

Article

Molecular Identification and Historic Demography of the Marine Tucuxi (*Sotalia guianensis*) at the Amazon River's Mouth by Means of Mitochondrial Control Region Gene Sequences and Implications for Conservation

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Abstract: In 2005, three fishermen, with artisan fishing vessels and drift gillnets, accidentally captured around 200 dolphins between Vigia and Salinópolis in the Amazon River estuary. The dolphins died and they then prepared their vaginas and penises in order to sell them in the Ver-ao-Peso market in the city of Belem within the Brazilian state of Pará. We randomly sampled a minimal quantity of tissue of these sexual organs from 78 of these 200 dolphins and we determined the following results after sequencing 689 base pairs (bp) from the mitochondrial control region gene: (1) 96.15% (75/78) of these dolphins belonged to the species *Sotalia guianensis*. The other species detected were *Steno brenadensis*, *Stenella coeruleoalba* and *Tursiops truncatus*; (2) The levels of gene diversity found in this sample of *S. guianensis* were high (33 haplotypes, haplotype diversity of 0.917 and nucleotide diversity of 0.0045) compared to gene diversities found in other Brazilian *S. guianensis* locations; (3) All the population genetics methods employed indicated a clear population expansion in this population. This population expansion could have begun 400,000 years ago; (4) The haplotype divergence within this population could have begun around 2.1

millions of years ago (MYA), with posterior splits around 2.0–1.8 MYA, 1.7–1.8 MYA, 1–1.5 MYA, 0.6–0.8 MYA, 0.4–0.2 MYA and 0.16–0.02 MYA, all during the Pleistocene.

Keywords: *Sotalia guianensis*; *Sotalia fluviatilis*; *Steno brenadensis*; Ver-ao-Peso market; mitochondrial control region gene; Amazon River mouth; population expansion; Pleistocene

1. Introduction

Sotalia guianensis is a small marine dolphin (with many local names such as boto-cinza or tucuxi in Brazil, tonina in Venezuela and Colombia, and lam in Nicaragua), with a seemingly continuous distribution from Honduras to the state of Santa Catarina, in southern Brazil [1–3]. Its distribution might be limited from extending southwards by low water surface temperatures [1].

Until recently, the separation of the marine form of *Sotalia* from the Amazon riverine one has been a subject of controversy. Historically, five different species of *Sotalia* have been described. In the late 1800s, three riverine specimens and two coastal specimens had been defined. The first riverine species was described by Gervais in 1853 as *Delphinus fluviatilis*, from a specimen collected in the Peruvian Amazon close to Pebas in the Amazon River. Gray placed this individual in the genus *Sotalia* in 1866 [4]. In 1855, Gervais described *Delphinus pallidus* from a specimen collected near Nauta in the Peruvian Marañón River. Gray described a third riverine species in 1856, originally named *Steno tucuxi*, from a specimen collected in the Brazilian Amazon near Santarem [5]. These specimens were later classified in the genus *Sotalia* by Flower [6].

In 1864, Van Bénédén described one coastal species, *Delphinus guianensis*, based on three specimens collected from the mouth of the Marowijna River in the border between Surinam and French Guiana [7]. This species was also reclassified by Gray as a member of the genus *Sotalia*. Van Bénédén described a second coastal species in 1857, from a specimen collected in Guanabara Bay, Brazil. This species was designated as *Sotalia brasiliensis*.

Cabrera [8] reduced the number of species to two, one riverine, *Sotalia fluviatilis*, and one coastal, *Sotalia guianensis*. Later, Mitchell [9] and Leatherwood and Reeves [10] argued that the differences between *S. fluviatilis* and *S. guianensis* were too subtle and attributable to phenotypic variability, and that *Sotalia* should be regarded as monotypic. This view was reinforced by the morphometric study of Borobia [11], which concluded that differences between marine and riverine *Sotalia* were mainly a consequence of size variation, and concluded that they should be considered a single species, without subspecific differentiation. Overall, coastal specimens tended to have larger skulls than riverine specimens, as well as larger body sizes. Since then, most authors adopted the name of *S. fluviatilis* but acknowledging marine and riverine populations as different ecotypes [1,3,12,13]. Other researchers preferred to distinguish the two *Sotalia* forms using the subspecific denomination *S. fluviatilis fluviatilis* and *S. fluviatilis guianensis*. At the end of the 20th century and in the beginning of the 21st century, new results indicated the existence of two *Sotalia* species. The first sign that the lumping of *Sotalia* species should be reassessed was given by Furtado-Neto [14]. A phylogenetic analysis of mitochondrial cytochrome b sequences showed that marine and riverine *Sotalia* were different, but that

