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How many lineages are there of the stingrays genus *Hypanus* (Myliobatiformes: Dasyatidae) and why does it matter?

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Stingrays genus *Hypanus* currently encompasses nine valid species from the Atlantic and Pacific oceans, though the phylogenetic relationships amongst some of them were based on a single mitochondrial gene and did not involve all putative *Hypanus* species. To address the monophyly of the genus and its relationship to other Dasyatinae genera, we sequenced the whole mitochondrial genomes of all species that supposedly belong to this genus and representatives of Dasyatinae, Neotrygoninae, and, as an outgroup, *Fontitrygon* (Urogymninae). Based on phylogenetic analyses, *Hypanus* is the sister-genus to all other Dasyatinae, and this subfamily is closely-related to Neotrygoninae within the family Dasyatidae. The species *F. geijskesi* is closely related to *H. guttatus* rather than to its congeners and should be allocated to *Hypanus* as *H. geijskesi* for the genus monophyly. After lineage delimitation analyses, we identified three species complexes composed of *H. americanus*, *H. guttatus*, and *H. say*, with two distinct evolutionary lineages within each, leaving the genus with 13 evolutionary units, of which six are currently under threat and only *H. sabinus* is of least concern. The urgency in identifying these new lineages lies in the fact they might already be under threat before being formally described.

Keywords: Atlantic Ocean, Conservation, Cryptic species, Diversification, Elasmobranchs.

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As raias com ferrão do gênero *Hypanus* atualmente compreendem nove espécies válidas nos oceanos Atlântico e Pacífico, embora as relações filogenéticas entre algumas delas tenha sido baseada em apenas um gene mitocondrial e não envolvia todas as possíveis espécies de *Hypanus*. Para avaliar o monofiletismo do gênero e sua relação com outros Dasyatinae, sequenciamos os genomas mitocondriais de todas as espécies que supostamente compõem o gênero e representantes de Dasyatinae, Neotrygoninae e, como grupo externo, *Fontitrygon* (Urogymninae). Baseados em análises filogenéticas, *Hypanus* é o gênero-irmão de todos os outros Dasyatinae e essa subfamília é proximamente relacionada à Neotrygoninae dentro da família Dasyatidae. A espécie *F. geijskesi* é mais relacionada a *H. guttatus* que a outras congêneres e deve ser alocada em *Hypanus* como *H. geijskesi* para que o gênero seja monofilético. Após análises de delimitações de linhagens, identificamos três complexos de espécies formados por *H. americanus*, *H. guttatus* e *H. say*, com duas linhagens evolutivas distintas em cada, deixando o gênero com 13 unidades evolutivas, das quais seis estão atualmente sob risco de extinção e somente o estado de *H. sabinus* é pouco preocupante. A urgência na identificação dessas linhagens reside no fato que podem já estar ameaçadas antes de serem formalmente descritas.

Palavras-chave: Conservação, Diversificação, Elasmobrânquios, Espécies crípticas, Oceano Atlântico.

INTRODUCTION

Speciation in marine environments is usually a complex process involving geographic isolation and ecological adaptation (Bowen *et al*., 2013) mediated by an organism's life history characteristics and biology (Craig *et al*., 2006). Some para- or sympatric lineages with incipient genetic differentiation might be considered as different species, not only populations (Avise, 2000; Potkamp, Fransen, 2019). This scenario has already been documented in some sharks (Corrigan, Beheregaray, 2009) and rays (Kashiwagi *et al*., 2012) that move extensively but are genetically restrained by environmental forces (Bowen *et al*., 2013). During the process of divergence, lineages incrementally acquire genotypic and phenotypic characteristics that make them distinct from each other, creating a grey zone where the definition of a species is ambiguous (De Queiroz, 2007). In such cases, a limited number of genetic loci are often insufficient to resolve taxonomic issues and semi-isolated lineages prevail until genomic studies are accomplished, making the delineation of species a challenging task. So, conservation should be a priority and not be constrained by a lack of clarity in species boundaries (Roux *et al*., 2016).

Dasyatid stingrays are globally distributed batoids that vary in size (from 23 cm to 2.2 m disc width), weigh up to 600 kg, and vary in disc shape from circular to rhombic. They primarily occur in coastal marine environments (down to 400 m), but can also be found in freshwater (Last *et al*., 2016b). Until recently, the genus *Dasyatis* Rafinesque, 1810 was indicated as a paraphyletic group of stingrays based on morphological data (Rosenberger, 2001), and subsequent studies based on the mitochondrial gene NADH

dehydrogenase 2 (*mt-nd2*) corroborated this hypothesis (Naylor *et al*., 2012). Last *et al*. (2016a) and Lim *et al*. (2015) revised Dasyatidae based on morphological and molecular data and divided it into four subfamilies (Dasyatinae, Hypolophinae, Neotrygoninae, and Urogymninae). Moreover, what was previously known as *Dasyatis* was separated into eight genera (*Dasyatis*, *Pteroplatytrygon* Fowler, 1910, *Taeniurops* Garman, 1913, *Bathytoshia* Whitley, 1933, *Hemitrygon* Müller & Henle, 1838, *Hypanus* Rafinesque, 1818, *Telatrygon* Last, Naylor & Manjaji-Matsumoto, 2016, and *Megatrygon* Last, Naylor & Manjaji-Matsumoto, 2016) in the subfamily Dasyatinae, grouped by morphological similarities and molecular clusters.

Despite the resurrection of a monophyletic *Hypanus*, the most species-rich Dasyatinae genus around the American continent, relationships among its species and their phylogenetic position within Dasyatidae were based on a single mitochondrial marker (*mt-nd2*), with few representatives per independent evolutionary lineage, and some missing ones due to lack of sampling (Last *et al*., 2016a).

Currently, *Hypanus* encompasses nine recognized species: *H. americanus* (Hildebrand & Schroeder, 1928), *H. berthalutzae* Petean, Naylor & Lima, 2020, *H. dipterurus* (Jordan & Gilbert, 1880), *H. guttatus* Bloch & Schneider (1801), *H. longus* (Garman, 1880), *H. marianae* (Gomes, Rosa & Gadig, 2000), *H. rudis* (Günther, 1870), *H. sabinus* (Lesueur, 1824), and *H. say* (Lesueur, 1817). Except for *H. rudis* from Guinea Gulf, on the western coast of the African continent, and *H. dipterurus* and *H. longus* from the Pacific Ocean, all other six species occur on the Atlantic coast of America. Even though six of these species were sampled and included in the analysis by Last *et al*. (2016a), the placement of *H. marianae* was not tested, and it was considered a *Hypanus* species based on morphological data, as well as *H. rudis*, which was recently corroborated as a *Hypanus* species by Petean *et al*. (2020), who also described a new one (*H. berthalutzae*).

Precise delimitation and identification of these stingrays are crucial for their conservation since they are frequently the targets of fisheries where they are harvested for food and clothing (Costa *et al*., 2015; Last *et al*., 2016b; Ceretta *et al*., 2020; Oliveira *et al*., 2021). More than half of *Hypanus* species are evaluated as threatened in the Red List of Threatened Species by IUCN: three are Vulnerable (*H. berthalutzae*, *H. dipterurus*, and *H. longus* (Charvet *et al*., 2020; Pollom *et al*., 2020a,c)), one is Endangered (*H. marianae* (Pollom *et al*., 2020b)), and one is Critically Endangered (*H. rudis* (Jabado *et al*., 2021c)); three are classified as Near Threatened (*H. americanus*, *H. guttatus*, and *H. say* (Carlson *et al*., 2020a,b,c)); and only one is clearly under no risk of extinction: *H. sabinus* (Least Concern, Carlson *et al*., 2020d)).

Another dasyatid genus, in the subfamily Urogymninae, with a similar pattern of species diversification in the Atlantic Ocean and facing risks of extinction is *Fontitrygon* Last, Naylor & Manjaji-Matsumoto, 2016, which currently contains six species (Last *et al*., 2016a). Four occur in western Africa: *Fontitrygon margarita* (Günther, 1870) (Vulnerable, Jabado *et al*., 2021a), *F. margaritella* (Compagno & Roberts, 1984) (Near Threatened, Jabado *et al*., 2021b), *F. ukpam* (Smith, 1863) (Critically Endangered, Jabado *et al*., 2021d), and *F. garouaensis* (Stauch & Blanc, 1963) (Critically Endangered, Jabado *et al*., 2021e) while two occur along the Northern coast of South America: *F. colarensis* (Santos, Gomes & Charvet-Almeida, 2004) (Santos *et al*., 2004) (Critically Endangered, Pollom *et al*., 2020d) and *F. geijskesi* (Boeseman, 1948) (Critically Endangered, Pollom *et al*., 2020e). Nevertheless, only three of these species were included in Last *et al*. (2016a)

Dasyatidae revision due to a lack of samples, and both American species were provisionally positioned in *Fontitrygon*. Despite the incomplete taxon sampling represented, the phylogenetic relationships provided by those authors indicated that some members of *Fontitrygon* might be misclassified, leaving it as a possible paraphyletic genus.

A useful genetic marker for investigating phylogenetic relationships and species identities is the mitochondrial DNA (mtDNA) due to its high evolutionary rate, maternal inheritance, intraspecific polymorphisms, and genes arrangement (Avise *et al*., 1987; Harrison, 1989; Boore, Brown, 1998; Satoh *et al*., 2016). Even though a phylogeny based on mtDNA is a story of modifications on a small portion of DNA of maternal transmission, it has not been puzzled by recombination (Avise *et al*., 1987). Species in which females are more stationary than males, mtDNA can provide distinct information than nuclear markers due to biased dispersal by sexes (Moritz *et al*., 1987). However, studies on *H. americanus* from Central America have shown little to no philopatric behavior, with both males and females contributing to gene flow (Corcoran *et al*., 2013; Flowers *et al*., 2016; Schwanck *et al*., 2020). So, evolutionary studies on *Hypanus* stingrays based on mtDNA might tell a similar story to nuclear markers, to be further assessed. Recently, mitogenomes have been widely used for phylogenetic inferences in distinct metazoan clades: Diptera (da Silva *et al*., 2020), Rodentia (Abramson *et al*., 2021), Coleoptera (Nie *et al*., 2021), and Elasmobranchii (Palacios-Barreto *et al*., 2023).

