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Evolutionary Dynamics of American Manatee Species on the Northern Coast of South America: Origins and Maintenance of an Interspecific Hybrid Zone

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Abstract

Interspecific hybridization has been historically neglected in research and conservation practice, but it is a common phenomenon in nature, and several models have been developed to characterize it genetically. Even though *Trichechus inunguis* (Amazonian manatee) and *T. manatus* (West Indian manatee) exhibit large morphological, karyotypic, and molecular differences, a hybrid zone was identified on the northern coast of South America, from the Amazon River estuary toward the Guianas coastline. Two major populations or evolutionarily significant units (ESUs) within *T. manatus*, namely, the Caribbean and Atlantic, were separated and their differentiation was likely promoted or reinforced by the interspecific hybridization zone. We used nuclear and mtDNA sequences to reconstruct manatee speciation, population diversification through time and space, and secondary contact, which resulted in a hybrid zone. In this hybrid zone, the genetic contribution of each parental species was estimated, and different models for generating the current scenario were tested using statistical phylogeographic tools. All the results suggest a long hybridization history, during which a stable and structured hybrid swarm is generated. The coastline hybrid zone is composed of individuals with a lesser genomic contribution from *T. inunguis*; this zone works as a genetic sink that restricts gene flow between neighbouring Atlantic (Brazil) and Caribbean (all others) *T. manatus* populations, which further reinforces the isolation and differentiation of the Brazilian manatees.

Keywords Hybrid zone · Introgression · Approximate Bayesian computation · Sirenians · *Trichechus manatus* · *Trichechus inunguis*

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Introduction

Most instances of new species formation arise from allopatry, where two populations undergo independent evolution in separate geographical locations, ultimately leading to reproductive isolation (Network, 2012). This allopatric differentiation can also give rise to populations with incipient reproductive barriers, facilitating interbreeding after secondary contact between deeply divergent lineages that may subsequently merge into a single population or form an interspecific hybrid zone (Abbott et al., 2013). In fact, hybridization is a common phenomenon in the animal kingdom, often leading to the generation of partially viable descendants in sympatric regions (Adavoudi & Pilot, 2022). Despite its common occurrence, interspecific hybridization has historically been overlooked in research and conservation practice, mainly due to misunderstandings surrounding the phenomenon of reproductive isolation, which is fundamental to the concept of biological species (Mayr, 1982; Fitzpatrick et al., 2015).

While hybridization is a natural part of the evolutionary process, it can pose a threat to population persistence, particularly when influenced by anthropogenic factors (Allendorf et al., 2001). Such factors may include the translocation of animals and plants, the consequences of habitat changes (Payseur, 2010) and recent population declines (Pinto et al., 2016). Furthermore, hybrid zones can reveal introgression, wherein F1 hybrids produce offspring with individuals of one or both parental species (Allendorf et al., 2010). Introgression creates individuals with mosaic genomes, resulting in unpredictable fitness effects depending on the genetic background and local environment, varying from deleterious incompatibilities to local adaptation or even speciation events (Martin & Jiggins, 2017). In certain hybrid zones, parental forms may be entirely replaced by introgressed individuals, resulting in the formation of a self-sustaining hybrid swarm population that does not require continuous gene flow from parental sources to maintain novel genetic combinations (Shurtliff, 2013).

In the evolutionary context, both American manatee species constitute excellent models for studying hybridization between two deeply divergent mammal species. The divergence time between *Trichechus inunguis* (Amazonian manatee) of the Amazon basin and *T. manatus* (West Indian manatee) of the Caribbean and Atlantic coasts has been estimated to have occurred between the middle Pliocene and middle Pleistocene (Santos et al., 2016; Souza et al., 2021). Both species present unique morphological, ecological, karyotypic, genetic and adaptive characteristics, but many interspecific hybrids have been described around the mouth of the Amazon River and toward the Guianas coastline (Garcia-Rodriguez et al., 1998; Vianna et al., 2006;

Santos et al., 2016; Oliveira et al., 2022; Noronha et al., 2022). Hybrids may exhibit morphological characteristics similar to those of both species; for example, the general morphology of *T. manatus* is frequently associated with a white spot in the abdomen and the absence of some nails, as observed for *T. inunguis* (Vilaça et al., 2019). Additionally, some individuals from the Amapá coast and Marajó Island in Brazil presented karyotypes with intermediate numbers of chromosomes (2n=49 and 2n=50) between *T. inunguis* (2n=56) and *T. manatus* (2n=48) (Vianna et al., 2006; Luna et al., 2021; Oliveira et al., 2022). A previous study using analyses of chromosome structure and skull morphology also detected important differences between Brazilian and other *T. manatus* populations and an intermediate skull morphology in the Guianas coastline hybrid zone (Barros et al., 2017). Other recent studies have also revealed differences in G-banding patterns, autosomal fundamental numbers, and distinct chromosomal pairs in manatees from this hybrid zone (Oliveira et al., 2022; Noronha et al., 2022).

Two of our previous studies reported incongruence between mitochondrial and nuclear DNA in the manatee population of French Guiana, suggesting long-term admixture (introgression) between the two species in this region (Lima et al., 2019; Vilaça et al., 2019). In addition, although *T. inunguis* is considered an endemic freshwater mammal of the Amazon basin, Vilaça et al. (2019) reported the presence of an Amazonian manatee in a river in French Guiana (50 km away from the coastline), indicating the transit of at least one of the parental species in the hybrid zone through the Amazon River plume. Bonvicino et al. (2020) confirmed that the Amazonian manatee is also found at the mouth of the Amazon River. More recently, Luna et al. (2021) suggested continuous gene flow between *T. manatus* populations from the northwestern and southeastern sides of the Amazon River using microsatellite and mitochondrial sequences, even though microsatellite data also provided evidence for the existence of introgressed individuals in the hybrid zone that should extend further to eastern Venezuela.