result needed further confirmation, since only a single riverine sample was analyzed. Later, a tridimensional morphometric analysis of skull shape of 92 coastal and 13 riverine specimens showed significant differences between riverine and coastal specimens of *Sotalia* suggesting both forms as separate species again [15]. However, the first conclusive genetic work in favor of two species (*S. fluviatilis* and *S. guianensis*) was that of Cunha *et al.* [16]. These authors clarified the taxonomic status of *Sotalia* dolphins using sequences of the mitochondrial control region and the cytochrome b of 56 samples (12 riverine and 44 marine exemplars). This was the first study to include samples of the Amazon estuary in analyses of differentiation between *Sotalia* ecotypes. Later, Caballero *et al.* [17] confirmed the discovery of Cunha *et al.* [16]. They analyzed sequences from introns of three nuclear genes (lactalbumin, actin and glucocerebrosidase) and another mitochondrial marker (ND2) including South America and Caribbean samples, giving support to the conclusion of Cunha *et al.* [16] and confirming the specific status of *S. guianensis* and *S. fluviatilis*.

S. guianensis is listed in the Appendix I of the Convention in International Trade of Endangered Species (CITES) and as Data Deficient (2008) by the World Conservation Union (IUCN). Although this is mainly a marine species, some records have been obtained in freshwater systems. For instance, there are some records of *Sotalia* dolphins in the Orinoco River, up to 800 km inland, as well as some disputed reports in the Upper Orinoco [1,18]. Those sightings may be attributed to marine *Sotalia*, since it inhabits bays and estuaries and is frequently seen entering rivers along the South American coast [3].

Only a few genetics studies have been undertaken for *S. guianensis* trying to elucidate its genetic structure across the Atlantic Ocean coasts of Central and South America. Cunha *et al.* [16,19] and Cunha [20] showed, by using mtDNA control region sequences, evidence for at least six MUs (Management Units) in Brazil, specifically the areas of Pará, Ceará, Rio Grande do Norte, Bahia, Espírito Santo and the South-Southeastern area (from Rio de Janeiro to Santa Catarina states). Those MUs were highly differentiated ($\Phi_{CT} = 0.485$, $p < 10^{-5}$), indicating severe restrictions to gene flow among them. An interesting finding was a lack of variation in the control region of dolphins from South-Southeastern Brazil (between parallels 22° and 25° S, extending 900 km). Genetic diversity patterns suggest that this homogeneity might have been caused by a recent colonization of the Brazilian coast through a range extension from north to south, which could be linked to a warming up of the Western Atlantic during the Holocene. Thus, the observed homogeneity is probably not due to gene flow within the region, but a consequence of recent foundation [20,21]. Another more recent work in Brazil is that of Hollatz *et al.* [22]. They examined the fine scale population structure for the largest populations of *S. guianensis* inhabiting Sepetiba and Paraty embayments at the southeastern coast of Rio de Janeiro. The mtDNA control region sequences failed to detect variability among sequences. Conversely, evidence of significant male population structure was found on the basis of ten nuclear microsatellite loci. Surprisingly, the microsatellite markers were able to distinguish between individuals from the two embayments located 60 km apart. They suggested that differences in habitat type and behavioral specializations are likely to explain the patterns of genetic structure.

Populations of *S. guianensis* from the northern part of South America and the Caribbean were analyzed by Caballero *et al.* [23], who proposed two MU for that area, one for Central American, Colombian and Venezuelan coasts, and another for Guyana, Surinam and French Guiana. These authors claimed that dolphins from Maracaibo Lake, despite being included in the first MU, had some unique mitochondrial haplotypes and their genetic distinctiveness should be further investigated.

However, only three individuals from southern Maracaibo were analyzed: the others were from the northern portion of the lake, where it opens to the Gulf of Venezuela.