Since the massive use of molecular methods, such as DNA barcoding (Hebert, Gregory, 2005), to identify and classify species, organisms with comparable phenotypes that could have been considered as unique species are recognized as genetically diverse, a concept known as "cryptic species". Sáez, Lozano (2005) described these as "groups of organisms that are morphologically indistinguishable from each other, yet found to belong to different evolutionary lineages". *Sphyrna gilberti* Quattro, Driggers, Grady, Ulrich & Roberts, 2013 and *Squalus suckleyi* (Girard, 1855) are examples of shark species with circumtropical distributions in which morphological analyses subsequently to identifications of genetic lineages corroborated the existence of more than one entity (Ebert *et al*., 2010; Quattro *et al*., 2013; Gaither *et al*., 2016).

A concept to be explored is that of taxonomic gap, in which there is a space between the extant biodiversity and what is actually known about it (Dubois, 2010; Raposo *et al*., 2020). This gap regards both the universe of unknown species and those susceptible to changes due to more studies. As many authors have said, "taxonomic stability is ignorance" (Dominguez, Wheeler, 1997; Benton, 2000; Dubois, 2010;) since with more data and analyses the gap might increase or decrease in a continuous progress of Science. It is indisputable that the lack of specimens that could serve as vouchers for each molecular sample could have consequences for taxonomy (Amorim *et al*., 2016) due to the impossibility of checking the morphology of all individuals. However, given the urgency in closing this taxonomic gap to recognize the world's biodiversity before more extinctions take place, even tissue samples could corroborate the once-existing variety of species (Engel *et al*., 2021).

Due to the taxonomic uncertainties in Dasyatinae (Lim *et al*., 2015; Last *et al*., 2016a; Pavan-Kumar *et al*., 2022), the absence of a complete sampling of all *Hypanus* species in other published works, and the risks of extinction these stingrays are facing, our goal is to use mitogenomes to define the relationships among *Hypanus* species, identifying possible cryptic ones, and their relationships to other Dasyatinae genera. Afterward, species can be properly identified and (re)evaluated for adequate conservation measures.

MATERIAL AND METHODS

Sampling, DNA isolation, and sequencing. To test the monophyly of the genus *Hypanus* and the subfamily Dasyatinae, we sampled 124 specimens from all nine valid species belonging to *Hypanus*, six representatives of almost all Dasyatinae genera (*Hemitrygon akajei* (Bürger, 1841), *Telatrygon acutirostra* (Nishida & Nakaya, 1988), *Pteroplatytrygon violacea* (Bonaparte, 1832), *Batytoshia lata* (Garman, 1880), *Taeniurops grabatus* (Geoffroy St. Hilaire, 1817), *Dasyatis hypostigma* Santos & Carvalho, 2004; except *Megatrygon*), and both Neotrygoninae genera (*Taeniura lymma* (Fabricius, 1775) and *Neotrygon kuhlii* (Müller & Henle, 1841)). As outgroup, we included representatives of each *Fontitrygon* species, subfamily Urogymninae (one sample of *F. margarita*, *F. margaritella*, *F. garouaensis*, and six of *F. geijskesi*; except *F. colarensis* and *F. ukpam*). Species distributions and sampling localities are provided in Tab. 1 (details in Tab. **S1**) and sample locations of *Hypanus* and *Fontitrygon* in Fig. 1. Valid names and distributions were obtained from Last *et al*. (2016a) and Eschmeyer's Catalog of Fishes (Fricke *et al*., 2023). Nearly all tissues were collected in fish markets, making it unfeasible to preserve most of the specimens; however, we performed barcode analyses (described below) to compare clades to examined specimens deposited in collections. Lineages for which we could provide vouchers are *H. americanus*, *H. berthalutzae*, *H. geijskesi*, *H. guttatus*, *H. sabinus*, and *H. say*; even though there is no voucher for *H. marianae*, tissues came from the specimens identified and collected by Costa *et al*. (2022). Before mitochondrial gene capture, samples were genetically identified based on Sanger sequencing of the mitochondrial marker *mt-nd2*, as described by Petean *et al*. (2020), and compared to the database from Naylor *et al*. (2012). After capture, we performed barcode analyses comparing to data from GenBank (detailed as it follows) as another approach to verifying species' identities. When we directly removed tissues from specimens through diving or trawling, we morphologically identified them. Data collection was under SISBIO permit 54254-3 and supported by Atlantis Divers in Fernando de Noronha, Brazil.

From genomic DNA extraction to the alignment of protein-coding gene sequences of mitochondrial genomes, all protocols and procedures followed Li *et al*. (2013, 2015) and Petean *et al*. (2020). Extracted DNA was sheared to 500 bp in an M220 Focused-ultrsonicator (Covaris, Inc., Wobuern, Massachusetts, USA) as the first step for library preparation, followed by the selection of > 200 bp fragments with solid-phase reversible immobilization beads (Li *et al*., 2015). We performed a series of reactions in each sample for mitochondrial gene capture using biotinylated RNA baits (Mycroarray, Ann Arbor, Michigan) (Li *et al*., 2013). For sequencing, we deployed an Illumina MiSeq Next Generation Sequencer and, from each read, removed low-quality reads (with Phred quality scores lower than 30; Illumina 2011, https://www.illumina.com/documents/products/technotes/technote_Q-Scores.pdf) and adaptors using Trim Galore 0.6.4 (Krueger, 2020) then mapped to the mitochondrial genome of a closely-related species, *Hemitrygon akajei* (NC_021132), from the GenBank using Geneious 7.9.1 (http://www.geneious.com). Finally, we used a pipeline (MitoAnnotator, (Iwasaki *et al*., 2013)) to annotate sequences, which are available on GenBank under accession numbers provided in Tab. **S1**.

TABLE 1 | Sampled species of the genera *Hypanus*, *Telatrygon*, *Hemitrygon*, *Taeniurops*, *Pteroplatytrygon*, *Bathytoshia*, *Dasyatis*, *Neotrygon*, *Taeniura*, and *Fontitrygon*, their location and geographic distributions. *non-sampled species by Last *et al.* (2016). EA: Eastern Atlantic, NWA: Northwestern Atlantic, SWA: Southwestern Atlantic, EP: Eastern Pacific, WA: Western Atlantic, WP: Western Pacific.

Species	N	Sampled locality	Distribution
Hypanus americanus (Hildebrand & Schroeder, 1828)	8	Virginia (USA) to Nicaragua	NWA: Massachusetts (USA) North of South America
**Hypanus berthalutzae* Petean, Naylor & Lima, 2020	23	From Pará to Bahia (Brazil)	SWA: Pará to São Paulo (Brazil)
Hypanus dipterurus (Jordan & Gilbert, 1880)	4	Baja California (Mexico)	EP: Hawaii (USA), California (USA) to northern Chile, including the Galápagos Islands
**Hypanus geijskesi* (Boeseman, 1948)	6	North of Brazil	WA: Venezuela and Suriname to Northern Brazil
Hypanus guttatus (Bloch & Schneider, 1801)	34	From Belize to Bahia (Brazil)	EA: Gulf of Mexico to Paraná (Brazil)
Hypanus longus (Garman, 1880)	4	Baja California (Mexico)	EP: Baja California (Mexico) to Ecuador, including the Galápagos Islands
**Hypanus marianae* (Gomes, Rosa & Gadig, 2000)	29	From Ceará to Bahia (Brazil)	SWA: Northeastern Brazil
Hypanus rudis (Günther, 1870)	4	Senegal and Ghana	EA: Gulf of Guinea
Hypanus sabinus (Lesueur, 1824)	4	South Carolina and Mississippi (USA)	NWA: Delaware (USA) to Gulf of Mexico
Hypanus say (Lesueur, 1817)	8	South Carolina and Mississippi (USA)	WA: Massachusetts (USA) to Brazil
Telatrygon acutirostra (Nishida & Nakaya, 1988)	1	Ariake Bay (Japan)	WP: China and southern Japan
Hemitrygon akajei (Müller & Henle, 1841)	1	Ariake Bay (Japan)	WP: China and Japan to Malasia
Taeniurops grabata (Geoffroy St. Hilaire, 1817)	1	Senegal	EA: Mediterranean Sea, Madeira, and Canary Islands to Angola
Pteroplatytrygon violacea (Bonaparte, 1832)	1	California (USA)	Cosmopolitan in tropical and warm temperate seas
Bathytoshia lata (Garman, 1880)	1	Hawaii (USA)	Indo-West Pacific, Hawaii (USA), Eastern Atlantic, and Mediterranean Sea
Dasyatis hypostigma Santos & Carvalho, 2004	1	Uruguay	SWA: South of Brazil
Neotrygon kuhlii (Müller & Henle, 1841)	1	Malasia	WP: Solomon Islands, Red Sea, Indo-West Pacific: East Africa, east to the Philippines and Mariana Islands, north to Japan, Australia, and New Caledonia
Taeniura lymma (Forsskål, 1775)	1	Indonesia	Red Sea, Indo-West Pacific: East and South Africa, east to the Philippines and Papua New Guinea, north to the Philippines, south to northern Australia
**Fontitrygon garouaensis* (Stauch & Blanc, 1962)	1	Nigeria	EA: Nigeria and Cameroon
Fontitrygon margarita (Günther, 1870)	1	Senegal	EA: Senegal to Congo
Fontitrygon margaritella (Compagno & Roberts, 1984)	1	Senegal	EA: Mauritania to Angola
Total	**135**		