The West Indian manatee is distributed in coastal areas from the larger Antilles and Florida (USA) to Alagoas (Brazil). In 1986, Domning and Hayek employed cranial analysis to divide *T. manatus* into two subspecies, *T. manatus latirostris* (restricted to Florida) and *T. m. manatus* (other locations), a morphological subdivision not supported by a more recent and detailed cranial analysis (Barros et al., 2017). Numerous molecular studies have evaluated intraspecific divisions within *T. manatus* (Garcia-Rodriguez et al., 1998; Hunter et al., 2010, 2012; Vianna et al., 2006), and most of them have presented divergent results from the current taxonomy. Indeed, *T. manatus* shows an intraspecific structure with two major populations displaying distinct traits: ESU 1, located in the Caribbean (Caribbean

coastline, Mexico Gulf, Antilles, and Florida); and ESU 2, located in the Atlantic (Brazilian) (Barros et al., 2017; Santos et al., 2016). These two populations are separated by an interspecific hybrid zone (Vilaça et al., 2019; Lima et al., 2021), which may have promoted or reinforced the differentiation observed between them, as observed for other species (Abbott et al., 2013). Moreover, a detailed study that combined cranial and chromosome structure variation in West Indian manatees (Barros et al., 2017) showed that manatees on the Brazilian coastline are markedly different from all others, including the Florida and Puerto Rican manatees (very similar between them), challenging the currently accepted taxonomy.

Despite various independent morphological, karyotypic, and molecular evidence indicating the significant intraspecific division of *T. manatus* separated by an interspecific hybrid zone, no study has focused on the population dynamics and speciation of American manatees or the origin of the extensive hybrid zone distributed from the Amazon River estuary toward the Guianas coastline. Presumably, the relative isolation of the Brazilian ESU was reinforced by the hybrid zone separating the two ESUs, as this area hosts a large hybrid (introgressed) population in a vast estuary-like environment with distinct habitat characteristics compared to the coastal environments where *T. manatus* typically resides (Artigas et al., 2003; Castelblanco-Martínez et al., 2017; Meirelles et al., 2018; Vilaça et al., 2019).

Nonetheless, characterizing admixture events can be challenging due to the retention of ancestral polymorphisms caused by incomplete lineage sorting in related species or differentiated populations (Zhou et al., 2017). This can hinder the reconstruction of interspecific hybridization in hybrid zones (Toews & Brelsford, 2012). Given the observations made across the entire tree of life, several coalescent models have been developed to aid in the estimation of the history of related taxa (Leaché et al., 2014). These models can be applied in statistical phylogeography to distinguish between gene flow and incomplete lineage sorting and estimate other demographic parameters (Knowles & Maddison, 2002). While available evidence points to the complex history of the hybrid zone between *T. manatus* and *T. inunguis*, many questions about its formation and maintenance remain unanswered (Santos et al., 2016; Vilaça et al., 2019).

By employing specific diagnostic genes and statistical phylogeographic tools, we aimed to test the hypothesis that if a hybrid swarm exists in this area, it may function as a genetic sink of diversity from both parental species and divergent *T. manatus* populations. Our study sought to reconstruct the process of formation and maintenance of the hybrid zone, considering its geographic position and ecological distinctness, with the goal of comprehending the

importance of this phenomenon for the evolution and conservation of both species and their habitats.

Materials and Methods

Sampling

DNA samples were obtained from previously collected tissues (skin and muscle) of *T. inunguis*, *T. manatus*, and putative hybrids between these two species (from the hybrid zone) that were already deposited in the Tissue Collection of the *Centro de Coleções Taxonômicas of Universidade Federal de Minas Gerais*, Belo Horizonte, MG, Brazil. To characterize the genetic composition of the hybrids, we compared them with "pure" individuals from parental species following Vilaça et al. (2019). *Trichechus inunguis* controls ("pure") were from the interior Amazon River ($n=44$), and *T. manatus* controls were from the states of Alagoas, Pernambuco, Paraíba, Rio Grande do Norte, Ceará and Piauí, Brazil ($n=43$); Florida, USA ($n=23$); and Puerto Rico ($n=5$). The "putative" hybrid/introgressed individuals were obtained from all samples obtained from the Amazon Estuary and Amapá coast of Brazil ($n=3$) and from French Guiana ($n=12$) (Fig. 1); these individuals were originally collected from stranded animals found dead on beaches and/or in mangroves along the coast, with no distinction of their phenotype. All deposited tissue samples were preserved in 100% ethanol, and DNA extraction was performed by a standard phenol–chloroform protocol (Sambrook et al., 1989). The complete set of manatee samples used in this study is shown in Tables S1 and S2.

PCR and Sequencing

We sequenced a control region (CR) fragment of mitochondrial DNA (mtDNA) (Vianna et al., 2006) and five nuclear (nuDNA) genes (APOB, APP, BMI1, CREM, and RAG1), both of which had diagnostic haplotypes for each parental species (Vilaça et al., 2019). PCR was performed following Vilaça et al. (2019) and Lima et al. (2019), and the amplicons were purified by the polyethylene glycol (PEG) method (20% PEG 8000, 2.5 M NaCl) with modifications (Santos Júnior et al., 2015). The fragments were sequenced on an ABI 3130xl Genetic Analyser (Applied Biosystems) using the BigDye Terminator 3.1 Cycle Sequencing Kit. The same amplification primers were used for DNA sequencing. Chromatograms were analysed in SeqScape 2.6, and consensus sequences were aligned using the ClustalW algorithm (Larkin et al., 2007) implemented in the software MEGA 7 (Kumar et al., 2016).