Taking all of this into account, the current study analyzed and discussed the following aims: (1) We obtained 78 samples of dolphins killed by fishermen at the Amazon mouth in 2005. The samples consisted of small pieces of penis and vagina, which the fishermen sell to self-declared witches at the Ver-o-peso at the Belem market. Since the rest of the bodies had been discarded by the fishermen, no physical identification of these dolphins was possible. Therefore, our first aim was to identify by means of mtDNA control region sequences which dolphins species were killed by the fishermen and if they were mainly from *S. guianensis* as we suspected; (2) Some authors have claimed that there is uncertainty in the evidence for the overlap in the distribution (sympatry) of *S. fluviatilis* and *S. guianensis* in the mouth of the Amazon River [16]. They asked for surveys and additional collection of genetic samples in order to clarify boundaries in the distribution of each species in this region. Thus, this study represented a good chance to detect if animals of both species were presented in the animals captured by these fishermen in the Amazon River mouth; (3) A large fraction of the samples analyzed were effectively from *S. guianensis* captured in a determined transect in the Amazon River delta. Thus, we determined the levels of gene diversity for mtDNA of *S. guianensis* at the Amazon mouth and we compared this observed gene diversity with other *S. guianensis* populations analyzed in other areas of the Brazilian coast; (4) We also wanted to determine if the population of *S. guianensis* in the Amazon River estuary had crossed bottlenecks or population expansions during its history and eventually to also determine when these historical changes occurred; (5) To determine by means of a Bayesian analysis and Median Joining Network when the divergence splits occurred among the different haplotype lineages found for *S. guianensis* at the mouth of the Amazon River and to correlate these possible splits with climatic changes during the Pleistocene.

2. Material and Methods

2.1. Samples and Molecular Procedures

In 2005, three different fishermen with drift-gillnets and artisan fishing vessels accidentally captured around 200 dolphins at the Amazon River estuary in different points between Vigia and Salinópolis. Because the dolphins died, they meticulously removed their sexual organs and brought them to the Ver-ao-Peso market in the city of Belem within the Brazilian state of Pará. This particular market is also used by self-declared witches who sell reproductive organs of dolphins as love charms. These three fishermen are the main suppliers of dolphin tissues of the witches in that market, which in turn is the most important market in Brazil in that type of products. We randomly sampled 78 of these around 200 penises and vaginas (the 78 samples in the best conditions to extract DNA). Witches voluntarily gave us small samples of these sexual organs without any financial retribution. We introduced three additional samples from *S. fluviatilis* for molecular comparisons. One of these samples was obtained from the Curaray River (an affluent of the Napo River) in the Peruvian Amazon (captured animal). Another sample was obtained from Patrulleros, near Puerto Nariño in the Colombian Amazon on the Amazon River (dead animal). We obtained the third sample from the Lago do Janauari near Manaus in the Negro River in central Brazilian Amazon (dead animal). Thus, 81

samples were sequenced for the mtDNA control region. The sequences of these samples were deposited in the Genbank (accession numbers: Bankit 1652621 to 1652702).

DNA extraction was performed by the phenol-chloroform method [24] from small muscle pieces. For the analysis of the mitochondrial control region gene and some fraction of the tRNA gene (689 bp), we used the primers H16498 and TRO [25]. The sequences of these primers are 5' CCT GAA GTA AGA ACC AGA TG3' for H16498 and 5' CCT CCC TAA GAC TCA AGG 3' for TRO. The PCR reactions were undertaken in a final 25 μ L volume with the following conditions: 4 μ L of 10 $\times$ buffer, 6 μ L of 3 mM MgCl₂, 2 μ L of 1 mM dNTPs, 2 μ L (8 pmol) of each primer, 1 units of Taq DNA polymerase, 6 μ L of H₂O and 2 μ L (20–50 ng/ μ L) of DNA. The amplifications were carried out in a BioRad thermocycler with the following protocol: 95 °C for 5 min, 30 cycles at 95 °C for 45 s, at 52 °C for 45 s and at 72 °C for 45 s and at 72 °C for 10 min for final extension. All amplifications, including positive and negative controls, were checked in 2% agarose gels, using the molecular weight marker ϕ 174 DNA digested with *Hind* III and *Hinf* I. The samples amplified were purified using membrane-binding spin columns (Qiagen). The double-stranded DNA was directly sequenced in a 377A (ABI) automated DNA sequence. The samples were sequenced in both directions using the BigDye TM kit and all the samples were repeated to ensure sequence accuracy. Sequence alignments and editing were performed by using Sequencer 3.0 (Gene Codes Corp.), DNA Alignment (Fluxus Technology Ltd., Suffolk, England) and Clustal X [26].

2.2. Data Analysis

To determine what dolphin species were sampled, we compared the sequences obtained (query sequences) against the Genbank sequences by means of the program BLAST (Basic Local Alignment Search Tool). Also the sequences were contrasted with the sequences obtained by Cunha *et al.* [16] and Caballero *et al.* [17] to determine the correct dolphin species analyzed.

2.2.1. Gene Diversity

We used the number of polymorphic sites (S), the number of haplotypes, the haplotypic diversity (H_d), the nucleotide diversity (π), the average number of nucleotide differences (k) and the θ statistic by sequence to determine genetic diversity in this sample of *S. guianensis*. These statistics were estimated with DNAsp 5.10 software [27].

