabbanelli et al., 2018). ENM can be used to generate the biogeographic hypotheses that are subsequently tested with genetic and morphological data and to detect areas of unsuitable habitat that might serve as geographic barriers or that restrict species' distribution (Alvarado-Serrano & Knowles, 2014), such as the Amazon River discharge as a barrier for *H. americanus* (Hoorn et al., 2010; Rocha, 2003).

Although there is almost universal agreement among the scientific community on the importance of conserving biodiversity, implementing practical conservation measures can run into problems because of the lack of integration among sub-disciplines of biology relevant to conservation efforts (Diniz-Filho et al., 2013). Four shortfalls are identified: (a) Linnean shortfall (Brown & Lomolino, 1998), which corresponds to the lack of species' descriptions, necessary to document the biodiversity; (b) Wallacean shortfall (Lomolino, 2004), where species' geographical distributions are unknown; (c) Hutchinsonian shortfall (Mokany & Ferrier, 2011), where the relation of the species to its environment is unknown; and (d) Darwinian shortfall (Diniz-Filho et al., 2013), which corresponds to the absence of knowledge of a species' phylogenetic placement (Cardoso et al., 2011; Diniz-Filho et al., 2013; Whittaker et al., 2005). These four shortfalls are addressed in this study.

To determine if *H. americanus* constitutes a broadly distributed species or a species complex, samples, specimens and records of all putative *H. americanus*, along with the closely related *H. longus* and *H. rufus*, were assessed. This information will be useful to evaluate the conservation status and to support fisheries management policies of Atlantic stingrays to be effective (Almeida Marques et al., 2019; Dulvy et al., 2017; Lima et al., 2019).

## 2 | MATERIALS AND METHODS

### 2.1 | Ethical statement

The care and use of animals complied with the Brazilian Ministry of the Environment animal welfare laws, guidelines and policies as approved by Chico Mendes Institute for Biodiversity Conservation, licence SISBIO 54254-3. Most materials were obtained in fish

collections, fish markets and landings. A few were obtained using a tissue spur to collect superficial flesh from the dorsal pectoral fin of live specimens in free diving, scuba dives or from small captured specimens that were immediately released. Only one stingray from Fernando de Noronha Marine National Park was euthanized to be the holotype of the newly described species.

## 2.2 | Ecological niche modelling

Two ecological niche models (ENM) were developed in compliance with the ODMAP protocol (overview/conceptualisation, data, model fitting, assessment and prediction) suggested by Zurell *et al.* (2020). The first was for the currently known species *H. americanus* throughout its distribution ( $100^{\circ}$  W to  $21^{\circ}$  E and  $33^{\circ}$  S to  $37^{\circ}$  N) to identify possible ecological barriers to these stingrays and raise hypotheses to be tested by molecular and morphological data. After these analyses were conducted, a second ENM was developed but using only location data from the SWA *H. americanus* clade; both modelling approaches used the maximum entropy modelling (MaxEnt) methodology (Phillips *et al.*, 2006) implemented in R software (R Core Team, 2019). The authors used the R package ENMeval (Muscarella *et al.*, 2014) to select the checkerboard1 partition method for training (75% of the data) and testing (25%), to choose the best combination of feature classes (linear, quadratic, hinge, product and threshold) and regularization multiplier (from 0.5 to 4, in 0.5 increments) and to use evaluation metrics to select the best model performance for each data set, such as deltaAICc that the closer to zero the best fitted the model, with low omission rates (Muscarella *et al.*, 2014). To run the models,  $10^{-5}$  convergence threshold, 10,000 maximum iterations and 10,000 maximum background points were used. A total of 15 replicates were run on each data set, and the average values were plotted. Also Jackknife tests were performed to evaluate the importance of each environmental variable in the models. Maxent raster files were edited in QGIS 3.2.3 (QGIS.org, 2020).

This study used 241 location records of *H. americanus* sensu lato and, of those, selected 38 location records of *H. americanus* (SWA) deposited in scientific collections from the online databases speciesLink (CRIA, 2019), FishNet2 (FishNet2, 2020) and GBIF (GBIF.org, 2020), as well as locations of examined specimens from morphological and molecular analyses [Table S2 (Supporting Information)]. Records went through a spatial thinning method to reduce overfitting (Boria *et al.*, 2014) using the package spThin (Aiello-Lammens *et al.*, 2015).

Environmental variables with predictive potential for species' distribution were extracted from the databases MARSPEC (Sbrocco & Barber, 2013) and Bio-ORACLE (Assis *et al.*, 2018) in a spatial resolution of 5 arcmin (9.2 km) in ESRI ASCII format. All layers were cropped according to the Marine Ecoregions of the World (Spalding *et al.*, 2007) using the function CropRaster of the package ENMGadgets (Barve & Barve, 2019) in R software (R Core Team, 2019). The data were then subjected to a Pearson correlation analysis among 41 variables to identify and remove highly correlated variables ( $>|0.8|$ ) (Dormann *et al.*, 2013). The variables used for assessing the ecological

niche of both data sets were benthic salinity, distance to shore, sea-surface salinity mean, sea-surface salinity range, sea-surface temperature mean, sea-surface temperature range, surface calcite, surface pH and surface silicate; for *H. americanus* sensu lato, bathymetric slope, bathymetry, benthic phosphate, benthic primary productivity, surface nitrate and surface phytoplankton were also included; and for the SWA group, benthic dissolved oxygen, surface phosphate and surface primary productivity were included.

## 2.3 | Molecular data

Based on the definition of the *Hypanus* genus by Last, Naylor and Manjaji-Matsumoto (2016), the most closely related species to *H. americanus* was selected to better define this species and its relationships. For the protein-coding mitochondrial analyses (mtDNA), in total, 40 specimens were sampled, of which 8 are *H. americanus* from Massachusetts (United States) to Belize (NWA) and 23 from Pará to Bahia (Brazil) (SWA); this division of *H. americanus* is based on the results from the ENM. Also four specimens of *H. longus* were sampled from Baja California Sur (Mexico) (EP) and four *H. rufus* from Senegal and Ghana [Eastern Atlantic (EA)], which had not been sampled by Last, Naylor, and Manjaji-Matsumoto (2016). *H. guttatus* was selected as an out-group representing the most closely related species to the in-group under investigation (*H. americanus*, *H. longus* and *H. rufus*). As such, it provides a reference against which to understand evolutionary changes inferred to have occurred. One specimen of *H. guttatus* from Pará (Brazil) was included in mtDNA analyses.

These specimens, except *H. guttatus*, were used for analyses using *mt-nd2* in addition to 12 representatives of *H. americanus* from Fernando de Noronha oceanic archipelago, Rio de Janeiro and São Paulo states (Brazil) [Table Supporting Information S1 (Supporting Information)], adding up to 51. The samples were provided by collaborators, collected in fish markets, or obtained by non-lethal sampling of specimens (SISBIO 54254-3, SISGEN A88B728). Other *Hypanus* species were not included given their morphological and molecular differences to this *H. americanus* species group (Last, Naylor, & Manjaji-Matsumoto, 2016).

Genomic DNA was extracted from muscular tissue samples using the E.Z.N.A Tissue DNA Kit (Omega Bio-Tek, Georgia, USA), and the amount of DNA was quantified using the Qubit 2.0 Fluorometric (Life Technologies Corporation, Grand Is, New York, USA) to obtain from 0.5 to 3  $\mu$ g of genomic DNA. For the first step of library preparation, the DNA was sheared to 500 bp using an M220 Focused-ultrasonicator (Covaris, Inc., Woburn, Massachusetts, USA). Sheared DNA went through a size-selection process, solid-phase reversible immobilization beads (for details, refer to Li *et al.*, 2013), to select fragments with more than 250 bp. Then, samples went through a series of reactions: blunt-end repair, adapter ligation, fill-in and pre-hybridization PCR with indexing primers for each sample, where all steps are carefully described by Li *et al.* (2015). Then, the method of mitochondrial gene capture proceeded, and an Illumina MiSeq Next Generation Sequencer was used for mitochondrial genome sequencing. For reaction details, refer to Li *et al.* (2013).

### 2.3.1 | Assembly and mitogenome annotation

Adaptors P5 and P7 (Li *et al.*, 2013) were trimmed from the MiSeq reads using Trim Galore (Krueger, 2020) script with the tool Cutadapt (Martin, 2011) based on a Phred quality score of 30. Then, reads from an individual were assembled by mapping it to a known mitochondrial genome reference using Geneious 7.9.1 (<https://www.geneious.com>). The sequences were annotated using a MitoAnnotator pipeline at MitoFish website (Iwasaki *et al.*, 2013), and RNA regions were excluded, leaving only protein-coding genes.

### 2.3.2 | Alignments and phylogenetic reconstructions

Mitochondrial genomes of the 40 specimens were aligned in Geneious 7.9.1 (<https://www.geneious.com>) using the MUSCLE software (Edgar, 2004). Mitochondrial control regions were excluded from all sequences because this region is highly variable among individuals and the coverage after mitochondrial gene capture was too low. The final alignment comprised 11,471 base pairs based on 13 protein-coding genes (GenBank accession numbers MT326596–MT326635). The best-fitting molecular evolution model was GTR + gamma + invariant based on both likelihood criteria jModelTest2 (Darriba *et al.*, 2012; Guindon & Gascuel, 2003) in CIPRES Science Gateway (Miller *et al.*, 2010) and Bayesian inference criterion (Schwarz, 1978). Phylogenetic analyses were conducted using maximum likelihood (RAxML) (Stamatakis, 2014) in CIPRES Science Gateway with 1000 bootstrap replicas and Bayesian inferences in BEAST 2.5 (Bouckaert *et al.*, 2019) using the GTR + gamma (1.62) + invariant site (0.52) model, Yule model as the prior tree, and 10,000,000 generations were sampled every 1000. Genetic p-distances (Jukes & Cantor, 1969) were calculated in MEGA X (Kumar *et al.*, 2018) to analyse intra- and inter-specific genetic differences between closely related taxa.

### 2.3.3 | Lineage delimitation

This study conducted six lineage delimitation analyses within the *H. americanus* species group using the mtDNA data set: multiple- and single-threshold generalized mixed Yule coalescent (m-GMYC and s-GMYC, Fujisawa & Barraclough, 2013), multi-rate Poisson tree process (mPTP, Kapli *et al.*, 2017), Bayesian Poisson tree process (bPTP, Zhang *et al.*, 2013), automatic barcode gap discovery (ABGD, Puillandre *et al.*, 2012) and Bayesian phylogenetics and phylogeography (BPP, Flouri *et al.*, 2018). Both tree topologies obtained using the aforementioned methods were used in the following methods.

GMYC calculates the maximum likelihood for a model that merges diversification among species and cladogenesis within species on an ultrametric time-tree. m-GMYC and s-GMYC were performed in R software using the package *splits* (Ezard *et al.*, 2017). PTP uses nucleotide substitutions to estimate intra- and interspecific processes. It

identifies the shift among intra- and interspecific processes by using one parameter for coalescence and another for speciation, and mPTP uses distinct branch rates for coalescence and speciation. For mPTP and bPTP analyses, the web server <https://species.h-its.org/ptp/>, was used. ABGD separates lineages into possible species based on a barcode gap (between intra- and interspecific pair-wise genetic distances). These analyses were also performed through a web server (<https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html>) using three distinct distance options (Jukes-Cantor, Kimura 2-parameter and Simple), intraspecific prior ranges from 0.001 to 0.25 in 10 steps and a relative gap width of 1.5.