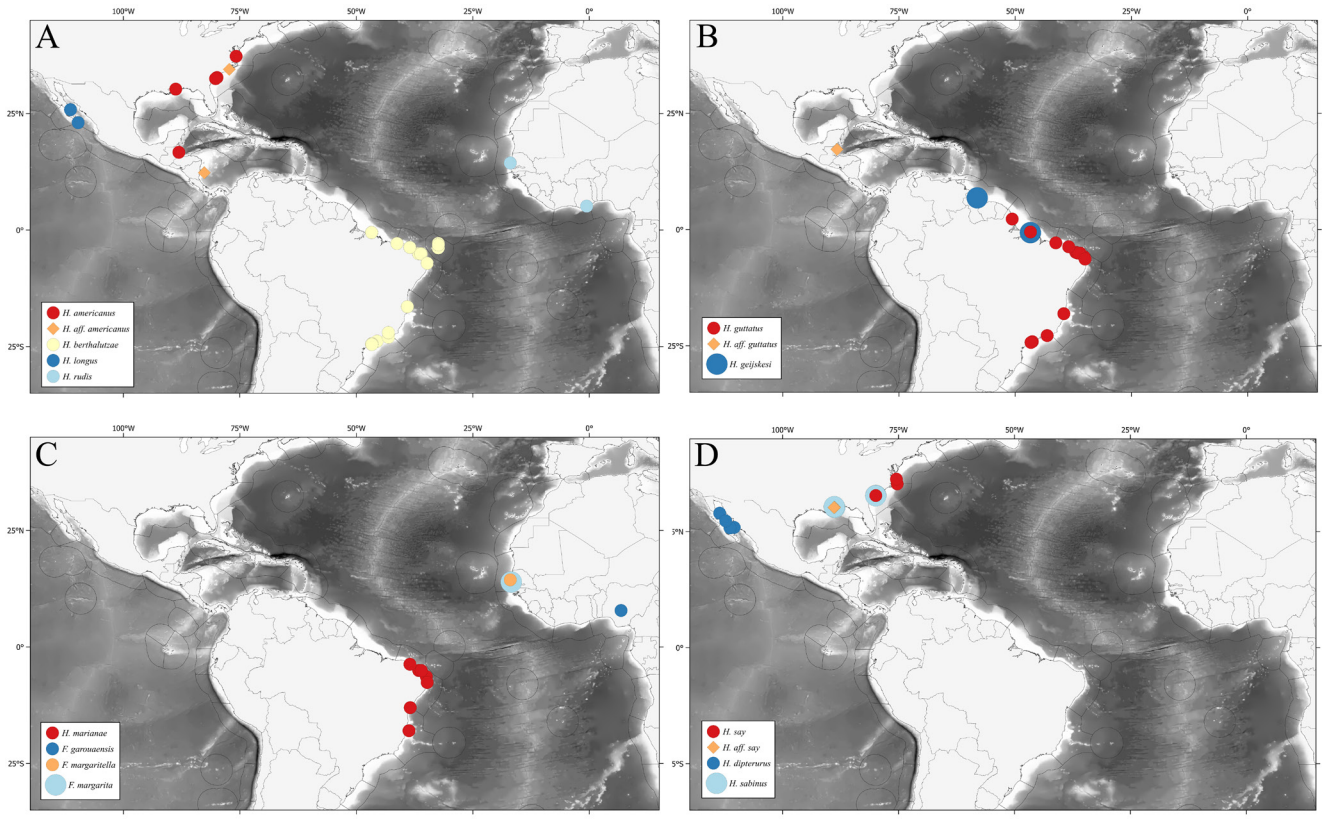

FIGURE 1 | Sampling locations of all *Hypanus* and *Fontitrygon* lineages (new name combinations are used in the figure, as discussed in the text). **A.** *Hypanus americanus*, *H.* aff. *americanus*, *H. berthalutzae*, *H. longus*, *and H. rudis*; **B.** *H. guttatus*, *H.* aff. *guttatus*, and *H. geijskesi*; **C.** *H. marianae*, *Fontitrygon garouaensis*, *F. margarita*, and *F. margaritella*; **D.** *H. say*, *H.* aff. *say*, *H. dipterurus*, and *H. sabinus*.

Phylogenetic reconstructions. To study the relationships within the genus *Hypanus*, its relationships within the subfamily Dasyatinae, and to Neotrygoninae, the whole mitogenome sequences of all 135 specimens were aligned in GENEIOUS 7.9.1 using the MUSCLE algorithm (Edgar, 2004). After annotation we identified and excluded from all sequences the mitochondrial control region (CR), tRNA, and rRNA. Control region was deleted because it is highly variable among individuals and its coverage after mitochondrial capture was too low; RNAs regions were eliminated because the indels present in these regions make alignment difficult. The final alignment had 11,471 base pairs in 13 protein-coding genes.

To select the best-fitting model of molecular evolution we used PartitionFinder2 (Lanfear *et al.*, 2017) and selected the best scheme for each protein-coding gene under Bayesian Inference Criteria: GTR+gamma+invariant sites for *mt*-nd1 and *mt*-nd5, HKY+gamma for *mt-nd2*, *mt-atp8*, *mt-atp6*, *mt-coiii*, *mt-nd6*, and *mt-cytb*, HKY+gamma+invariant sites for *mt-nd3*, *mt-nd4l*, and *mt-nd4*, and TN93+gamma for *mt-coi* and *mt-coii*. Maximum Likelihood analyses were conducted using RAxML version 8. (Stamatakis, 2014) in CIPRES Science Gateway (Miller *et al.*, 2010), with bootstrap and consensus calculations based on a 1000-generation search of tree space.

Bayesian Inferences were carried out in BEAST 2.5 (Bouckaert *et al.*, 2019) using Yule model as the prior tree as we are not considering known extinctions ($\mu = 0$) and

there is a reasonable sampling for analyses (ρ = 1) (Drummond, Bouckaert, 2015) in 1,000,000,000 generations with 5 chains resampled every 10,000. The software MEGA X (Kumar *et al*., 2018) was used to calculate uncorrected genetic p-distances and analyze intra- and interspecific genetic differences between *Hypanus* lineages.

Lineage delimitation methods. By analyzing the relationships among species, we noticed some valid species could be either paraphyletic or have long branches within them, suggesting possible distinct lineages. Therefore, we decided to do five species delimitation analyses within the clades of *H. guttatus* and *H. say* independently: 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), and Assemble Species by Automatic Partitioning (ASAP, (Puillandre *et al*., 2021). For each clade's data (*H. guttatus* and *H. say*) we selected an outgroup based on the results of our phylogenetic analysis and Last *et al*. (2016a): *Hypanus marianae* for *H. guttatus* and *H. sabinus* for *H. say* analyses. Most of these analyses (except ASAP) are performed on a tree topology: both GMYC methods rely on an ultrametric tree, which was built under a Bayesian Inference analysis in BEAST 2.5, and both PTP on a tree with nucleotides' substitutions, built with a Maximum Likelihood analysis in RAxML version 8. For such phylogenetic analyses before delimitation ones, we used jModelTest2 (Darriba *et al*., 2012) to select the best molecular evolution model for each clade. The ASAP method depends on an alignment matrix instead of a tree. For more details on delimitation methods, refer to Petean *et al*. (2020).

DNA Barcode analyses. The mitochondrial protein-coding gene region cytochrome c oxidase subunit I (*mt-co1*) was identified and extracted from some sequences for comparisons to those available at GenBank (Tab. **S2**). The goal was to use the molecular clusters as support for the verification of taxonomic status when vouchers were available for at least one sequence in a clade. Sequences of *Fontitrygon geijskesi* sampled by this study were aligned with a sample from Guyana (GN17902) and four samples from Rodrigues-Filho *et al*. (2020) (GenBank numbers MN105749, MN105812, MN105813, MN105819), who provided a voucher for the species. The alignment was performed in MEGA X using the MUSCLE algorithm, which had 587 base pairs, 12 *F. geijskesi* samples, and one outgroup. The genetic p-distance was also performed in MEGA X and, to estimate species identities based on sequences' similarities, a Neighbor-Joining (Saitou, Nei, 1987) tree was built in GENEIOUS 7.9.1 with 1,000 bootstrap replicas and edited in FigTree v. 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

The same barcode analyses were performed for the three clades containing species-complexes (*H. guttatus*, *H. say*, and *H. americanus*) identified by abovementioned analyses and Petean *et al*. (2020). *Hypanus berthalutzae* (GN18496) was used as an outgroup for all independent analyses, except for *H. americanus*, for which it was also the ingroup and *H. guttatus* (GN18434) was then used as an outgroup.

For *H. guttatus*, besides the 33 samples from this study, we included 52 *mt-co1* sequences from GenBank, totaling 85 samples (and one outgroup) in 524 base pairs. To analyze the clade containing *H. say*, we extracted the *mt-co1* region from mitogenomes of this species, *H. dipterurus*, and *H. sabinus* and added 13 *mt-co1* sequences from *H. say*,

two *H. dipterurus*, and eight *H. sabinus* from GenBank. The alignment had 40 samples (and one outgroup) in 547 base pairs. Finally, to investigate *H. americanus*, we not only added 38 *mt-co1* sequences from GenBank, but we also included 23 *H. berthalutzae*, four *H. longus*, and four *H. rudis*, in a total of 77 samples as the ingroup in 599 base pairs.

RESULTS

Phylogenetic inferences. The genus *Hypanus sensu* Last *et al*. (2016a) was recovered as monophyletic and sister to all other genera (except *Megatryon*, not sampled for this study) within the subfamily Dasyatinae (Fig. 2), which is a sister-group to Neotrygoninae. These results were already suggested by Last *et al*. (2016a) and are now corroborated by mitogenomes through the same resulting topologies by Maximum Likelihood and Bayesian Inference phylogenetic analyses with all nodes' values higher than 88% bootstrap and 0.995 of posterior probability. Maximum Likelihood and Bayesian Inference trees topologies with all taxa and nodes values are available in Figs. **S3** and **S4**, respectively.