2.2.2. Demographic Changes

We used the following procedures to determine possible historical population changes for the samples of *S. guianensis* studied by means of the mtDNA control region sequences (it is interesting to compare diverse procedures to determine if all them yielded the patterns of results): (1) The mismatch distribution (pairwise sequence differences) was obtained following the method of Rogers and Harpending [28] and Rogers *et al.* [29]. We compared the curves obtained assuming constant and non-constant sizes to the empirical observed distribution. We used the raggedness rg statistic [30,31] and the R2 statistic of Ramos-Onsins and Rozas [32] to determine the similarity between the observed and the theoretical curves; (2) We used the Fu & Li D* and F* tests [33], the Fu F_s statistic [34] and the Tajima D test [35] to determine possible changes in the *S. guianensis* population studied [32,36];

(3) A Bayesian skyline plot (BSP) was obtained by means of the BEAST v. 1.6.2 and Tracer v1.5 software [37,38]. This Bayesian analysis was performed using a HKY (Hasegawa-Kishino-Yano) model of nucleotide substitution. We used the BEAST v. 1.6.2 program for Bayesian analyses and incorporated estimated base frequencies, a gamma site heterogeneity model, rates varying among sites, four rate categories (because it was determined to be the better model using the Modeltest software) and a relaxed molecular clock with an uncorrelated log-normal rate of distribution [39]. The Coalescent-Bayesian skyline option in the tree priors was selected with four steps and a piecewise-constant skyline model with 10,000,000 generations (the first 1,000,000 discarded as burn-in). In the Tracer v1.5, the marginal densities of temporal splits were analyzed and the Bayesian Skyline reconstruction option was selected for the trees' log file. A stepwise (constant) Bayesian skyline variant was selected with the maximum time as the upper 95% highest posterior densities (HPD) and the trace of the root height as the treeModel.rootHeight. For *S. guianensis*, this analysis was carried out for the last 2.3 million years with a standard deviation of ± 0.5 MYA (3.4–1.4 MYA) for the split between *S. guianensis* and *S. fluviatilis* [40].

2.2.3. Phylogenetic Analyses and Temporal Split Times

The sequence alignments were carried out manually and with the software previously quoted. The Modeltest software [41] was applied to determine the best evolutionary nucleotide model for the analyzed sequences of *S. guianensis* from different nucleotide substitution models. Additionally, we relied on Mega 5.1 software [42] to determine the best model among 24 different evolutionary mutation models.

Several phylogenetic trees were obtained by means of genetic distances (maximum composite likelihood), maximum likelihood and maximum parsimony (with close-neighbor-interchange with search level 1 and random addition with 100 replicates) with the program PAUP* 4.0b8 [43] and MEGA 5.1 to determine the relationships among the *S. guianensis* haplotypes found. In these analyses, the three *S. fluviatilis* sequences and the sequences of *Steno brenadensis*, *Stenella coeruleoalba* and *Tursiops truncatus* were added.

To determine the temporal splits among the different haplotype clades within *S. guianensis*, a Median Joining Network (MJN) [44] was carried out. It was constructed with Network 4.6 software (Fluxus Technology Ltd, Suffolk, England). Additionally, the ρ statistic [45] was estimated and it was transformed into years. The standard deviation of ρ was also calculated [46], which is unbiased and highly independent of past demographic events. The mutation rate employed for this work was one mutation each 145,138 years, which corresponded to 1%/MYA for the mtDNA control region. Furthermore, a star-form of the MJN agrees quite well with a population expansion.

3. Results

Out of the 78 dolphin samples analyzed, 75 (96.15%) were samples corresponding to *S. guianensis*. The three samples, which did not correspond to *S. guianensis*, belonged to *Steno brenadensis* (1.28%), *Stenella coeruleoalba* (1.28%) and *Tursiops truncatus* (1.28%). The BLAST program was employed to determine these species. Although the point where all of these animals were caught corresponded to a freshwater area in the Amazon River's estuary, no *S. fluviatilis* was present. This is a strong indication that the *Sotalia* inhabiting the mouth of the Amazon River is basically *S. guianensis*.

3.1. Gene Diversity in the Population of *S. guianensis* at the Mouth of the Amazon River

Our analysis, of the 689 bp from the mtDNA control region of 75 *S. guianensis* individuals, revealed 29 polymorphic sites, with a total number of 29 mutations, with 15 singleton variable sites and 14 parsimony informative sites. There were no variable sites for three or four variants. We noted 33 haplotypes, a haplotype diversity (H_d) of 0.917 ± 0.020 , a nucleotide diversity (π) of 0.00455 ± 0.00038 , an average number of nucleotide differences (k) of 3.129 ± 1.641 and a θ per sequence of 5.933 ± 1.840 .