BPP uses genomic data sets to adapt ancestral genetic polymorphisms and coalescent processes by using Markov chain Monte Carlo (MCMC) to calculate distinct species trees' posterior probabilities (Flouri *et al.*, 2018). For BPP analyses, this study evaluated four scenarios combining large and small ancestral populations and deep and recent divergences which were performed thrice each,  $5 \times 10^5$  generation samples and a burnin of 5000: (a) a large ancestral population with deep divergences,  $\theta = \text{invgamma}(2, 0.1)$  and  $\tau = \text{invgamma}(2, 0.1)$ ; (b) a large ancestral population with recent divergences,  $\theta = \text{invgamma}(2, 0.1)$  and  $\tau = \text{invgamma}(3, 0.002)$ ; (c) a small ancestral population with deep divergences,  $\theta = \text{invgamma}(3, 0.002)$  and  $\tau = \text{invgamma}(2, 0.1)$ ; and (d) a small ancestral population with recent divergences,  $\theta = \text{invgamma}(3, 0.002)$  and  $\tau = \text{invgamma}(3, 0.002)$ .

### 2.3.4 | Amplification, sequencing and analyses of mt-nd2

PCRs were carried out to amplify the mitochondrial gene NADH dehydrogenase 2, mitochondrial (mt-nd2) for 51 samples [Table SUPPORTING INFORMATION S1 (Supporting Information)] using a pair of primers proposed by Naylor *et al.* (2005), Ilem-Mustelus and Asn-Mustelus. The reactions were performed in a volume of 25  $\mu\text{l}$  containing 0.3  $\mu\text{M}$  primers, 2.5 mM MgCl<sub>2</sub>, 200  $\mu\text{M}$  each of deoxynucleotide triphosphate, 10x Ex Taq buffer (20 mM Tris-HCl, pH 8.0; 100 mM KCl; 0.1 mM EDTA; 1 mM dithiothreitol; 0.5% of Tween 20; 0.5% of Nonidet P-40; and 50% of glycerol), 0.25 UTaKaRa Ex Taq (Takara, Mountain View, CA, USA) and 50–100 ng of template DNA. The reactions were denatured at 94°C for 3 min and then went through 35 cycles of denaturation at 94°C for 30 s, annealing at 48°C for 30 s and extension at 72°C for 90 s. PCR products were bi-directionally Sanger sequenced at DNA Sequencing Facility (San Diego, CA, USA) and Macrogen Inc. (Seoul, Korea). DNA sequences were edited using Geneious 7.9.1 (<https://www.geneious.com>) (GenBank accession numbers: MT319677–MT319728).

All 51 sequences were aligned using the MUSCLE software implemented in Geneious 7.9.1. The alignment included most of the coding sequence of mt-nd2, flanked by tRNA-Gln and tRNA-Met at the 5' end and tRNA-Trp and tRNA-Ala at the 3' end. An 80 bp segment at the 3' end of mt-nd2 was removed as poor sequence resolution prevented reliable alignment. The software package MEGA X (Kumar *et al.*, 2018) was used to determine the best-fitting model of molecular evolution to the data (HKY + G).

The software DNAsp (Rozas *et al.*, 2017) was used for the generation of a haplotype network. Haplotypes and location information were combined in the software PopArt (Leigh & Bryant, 2015) using the algorithm TCS (Clement *et al.*, 2002) to build haplotype networks, which is a statistical parsimony method that incorporates unobserved haplotypes aiding the identification of microevolutionary events (Paradis, 2018; Posada & Crandall, 2001). The software BAPS 6.0 (Bayesian Analysis of Population Structure) (Corander & Marttinen, 2006) was used to estimate the number of genetic clusters and test admixture levels associated with different geographic locations. Because this analysis works at the population level, a small clade was chosen to evaluate their differences, instead of analysing the whole *H. americanus* species group.

## 2.4 | Morphology

Seventy-nine preserved specimens, determined to be distinctly based on genetic analyses, of *Hypanus* were examined: 59 of *H. americanus* (42 from NWA and Gulf of Mexico and 17 from SWA), 15 of *H. longus* and 5 of *H. rufus* from the collections: Laboratório de Ictiologia de Ribeirão Preto, Universidade de São Paulo (LIRP), Museum of Comparative Zoology (MCZ), Harvard University, Museu Nacional, Universidade Federal do Rio de Janeiro (MNRJ), Museu de Zoologia da Universidade de São Paulo (MZUSP), Biodiversity Research and Teaching Collections, Texas A&M University (TCWC), Universidade do Estado do Rio de Janeiro (UERJ), University of Florida, Florida Museum of Natural History (UF), National Museum of Natural History, Smithsonian Institution (USNM) (collections' codes follow Sabaj, 2016). The holotypes of *H. americanus* and syntypes of *H. longus* were analysed, whereas the holotype of *H. rufus* was lost (Séret, 1990). A list of the examined comparative material can be found in Supporting Information S1 encompassing representatives from an extensive area of distribution, including ontogenetic stages for each species whenever specimens were available in collections. Measurements and their abbreviations are presented in Figure Supporting Information S1 (Supporting Information), except the tail origin to spine origin length (TSL) and caudal height at pelvic fin end (CH), which were measured in lateral view.

The specimens were measured following Last *et al.* (2009) and Manjaji (2004) in a total of 42 measurements. External morphology descriptions followed Last and Stevens (2009) and Manjaji (2004); clasper descriptions were in accordance with de Moreira *et al.* (2018). The morphometric data were standardized to reduce the allometric influence on data using the equation  $M_s = M_0(L_s/L_0)^b$  (Konan *et al.*, 2010), where  $M_s$  is the specimen's standardized measure,  $M_0$  is the original measure,  $L_s$  is the arithmetic average of the disc width of all measured specimens and  $L_0$  is the disc width of the examined specimen. The allometric coefficient ( $b$ ) is calculated by the linear regression of  $M_0 \log$  vs.  $L_0 \log$ .

On the data set in which the allometry has been corrected, a variance inflation factor (Zuur *et al.*, 2009) analysis was done to remove autocorrelated variables with a value higher than 5, because 3, as

suggested by Zuur *et al.* (2009), would remove many variables relevant to the distinction of these stingrays we opted to take our knowledge of the group into account (O'brien, 2007). Then, the presence of outliers was detected, and those specimens were removed from the data set. Before statistical analyses were conducted, the data were tested for parametricity (homogeneity and homoscedasticity) using the function "mvn" of the R package MVN (Korkmaz *et al.*, 2014). A principal component analysis (PCA) of the final data set, with uncorrelated measurements and absence of outliers, was conducted to characterize the variation in the samples (Schlager, 2017) and to designate specimens to groups based on their characteristics. A canonical variate analysis (CVA) that maximizes the distances between groups (Mitteroecker & Bookstein, 2011; Schlager, 2017) was also performed.

Finally, a MANOVA for the parametric data set or permutational multivariate analysis of variance for the non-parametric data set using the R package vegan (Oksanen *et al.*, 2019) was applied to test the differences in the overall means across the species being compared, in which a significant result ( $P < 0.05$ ) supports morphological differences among the groups being tested. Univariate ANOVA was also carried out using the R package vegan to identify which are the most relevant measurements that reinforce the separation of the groups. All analyses were developed using the R software (R Core Team, 2019). Illustrations and images were edited in Adobe Photoshop CC 20.0.6.

## 3 | RESULTS

### 3.1 | Ecological niche modelling of *H. americanus* *sensu lato*

This study used 124 records of *H. americanus* *sensu lato* after spatial thinning. The ENMeval results pointed the feature classes linear-quadratic-hinge with a regularization multiplier of 1.5 as the best parameters for running the analysis, with a deltaAICc equal to zero. After 15 replicates, the test of omission rate vs. predicted area illustrated that the average omission rate is close to the predicted omission, and the average training AUC for the replicate runs is 0.980 (s.d. 0.003). Therefore, based on a good performance of the model, a clear break near the Amazon River outflow was observed (Figure 1). This ENM was used as *a priori* test to generate the hypotheses to be tested by the integration of morphological and molecular data, as suggested by Alvarado-Serrano and Knowles (2014). The most relevant environmental variables predicting the occurrence are (a) low depth (34.7% of permutation importance), (b) benthic phosphate values close to  $0 \text{ mmol m}^{-3}$  (27.1%), (c) closeness to coast (13.5%) and (d) values of benthic salinity higher than 35 PSU (5.4%). The Amazon River discharge in the Atlantic Ocean provides a salinity decrease to *ca.* 20 PSU (Gouveia *et al.*, 2019), which is a value these stingrays may not tolerate, and therefore the distribution discontinuity. Based on this, it is reasonable to assume both allopatric lineages north and south of this barrier, because the habitat is not adequate for them. As

a consequence of the lack of gene flow, they could be undergoing independent evolutionary paths. This model can be used to test the hypothesis that *H. americanus* is not a natural group.

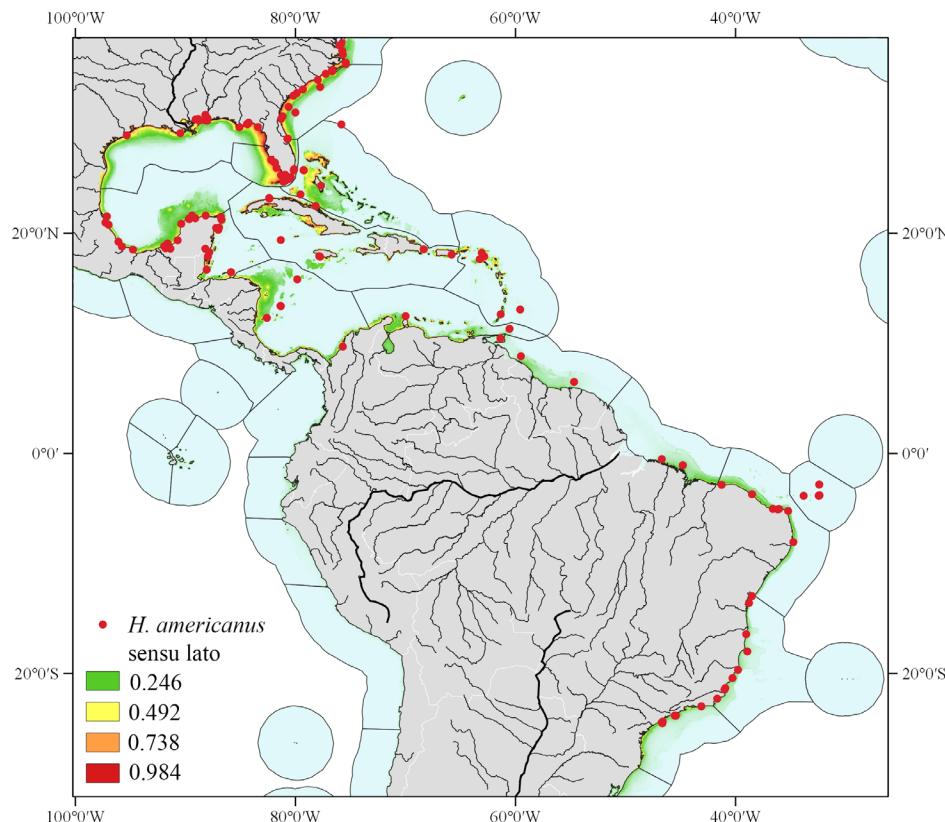
### 3.2 | Molecular phylogeny and lineage delimitation

Both maximum likelihood and Bayesian inference phylogenetic analyses of the *H. americanus* species group using the mtDNA, which resulted in the same tree topology with bootstrap values of nodes higher than 95 and posterior probabilities higher than 0.85, and the combination of the six lineages' delimitation protocols suggest five lineages within this species group (Figure 2). Even though GMYC analyses suggest more delimitations within the data set, the authors decided to be conservative and accept only lineages congruently delimited by the six analyses. Therefore, the currently known species *H. americanus* comprises two distinct clades, one in NWA, *H. americanus sensu stricto*, and the other in SWA, described here as a new species, *H. rufus*, which had been provisionally assigned to the genus (Last, Naylor, & Manjaji-Matsumoto, 2016), is indeed a *Hypanus* species and found to be the sister to *Hypanus berthalutzae sp. nov.* from SWA.