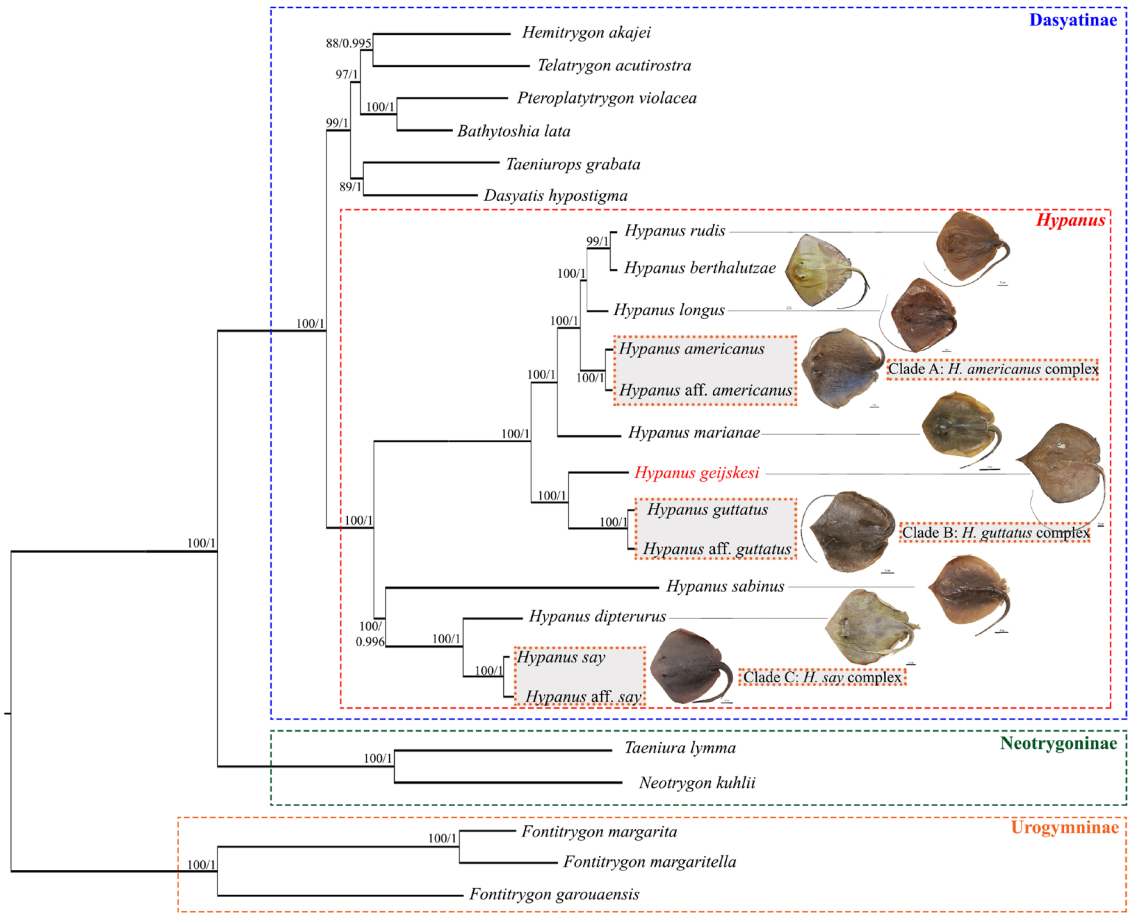

FIGURE 2 | Maximum Likelihood tree topology of mtDNA with representatives of *Hypanus* species, dasyatine genera, neotrygonine genera, and urogymnine genus *Fontitrygon* as an outgroup. New name combinations are used in the figure in red, as discussed in the text. For each node, the maximum likelihood bootstrap value is given first, followed by the Bayesian inference posterior probability. Clade A (*H. americanus* complex) taken from Petean *et al*. (2020).

There are clear unique lineages that correspond to valid species names: *Hypanus berthalutzae*, *H. dipterurus*, *H. longus*, *H. marianae*, *H. rudis*, and *H. sabinus*. *Hypanus americanus*, the Southern stingray, nonetheless, is not a single evolutionary lineage (Clade A in Fig. 2), as suggested by previous mitogenomes delimitation analyses and haplotype network based on *mt-nd2* (Petean *et al*., 2020; Figs, 2–3, respectively), which showed 8 unsampled haplotypes and mutational steps between both lineages, while there are four between *H. berthalutzae* and *H. rudis*; and a phylogeographic study based on the mitochondrial control region by Richards *et al*. (2019) that found three populations of this species in the USA's coast and Caribbean. The species *H. marianae*, which was not included in the previous molecular study (Last *et al*., 2016a), is a monophyletic lineage and sister to the clade containing *H. americanus sensu lato*, *H. longus*, *H. berthalutzae*, and *H. rudis*. This clade is, then, closely-related to a group containing *H. guttatus*, which now is suggested to harbor two lineages: one distributed from Central America to the south of Brazil and another of specimens from Belize (Clade B in Fig. 2).

Two species of *Hypanus* occur along the Pacific coast, *H. dipterurus* and *H. longus*, both with similar evolutionary histories since they are independent sister-groups to Atlantic clades: *H. say* and *H. berthalutzae* + *H. rudis*, respectively. Moreover, within the clade *H. say*, there is a clear divergence of two lineages separated by the Peninsula of Florida (Clade C in Fig. 2).

Six representatives of *Fontitrygon geijskesi*, subfamily Urogymninae, which was not included in the Dasyatidae revision by Last *et al*. (2016a), formed a sister-clade to *H. guttatus*, within the genus *Hypanus*, but not *Fontitrygon*. So, for *Hypanus* to be monophyletic, this species should be reclassified as *Hypanus geijskesi*. The subfamily Urogymninae is then represented by three *Fontitrygon* species occurring in Africa, which formed a cluster: *F. margarita*, *F. margaritella*, and *F. garouaensis*.

Delimitation of lineages. Candidate species of both species complexes, *H. guttatus* and *H. say*, were analyzed by combining all five delimitation methods (Figs. 3–4); analyses of *H. americanus* complex were performed by Petean *et al*. (2020). The observed new lineages, sister to known species, are named as *affinis* to those they are closely related to. We kept the valid name according to the type-locality of each species: type of *H. guttatus* from Brazil, so *H.* aff. *guttatus* from Central America; and type of *H. say* from Egg Harbor (USA), *H.* aff. *say* from the Gulf of Mexico.

We selected those results which were more consistent among methods, with similar branches' division, and those that provided the least number of lineages within a species complex to avoid over-splitting taxa due to mere genetic structure. mPTP and ASAP were the most conservative analyses suggesting only two lineages within each species complex; however, while bPTP agreed with mPTP in delimiting *H. say* two lineages, it was a less stringent method when analyzing *H. guttatus* data, pointing to 25 entities (almost one per individual). Both GMYC methods, single and multiple thresholds, resulted in similar groupings within each complex and proposed only one or two lineages more than we accepted.

Intraspecific average pairwise distances vary from 0.036% in *H. longus* to 0.26% in *H.* aff. *guttatus* (Tab. 2), while in interspecific average pairwise, the smallest distances are 0.82% between *H. rudis* and *H. berthalutzae*, 0.83% between *H. americanus* and *H.* aff. *americanus*, and 0.95% between *H. say* and *H.* aff. *say* (Tab. 3). Interestingly, the distance

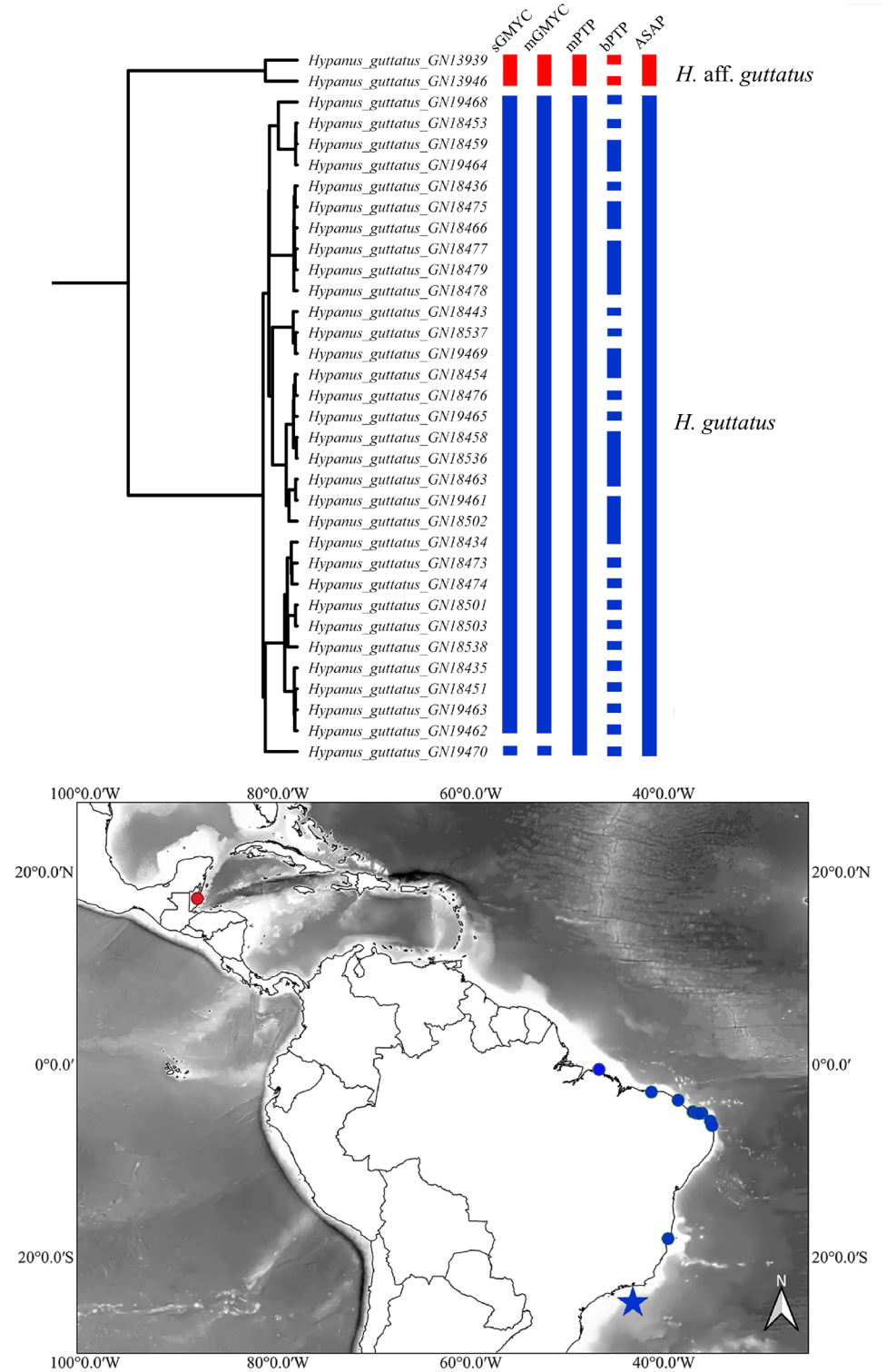

FIGURE 3 | Candidate species of the clade *Hypanus guttatus* species complex (Clade B), according to five lineage delimitation analyses using the mtDNA. Possible species found in each analysis are portrayed as colored boxes in columns. In blue, *H. guttatus*; red, *H.* aff. *guttatus*. The same colors are used to represent sampled specimens in the map to the right: *H. guttatus*, blue circles in the Brazilian coast; *H.* aff. *guttatus*, red circles in Central America. Blue star is the holotype location of the valid species, which was not sampled, in southeastern Brazilian coast.