3.2. Possible Demographic Changes in the History of the *S. guianensis* Population within the Amazon River Estuary

All the analyses carried out to determine possible historical demographic changes revealed that the *S. guianensis* population analyzed underwent an important population expansion. The mismatch analysis clearly revealed an expansion of this population (Figure 1). The associated statistics revealed that the observed distribution is much more similar to the expected distribution when a population expansion is present than to an expected distribution of a constant population ($rg = 0.016$, $p = 0.02$; $R^2 = 0.053$, $p = 0.064$). The other four statistics employed to determine possible demographic changes also showed demographic expansion for this population ($D^* = -2.822$, $p = 0.016$; $F^* = -2.771$, $p = 0.015$; $F_s = -26.822$, $p = 0.000001$; $D = -1.484$, $p = 0.053$). Median and mean data with the Bayesian skyline plot analysis also indicated a population expansion. With the median (Figure 2a), the population showed a constant size until around 0.3–0.4 MYA, when a strong population expansion began and continued to current times. With the mean (Figure 2b), this population expansion began around 1.25 MYA and its growth was more linear than the explosive growth obtained with the median analysis. It also continued to the present time. Therefore, all the different analyses undertaken showed the same trend: a very important population expansion for the population of *S. guianensis* analyzed independently of the mathematical properties of each analysis employed.

Figure 1. Mismatch distribution (pairwise sequence differences) for the mitochondrial control gene for the *Sotalia guianensis* population analyzed. (A) Assuming a constant population size; (B) Assuming a population expansion. The analysis showed a clear and significant population expansion.

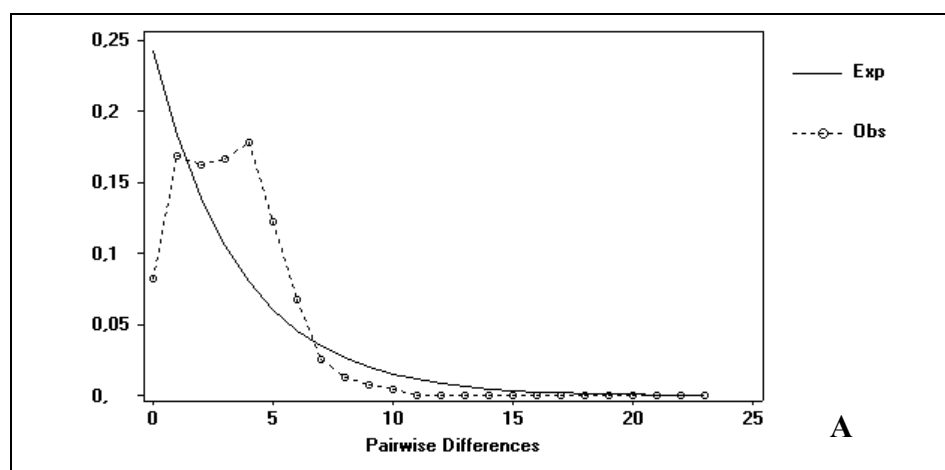


Figure 1. Cont.

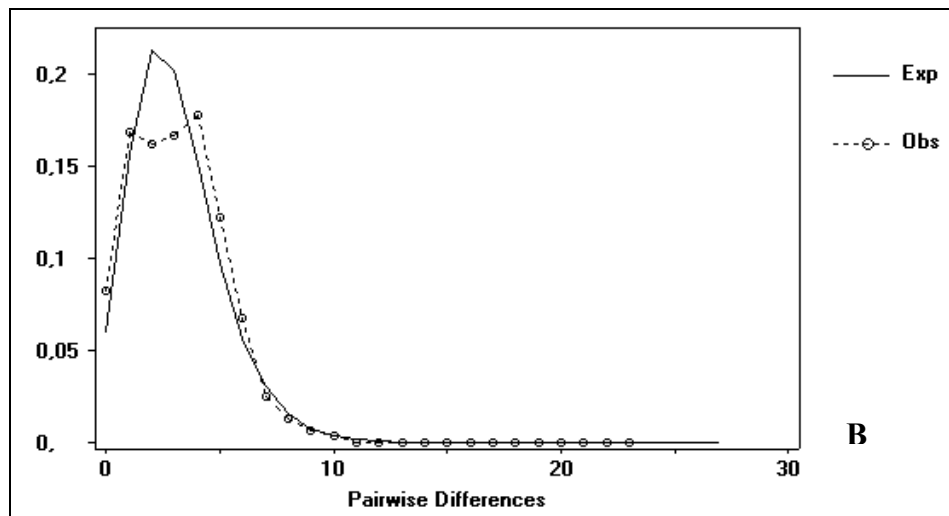


Figure 2. Two Bayesian skyline plot (BSP) analyses applied to the *Sotalia guianensis* population studied. **(A)** With the median; **(B)** With the mean. In both cases, although in different times, population expansion was detected in this population.