Haplotype Inference and Characterization of Hybrids

The haplotype phases were inferred via DNAsp 5 software using the algorithm "Phase" (Rozas et al., 2003) under default parameters, and parsimony relationships between them were estimated via PopART software (Leigh & Bryant, 2015) via the median-joining algorithm (Bandelt et al., 1999). This analysis was performed to identify diagnostic haplotypes of each manatee species and potential hybrid individuals that presented mixed species-specific alleles. To estimate the relative genomic contribution of both parental species to the hybrids through nuclear DNA haplotypes, we used the software STRUCTURE 2.3.4 (Pritchard et al., 2000) with K = 2 (two species) and treated the haplotypes as alleles. The analysis was performed under the admixture model and the assumption of uncorrelated allele frequencies with ten replicates of 10×10^6 iterations each and a burn-in of 2.5×10^4. The software CLUMPAK (Kopelman et al., 2015) was used to summarize the STRUCTURE results, which were visualized in DISTRUCT (Rosenberg, 2004). Additionally, we performed a factorial correspondence analysis (FCA) implemented in GENETIX 4.02 (Belkhir et al., 2000) to covalidate the admixture results identified in the STRUCTURE analysis, treating distinct haplotypes as alleles. To evaluate the mtDNA and nuDNA sequences, we estimated the multigenic nuclear distance matrix between the individuals in the program POFAD (Joly & Bruneau, 2006) using the genpofad algorithm (Joly et al., 2015). Finally, this genetic dissimilarity matrix was visualized with SplitsTree4 software (Huson, 1998; Huson & Bryant, 2006) using the Neighbor-Net algorithm.

Phylogenetic Estimation and Dating

To gain insights into the minimum time of hybrid zone formation and maintenance, we reconstructed a dated species tree of *Trichechus* using BEAST 2.5 software (Bouckaert et al., 2014). Our analysis included three manatee species (*T. inunguis*, *T. senegalensis*, and *T. manatus*), with *T. manatus* divided into two distinct lineages or demic populations, namely, the Caribbean and Atlantic ESUs of West Indian manatees. This division was considered to be due to the presence of mtDNA haplotypes from the two ESU lineages, as well as *T. inunguis* mtDNA, within the hybrid zone (Lima et al., 2021). Additionally, two outgroups, the dugong (*Dugong dugon*) and the sea cow (*Hydrodamalis gigas*), were also included in the analysis. We retrieved the mtDNA and nuDNA sequences of *D. dugon*, *H. gigas*, and *T. senegalensis* from GenBank (accession numbers: JN413953, JN632735, JN633472, JN633590, KR827286, KR827273, KR827274, KR827287, KR827336, KR827337, KR827277,

KR827278, KR827275, KR827276, KR827280, KR827288, KR827289, KR827323, KR827324, KR827335, KR827338, and AY963894-98). After aligning these sequences and adding them to our data matrix, we estimated the best substitution model for each fragment using the AICc criterion in jModeltest2 (Darriba et al., 2012). The molecular clock model for each locus was tested using the maximum likelihood method (ML) with the best substitution model selected in MEGA7 (Kumar et al., 2016).

To calibrate the molecular clock, we employed the calibrated Yule model (Steel & Mckenzie, 2001) along with two fossil calibration intervals: one for the Sirenia ancestor (minimum 28.1 MYA, maximum 38.0 MYA) (Velez-Juarbe et al., 2012) and another for the Dugongidae ancestor (minimum 41.3 MYA, maximum 59.2 MYA) (Vélez-Juarbe & Domning, 2014), as implemented by Springer et al. (2015). Subsequently, we conducted three distinct analyses with 5×10^8 generations and thinning 5×10^4 each, and we ensured the stationarity and convergence of the parameters using Tracer 1.7 (Rambaut et al., 2018). The tree files were combined in LogCombiner and summarized in TreeAnnotator, both of which are available in the BEAST 2.5 package (Bouckaert et al., 2014).

With the substitution rates estimated in the previous analyses, we reconstructed a species network under the multispecies network coalescent model (MSNC) (Yu et al., 2014), which takes reticulations as hybridization and introgression events into account. This analysis was conducted using the SpeciesNetwork package (Zhang et al., 2017) within BEAST 2.5. After several pilot tests, we adopted the JC96 substitution model and the strict clock for all fragments, as this was the only way to achieve convergence among the independent runs and proper mixing of the estimated parameters. We ran two distinct analyses with 2.5×10^8 generations and thinning of 2.5×10^4 each. The stationarity and convergence were verified as previously described. To visualize the multispecies phylogenetic network, we utilized the IcyTree program (Vaughan, 2017) and Dendroscope 3 (Huson & Scornavacca, 2012).

Results

We obtained a total of 2,768 bp high-quality sequences for nuDNA (APOB = 644 bp, APP = 642 bp, BMI1 = 401 bp, CREM = 767 bp, and RAG1 = 767 bp) and 410 bp for mtDNA. All diagnostic haplotypes were exclusive to each parental species (Fig. S2). On the other hand, all of these diagnostic haplotypes were found to cooccur in individuals within the putative hybrid zone. Additionally, we discovered eleven new *T. inunguis* mtDNA haplotypes (Q5, Q6, Q7, Q7a, S4, S7, T17, U3, V3, V4, and W2) (Fig. S1). Fourteen

out of fifteen individuals from the hybrid zone showed signs of introgression (Table S2). The locations of the samples and the proportions of haplotypes belonging to each species are shown in Fig. 1.