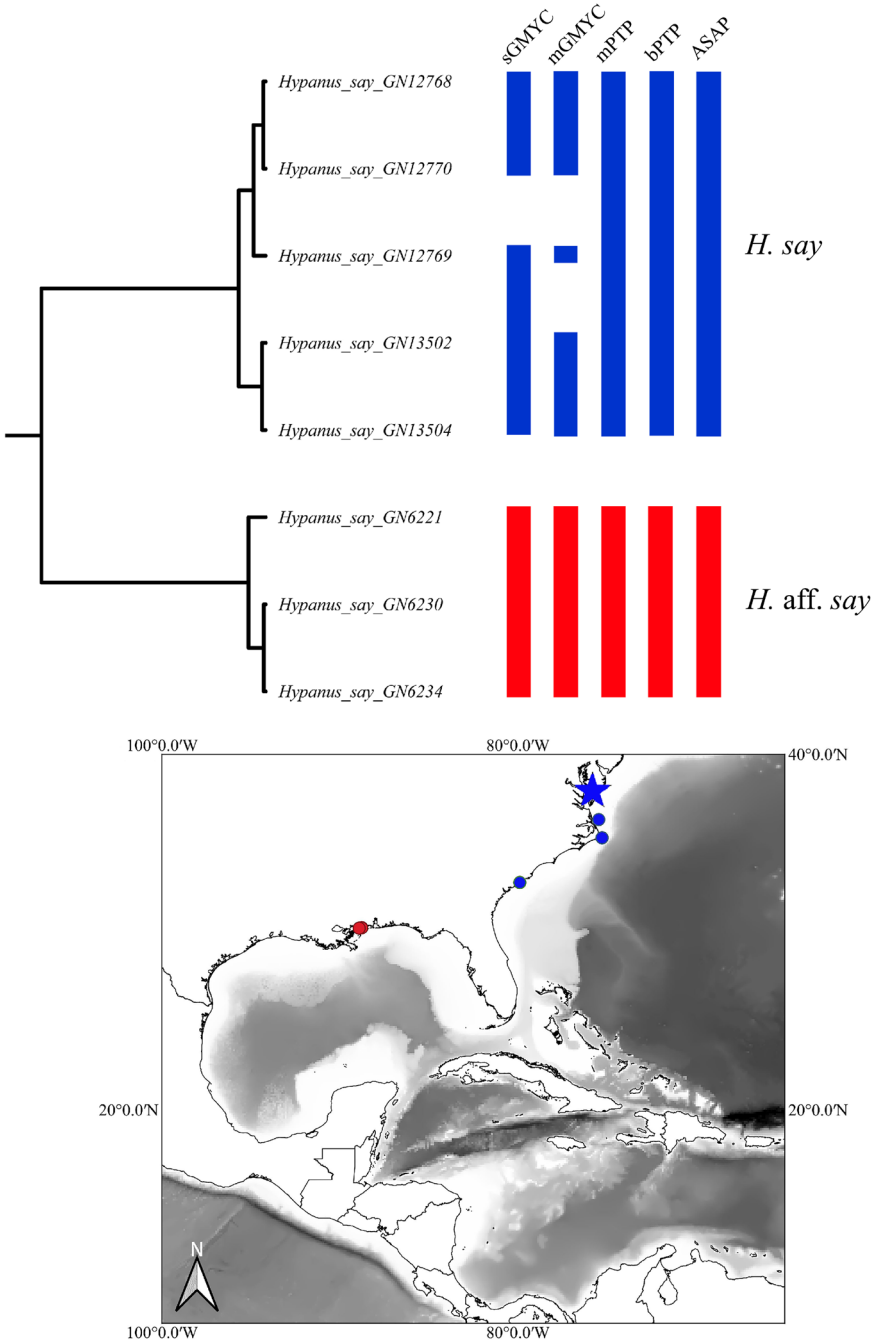

FIGURE 4 | Candidate species of the clade *Hypanus say* species complex (Clade C), according to five lineage delimitation analyses using the mtDNA. Possible species found in each analysis are portrayed as colored boxes in columns. In blue, *H. say*; red, *H.* aff. *say*. The same colors are used to represent sampled specimens in the map to the right: *H. say*, blue circles in USA's Eastern coast; *H.* aff. *say*, red circles in Gulf of Mexico. Blue star is the holotype location of the valid species, which was not sampled, in USA's Northeastern coast.

between two geographically distant species as *H. longus*, from the Pacific, and *H. rudis*, from Africa is only 2.4% (Petean *et al*., 2020), while *H. sabinus* has the highest distances to all other *Hypanus* lineages (11.74% from *H. dipterurus* is the smallest).

TABLE 2 | Pairwise average distances of mitogenome within *Hypanus* species in %.

Species	% of average divergence within each species
H. americanus	0.092
H. aff. *americanus*	0.070
H. longus	0.036
H. rudis	0.098
H. berthalutzae	0.121
H. marianae	0.068
H. guttatus	0.200
H. aff. *guttatus*	0.262
H. geijskesi	0.167
H. dipterurus	0.163
H. say	0.061
H. aff. *say*	0.047
H. sabinus	0.134

TABLE 3 | Pairwise distances of mitogenome between pairs of species in %. Hamer, *Hypanus americanus*; Haffamer, *H.* aff. *americanus*; Hlon, *H. longus*; Hrud, *H. rudis*; Hbert, *H. berthalutzae*; Hmari, *H. marianae*; Hgut, *H. guttatus*; Haffgut, *H.* aff. *guttatus*; Hgeij, *H. geijskesi*; Hdipt, *H. dipterurus*; Hsay, *H. say*; Haffsay, *H.* aff. *say*; Hsab, *H. sabinus*; Dhypo, *Dasyatis hypostigma*.

%	Hamer	Haffamer	Hlon	Hrud	Hbert	Hmari	Hgut	Haffgut	Hgeij	Hdipt	Hsay	Haffsay	Hsab
Haffamer	0.83												
Hlon	2.69	2.69											
Hrud	3.11	3.12	2.40										
Hbert	3.08	3.14	2.43	0.82									
Hmari	4.91	4.94	4.61	4.96	4.95								
Hgut	6.84	6.86	6.72	7.01	6.94	7.19							
Haffgut	7.02	7.04	6.88	7.21	7.11	7.24	1.37						
Hgeij	6.49	6.44	6.40	6.67	6.56	6.97	5.35	5.53					
Hdipt	11.24	11.20	11.02	11.16	11.15	11.14	11.57	11.75	11.71				
Hsay	10.89	10.89	10.60	10.80	10.85	10.66	11.13	11.28	11.08	4.57			
Haffsay	10.97	11.01	10.80	11.00	11.06	10.83	11.35	11.53	11.39	4.76	0.95		
Hsab	13.05	13.03	12.80	12.93	12.96	13.55	13.21	13.38	13.01	11.74	11.81	11.89	
Dhypo	14.06	14.09	13.85	14.09	14.07	13.91	14.20	14.32	14.02	13.50	13.06	13.19	14.77

DNA Barcoding. By analyzing the protein-coding region *mt-co1* of 11 samples of "*Fontitrygon geijskesi*", we obtained a monophyletic group by a Neighbor-Joining analysis with 100% of bootstrap value (Fig. 5), a result similar to that using mitogenomes. Besides, the genetic distances among all sequences varied from 0 to 0.17%, with an average of 0.03% (Tabs. 4, **S5**). Given the genetic similarity of these sequences and since Rodrigues Filho *et al*. (2020), from which came four of those samples, could provide a voucher specimen for one of them, we have enough support to suggest that it is indeed a valid species. However, it should be considered as *Hypanus geijskesi* due to its close relationship to *H. guttatus*, as suggested by the abovementioned phylogenetic analyses.

Regarding the species *H. guttatus*, the scenario is convoluted: the lineage *H.* aff. *guttatus* identified by mitogenomic delimitation analyses was supported by the inclusion of more samples, as shown by the Neighbor-Joining tree with 89.2% of bootstrap value (Fig. 6). The genetic distance between these two samples (GN13939, GN13946) was 0.38%, which was the same distance between GN13946 and ten other samples (GN19470, MN105788, MN105792, MN105794, MN105808, MN105817, MN105869–71, MN105875), while the distance between GN13939 and the same ten samples was

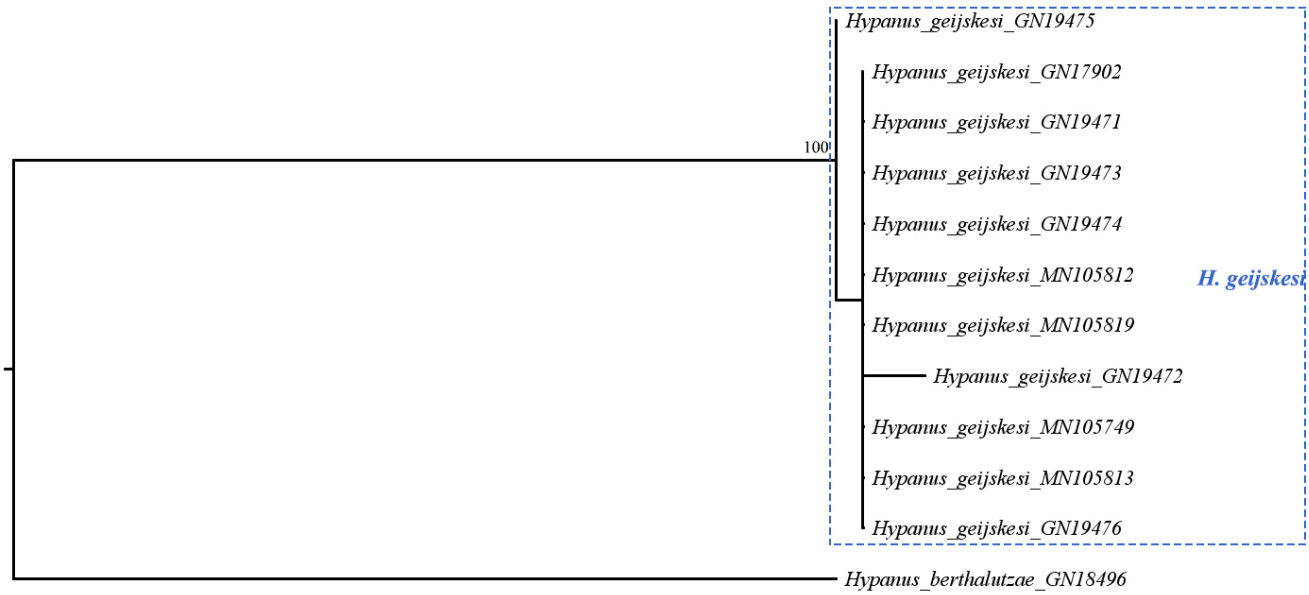

FIGURE 5 | Neighbor-Joining tree based on *mt-co1* from samples identified as *Hypanus geijskesi*, with *H. berthalutzae* as an outgroup. Nodes' numbers correspond to bootstrap values in percentage; only those higher than 85% are shown.