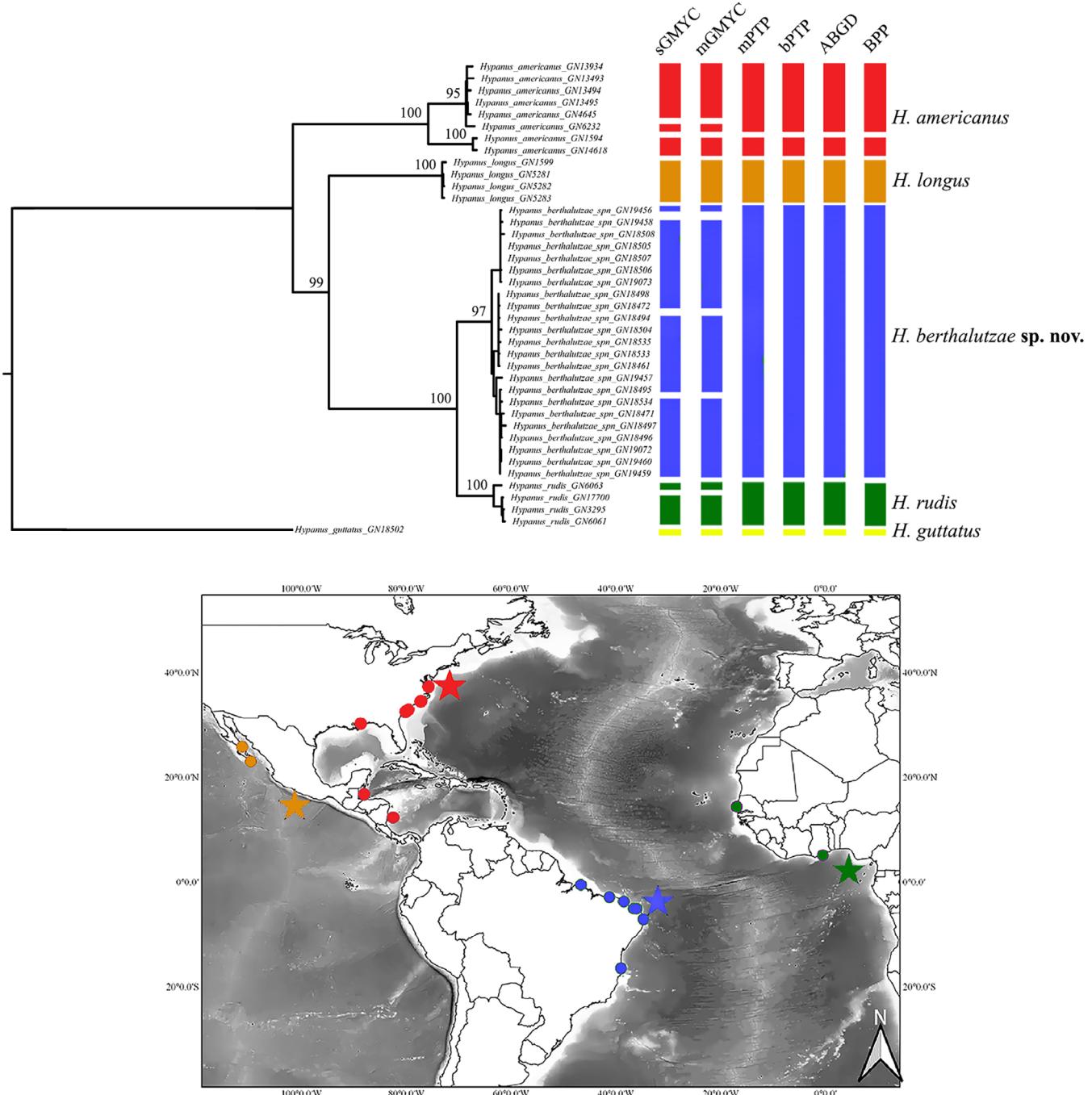
Despite observing genetic differences between two sympatric lineages of what is left of *H. americanus sensu stricto* in NWA, this study did not find any morphological or ecological dissimilarities between them (see the "Ecological niche modelling of *H. americanus* sensu lato"

and "Morphometric comparisons" sections). Therefore, this study suggests the maintenance of the species name *H. americanus* for both lineages until further analyses are carried out (Padial et al., 2010). The authors did not proceed with population genetic analysis within this clade. Genetic distances, based on mitochondrial protein-coding genes, between and within the lineages (Table 1), show interspecific variation from 0.82% (*H. rufus* × *H. berthalutzae sp. nov.*) to 3.11% (*H. americanus* × *H. rufus*) and intraspecific variation from 0.04% (*H. longus*) to 0.41% (*H. americanus*). The highest observed within-lineage distance occurs in *H. americanus*, supporting the aforementioned delimitation analyses.

Even though the haplotype network of *mt-nd2* was built with less genetic information, 1122 bp instead of 11,471 bp of mitogenomes, it also revealed that *H. americanus* sensu lato comprises more than one lineage (Figure 3). Representatives of *H. berthalutzae sp. nov.* from Fernando de Noronha Archipelago and southeastern Brazil were included and shown to belong to the same clade as suggested by mitogenome analyses, with 12 haplotypes along the Brazilian coast and Fernando de Noronha. All specimens of *H. longus* and *H. rufus* exhibit species-specific *mt-nd2* haplotypes. Even though there are only six mutations between *H. rufus* and representatives of *H. berthalutzae sp. nov.*, it is higher than the observed intraspecific values. In addition, the accumulation of mutations between *H. americanus* sensu stricto (NWA) and *H. berthalutzae* sp. nov. (SWA) is conspicuous, corroborating the hypothesis of the Amazon River outflow as a barrier for their distribution.



**FIGURE 1** Ecological niche modelling of *Hypanus americanus* sensu lato. Legend colours indicate habitat suitability for its occurrence. Marine ecoregions are highlighted in black lines following Spalding et al. (2007). Mississippi, Amazon and Parana-Paraguay-La Plata Rivers are shown in thick black lines in the American continent, from north to south. Location records of *H. americanus* and *Hypanus berthalutzae* sp. nov. in Table S2 (Supporting Information). (●) *H. americanus* sensu lato, (■) 0.246, (■) 0.492, (■) 0.738, (■) 0.984



**FIGURE 2** Phylogenetic relationships among *Hypanus americanus* species group, estimated using maximum likelihood on the mtDNA data set; bootstrap values shown for each node; *Hypanus guttatus* used as out-group; candidate species according to delimitation analyses using the mitochondrial genome. Possible species found in each analysis are portrayed as coloured boxes in columns. In red, *H. americanus*; orange, *Hypanus longus*; blue, *Hypanus berthalutzae* sp. nov.; green, *Hypanus rufus*; grey, *H. guttatus*. The same colours are used to represent sampled specimens in the map below. Stars are the holotypes' locations of each valid species, which were not sampled, except *H. berthalutzae* sp. nov.

BAPS analysis (Figure 4) based on *mt-nd2* sequences of *H. rufus* and *H. berthalutzae* sp. nov., including representatives from the whole distribution, suggests the best clustering for  $K = 3$ , with all *H. rufus* specimens grouped in a lineage, and two clusters for *H. berthalutzae* sp. nov. (NO + NE + FN and NE + SE), with the northeastern region of Brazil encompassing putatively two populations but without geographical congruence to genetic analysis.

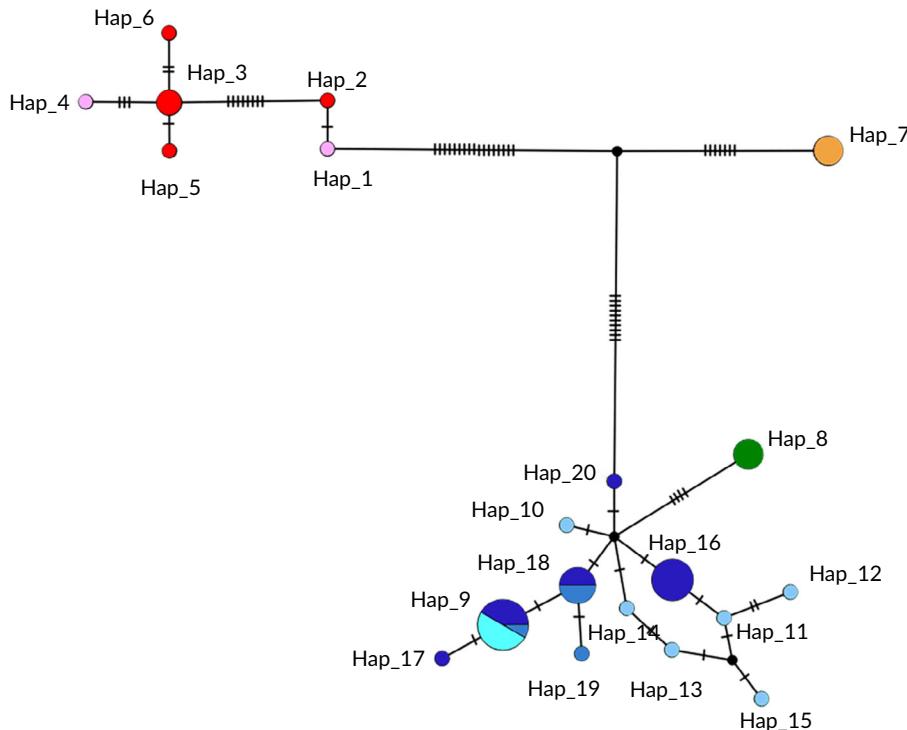
### 3.3 | Morphometric comparisons

Morphometric measurements of 79 specimens of the *H. americanus* species group yielded the CVA and PCA ordinations shown in Figure 5. Because no morphological differences were observed within the *H. americanus* *sensu stricto* clade (NWA), it was considered as one species for taxonomic stability (Padial et al., 2010). The measured

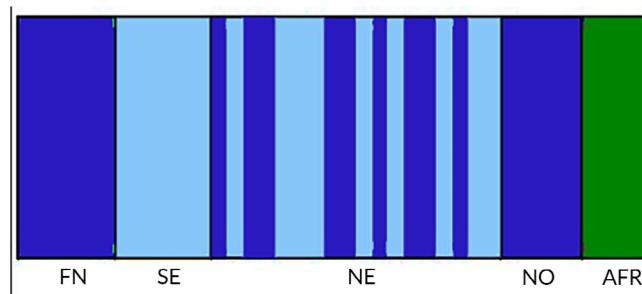
**TABLE 1** Mitochondrial genomic distances between *Hypanus americanus* species group lineages below diagonal and within each lineage in bold-face diagonal

	<i>H. americanus</i>	<i>Hypanus berthalutzae</i> sp. nov.	<i>Hypanus longus</i>	<i>Hypanus rufus</i>
<i>H. americanus</i>	<b>0.41</b>			
<i>H. berthalutzae</i> sp. nov.	3.1	<b>0.12</b>		
<i>H. longus</i>	2.69	2.43	<b>0.04</b>	
<i>H. rufus</i>	3.11	0.82	2.4	<b>0.1</b>

Note. Distances are given as percentages.