As seen in STRUCTURE, admixed individuals were found only in the hybrid zone (Fig. 2A, Table S2), with the average proportions of individual admixtures in this area being 20.3% and 79.7% of *T. inunguis* and *T. manatus*, respectively. The results of the factorial correspondence analysis (FCA) revealed separate clustering between the *T. inunguis* and *T. manatus* populations. On the other hand, individuals from the hybrid zone were graphically distributed between the two species clusters (Fig. 2B), confirming

the STRUCTURE results. Finally, the neighbour net also showed a clear separation between *T. inunguis* and *T. manatus*, with most hybrids in the torso of the network (Fig. 2C). Eight hybrids formed a new group and were more similar among them than to the parental species. Two putative hybrids (from Amapá, Brazil, and French Guiana) were grouped with *T. manatus*, likely due to a large amount of missing data, and other hybrids were grouped with *T. inunguis* (Fig. 2C and Table S2). In addition, it was possible to observe a subdivision within *T. manatus*, which formed two population groups equivalent to the two known ESUs (Fig. 2C), with some hybrids closer to the Brazilian (Atlantic) ESU (Ellipse 1).
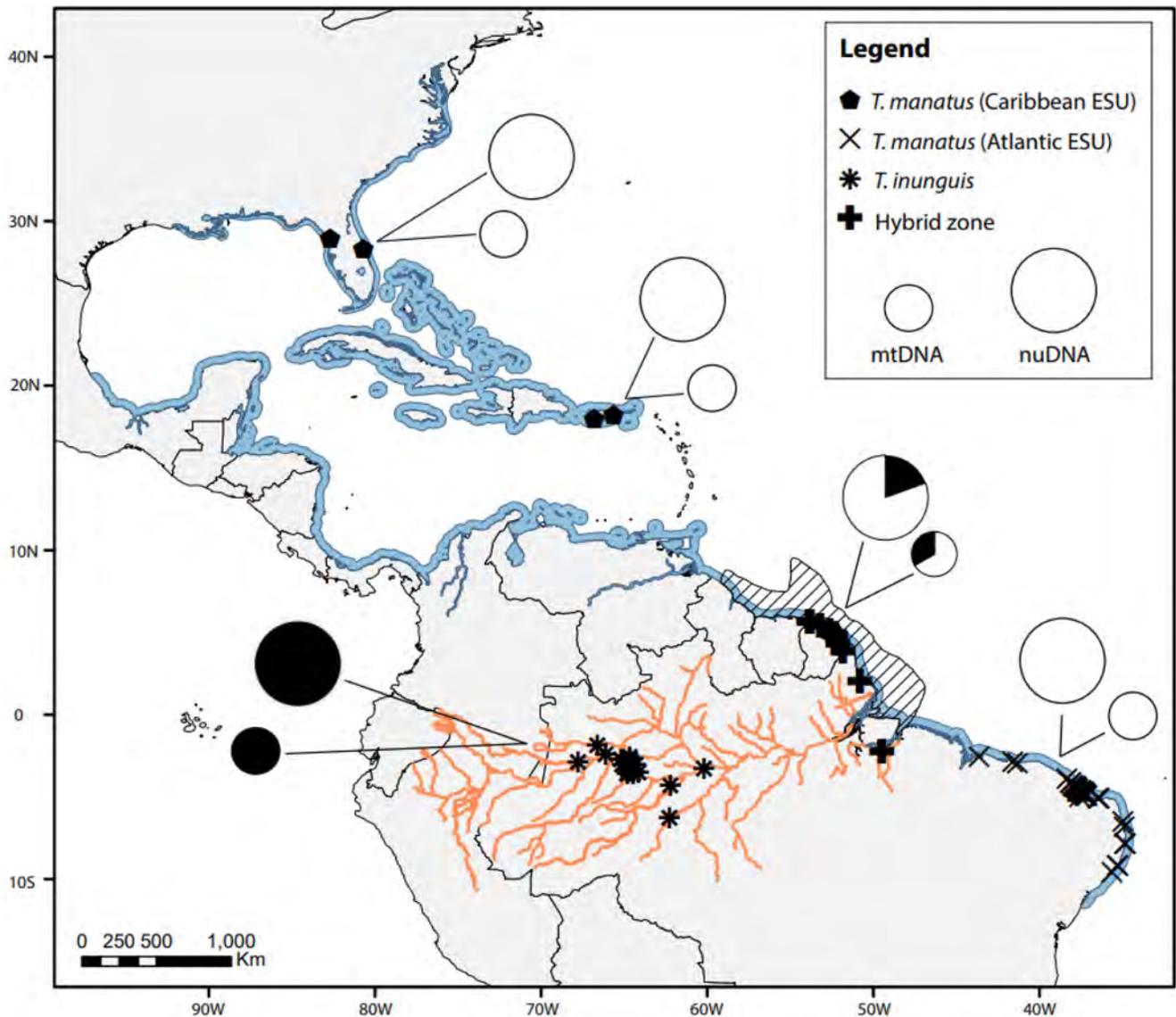
Fig. 1 The geographic ranges of *Trichechus manatus* (blue) and *Trichechus inunguis* (orange) according to the International Union for Conservation of Nature (IUCN) Red List of Threatened Species (version 2023-1) and hybrid zone delimitation (striped) according to known hybrid locations and the Amazon River plume visualized in Google Earth (Dec 2015). The pie chart represents the proportion of diagnostic haplotypes (mitochondrial and nuclear) of each species found in our sample locations
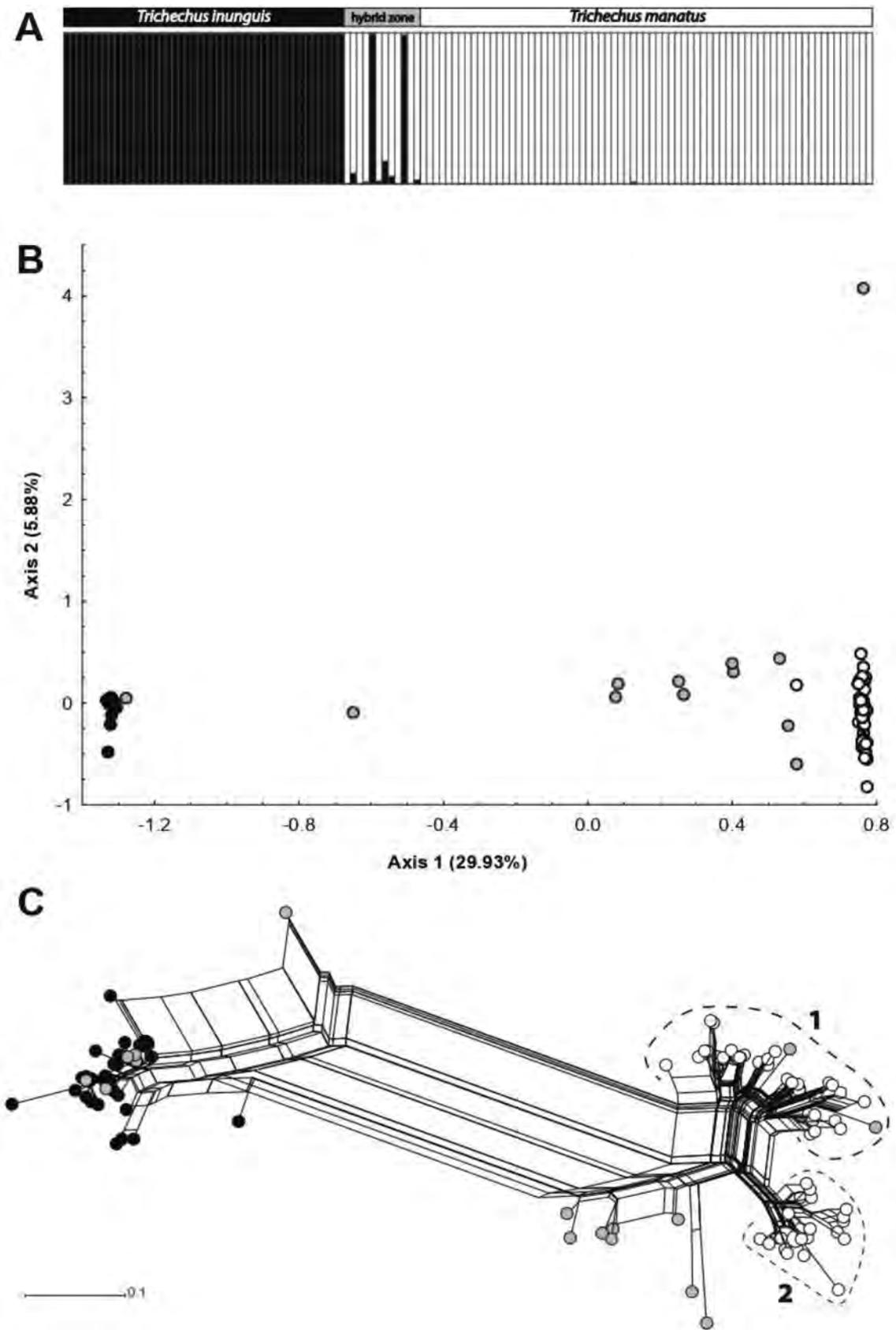
Fig. 2 Interindividual analyses of American manatees. **A** Q-plot showing the genome composition (admixture) of each individual from the hybrid zone and both parental species. **B** Factorial correspondence analysis (FCA) based on five nuDNA haplotypes showing clustering between *T. inunguis* (black) and *T. manatus* (white). Samples of the hybrid zone (gray) occupied intermediate positions. **C** Neighbor-net based on genpofad distances. Ellipse 1 highlights the samples of *T. manatus* from Brazil (ESU1), while Ellipse 2 includes samples from Florida and Puerto Rico (ESU2). Some hybrid individuals were grouped with "pure" individuals likely due to missing data