TABLE 4 | Average pairwise distances of the mitochondrial marker *mt-co1* between eleven samples of *Hypanus geijskesi*, with *H. berthalutzae* as an outgroup for comparison. Values in %: interspecific in first cell; intraspecific in second bold cell (with standard error estimate in parenthesis).

%	*H. berthalutzae*	*H. geijskesi*
H. geijskesi	4.6	**0.03 (±0.03)**

0.76%. However, the distances between these two (GN13939, GN13946) and the other 73 were higher than 1.15%, distances between nine of those abovementioned (except GN19470) and the others varied from 0.76% to 0.95%, and distances between those 73 samples varied from 0 to 0.19% (Tabs. 5, **S6**).

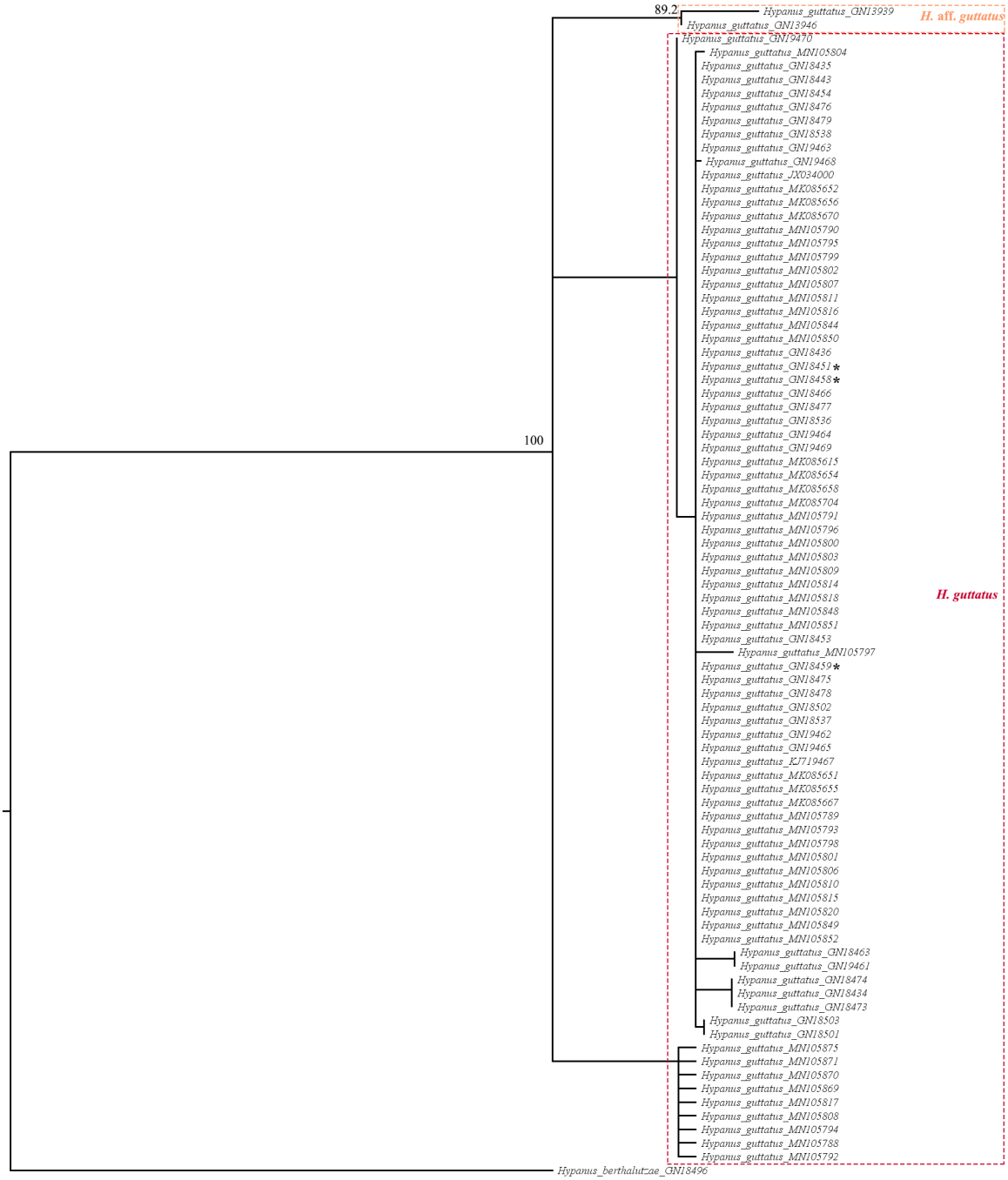

FIGURE 6 | Neighbor-Joining tree based on *mt-co1* from samples identified as *Hypanus guttatus*, with *H. berthalutzae* as an outgroup. Nodes' numbers correspond to bootstrap values in percentage; only those higher than 85% are shown. Examined vouchers with an asterisk.

TABLE 5 | Average pairwise distances of the mitochondrial marker *mt-co1* between 85 samples of *Hypanus guttatus* and *H.* aff. *guttatus*, with *H. berthalutzae* as an outgroup for comparison. Values in %: interspecific below diagonal; intraspecific bold diagonal (with standard error estimate in parenthesis).

%	*H. berthalutzae*	*H.* aff. *guttatus*	*H. guttatus*
H. aff. *guttatus*	5.73	**0.38 (±0.26)**	
H. guttatus	5.92	1.27	**0.19 (±0.07)**

Therefore, based on these results, we suggest that, besides the lineage *H.* aff. *guttatus*, there could also be some hybridization or incomplete lineage sorting driving the evolution of *H. guttatus*, which could be undergoing diversifications into distinct ecological niches (Nosil, Harmon, 2009); such processes could only be understood through more sampling and markers. Given the data we have, we can corroborate the lineage *H. guttatus* by examination of three vouchers by one of us (FFP) (GN18451, GN18458–9; deposited at the fish collection at Universidade Federal do Rio Grande do Norte under respective codes CIUFRN 4442, 4449–50).

Through the extraction of the *mt-co1* region from samples of the species-complex *H. say*, the result agrees with our previous outcome: groups separated by the Peninsula of Florida (Fig. 7), with high bootstrap values for each clade, *H. say* and *H.* aff. *say*, of 97% and 97.5%, respectively. The genetic p-distances between both lineages varied from 0.93% to 1.09%, while within lineage values ranged from 0 to 0.31% (Tabs. 6, **S7**). For the lineage occurring on USA's East coast, which should bear the name *H. say*, one of the samples (MH378605) came from a specimen deposited at the Smithsonian Fish Collection (USNM433289), from a place close to type's location. This specimen was examined by FFP, who identified it as *H. say*, thus serving as a voucher for the lineage.

Sequences of the species *H. dipterurus* and *H. sabinus* were analyzed together with *H. say* complex, and analyses suggested the southernmost sample of *H. dipterurus* (MH 194454, from the Peruvian coast, Pacific Ocean) could be another lineage since it has an average of 3.84% of genetic distance to the other five samples of *H. dipterurus* from Baja California and California coast. Moreover, the inclusion of eight sequences of *H. sabinus* still leaves it monophyletic, with samples from both the East coast of the USA and the Gulf of Mexico. One of these samples (MT 455431) was extracted from a specimen deposited at the Smithsonian Fish Collection (USNM 426256), which was examined by FFP, hence could serve as a voucher for the clade.

Within *H. americanus* species-complex there seem to be two sympatric clades: one that should bear the species name, *H. americanus* (82.9% of bootstrap value) (Fig. 8), since *mt-co1* sequences of some analyzed specimens by FFP at the Smithsonian Fish Collection (USNM 433102, USNM 433338–9) fall within it (KT 075327, MH 378683–4) and they were collected close to the species type-locality. The other clade is composed of three samples (two previously identified as *H.* aff. *americanus* by Petean *et al.* (2020), and one sample deposited at GenBank, MG837920). Genetic distance among these three *H.* aff. *americanus* samples is 0, and their distance to other *H. americanus* vary from 0.33% to 0.67%; while distances within *H. americanus* vary from 0 to 0.17%. Regardless of which *H. americanus* clade, sequences belonging to this species-complex have more than 1.5%

distance to any other sequence belonging to *H. berthalutzae*, *H. longus*, and *H. rudis* (Tabs. 7, **S8**). Besides, some sequences previously identified and submitted to GenBank as *H. americanus* should be reallocated to *H. berthalutzae* (MK085594, MK085604, MK085629, MK085636, MK085638, MK085641, MK085657, MK085659, MK085662, MK085669, MK085672, MK085684, MK085742, MN105805, MN105821, MN105822, MN105823, MN105824, MN105839, MN105842, MN105845, MN105846, MN105847). Some of