**FIGURE 3** Haplotype network of *Hypanus americanus* species group: *H. americanus* from northwestern Atlantic (NWA) and Central America (CAM) in red and pink, respectively (haplotypes 1–6); *Hypanus longus* from Baja California in the Pacific coast (BCA) in orange (haplotype 7); *Hypanus rufus* from Africa (AFR) in green (haplotype 8); and *Hypanus berthalutzae* sp. nov. from northern, northeastern, Fernando de Noronha Archipelago and southeastern Brazil (NO, NE, FN, SE) in shades of blue (haplotypes 9–20). Black circles represent non-sampled haplotypes and trace mutational steps. (◎) 10 samples, 1 sample; (○) CAM; (●) NWA; (○) BCA; (●) AFR; (○) SE; (●) NE; (○) FN; (●) NO



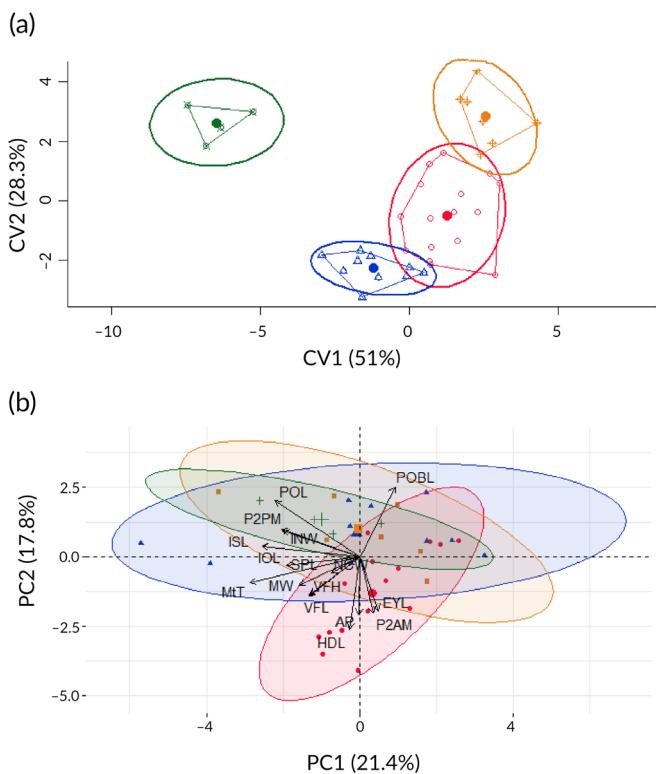
**FIGURE 4** Genetic structure between *Hypanus berthalutzae* sp. nov. (FN, SE, NE, NO in dark and light blue) and *Hypanus rufus* (AFR, in green). Bayesian Analysis of Population Structure admixture based on ND2 sequences

morphometric data met the assumptions of parametricity, and MANOVA suggested that there are significant ( $P < 10^{-9}$ ) differences among the averages of the measurements of the four lineages, agreeing with genetic analyses that it is indeed a species group: *H. americanus* sensu

*stricto* (NWA), *H. longus* (EP), *H. rufus* (EA) and *H. berthalutzae* sp. nov. (SWA).

The most influential variables for distinguishing among the four species are anterior projection (AP), ventral caudal fold length (VFL) and ventral caudal fold height (VFH), pelvic fin anterior margin (P2AM), head length (HDL), internasal width (INW), interorbital length (IOL), interspiracle length (ISL) and spiracle length (SPL) (Table 2), with superposition of values due to the presence of small and large specimens, whose proportions vary throughout their development.

Both clasper external length (CEL) and clasper internal length (CIL) by pelvic fin length (P2L) are statistically significant (MANOVA's  $P = 4.27 \times 10^{-5}$  and  $P = 6.62 \times 10^{-5}$ , respectively) (Figure 6) in differentiating male specimens of this clade. Claspers of *H. berthalutzae* sp. nov. have an intermediate size when compared to *H. americanus* (smaller) and *H. longus* (larger). In a data set with corrected allometry and outliers removed, the measurement CEL by P2L is larger in *H. berthalutzae* sp. nov. [48.36% (47.42%–49.24%)] than in *H. americanus* [34.6% (24.52%–45.69%)] and smaller than *H. longus* [88% (82.11%–100.51%)], and CIL by P2L is also larger in



**FIGURE 5** Visual representations of morphometric measurements of *Hypanus americanus* (red circles), *Hypanus berthalutzae* sp. nov. (blue triangles), *Hypanus longus* (orange squares) and *Hypanus rufus* (green crosses). (a) Canonical variance analysis, with first canonical variate (51%) in the x-axis and the second canonical variate (28.3%) in the y-axis and (b) Principal component analysis, with the first principal component (21.4%) in the x-axis and the second principal component (17.8%) in the y-axis. (■) *H. americanus*, (▲) *H. berthalutzae* sp. nov., (■) *H. longus*, (■) *H. rufus*

*H. berthalutzae* sp. nov. [34.76% (34.44%–35.06%)] than in *H. americanus* [24.61% (18.16%–33.19%)] and smaller than *H. longus* [74.78% (63.25%–86.58%)]. The ventral cover piece is dorsally visible in *H. berthalutzae* sp. nov., but it is larger in *H. longus*, and only its proximal portion is dorsally observable in *H. americanus* (Figure 7). In addition, in male adults, the distance from hypopyle (hy) to the clasper distal portion is 46.15 mm in *H. berthalutzae* sp. nov. (UERJ 2045) [vs. 40.65 mm in *H. americanus* (USNM 395424) vs. 45.45 mm in *H. longus* (TCWC 12102.01)], showing the clasper gland is proportionately smaller in *H. berthalutzae* sp. nov. than in *H. americanus* and *H. longus* (33.61% vs. 36.07% vs. 37.66% of distance from apopyle to clasper tip, respectively).

Morphometric comparisons between *H. berthalutzae* sp. nov. from the Brazilian coast and *H. rufus* from West Africa were carried out. Comparisons were restricted to specimens with disc width (DW) up to 400 mm as larger specimens of *H. rufus* were unavailable in collections. Five specimens of *H. berthalutzae* sp. nov. and four of *H. rufus* were used to generate the plots of histogram of CVA and PCA depicted in Figure 8. A MANOVA test for

differences in the average between groups was significant ( $P < 10^{-3}$ ), suggesting they are statistically distinct.

The measurements that best discriminated the two forms based on morphology were IOL and SPL in percentage of HDL, HDL in percentage of AP and SPL in percentage of pre-orbital length (POBL) (Table 3). Both the multivariate ordination and the statistical analyses of the most discriminating morphometric measurements support the description of a new species for the Brazilian coast, distinct from *H. rufus*, its genetically closest-related species.

### 3.4 | Ecological niche modelling of *H. berthalutzae* sp. nov

Records of *H. berthalutzae* sp. nov. from 28 locations were employed to conduct an ENM assessment (after spatial thinning). ENM eval results pointed the hinge feature class with a regularization multiplier of 4 as the best parameter for running the analysis, with a deltaAICc value equal to 0. After 15 replicates, the test of omission rate vs. predicted area illustrated that the average omission rate is close to the predicted omission, and the average training AUC for the replicate runs is 0.994 (S.D. 0.002). Therefore, based on a good performance of the model, a low suitability area near the Amazon River outflow was observed (Figure 9) consistent with results from both molecular and morphological data analyses and the ENM of the clade *H. americanus* sensu lato. The environmental variables that are most predictive of its occurrence were (a) closeness to shore (61.4% of permutation importance), (b) high benthic salinity values above 35 PSU (13.6%), (c) low surface phosphate values of  $0.1 \text{ mmol m}^{-3}$  (13.1%) and (d) surface primary productivity values close to  $0.02 \text{ g m}^{-3} \text{ day}^{-1}$  (6.6%), and in addition to analysing the environmental variables suitable for its occurrence, the species' distribution could be suggested, and its geographic map could be updated based on verified occurrence records using morphological, molecular and ecological tools.

### 3.5 | Description of a new *Hypanus* species from the SWA

Based on the congruence of molecular, morphological and ecological data, there is enough evidence to describe a new species of *Hypanus* from the SWA, at the Brazilian coast, oceanic archipelago of Fernando de Noronha, and Rocas Atoll. These stingrays used to be identified as *H. americanus*, but they will be described under a new specific epithet because the examined holotype of *H. americanus* is from Chesapeake Bay (Maryland, USA) (Hildebrand & Schroeder, 1928). A specimen from Fernando de Noronha oceanic archipelago in northeastern Brazil is suggested as a holotype of the new species, which protects a large and conspicuous population.

Order Myliobatiformes Compagno (1973)

Family Dasyatidae Jordan and Gilbert (1879)

**TABLE 2** Most statistically relevant morphometric measurements (all in %) to distinguish the species *Hypanus americanus*, *Hypanus berthalutzae* sp. nov., *Hypanus longus* and *Hypanus rufus*, with mean, range (minimum and maximum) and holotype values, except *H. rufus* whose holotype was lost

	<i>H. americanus</i>			<i>H. berthalutzae</i> sp. nov.			<i>H. longus</i>			<i>H. rufus</i>	
	Mean	Range	USNM 88378	Mean	Range	MNRJ 51963	Mean	Range	MCZ 126	Mean	Range
AP**	38.75	32.12	37.04	35.86	29.1	41.42	39.08	35.34	39.12	35.37	32.66
		50.28			41.42			43.24			39.97
VFL**	53.4	40.24	54.17	45.53	40	44.98	50.49	42.24	51.24	47.92	45.72
		69.47			54.76			56			49.4
VFH***	1.74	0.83	1.68	1.92	1.37	1.96	1.39	0.75	0.82	1.55	1.1
		2.83			2.3			2.71			1.81
P2AM*	15.43	11.57	16.99	14.45	12.22	15.81	14.14	12.47	16.33	13.35	12.97
		28.02			15.81			16.33			13.73
HDL*	32.3	30.07	31.56	31.73	30.22	33.98	31.14	29.55	64.15	31.05	30.13
		35.13			34.43			33.31			31.87
INW*	31.39	29.26	31.66	32.35	28.16	28.16	31.84	28.9	30.14	32.19	31.16
		35.45			36.7			35.69			33.59
IOL*	53.39	45.93	51.48	52.07	48.5	51.42	50.26	47.23	48.17	58.03	54.39
		70.83			55.69			52.73			59.62
ISL**	49.91	45.8	47.61	49.56	46.71	48.52	51.09	48.08	51.17	51.79	48.58
		59.55			52.49			53.94			55.43
SPL***	19.61	15.85	19.85	18.62	16.21	16.21	18.37	17.03	17.09	24.79	23.55
		32.81			22.47			21.89			26.11
CIL***	33.44	18.78	23.30	49.57	16.72	-	84.38	73	-	22.75	-
		66.27			111.97			94.61			
CEL***	49.62	24.55	29.84	63.34	25.19	-	95.71	90.68	-	32.48	-
		117.11			134.74			106.88			

Note. AP (anterior projection), VFL (ventral caudal fold length), VFH (ventral caudal fold height), P2AM (pelvic fin anterior margin) and HDL (head length) in %DW (disc width); INW (internasal width), IOL (interorbital length), ISL (interspiracle length) and SPL (spiracle length) in %HDL; CIL (clasper internal length) and CEL (clasper external length) in %P2L (pelvic fin length). \*P < 0.05; \*\*P < 10<sup>-2</sup>; \*\*\*P < 10<sup>-3</sup>. MCZ: Museum of Comparative Zoology; MNRJ: Museu Nacional, Universidade Federal do Rio de Janeiro.