A species tree (Fig. 3) recovered almost all the nodes fully supported (PP = 1). Nevertheless, the relationship between the *Trichechus* branches was weakly supported (PP = 0.4237). The mean length of the most recent common ancestor (TMRCA) of *H. gigas* and *D. dugon* was 32.18 MYA, while the mean TMRCA of the Sirenian ancestor was 36.50 MYA. Our results recovered the TMRCAs of *T. inunguis* and *T. manatus* in the late Miocene, known as the Messinian Age of the Neogene (6.26 MYA). The time of the ancestor of all trichechids was estimated to be 7.48 MYA (late Miocene), at the end of the Tortonian Age of the Neogene. The intraspecific lineages (ESUs 1 and 2) of *T. manatus* share a recent ancestor dated to approximately 0.26 MYA in the late Pleistocene. The substitution rates for each fragment were as follows (mean ± standard deviation): APOB ($1.766 \times 10^{-4} \pm 1.7473 \times 10^{-5}$), APP ($2.105 \times 10^{-4} \pm 5.3075 \times 10^{-5}$), BMI1 ($2.821 \times 10^{-4} \pm 7.9405 \times 10^{-5}$), CREM ($2.141 \times 10^{-4} \pm 5.8317 \times 10^{-5}$), CR ($1.064 \times 10^{-2} \pm 3.2226 \times 10^{-3}$), and RAG1 ($3.2548 \times 10^{-4} \pm 6.8261 \times 10^{-5}$).

Finally, the SpeciesNetwork analysis (Fig. 4) revealed that both *T. inunguis* and the two lineages of *T. manatus* (ESUs 1 and 2) contributed to the formation of the hybrid zone, with a greater probability of inheritance from the Amazonian manatee (γ = 0.583) than from *T. manatus* (γ = 0.296), in addition to being older. Furthermore, the MSNC analysis indicated an ancient gene flow event between both lineages of *T. manatus* (γ = 0.512).

Discussion

Few algorithms have been developed to characterize F1 and F2 hybrids and further generations resulting from backcrossing (Anderson, 2003; Gompert & Buerkle, 2010). However, these analyses do not consider additional genomic information, leading to limitations in cases of long-term introgression and heightened sensitivity to missing data. As evident in our results, employing at least two integrative methods for identification, such as geography and DNA analyses (both mitochondrial and nuclear), proved crucial for effective diagnosis of hybrids. For instance, some hybrids with missing data were identified by displaying diagnostic haplotypes from one species inhabiting a typical environment of

another. Despite the increasing number of genomic tools and the application of high-throughput sequencing for hybrid identification (Vilaça et al., 2019), our Sanger sequencing of six loci has been shown to be reliable, cost effective, and applicable to both population and interindividual analyses for diagnosing the genome composition of hybrids, assessing population structure, and estimating gene flow. This information can be valuable in conservation planning and monitoring for both American manatee species, as species-specific Sanger markers may be accessible in laboratories with limited resources.