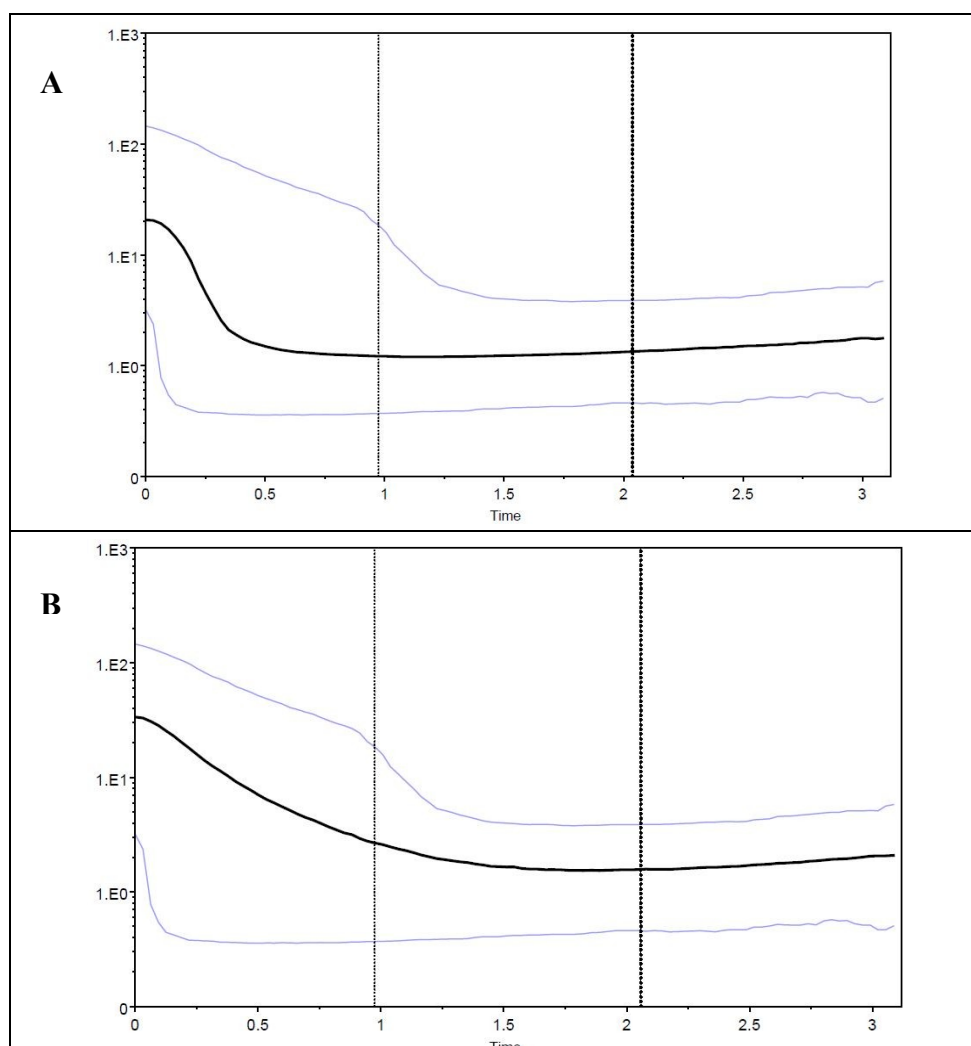
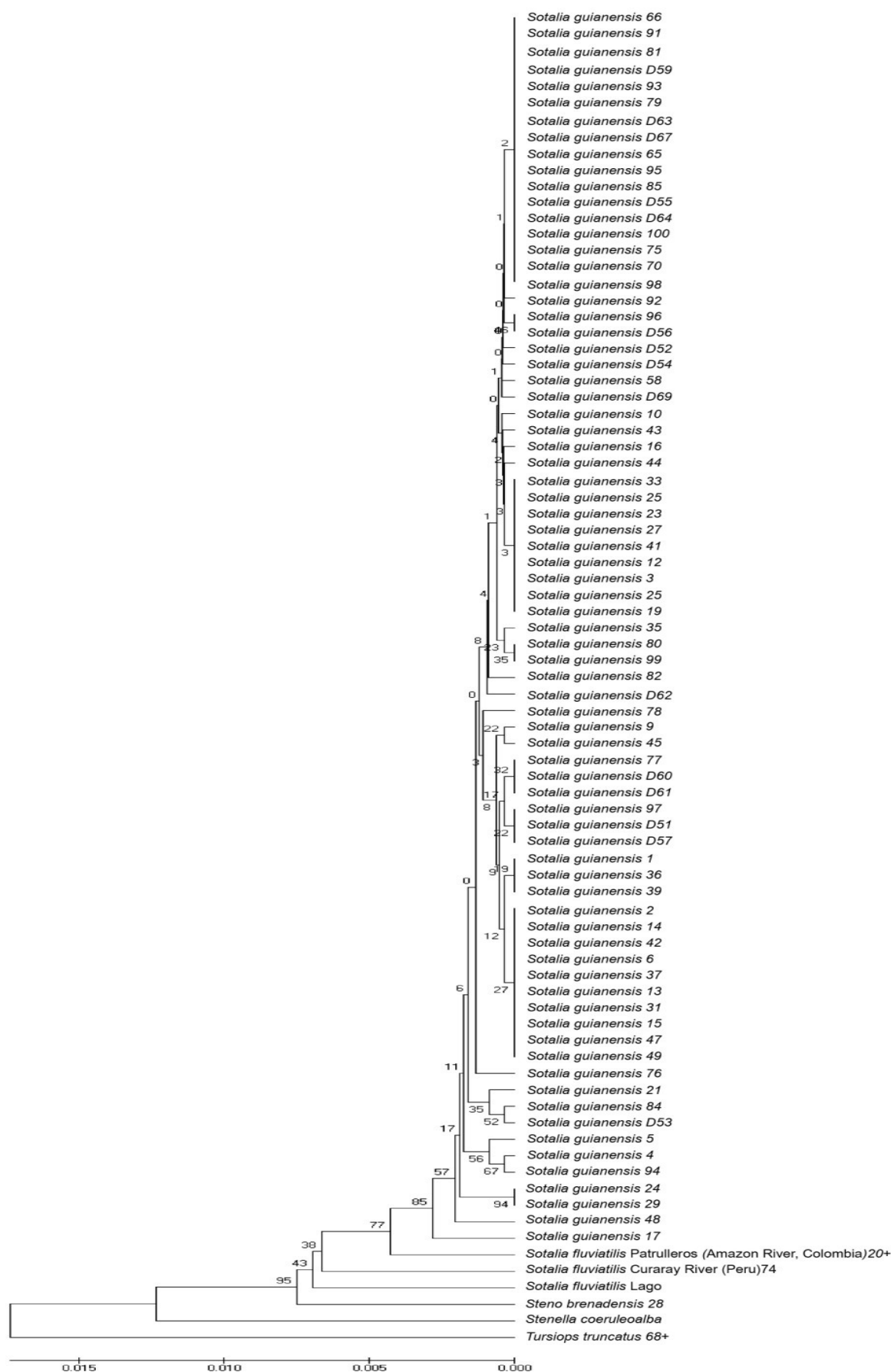