#### Genus *Hypanus* Rafinesque (1818)

##### *Hypanus berthalutzae* sp. nov.

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E8638AA16696

(Figure 10; Table 4)

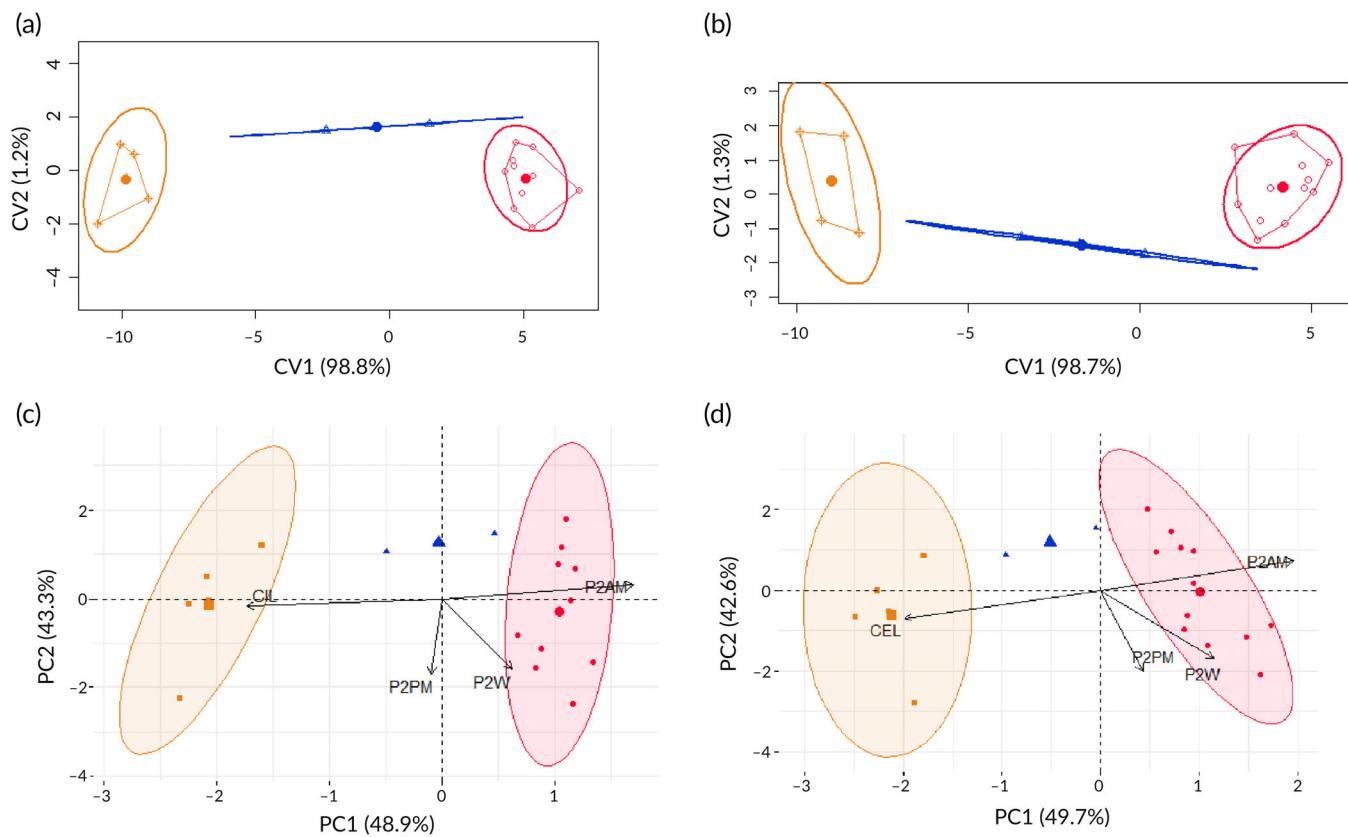
#### 3.5.1 | Holotype

MNRJ 51963, female, 618 mm DW, Praia do Porto, Fernando de Noronha, Pernambuco, Brazil, 3° 50' 02.5" S, 32° 24' 05.3" W, Col. date: 27 July 2019, Cols.: Petean, F. F., Lima, S. M. Q., Costa, T. A., Di Dario, F., Mendes, L. F., Araújo, T. F. P.

#### 3.5.2 | Paratypes

Brazil. USNM 216825, female, 239 mm DW off the coast of north-eastern Brazil. Maranhão, 1° 04' 48.0" S, 44° 48' 00.0" W, Col. date:

17 May 1975, Col.: Collette, B. B.; MNRJ 18910, female, 591 mm DW, Praia de Iracema, Fortaleza, Ceará; UERJ 2045, male, 680 mm DW, Praia de Iracema, Fortaleza, Ceará; MZUSP 9925, male, Atol das Rocas, Rio Grande do Norte, Col. date: February 1972, Col: N. A. Menezes; UERJ 373, female, 536 mm DW, Bahia, 13° 36' S, 38° 47' W, Col. date: 24 July 1999; UERJ 1880, female, 501 mm DW, Ilha Grande, Rio de Janeiro; UERJ 2047, male, 364 mm DW, Muriqui, Mangaratiba, Rio de Janeiro, Col. date: 1 February 2004, Col.: L. R. G. Rodrigues; UERJ 2048, male, 370 mm DW, Muriqui, Mangaratiba, Rio de Janeiro, Col. date: 18 March 2002, Col.: L. R. G. Rodrigues; UERJ 2051, female, 364 mm DW, Muriqui, Mangaratiba, Rio de Janeiro, Col. date: 8 March 2004, Col.: L. R. G. Rodrigues; MNRJ 17709, female, 345 mm DW, Col. date: 21 August 1998; LIRP 1554, female, 668 mm DW, Ponta do Jarobá, Canal de São Sebastião, Município de São Sebastião, São Paulo, 23.83° S, 45.42° W, Col. date: 4 May 1996, Col.: Lev. Canal S. Seb. 1993–1996; LIRP 1555, female, 565 mm DW, Praia das Pitangueiras, Canal de São Sebastião, São Paulo, 23.83° S, 45.42° W, Col. date: 14 March 1996, Col.: Lev. Canal S. Seb. 1993–1996; LIRP 1556, female, 536 mm DW, Ponta do



**FIGURE 6** Visual representations of morphometric measurements of internal (a and c) and external (b and d) lengths of pelvic fins and claspers in terms of pelvic fin length of *Hypanus americanus* (red circles), *Hypanus berthalutzae* sp. nov. (blue triangles) and *Hypanus longus* (orange squares). (a and b) Canonical variance analysis and (c and d) PCA. (■) *H. americanus*, (▲) *H. berthalutzae* sp. nov., (□) *H. longus*

Jarobá, Canal de São Sebastião, Município de São Sebastião, São Paulo, 23.83° S, 45.42° W, Col. date: 25 July 1995, Col.: Lev. Canal S. Seb. 1993-1996; LIRP 1557, female, 515 mm DW, Ponta do Jarobá, Canal de São Sebastião, Município de São Sebastião, São Paulo.

Additional examined specimens are provided in Supporting Information Supporting Information S1.

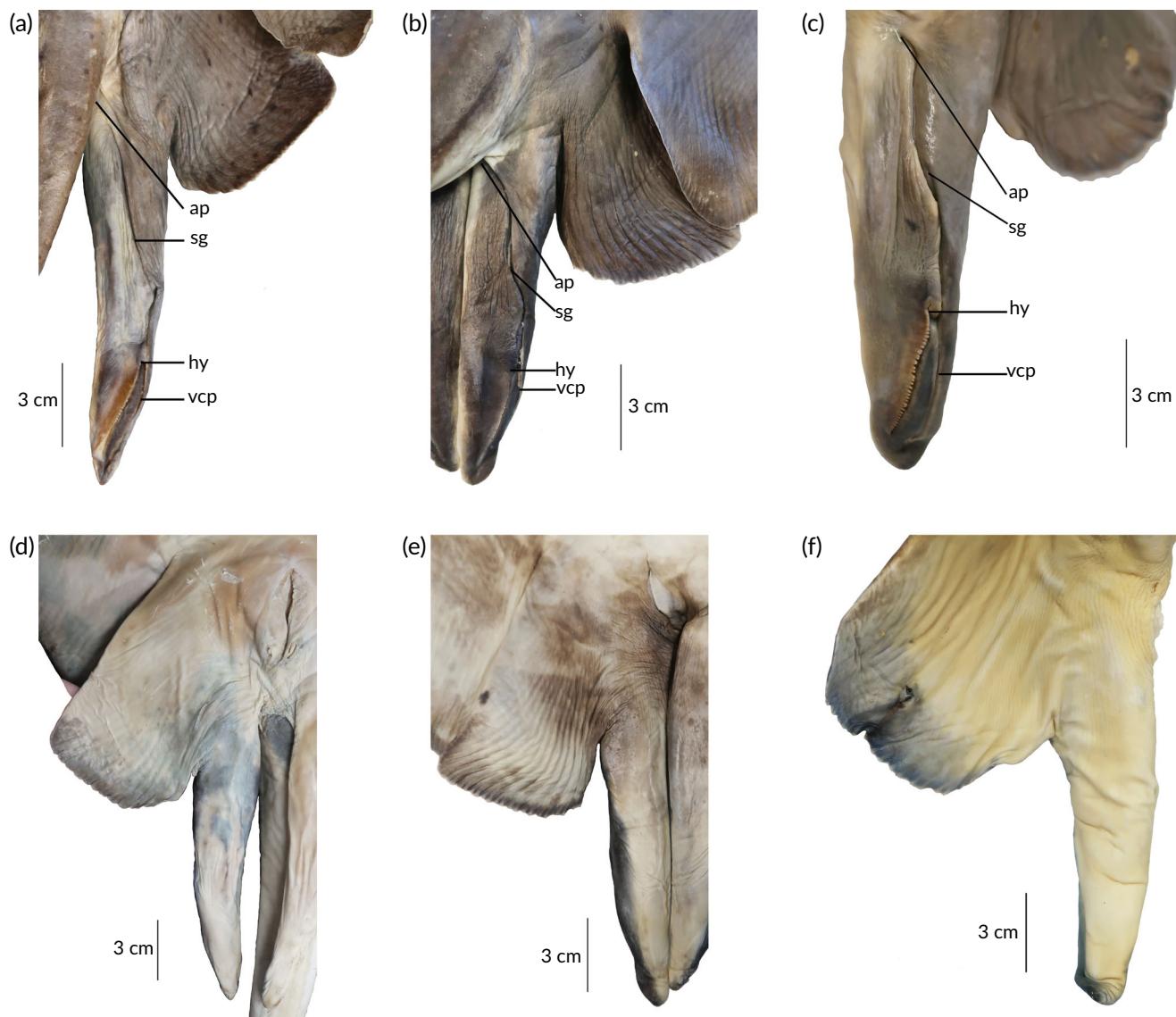
### 3.5.3 | Synonymy

*Pastinaca hastata* (DeKay, 1842): p. 373 (in part; original description); *Dasibatis hastata* (Garman, 1883): p. 70 (in part; brief description); *Dasyatis hastata* (Jordan & Evermann, 1896): p. 83 (in part; brief description); *Dasybatus hastatus* (Garman, 1913): p. 391 (in part; brief description); *Dasyatis americana* (Hildebrand & Schroeder, 1928): p. 64 (in part; original description); *H. americanus* (Last, Naylor, Séret, et al., 2016): p. 356 (in part; brief description).