The genetic admixture found exclusively in individuals from the hybrid zone likely results from backcrossing events between hybrids in this region and West Indian manatees from the eastern (Brazil) and western (Venezuela) coastlines. The overall results also indicate that *T. inunguis* and both *T. manatus* ESUs contributed genes to the hybrids, which is also supported by mtDNA evidence (Lima et al., 2021). Even though our nuDNA data are inconclusive, a greater contribution of Brazilian *T. manatus* genes to the hybrid zone is expected because the primary contact zone of both species is in the Amazon River mouth, where the North Brazil Atlantic Current drives the Amazon River plume westwards (Lima et al., 2019). The establishment of a putatively locally adapted hybrid population on the Guianas coastline influenced by the Amazon River plume (Vilaça et al., 2019) may have reinforced the barrier between the Atlantic and Caribbean ESUs of *T. manatus* (Vianna et al., 2006; Santos et al., 2016; Lima et al., 2019, 2021), leading to relative isolation and genetic, chromosomal and morphological differentiation of Brazilian *T. manatus* ESU/population (Barros et al., 2017; Santos et al., 2016). In addition to the hybrid zone barrier, the Amazon River plume flows along the Atlantic coastline of the Guianas Shield and serves as a well-known biogeographic barrier for many marine organisms, promoting genetic (and taxonomic) differences between animal communities from the Caribbean Sea and Northeast Brazil continental shelves (Luiz et al., 2012; Spalding et al., 2007; Tosetto et al., 2022). This region experiences changes in salinity and sediments that affect sunlight incidence and form a physical obstacle due to the westwards water flow of the Amazon River plume, guided by the North Atlantic Brazil current (Geyer et al., 1996). Therefore, as suggested by Vilaça et al. (2019), manatees from the hybrid zone may possess some local adaptive advantages compared to both parental species. This apparently stable hybrid population appears to be working as a barrier to manatees of the Caribbean and Atlantic *T. manatus* ESUs. Interestingly, most of the maternal lineages (mtDNAs) of individuals from the hybrid zone originated from *T. inunguis*, indicating that the majority of the initial crossings generating F1 hybrids (not yet observed) involved male West Indian and
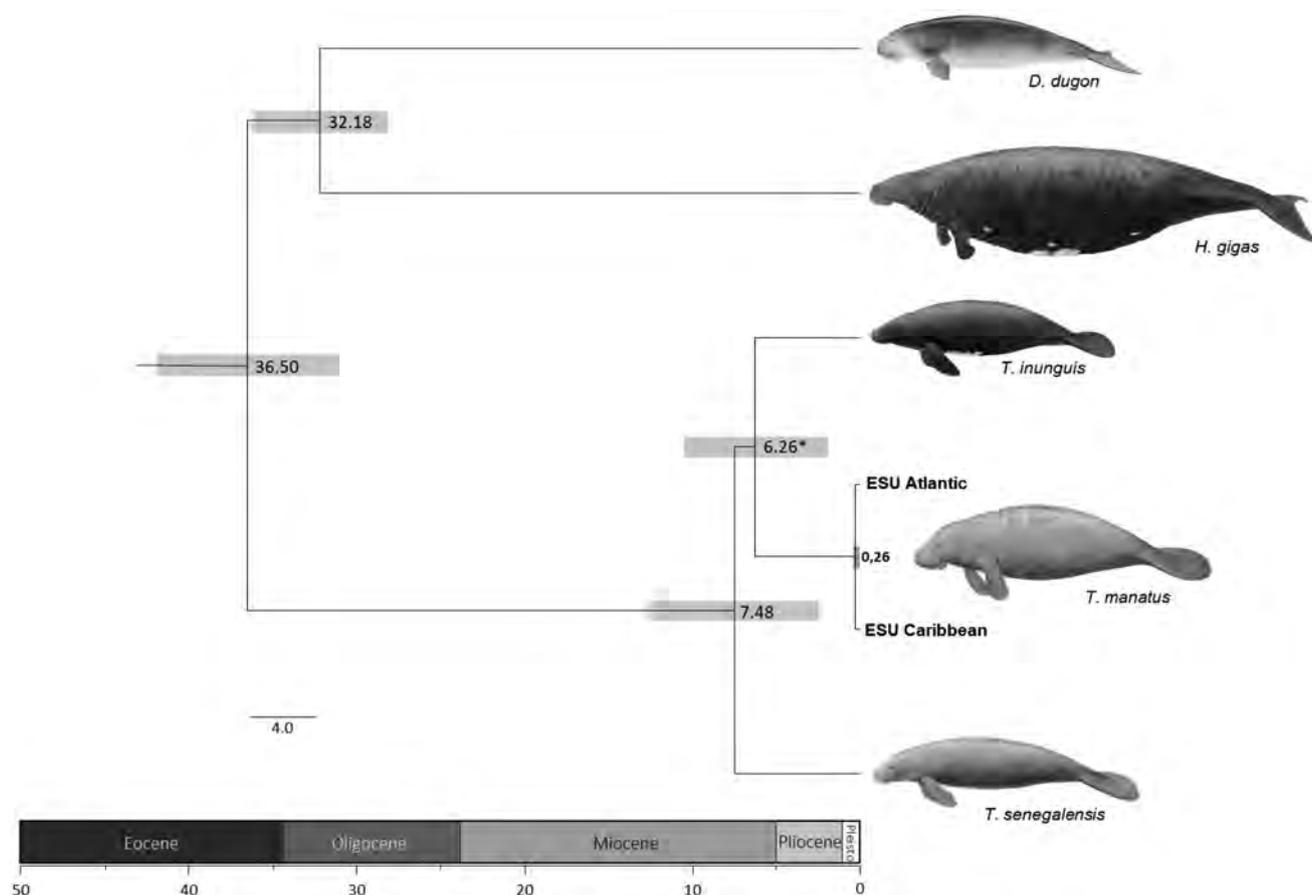
Fig. 3 Bayesian phylogeny of species in the order Sirenia using calibration by fossils and mtDNA (CR) and five nuDNA loci (APOB, APP, BMI1, CREM, and RAG1). The node dates refer to millions of years ago (MYA), and the gray bar on nodes represents the 95% confidence interval of the dating. All nodes have a posterior probability (PP) equal to 1, except for the node signalized with an asterisk (0.4237). This analysis excluded individuals from the interspecific hybridization zone