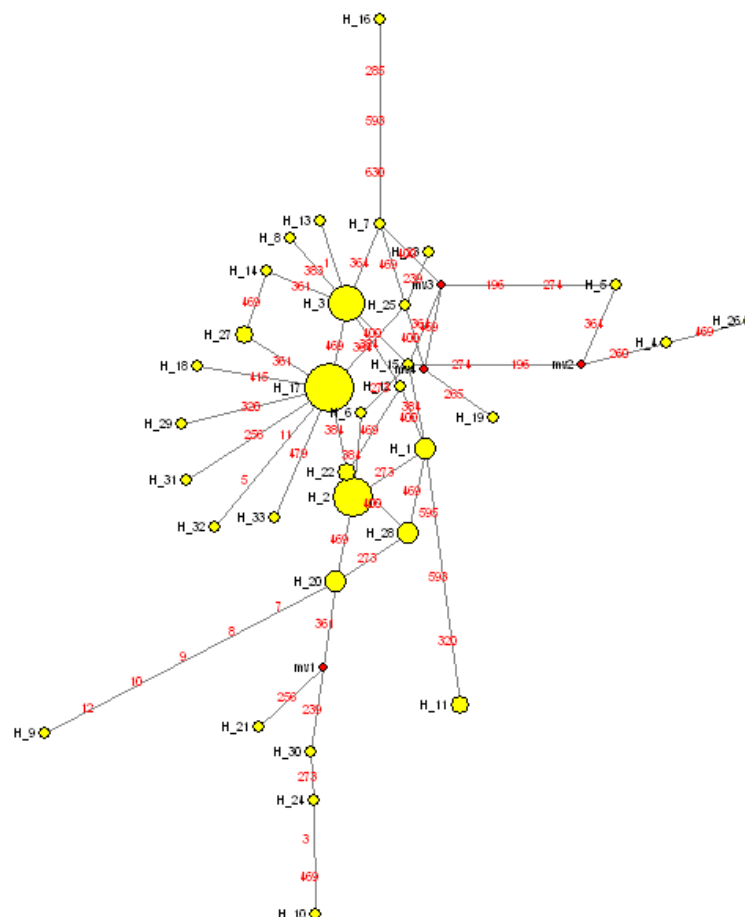
Figure 3. Maximum likelihood tree at the mitochondrial control region gene for *Sotalia guianensis*, *Sotalia fluviatilis*, *Steno brenadensis*, *Stenella coeruleoalba* and *Tursiops truncatus*.