### 3.5.4 | Diagnosis

The species *H. berthalutzae* sp. nov. can be diagnosed by a combination of characters. It is distinguished from non-*Hypanus* Dasyatidae

species that occur in the western Atlantic by the presence of dorsal caudal ridge [vs. absence in *Bathytochia centroura* (Mitchill, 1815), vs. dorsal caudal fold in *Dasyatis hypostigma* (Santos & Carvalho, 2004)]; absence of a w-shaped notch in the central ventral disc (vs. its presence in *D. hypostigma*); and anterior portion of the disc with angle varying from 125° to 135° and a clear ventral disc [vs. 150° and dark ventral disc in *Pteroplatytrygon violacea* (Bonaparte, 1832)]. From most *Hypanus* species, except those from the *H. americanus* species group, it can be discriminated by the diamond-shaped body with a short snout (vs. a long snout in *H. guttatus* and *H. sabinus*); presence of ventral caudal fold and dorsal caudal ridge (vs. ventral and dorsal caudal folds in *H. marianae*, *H. dipterurus*, *H. say* and *H. sabinus*); and a white spot between the eyes anterior to the precerebral fontanelle (vs. absence of this spot in *H. marianae*, *H. guttatus*, *H. dipterurus*, *H. say* and *H. sabinus*). It differs from the species of the *H. americanus* clade (*H. americanus*, *H. longus* and *H. rufus*) by the presence of sparse black spots on the dorsal disc of live specimens (vs. absent) and by 11 morphometric measurements (Table 2), the most relevant being SPL [18.62% (16.21%–22.47%) of HDL vs. 19.61% (15.85%–32.81%) in *H. americanus*, 18.37% (17.03%–21.89%) in *H. longus* and 24.79% (23.55%–26.11%) in *H. rufus*] and caudal VFH [1.92% (1.37%–2.3%) of DW vs. 1.74% (0.83%–2.83%) in *H. americanus*, 1.39% (0.75%–2.71%) in *H. longus* and 1.55% (1.1%–1.81%) in *H. rufus*]. In male adults of



**FIGURE 7** Right clasper images in dorsal (a, b, c) and ventral views (d, e, f) of *Hypanus berthalutzae* sp. nov. [UERJ 2045 from Ceará, Brazil, 680 mm of disc width (DW)] in (a) and (d); *Hypanus americanus* (USNM 395424 from the Gulf of Mexico, 602 mm of DW) in (b) and (e); and *Hypanus longus* (TCWC 12102.01 from the Gulf of California) in (c) and (f). ap: apopyle; hy: hypopyle; sg: spermatic groove; vcp: ventral cover piece

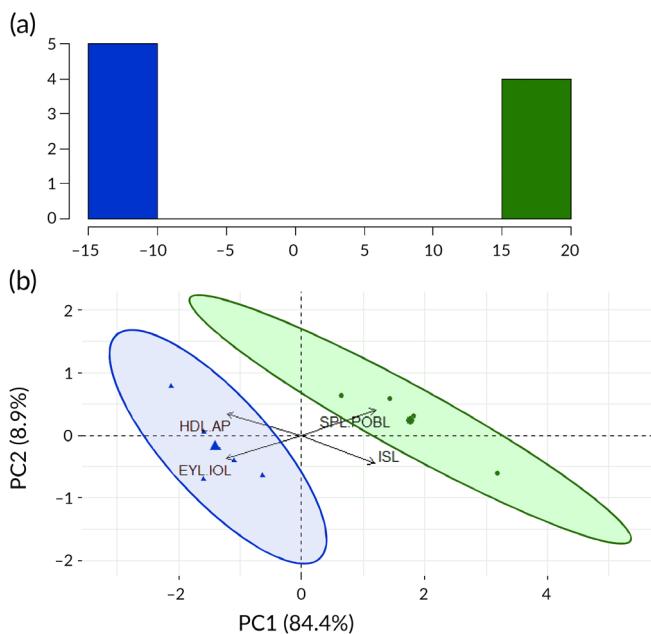
*H. berthalutzae* sp. nov. and *H. americanus*, the P2L is smaller than the distance from apopyle to clasper tip (ap-tip), whereas it is larger in *H. longus*. The measurement P2L/ap-tip is 54.16% in *H. berthalutzae* sp. nov. [vs. 49.43% (41.78%–56.33%) in *H. americanus* and 119.44% (113.20%–124.15%) in *H. longus*].

### 3.5.5 | Description

Angular snout (in contrast to rounded in other *Hypanus* species), pectoral fins with straight anterior margins (in contrast to undulated), moderately angular lateral (in contrast to rounded) and angular free rear tips. Disc wider than longer; disc length 86% of its width (89% in holotype, 81.62%–96.11% in paratypes). Sinuous ventral gill openings, first 8.8% (8.06%, 7.72%–10.53%), third 0.36% (8.67%, 7.74%–

10.65%) and fifth 6.09% (6.16%, 5.15%–7.32%) of HDL. Presence of dorsal caudal ridge and ventral caudal fold of almost same length, which begins below spine origin and measures 45.53% (44.98%, 40%–54.76%) of DW (Figure 11). When complete, tail can measure up to 146% (103.56%, 84.43%–146.02%) of DW and can either taper towards a thin or thick caudal at the end depending on its length.

Protruded eyes above head; laterally positioned spiracles with rectangular shape in diagonal direction with upper portion more posterior than lower. SPL 37.59% (33.41%–45.98%) smaller than ISL; eye length (EYL) 23.86% (20.54%–27.60%) smaller than IOL. Lower jaw slightly convex with corners somewhat posterior than medial portion; subtle medial indentation. Upper teeth fairly exposed with closed mouth. Mouth floor with three central papillae and two smaller ones at the corners.



**FIGURE 8** Histogram of (a) canonical variate scores and (b) principal component analysis of *Hypanus berthalutzae* sp. nov. (blue triangles) and *Hypanus rufus* (green circles). Specimens of *H. berthalutzae* sp. nov. from southeastern Brazil (SE Bra) and southwestern Atlantic and *Hypanus rufus* from Guinea, eastern Atlantic. (a) (■) Guinea, (■) SE Bra; (b) (■) *Hypanus rufus*, (▲) *H. berthalutzae* sp. nov.

**TABLE 3** Most statistically relevant ( $P < 0.05$ ) morphometric measurements to distinguish the species *Hypanus berthalutzae* sp. nov. and *Hypanus rufus*

	<i>H. berthalutzae</i> sp. nov.			<i>H. rufus</i>	
	Mean	Range	MNRJ 51963	Mean	Range
IOL/HDL	52.07	48.5	51.42	58.03	54.39
		55.69			59.62
SPL/HDL	18.62	16.21	16.21	24.79	23.55
		22.47			26.11
HDL/AP	115.04	103.48	121.90	87.91	84.35
		143.27			94.77
SPL/POBL	28.79	23.89	23.89	38.12	35.91
		35.40			39.93

Note. AP: anterior projection; HDL: head length; IOL: interorbital length; POBL: preorbital length; SPL: spiracle length.

Nasal curtain weakly concave with subtle invagination at the centre and rounded at corners; do not expand laterally beyond oral groove and the external limit of nostril. Anterior nasal distance almost same as free posterior margin of nasal curtain. Fringes at posterior margin of nasal curtain with subtle gap at midline; oval nostril (Figure 12). Adult teeth cusps acute in males and non-cuspidate in females. Teeth larger and in more rows medially, which decrease in size and number laterally.

Pelvic fin almost entirely dorsally exposed by the pectoral; squared shape with rounded corners, which are more laterally expanded in males. Dorsal terminal cartilage of clasper with serrated lateral margin and absence of pseudosiphon; CIL and CEL 7.79% (2.69%-17.56%) and 9.96% (4.05%-21.13%) of DW, respectively (Figure 7).

A few rostral and stellar interorbital and interspiracle thorns; a scapular thorn (only one) and two scapular rows (from 0 to 14 thorns on each side); dorsal disc covered by sparse thorns close to midline, and a row from mid-scapular to caudal sting (from 0 to 24 thorns) (Figure 13); caudal fin with minute sparse thorns. Smooth pelvic fins.

Dorsal disc colour greenish grey with small black spots distributed on surface of live specimens. Lateral and posterior margin of pectoral fins and posterior margin of pelvic with a white stripe dorsally (Figure 14). Ventral disc surface whitish, with darker pectoral and pelvic fins' margins. Preserved specimens in alcohol 70% are greyish or brownish, and the black spots usually disappear. Ventral surface keeps clearer than dorsal. White rounded mark anterior to fontanelle region half HDL. Largest disc width measured was 680 mm in a mature male and 668 mm in a mature female; largest immature male 370 mm. Larger specimens were observed alive in Fernando de Noronha Archipelago, but not measured, suggesting this species might reach larger sizes than mentioned here.

### 3.5.6 | Geographic distribution

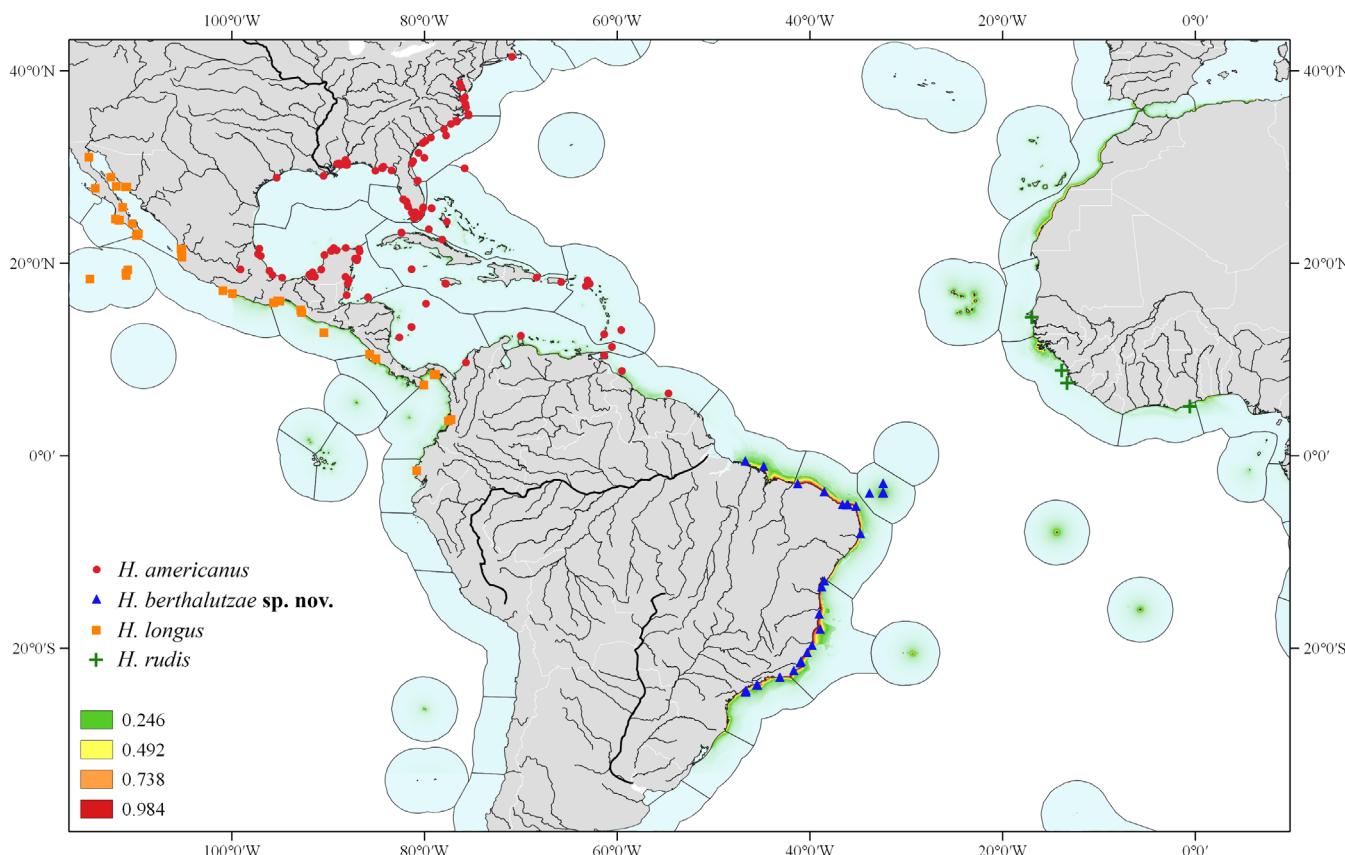
This species is distributed from the mouth of the Amazon River, with confirmed occurrence at Parcel de Manuel Luiz in Maranhão (Rocha & Rosa, 2001), to São Paulo State coast in Brazil (Menni & Stehmann, 2000), besides Fernando de Noronha and Rocas Atoll oceanic archipelagos (Rosa & Moura, 1997) at northeastern Brazil, being delimited at its southernmost distribution by the influence of the La Plata River. Despite occurring in localities up to 250 km off the coast (Fernando de Noronha), it is a mostly coastal species occurring usually at the continental shelf in marine environments. It ranges from Tropical Atlantic to Temperate South America Realms, at the Provinces of North Brazil Shelf, Tropical Southwestern Atlantic and Warm Temperate Southwestern Atlantic according to the marine delimitations by Spalding et al. (2007) (Figure 9).