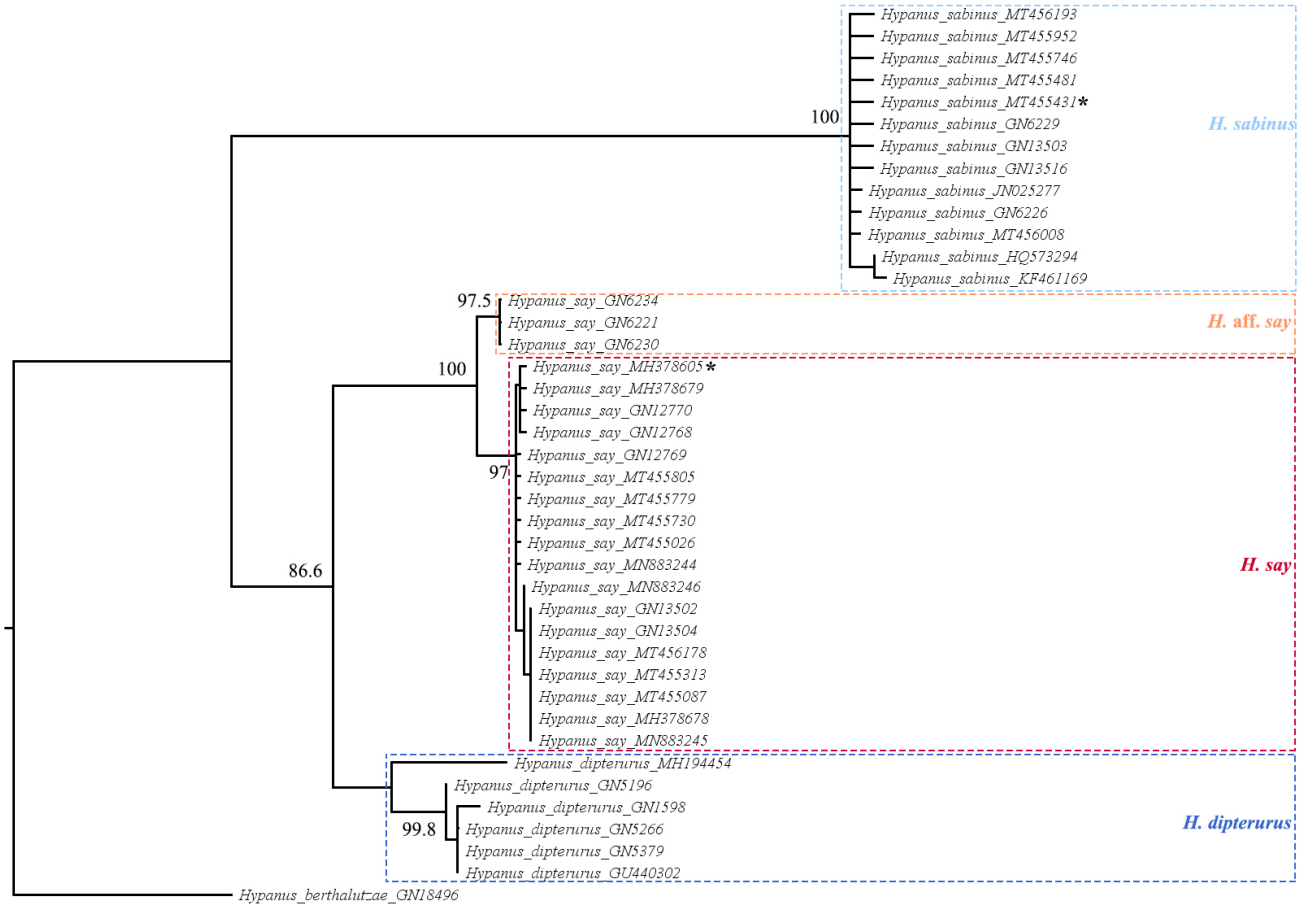

FIGURE 7 | Neighbor-Joining tree based on *mt-co1* from samples identified as *Hypanus say*, *H. dipterurus*, and *H. sabinus*, with *H. berthalutzae* as an outgroup. Nodes' numbers correspond to bootstrap values in percentage; only those higher than 85% are shown. Examined vouchers with an asterisk.

TABLE 6 | Average pairwise distances of the mitochondrial marker *mt-co1* between six samples of *Hypanus dipterurus*, 13 *H. sabinus*, 18 *H. say* and three *H.* aff. *say*, with *H. berthalutzae* as an outgroup for comparison. Values in %: interspecific below diagonal; intraspecific bold diagonal (with standard error estimate in parenthesis).

%	*H. berthalutzae*	*H. dipterurus*	*H. sabinus*	*H. say*	*H. aff. say*
H. dipterurus	10.76	**1.43 (±0.29)**			
H. sabinus	15.61	13.47	**0.19 (±0.11)**		
H. say	11.06	5.63	13.92	**0.15 (±0.1)**	
H. aff. say	10.60	5.12	14.05	1.21	**0**

these sequences were used by Rodrigues Filho *et al*. (2020) to suggest the existence of two lineages of *H. americanus* in Northern Brazil; and one of them was described as *H. berthalutzae* (*H. americanus* 1). The clade they called *H. americanus* 2 is what we identified as *H. americanus*. The lineage identified by Petean *et al*. (2020) and hereby sustained as *H.* aff. *americanus* was not sampled by those authors.

FIGURE 8 | Neighbor-Joining tree based on *mt-co1* from samples identified as *Hypanus americanus*, *H. berthalutzae*, *H. rudis* and *H. longus*, with *H. guttatus* as an outgroup. Nodes' numbers correspond to bootstrap values in percentage; only those higher than 85% are shown. Examined vouchers with an asterisk.

TABLE 7 | Average pairwise distances of the mitochondrial marker *mt-co1* between 46 samples of *Hypanus berthalutzae*, 20 *H. americanus*, three *H.* aff. *americanus*, four *H. rudis*, and four *H. longus*, with *H. guttatus* as an outgroup for comparison. Values in %: interspecific below diagonal; intraspecific bold diagonal (with standard error estimate in parenthesis).

%	*H. guttatus*	*H. berthalutzae*	*H. americanus*	*H.* aff. *americanus*	*H. rudis*	*H. longus*
H. berthalutzae	6.09	**0.17 (±0.08)**				
H. americanus	7.18	1.83	**0.04 (±0.02)**			
H. aff. *americanus*	6.68	1.69	0.50	**0.00 (±0.00)**		
H. rudis	6.47	0.59	1.96	1.79	**0.17 (± 0.11)**	
H. longus	5.51	1.48	2.34	2.17	1.29	**0 (± 0.00)**

DISCUSSION

Phylogenetic considerations. *Hypanus* was recovered as monophyletic by using mitochondrial genome sequences of all species previously attributed to it in addition to another species that was provisionally allocated in *Fontitrygon* by Last *et al*. (2016a) due to a lack of sampling and morphological similarities. These authors revised the family Dasyatidae and used the *mt-nd2* gene to identify chondrichthyans' lineages, as suggested by Naylor *et al*. (2012). Our results indicate the use of this marker is reliable for identifying Chondrichthyes species, especially when resources are unavailable for genome sequencing. Some lineages suggested to belong to *Hypanus*, but unsampled by Last *et al*. (2016a), were hereby included. *Hypanus marianae* was identified as a *Hypanus* species, as implied by morphological similarities by Last *et al.* (2016a), and it is a sister-species to the clade containing *H. americanus* species complex, *H. longus*, *H. rudis*, and *H. berthalutzae*, a recently described species (Petean *et al*., 2020) whose phylogenetic position was also corroborated by the inclusion of representatives of the whole genus.

For the monophyly of the genus *Hypanus*, the species "*Fontitrygon geijskesi*" should be considered a member of *Hypanus*, as it is found to be sister to *H. guttatus*. Due to morphological similarities, this species was expected to be related to its African congeners *F. margarita*, *F. margaritella*, and *F. garouaensis* (Last *et al*., 2016a). However, we suggest the reallocation of *F. geijskesi* to the genus *Hypanus* with a new name combination as *Hypanus geijskesi* (Boeseman, 1948). This result had already been observed by Rodrigues Filho *et al.* (2020) on a Neighbor-Joining analysis using the mitochondrial marker COI (*mt-co1*), in which "*F. geijskesi*" specimens resulted as a sister group to *H. guttatus* within the genus *Hypanus*; however, their analysis lacked a phylogenetic inference of its relationships to other *Hypanus* lineages. Through a combination of *mt-co1* sequences by Rodrigues Filho *et al*. (2020) to those hereby provided, we noticed *H. geijskesi* intraspecific distances ranging from 0 to 0.17% and, as they have provided a voucher for one of their samples, we have support for the species reallocation. This change leaves the genus *Fontitrygon* with five species, of which four occur in the African continent and one in South America (*F. colarensis*, not sampled by this study).

As already suggested by Lim *et al*. (2015), Last *et al*. (2016a), and Pavan-Kumar *et al*. (2022), but including more representatives of the subfamily Dasyatinae, we also recognized its monophyly and close relationship to Neotrygoninae, with *Hypanus* as the sister-group to all other genera within the first subfamily; and the subfamily Urogymninae, represented by the genus *Fontitrygon*, supporting the subfamilies' rooting.

Two *Hypanus* species that used to have the largest geographic distributions, *H. americanus sensu lato* (Petean *et al*., 2020) from Massachusetts (USA) to São Paulo (Brazil) and *H. guttatus* from Mexico to Southeastern Brazil, are now recognized to encompass more than one lineage each. The lineage of *H. americanus sensu* (Petean *et al*., 2020) occurring at the Brazilian coast was recently described as *H. berthalutzae*, a sister-species to *H. rudis* in Eastern Atlantic. What was left of *H. americanus* in Central and North America also represents more lineages, as suggested by our phylogenetic analyses (Clade A, Fig. 2), delimitations done by Petean *et al*. (2020) (Figs. 2–3), and phylogeographic studies by Richards *et al*. (2019) (Figs. 1–2). Richards *et al*. (2019) found three lineages of *H. americanus* occurring in the USA and Caribbean; however, based on our smaller sampling and distinct markers (that did not involve the mitochondrial control region used by those authors), we noticed two sympatric clades (Fig. 1A) where they named "Clade 3" (Richards *et al*., 2019) (Figs. 1–2) and we could not recover their "Clades 1 and 2".

The second species with a wide distribution, *H. guttatus*, has also been shown to contain two lineages, as presented here and by Rodrigues Filho *et al*. (2020). Therefore, two wide-ranging marine coastal species were recently shown to occupy smaller areas than previously described, with currently valid species probably harboring more than one lineage and increasing the known diversity within the genus *Hypanus*.

Lineage delimitations. Based on five distinct lineage delimitation methods (sGMYC, mGMYC, mPTP, bPTP, and ASAP), we analyzed two clades within *Hypanus* that showed deeper divergences in phylogenetic analysis than what is expected for intraspecific evolution, since branches are longer between these lineages than within each one of them (Schwartz, Mueller, 2010).

Within the clade composed of *H. guttatus*, a species that supposedly occurs from Mexico to Southeastern Brazil, all analyses suggested a deeper divergence separating Central America's samples from Brazilian ones than those within each area of occurrence. This scenario was also observed by Rodrigues Filho *et al*. (2020), besides a high genetic similarity among stingrays southern of the Amazon river mouth (0.19% of *mt-co1* intraspecific distance, Tab. 5, Fig. 3), which would be left under the valid name *H. guttatus* due to the species' type-locality: "Brazil". We infer that, even though *H. guttatus* is a marine and estuarine species that tolerates low salinities environments, the great freshwater and nutrients influx of the Amazon system may be a barrier for these stingrays, which resulted in isolated northern and southern lineages (Rocha, 2003; Hoorn *et al*., 2010). It was also shown by Tosetto *et al*. (2022) that the small number of species in common between the Caribbean Sea and the Brazilian coast demonstrates their high isolation by the Amazon River Plume. This differentiation suggested by genetic analysis might be supported by morphological dissimilarities as well, such as morphometric differences in nostrils, spiracles, and caudal structures (FFP, pers. obs.; review in progress).