female Amazonian manatees. According to the hypothesis that females are usually the limiting resource sex (Futuyma & Kirkpatrick, 2017), we would expect *T. inunguis* to be more abundant locally (in the Amazon Estuary) than *T. manatus*. Notably, if mammalian hybrid F1 males are often sterile, as expected by Haldane's rule (Haldane, 1922), then only F1 hybrid females could be capable of generating off-spring, spreading *T. inunguis* mtDNA haplotypes into the hybrid zone.

The historical population dynamics of West Indian mana-tees were likely influenced by climate change and sea level fluctuations (Edwards, 2013). Our multilocus analysis with fossil calibration provided reliable dating for the divergence of both evolutionary populations (ESUs) of *T. manatus* in the late Pleistocene. A low genomic contribution from *T. inunguis* was estimated (~20%), and no F1 hybrids have been identified thus far. This may indicate that the hybrid-ization process was ancient and possibly cyclical, likely related to Amazon estuary dynamics. The Amazon River basin reached its current state approximately 2.5 MYA,

and before that, the amount and force of water flow were lower (Hoorn et al., 2010), which could have facilitated the entrance of manatees into the Amazon River. In our spe-cies tree, only the node between *T. inunguis* and *T. manatus* showed low support, likely due to the absence of nuclear sequences of *T. senegalensis*. This phylogenetic uncertainty has also been found in other studies. For example, Santos et al. (2016) found a distinct topology using only the mtDNA Cyt-b fragment, with *T. manatus* being more related to *T. senegalensis* than to *T. inunguis*, a topology that was recov-ered with good support using morphological data (Springer et al., 2015). However, these findings contrast with those of a recent complete mtDNA phylogeny in which *T. manatus* and *T. inunguis* were classified as sister groups (Souza et al., 2021), which is similar to our tree (Fig. 4). Therefore, we conclude that this phylogeny requires further investiga-tion using additional independent markers, especially for *T. senegalensis*. However, we believe that our dated phyloge-netic tree, which utilizes genome-scale markers and more calibration points than those used by other studies, should
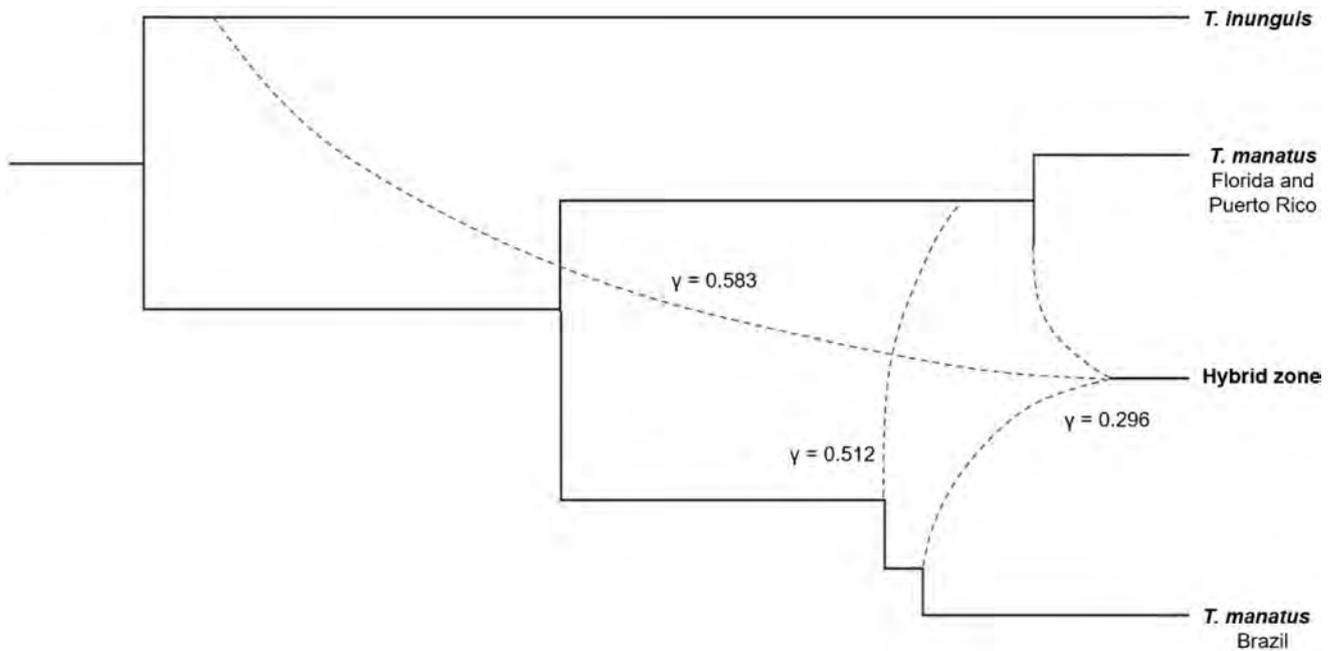
Fig. 4 The best-supported species network (PP = 87.27%). The numbers in dotted reticulations indicate inheritance probabilities of the hybridization event

be more accurate, at least considering the topology. On the other hand, Springer et al. (2015) showed more ancient divergence times than our dated phylogeny. This discrepancy could be attributed to the distinct sampling methods performed by these authors, which included many Afrotheria outgroups, whereas our tree used only Sirenians.