### 3.5.7 | Common names

In Brazil (Portuguese language) these stingrays are usually known by the names "Raia de Pedra" (rockray), "Raia Manteiga" (butterray) and "Raia Prego" (nailray).

### 3.5.8 | Etymology

The specific epithet is in honour of Bertha Lutz, a pioneering Brazilian woman zoologist, who was also involved in feminist issues and



**FIGURE 9** Ecological niche modelling of *Hypanus berthalutzae* sp. nov. Legend colours indicate habitat suitability for its occurrence. Marine ecoregions are highlighted in black lines following Spalding et al. (2007). Mississippi, Amazon and Parana–Paraguay–La Plata Rivers are shown by thick black lines in the American continent, from north to south. Location records of *Hypanus americanus*, *H. berthalutzae* sp. nov., *Hypanus longus* and *Hypanus rufus* in Table S2 (Supporting Information). (●) *H. americanus*, (▲) *H. berthalutzae* sp. nov., (■) *H. longus*, (+) *H. rufus*, (■■) 0.246, (■■■) 0.492, (■■■■) 0.738, (■■■■■) 0.984

created the Brazilian Federation for Feminine Progress, which led to female suffrage in Brazil. She represents women's strength in Brazil, who not only work for science development but also fight for women's rights. Because this species of stingrays is restricted to the Brazilian waters and most known specimens are females, it represents the recent feminine empowering, including in sciences (Blickenstaff, 2005; Van Oosten et al., 2017).

### 3.5.9 | Natural history

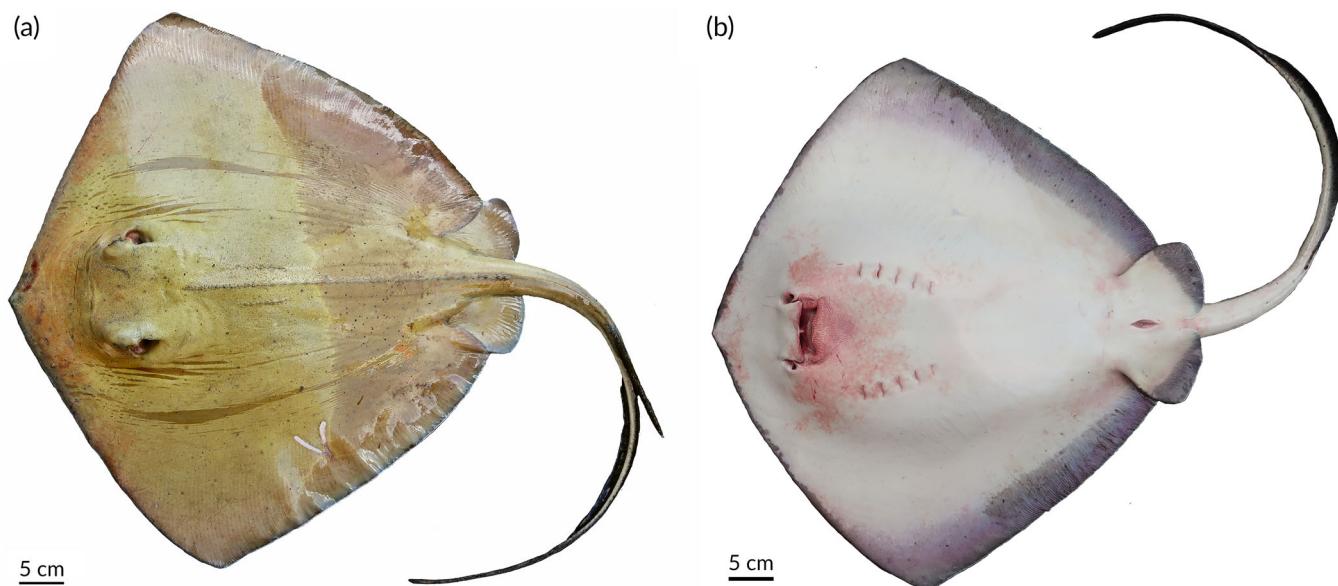
Most examined specimens of *H. berthalutzae* sp. nov. in fish collections and observed living stingrays in Fernando de Noronha, Pernambuco, Brazil, were female. This unequal sex ratio of 4:1 has already been observed in Fernando de Noronha by Aguiar (2005) and an even greater skew of 52:1 in Rocas Atoll (Agra, 2009). A ratio of 3:1 in Isla de Margarita, Venezuela (Tagliafico et al., 2013), and in Grovers Reef Atoll, Belize (Tilley & Strindberg, 2013), was also reported for *H. americanus*. Because all these observations were on shallow habitats, it is possible that female stingrays use these areas for reproduction or parturition (Pikitch et al., 2005). It is probably not a reflection

of unequal birth sex ratio, as neonates born in captivity have the same proportion of males and females (Henningsen & Leaf, 2010), suggesting the behavioural grouping of females could be the reason for such unequal ratios.

In addition, these stingrays are usually seen with an accompanying fauna such as the yellow jack *Caranx bartholomaei* Cuvier, 1833 (Figure 15), sergeant major *Abudefduf saxatilis* (Linnaeus 1758), nurse shark *Ginglymostoma cirratum* (Bonnaterre 1788), lemon shark *Negaprion brevirostris* (Poey 1868) and even the green sea turtle *Chelonia mydas* (Linnaeus 1758) (Agra, 2009).

### 3.5.10 | Remarks

For DeKay (1842) the genus *Pastinaca* Cuvier did not have any caudal fins, but specimens possessed edentate spines. He described the species *P. hastata* (DeKay, 1842) from Rhode Island (USA), which was synonymized to the currently valid species *B. centroura* by Bigelow and Schroeder (1953), Krefft and Stehmann (1973), Capapé (1977), and Capapé and Desoutter (1990), considered valid by Seret (1990) as *D. hastata* in EA, Africa, and then invalid by Compagno (1999, 2005).



**FIGURE 10** (a) Dorsal and (b) ventral views of the holotype MNRJ 51963, female, 618 mm disc width, of *Hypanus berthalutzae* sp. nov. from Fernando de Noronha Archipelago, Pernambuco State, Brazil

Garman (1883) wrote a list of *Dasibatis* species occurring in the Americas and added *D. hastata* to it, mentioning it was known from Florida (USA) to Rio de Janeiro (Brazil). Later, Hildebrand and Schroeder (1928) described *H. americanus* and suggested *D. hastata* as a synonym to the newly described species, besides expanding its distribution from the Chesapeake Bay (USA) to Brazil. Compagno and Roberts (1984) suggested that *H. americanus* also occurs in Western Africa, which was referred to as *D. hastata* by Séret (1990). The validity of *D. hastata* in Western Africa is yet to be studied; nonetheless, so far, it can be assumed that there are two similar morphotypes of *Hypanus* at the American Atlantic coast: *H. americanus* from New Jersey (USA) to the north of the Amazon River and *H. berthalutzae* sp. nov. from the mouth of the Amazon south to São Paulo (Brazil).

## 4 | DISCUSSION

Species descriptions based on limited evidence provoke taxonomic instabilities that compromise the utility of subsequent research (Padial & De La Riva, 2006). To avoid such pitfalls, more integrative research to infer relationships among lineages is needed (Padial et al., 2010). This present study has described a new species of stingray from the southwestern Atlantic Ocean, using molecular, morphological and ecological information for a group of animals that are sparsely represented in museum fish collections. The new species, previously identified as *H. americanus*, is more closely related to the African coastal species, *H. rufus*. *H. berthalutzae* sp. nov. is probably limited at its northern and southern distribution by the fresh water and sediments brought by the Amazon and La Plata Rivers to the Atlantic Ocean. The new taxonomic arrangement of *H. americanus* restricts this species to the NWA and Caribbean Sea.

### 4.1 | Genetic analyses

The phylogenetic inferences and lineage delimitation analyses of the mitochondrial genome and interpretation of the *mt-nd2* haplotype network suggest five lineages for a group of stingrays for which only three species were previously recognized: *H. americanus* in the western Atlantic, *H. longus* in EP and *H. rufus* in EA. In addition to the newly recognized *H. berthalutzae* sp. nov., there is a deeply divergent lineage within *H. americanus* in the NWA. Molecular differences in the mitochondrial control region between *H. americanus* from the eastern coast of the United States and Central America were previously noticed by Richards et al. (2018). Even though these results suggest either a hidden sympatric species or an ancestral hybridization event, because no morphological and/or ecological features have been identified to separate these two lineages, this study suggests the maintenance of *H. americanus* as a species restricted to Central and North America. As stated by Carstens et al. (2013), “It is better to fail to delimit species than it is to falsely delimit entities that do not represent actual evolutionary lineages.”

The mitochondrial genomic distances between *H. berthalutzae* sp. nov. and the other congeners vary from 0.82% (*H. rufus*) to 3.14% (*H. americanus*). White et al. (2018) used mitochondrial protein-coding genes to infer phylogenetic relationships among devil rays and obtained genetic pair-wise distances of 0.5% and 0.1% for pairs of species that were synonymized. Nonetheless, a distance of 0.4% between *Mobula birostris* (Walbaum 1792) and *Manta alfredi* (Krefft 1868) was not used as a threshold to synonymize these species because analyses using the nuclear marker *rag1* and the mitochondrial *mt-nd5* had already been done for this group by Kashiwagi et al. (2012), suggesting their divergence. The distance between *H. rufus* and the new species is twice that found in mtDNA of these devil rays.