With regards to *H. say*, the lineage from the eastern coast of the USA has a different evolutionary history than that from the Gulf of Mexico, which is supported by all genetic analyses conducted here. Morphological differences could also justify this finding, such as differences in snout and caudal morphometries (FFP, pers. obs.; review in progress), leaving the lineage from eastern USA under the valid species name *H. say* according to its type-locality (New Jersey, USA), and that occurring in the Gulf of Mexico as *H.* aff. *say* until further analyses can be performed, and its taxonomic status evaluated. These results agree with the biogeographic proposals of Spalding *et al.* (2007) as both populations occur in the Warm Temperate North Atlantic province but in distinct ecoregions and separated by the Floridian one. The southernmost portion of the Florida Peninsula is known to have a detached ecosystem from the adjacent USA coast (Bowen, Avise, 1990). This can also be seen in *H. americanus* in which samples from the Bahamas (~50 Km from the US coast) are more closely related to those from the US Virgin Islands than those from Florida (Richards *et al*., 2019). Despite these results, gene transfer among lineages cannot be ruled out, which could result in hybridization and species not achieving reproductive isolation. As a consequence, there would be a disagreement between gene trees and species trees, a situation that might be underlying the evolution of freshwater stingrays Potamotrygoninae, as well as incomplete lineage sorting and diversification times (Fontenelle *et al*., 2021). These hypotheses should be further tested with nuclear genetic data (Petean and collaborators, working in progress).

The phylogenetic analysis of manta rays based on mitogenomes and nuclear exons (White *et al*., 2018) found pairwise distances between *Mobula birostris* (Walbaum, 1792) and *M. alfredi* (Krefft, 1868) as 0.4%; even though this distance might seem small for two species, their taxonomic identities as distinct species had already been suggested by morphometric, meristic, two mitochondrial, and one nuclear gene (Marshall *et al*., 2009; Kashiwagi *et al*., 2012). Likewise, the pairwise distances between the three cryptic lineages of *Hypanus* and the valid species to which they currently belong are small: between *H. guttatus* and *H.* aff. *guttatus*, 1.37%, *H. say* and *H.* aff. *say*, 0.95%, and *H. americanus* and *H.* aff. *americanus*, 0.83%. Simultaneously, the largest distance between two *Hypanus* species is 13.55% in *H. sabinus* and *H. marianae*. Interspecific genetic distances within the genus vary from 0.82% (*H. berthalutzae* and *H. rudis*, Petean *et al*., 2020) to 13.55%, which is a high variation. However, intraspecific variations in *Hypanus* range from 0.047% in *H.* aff. *say* to 0.26% in *H.* aff. *guttatus*, which are values at least four times smaller than the interspecific distances.

There are many definitions of what "cryptic species" are and, even though there is still no consensus on their meaning (Struck *et al*., 2018), they could be "erroneously classified (and hidden) under one species name" (Bickford *et al*., 2007). Both sibling lineages to currently valid species *H. guttatus* and *H. say* are yet undescribed due to a lack of taxonomic studies and poor sampling. Subtle differences between species, with some of them being separated only by morphometrics, suggest a conservative morphology, making it more difficult to identify the species complex despite the allopatric pattern. Therefore, molecular markers such as mtDNA can be used to confirm species identification. Scenarios of cryptic speciation, with DNA sequences showing deep genetic divergences and morphological data revealing subtle diversity, have been observed in many non-elasmobranch fish clades: bonefish *Albula* Scopoli, 1777 (Colborn *et al*., 2001), catfish *Noturus* Rafinesque, 1818 (Egge, Simons, 2006), tubenose goby *Proterorhinus* Smitt, 1900 (Neilson, Stepien, 2009).

Genetic data have also been showing a higher species diversity in Elasmobranchs than formerly known, indicating the necessity of taxonomic work (Richards *et al*., 2009, 2019; Dudgeon *et al*., 2012; Borsa *et al*., 2016; Henderson *et al*., 2016; Sales *et al*., 2019; Fahmi *et al*., 2021; Gonzalez *et al*., 2021; Vilasboa *et al*., 2022; Kottillil *et al*., 2023). Through the combination of distinct tools, species' hypotheses have been corroborated by independent studies, such as *Gymnura* van Hasselt, 1823 (Yokota, Carvalho, 2017, morphology; Rodrigues Filho *et al*., 2020, genetics; Vilasboa *et al*., 2022, genetics), *Aetobatus* Blainville, 1816 (Richards *et al*., 2009, genetics; White *et al*., 2010, 2013, morphology and genetics; Sales *et al*., 2019, genetics), *Rhizoprionodon* Whitley, 1929 (Springer, 1964, morphology; Mendonça *et al*., 2011, genetics, *Pseudobatos* Last, Séret & Naylor, 2016 (Rutledge, 2019, morphology; Sandoval-Castillo, Beheregaray, 2020, genetics).

Our findings regarding these independent evolutionary units in *Hypanus* are only hypotheses of possible species as morphological and ecological data are recommended to be included since the use of exclusively molecular tools might lead to over or under-estimations of species (Carstens *et al*., 2013). This is due to species delimitation methods being unable to distinguish deep structure as a result of population-level processes or species boundaries (Sukumaran, Knowles, 2017). Therefore, to avoid failure, we are not describing any species until more data can be combined (Carstens *et al*., 2013).

Conservation. There is no threshold of genetic distances between lineages that should be regarded as populations and those that should receive a species status, since this is a faint boundary (De Queiroz, 2007; Roux *et al*., 2016). As mitochondrial evolution rates are slower in elasmobranchs than in other vertebrates (Martin *et al*., 1992), those mtDNA differences found here may represent distinct species. It is undoubtful that more studies are needed for a resolution of their taxonomic status; however, despite being categorized as species or populations, these lineages should be considered for conservation purposes (Henderson *et al*., 2016).

The urgency in identifying these lineages is because each entity in a threatened species complex might be even more endangered than the nominal species as a whole, and may need distinct conservation measures (Bickford *et al*., 2007). All current valid *Hypanus* species have been recently evaluated by elasmobranch specialists at IUCN (2020). Of the 13 evolutionary units identified in this study, only one is clearly under low risk, *H. sabinus* (Least Concern), while six are under some risk of extinction, with criteria used to evaluate each species threatened category in parenthesis: *Hypanus berthalutzae* (A2d), *H. dipterurus* (A2d), and *H. longus* (A2d) are Vulnerable, *H. marianae* (A2cd) is Endangered, and *H. rudis* (A2d) and *H. geijskesi* (A2d) are Critically Endangered. A concerning situation regards the three species-complexes (*H. americanus* (A2bd), *H. guttatus* (A2d), and *H. say* (A2bd)) since they had their threatened status recently evaluated and were considered as Near Threatened (Carlson *et al*., 2020a,b,c). However, after this and previous studies (Petean *et al*., 2020; Richards *et al*., 2019; Rodrigues Filho *et al*., 2020), we recognized each of them might be, at least, two evolutionary lineages. Therefore, the possible restriction of each clade's geographic range could have an impact on their threatened categories, with consequences on management proposals. The recently described *H. berthalutzae* is the most recent example of this scenario since it was considered *H. americanus* and encompassed the largest species distribution within

the genus. Soon after its description, the species was evaluated and already classified as Vulnerable (Charvet *et al*., 2020), thus demonstrating that lineages currently unknown can already be under threat before their formal description and evaluation as a species.

Throughout evolution, some lineages might be described as species due to population isolation, while others present high genetic variability and each may be named Evolutionarily Significant Unit (ESU) (Coates *et al*., 2018) in an attempt to identify independent entities for conservation and perpetuation of their evolutionary history (Diniz-Filho *et al*., 2013; Hoezel, 2023). These ESUs should be the focus of management efforts (Ryder, 1986; Waples, 1991, 1995; Moritz, 1994).

Currently, conservation aims mostly on valid species, ignoring genetic diversity. Due to the existence of several species concepts and species' delimitation methods, there are many conflicts within taxonomy; besides, scientists do not comply on how to deal with ESUs leading to difficulties in actually applying measurements (Coates *et al*., 2018). Therefore, we suggest the evolutionary lineages hereby identified, even if not formally described as species, to be treated as ESUs and thus be the target of threat evaluation.

To conclude, based on 13 protein-coding mitochondrial genes, there is enough support for the monophyly of the resurrected genus *Hypanus* by Last *et al*. (2016a) after the description of a new species (*H. berthalutzae*) and the transference of *Fontitrygon geijskesi* to *Hypanus*, becoming *Hypanus geijskesi* due to its close relationship to *H. guttatus*. Besides the recognition of a cryptic species within *H. americanus* by Petean *et al*. (2020), we have also identified evolutionary lineages that represent currently known species, as well as suggested two putatively new ones not detected until now, which are sister-lineages to *H. guttatus* and *H. say*, thus reducing their geographic distribution, with possible impacts on their conservation status.

These results leave the genus *Hypanus* with 13 independent evolutionary units, of which 10 are valid species and three "*affinis*" to their siblings (*H.* aff. *americanus*, *H.* aff. *guttatus*, and *H.* aff. *say*). Further formal descriptions of these new lineages will have consequences on their conservation status since current areas of distribution of valid species will decrease with their division into more than one entity, leading to an urgency in evaluating their threatened status and proposing conservation measures, actions that could already begin with ESUs before descriptions. Even though we have delimited some evolutionary lineages within the genus, maybe more could be found with wider sampling. Finally, to rigorously evaluate these species complexes, morphological studies, the examination of type series, and ecological niche modeling should be performed to better define these stingray species and their geographic distributions.

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ETHICAL STATEMENT

The care and use of animals within Brazilian territory followed the Ministry of the Environment animal welfare laws, guidelines, and policies as approved by Chico Mendes Institute for Biodiversity Conservation, license SISBIO 54254–3.

COMPETING INTERESTS

The author declares no competing interests.

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