In a recent study, Heritage and Seiffert (2022) combined morphological, molecular, temporal, and geographic data and applied Bayesian phylogenetic methods to elucidate the evolution of sirenians. Although they found that the Dugongidae-Trichechidae split might have occurred approximately 33.9 MYA during the Eocene–Oligocene, similar to our analysis, they estimated a division of crown Trichechidae in the Pliocene (~3.3 MYA), the same divergence date for *T. inunguis* and *T. manatus* (~1.3 MYA) was found by Souza et al. (2021). Additionally, according to their results, *T. senegalensis* was also recovered as the most basal lineage of the tree. Rull (2011) demonstrated that the sampling design can affect dating and resolution at different depths within a tree. Deep lineage sampling (i.e., crown dating) results in more ancient nodes, while shallow sampling (i.e., species dating) yields more recent nodes (Rull, 2011). Species dating, as in our study, is more suitable for investigating recent divergence events (Rull, 2011). Nevertheless, it is important to note that our HPD intervals included the means obtained by Springer et al. (2015).

Despite the long-term divergence between Amazonian and West Indian manatees, dating back from the late Miocene to the middle Pleistocene (this study, Souza et

al., 2021, Heritage & Seiffert, 2022), all analyses suggest introgression in the hybrid zone, with individuals exhibiting an admixed genome between *T. manatus* and *T. inunguis*. SpeciesNetwork analysis (Fig. 4) indicated that the *T. inunguis* contribution was greater than that of the two *T. manatus* lineages combined, which was unexpected given that most hybrids are found in the Guianas coastline and are usually morphologically identified as West Indian manatees (Vilaça et al., 2019; Meirelles et al., 2022). However, most of these records consist of carcasses of deceased animals, often in an advanced state of decomposition. In these cases, external features such as spots, fur, and nails can be lost; thus, these identifications should be appreciated with caution (Benoit de Thoisy, pers. obs.). Several factors should be considered in the interpretation of these results. First, the absence of samples from the Amazon River close to the mouth (e.g., Pará/Marajó and Amapá) may have contributed to the ancient contribution of *T. inunguis* to the hybrid zone. This is because the unsampled region could contain individuals with genomes more closely related to *T. inunguis*. Additionally, the connections between the branches of Brazil and Florida + Puerto Rico may also represent a ghost lineage, likely from the Caribbean/South American region. Furthermore, our results may be influenced by methodological limitations. Given that MSNC analyses are highly parametric (Yu et al., 2014) and that we had a limited number of independent markers, we had to simplify the model to estimate the species network, such as by adopting less-parametric nucleotide substitution and molecular clock models.

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It is well known that these simpler models reduce variance in phylogenetic reconstructions at the expense of increased biases (Burnham & Anderson, 2002; Kelchner & Thomas, 2007). Therefore, we advise interpreting the estimated parameters with caution. Additionally, the use of a larger number of independent genomic markers should provide a more robust understanding of the genomic contribution of each parental species to the formation of the hybrid zone. Nonetheless, the results indicate that both *T. inunguis* and Brazilian and Caribbean *T. manatus* (ESUs) have contributed to the formation of the hybrid zone occupying approximately 1,000 km along the Guianas coastline (Lima et al., 2019).

By analysing only West Indian manatees, we were able to distinguish two groups (Fig. 3C), corresponding to the two geographically separated ESUs proposed by Vianna et al. (2006) and confirmed by Lima et al. (2019, 2021). The first ESU (Caribbean) comprises populations from Venezuela, Colombia, Central America, Antilles, Florida, and the Gulf of Mexico (Mexico-USA), and the second ESU (Atlantic) consists of individuals from the northeastern Brazilian coast only, separated by a long hybrid zone in the Guianas coastline. Despite this difference, it is important to emphasize that the divergence between the two *T. manatus* lineages was recent, with no autosomal diagnostic variation found between them, and that *T. manatus* alleles contributed more to the present-day genetic diversity of the hybrid zone than did those of *T. inunguis*. It is important to emphasize that the nuclear loci were initially selected for the identification of interspecific genetic differences to identify hybrids (a likely ascertainment bias), as they showed little intraspecific variation. Once again, the inclusion of more variable and genomic-scale markers may aid in a more accurate reconstruction of the hybrid zone's formation history, with emphasis on the differentiation of both *T. manatus* ESUs.

This study sheds light on the formation of an interspecific hybrid zone through secondary contact between two divergent manatee species, warranting special attention, as conservation efforts for hybrids are still limited (Dong et al., 2003; Fitzpatrick et al., 2015; Grobler et al., 2011). The origin of the hybrid zone appears to be "natural", ancient, and complex, serving as a relative genetic barrier between neighbouring populations. However, it is not possible to disregard the potential role of the decline in manatee populations caused mainly by hunting and habitat loss, which could promote interspecific hybrid formation (Pinto et al., 2016). Nevertheless, the interspecific hybridization zone appears to result from evolutionary and adaptive responses to such a unique environment (Lima et al., 2019), where conservation efforts are needed, equivalent to those for nonhybrid threatened populations (Allendorf et al., 2001). Since the populations of both *T. manatus* ESUs were separated by

at least 0.25 MYA, our results support the hypothesis that this 1,000 km-long hybrid zone functions as a genetic sink where diversity is eroded, restricting gene flow between the Atlantic (Brazil) and Caribbean (all others) *T. manatus* populations. This further emphasizes the isolation and differentiation of Brazilian manatees, which deserve a special conservation status as an endemic and threatened population, as recently suggested (Meirelles et al., 2022).

Declarations

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