**TABLE 4** Morphometric measurements of specimens of *Hypanus berthalutzae* sp. nov

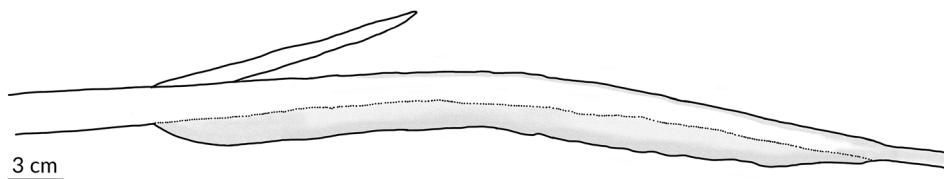
	MNRJ 51963		Mean			Range			
	mm	%	n	%	S.D.	mm		%	
						Minimum	Maximum	Minimum	Maximum
TL	1150		13	991.0714	227.20	661	1430		
HDL	210		15	158.4581	56.58	87.25	230		
DW	618		14	493	120.27	345	680		
%DW									
DL	550.00	89.00	15	86.01	3.66	231	642	81.62	96.11
AP	256.00	41.42	15	35.86	1.87	125	256	32.93	41.42
MtT	394.00	63.75	13	64.18	6.34	173	481	55.95	73.10
PCL	528.00	85.44	15	83.38	9.84	221	635	60.29	96.46
StC	470.00	76.05	14	74.76	5.38	199	556	69.03	84.60
CtCT	653.00	105.66	13	126.91	17.67	441	877	87.82	142.58
PSL	674.00	109.06	13	110.29	4.39	306	817	105.68	122.31
CL	640.00	103.56	13	125.14	17.66	431	844	84.43	146.02
CSW	18.83	3.05	14	2.87	0.32	7.66	22.25	2.38	3.47
TSL	160.00	25.89	13	28.25	2.54	78.37	225	24.86	33.68
CW	35.94	5.82	15	5.31	0.53	14.28	40.4	4.39	6.15
CH	24.13	3.90	15	3.76	0.41	10.17	28.76	3.20	4.32
VFL	278.00	44.98	14	45.53	4.33	119	282	40.00	54.76
VFH	12.13	1.96	14	1.92	0.27	3.73	14.2	1.37	2.30
CLPF	192.00	31.07	13	50.06	15.85	93	338	15.74	68.96
P2W	95.30	15.42	15	14.99	3.45	38.33	128.84	9.85	20.30
P2L	105.47	17.07	15	15.43	1.49	38.37	106.65	11.65	17.54
P2AM	97.71	15.81	15	14.45	1.02	38.43	100.81	12.22	15.81
P2PM	91.47	14.80	15	13.51	2.70	30.66	116.05	9.24	17.37
CIL	-		3	7.79	8.46	9.95	119.42	2.69	17.56
CEL	-		3	9.96	9.68	14.99	143.7	4.05	21.13
HDL	210.00	33.98	15	31.73	1.17	87.25	230	30.22	34.43
%HDL									
POBL	142.47	67.84	15	64.89	3.13	54.24	143	56.97	68.89
POL	130.20	62.00	15	62.07	6.50	49.83	157	52.44	75.76
PNL	98.15	46.74	15	48.42	4.92	36.27	117	41.57	57.30
INW	59.13	28.16	15	32.35	2.68	29.05	72.06	28.16	36.70
NCL	64.71	30.81	14	33.09	2.84	28.32	73.12	29.05	38.42
NCW	35.02	16.68	15	17.08	1.72	14.54	36.71	13.57	20.51
MW	49.95	23.79	15	26.74	2.20	24.35	59.81	23.79	31.23
EYL	23.18	11.04	15	12.44	1.26	13.19	24	10.38	15.12
IOL	107.98	51.42	15	52.07	2.41	47.79	116.21	48.50	55.69
ISL	101.90	48.52	15	49.56	1.64	45.8	115.86	46.71	52.49
SPL	34.04	16.21	15	18.62	1.51	18.07	40.04	16.21	22.47
O1G	148.05	70.50	15	74.75	5.14	63.45	180.33	68.60	84.61
O5G	98.29	46.80	15	49.12	3.09	43.12	115.71	45.40	55.65
1GW	16.93	8.06	15	8.80	1.02	7.31	22.39	7.72	10.53
3GW	18.21	8.67	15	9.36	0.89	7.8	22.91	7.74	10.65

**TABLE 4** (Continued)

MNRJ 51963				Mean			Range				
	mm	%	n	%	S.D.	mm	Minimum	Maximum	%	Minimum	Maximum
5GW	12.94	6.16	15	6.09	0.63	5.38	5.38	14.46	5.15	5.15	7.32
PFL	101.00	48.10	13	51.07	3.91	45.79	45.79	111	45.24	45.24	55.76

Note. AP: anterior projection; CEL: clasper external length; CH: caudal height at pelvic fin end; CIL: clasper internal length; CL: caudal length; CLPF: caudal length post ventral fold; CSW: caudal width at spine; CtCT: cloaca to caudal tip length; CW: caudal width at P2 end; DL: disc length; DW: body measurements in relation to disc width; EYL: eye length; 5GW: fifth gill slit width; HDL: head measurements in relation to head length; IOL: interorbital length; INW: internostiril width; ISL: interspiracle length; MNRJ 51963: holotype; MtT: mouth to tail; MW: mouth width; n: number of measured specimens for each measurement; NCL: nasal curtain length; NCW: nasal curtain width; 1GW: first gill slit width; PCL: pre-caudal length; PFL: pre-fontanelle length; PNL: pre-nasal length; POBL: preorbital length; POL: pre-oral length; PSL: pre-spine length; P2AM: pelvic fin anterior margin; P2L: pelvic fin length; P2PM: pelvic fin posterior margin; P2W: pelvic fin width; SPL: spiracle length; StC: snout to cloaca length; 3GW: third gill slit width; TL: total length; TSL: tail origin to spine origin length; VFH: ventral caudal fold height; VFL: ventral caudal fold length.

**FIGURE 11** Schematic lateral view drawing of *Hypanus berthalutzae* sp. nov. caudal fin showing ventral fold and dorsal ridge



**FIGURE 12** Oronasal region of the holotype of *Hypanus berthalutzae* sp. nov. (MNRJ 51963)



**FIGURE 13** Dorsal thorns of the holotype of *Hypanus berthalutzae* sp. nov. (MNRJ 51963). Dorsal region on top

## 4.2 | Morphological comparisons

This study investigated morphologically four of the five lineages identified genetically and obtained results that lent congruent support to their distinctiveness. The most decisive features for identifying *H. berthalutzae* sp. nov. are the presence of small black spots on the dorsal side of live stingrays (absent in the other species) and morphometric measurements (Table 2). There is a

significant difference in clasper morphology between *H. americanus*, *H. longus* and *H. berthalutzae* sp. nov., as already observed by Santos (2007).

*H. berthalutzae sp. nov.* and *H. rufus* are morphologically distinct, based on morphometric differences (Table 3) between IOL, ISL and SPL, and the proportion of EYL by the SPL [65.84% (59.61%–73.17%) in *H. berthalutzae sp. nov.* and 50.57% (47.94%–57.66%) *H. rufus*] is a relevant measurement.

#### 4.3 | Ecological niche modelling

The ENM of *H. berthalutzae sp. nov.* indicates that the most suitable habitats for this species are those closer to the coast (<100 m), with low phosphate values (<0.5 µmol l<sup>-1</sup>) and high benthic salinities (36 PSU). It is absent in most oceanic islands and seamounts, such as Saint Peter and Saint Paul Archipelago (Vaske et al., 2005), Vitória-Trindade Seamount Chain (Pinheiro et al., 2015), Ascension Island (Wirtz et al., 2017) and Cape Verde Islands (Wirtz et al., 2013) in the Mid-Atlantic Ridge, except Fernando de Noronha Archipelago and

Rocas Atoll, between the Brazilian and African coasts. It is unlikely that these stingrays cross the Atlantic to interact with *H. rufus* on EA, given their coastal habitat preferences (Last, Naylor, Sérét, et al., 2016) and the result of the ENM (Figure 9) analyses which indicate low habitat suitability in non-coastal areas.

Ocean basins have already been suggested to serve as barriers for the dispersal of benthic coastal stingrays (Le Port & Lavery, 2012). The present findings corroborate the isolation of these two stingrays, supporting a description of a new species in the Brazilian coast besides supporting a differentiation of this clade's species based on geographic locality. The outflow of the Amazon, as already suggested by Rodrigues Filho et al. (2020), and La Plata Rivers seems to be barriers also for the occurrence of this species, as hypothesized by the ENM of *H. americanus sensu lato*. This ENM, done as *a priori* test, includes occurrence data of what was later discovered to be more than one lineage. Although this violates the assumption that the data refer to a single species to run models (Peterson, 2006), this ENM was important as it allowed the authors to raise questions that were further tested. Nevertheless, the relevant ecological variables for the occurrence of this group and for *H. berthalutzae sp. nov.* alone are remarkably similar given that both species, the newly described one and *H. americanus sensu scripto*, are indeed much alike in terms of not only morphology but also habitat preferences.

The main difference prevails in the importance of bathymetry for *H. americanus sensu lato* (34.7%), which is overcome by other environmental features in *H. berthalutzae sp. nov.*, such as the primary productivity. Because this characteristic is causally related to the presence of micronutrients in water (Cermeño et al., 2016), due to the climatic condition of northeastern Brazil, with many intermittent rivers, the outflow of sediments and minerals to the western Atlantic is compromised (Souza & Knoppers, 2011). Therefore, the primary productivity in this coastal region is smaller than that in its adjacent northern and southern areas. As *H. berthalutzae sp. nov.* occurs largely in northeastern Brazil, this low primary productivity influences its ENM, which is not observed for *H. americanus sensu lato* as the used location records are distributed almost along the whole of America's coast.



**FIGURE 14** Details of *Hypanus berthalutzae sp. nov.* showing its dorsal colouration of small black spots and white pectoral margins in Fernando de Noronha, Pernambuco, Brazil. Photo by Tiego L. A. Costa



**FIGURE 15** A female specimen of *Hypanus berthalutzae sp. nov.* and two representatives of *Caranx bartholomaei* in Fernando de Noronha Archipelago, Brazil. Photo by Tiego A. L. Costa

In addition, based on the ENM of *H. americanus* *sensu lato* and on what is known of the ecological preferences of these stingrays (Last, Naylor, Sérét, et al., 2016), bathymetry was removed as a variable for the modelling of *H. berthalutzae* sp. nov. because it was highly correlated to other features that would need to be excluded. Therefore, because its relevance is known, this study opted for its exclusion to test the importance of others, such as the primary productivity and surface phosphate instead of benthic phosphate.

A literature review found only nine studies of ENM in elasmobranchs, most of them concentrated in the Gulf of Mexico, with a few species of concern in the southwestern Atlantic Ocean (Melo-Merino et al., 2020). In the present study, the ENM helped identify the main ecological drivers and barriers for the dispersal of this species, indicating the relevance of ENM in phylogeographic studies (Alvarado-Serrano & Knowles, 2014).

## 5 | CONCLUSION

Given the combination of multiple lines of evidence that suggest distinct geographic distribution, independent molecular evolutionary processes and morphological differences in claspers with likely consequences for reproduction, enough evidence exists to formally describe a new taxon. *H. berthalutzae* sp. nov. is here defined as a species in the genus *Hypanus* distributed from the mouth of the Amazon River to São Paulo State, as well as in Parcel de Manuel Luiz (Rocha & Rosa, 2001), Fernando de Noronha Archipelago and Rocas Atoll (Rosa & Moura, 1997), preferring habitats closer to the coast and shallow environments.

*H. americanus* is currently classified as “data deficient” in IUCN (Grubbs et al., 2016), and taxonomic studies were a priority research for better delimitation of the evolutionary unit and posterior evaluation of its conservation status. This study provides information that this species has a more restricted distribution than previously thought, occurring from New Jersey (USA) to the mouth of the Amazon River, and could even be two sympatric species to be morphologically and ecologically delimited in future studies. The new species, *H. berthalutzae* sp. nov., is fished in northeast Brazil but protected in Fernando de Noronha Archipelago and Rocas Atoll marine conservation units. In the former, it is conspicuous in shallow waters, visible even from the shore, and could be a symbol of the Brazilian endemic marine species. With its taxonomic and geographic delimitations, it can now have its conservation status evaluated and this information transmitted to fishermen, who are the most relevant people in promoting its conservation (Silva et al., 2020).

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## AUTHOR CONTRIBUTIONS

F.F.P., G.J.P.N. and S.M.Q.L. conceived of the idea. F.F.P. generated and analysed the data and prepared drafts of the manuscript. Funding was provided by all three authors.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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