3.3. Phylogenetic Analyses and Temporal Divergence Splits among the mt Haplotype Lineages Found in the *S. guianensis* Population Studied in the Amazon River Mouth

Both the Modeltest and Mega 5.1 programs showed that the best mutation model was HKY+G+I (Hasegawa-Kishino-Yano substitution model with G = gamma distributed rate variation among sites and I = extent of static, unchanging sites in a dataset) (BIC = 4147.677; AIC = 2805.988; LnL = -1243.716). The disparity index test of substitution pattern homogeneity basically showed that all the *S. guianensis* individuals analyzed showed the same pattern of nucleotide substitutions. All the phylogenetic analyses carried out showed the same tree topology, independently of the procedures employed. Herein, we show the tree with the maximum likelihood procedure (Figure 3). The 75 *S. guianensis* analyzed conformed a monophyletic group and clearly differentiable from *S. fluviatilis*. Several aspects are noteworthy to point out. Firstly, although only three samples of *S. fluviatilis* were enclosed in this study, it was clear that the genetic heterogeneity among the individuals of this last species was higher than among the samples of *S. guianensis*. All the *S. guianensis* individuals were coming from a transect of around 100 km (Vigia-Salinópolis), meanwhile the three *S. fluviatilis* exemplars were sampled in a distance around 2,000 km. And secondly, of the other genera enclosed in the analysis, *Steno* was the one most related to *Sotalia*, whereas *Tursiops* was the most distantly related.

Figure 4. Median Joining Network (MJN) for the mitochondrial haplotypes found at the control region gene for the *Sotalia guianensis* population at the Amazon River's mouth. The network showed a star-form, which agrees quite well with a population expansion.



The MJN showed a star-form which is again indicative of population expansion. Three haplotypes contained important numbers of the individuals analyzed: 17 for H17, 10 for H2, and 9 for H3. The remaining haplotypes derived from these three haplotypes. However, H17 seems to be the original haplotype of the three quoted haplotypes. The time split between H17 and H2 and H3 were $50,240 \pm 25,120$ YA ($\rho = 0.3461 \pm 0.3461$) and $215,019 \pm 53,755$ YA ($\rho = 1.481 \pm 0.3704$), respectively. The time split between H17 and all the other haplotypes was $327,044 \pm 92,342$ YA ($\rho = 2.2533 \pm 0.6368$). Time splits of H3 and H2 from the remaining haplotypes were $392,874 \pm 114,537$ YA ($\rho = 2.7069 \pm 0.7892$) and $0.7892 \pm 136,328$ YA ($\rho = 2.9483 \pm 0.9313$) respectively. These divergence times agree quite well with the population expansion detected by the BSP with the median. The time split between the two most differentiated haplotypes was $943,397 \pm 102,628$ YA ($\rho = 6.5 \pm 0.7071$) (Figure 4).

4. Discussion

A major finding in this study is the clear evidence of *S. guianensis* as the main dolphin species in bycatch collected by artisan fishing boats using gillnets in the estuary of the Amazon River. They are basically accidental captures as it was demonstrated by Beltran-Pedrerros *et al.* [47]. This is an important finding because it confirmed the presence of this marine species in a freshwater system because the Amazon River carries freshwater hundreds of kilometers into the sea [48]. In other words these *S. guianensis* caught in Pará were actually living in freshwater. No *S. fluviatilis* individuals were detected among the samples analyzed. This result agrees quite well with the work of Cunha *et al.* [16]. They included samples from the Amazon delta in their analyses of differentiation between the two ecotypes of *Sotalia*, although the number of samples that they studied for the estuary of the Amazon River was very small compared with the current study. However, the results of Cunha *et al.* [16] were the first that revealed that the *Sotalia* from the mouth of the Amazon River were genetically much closer to dolphins from Santa Catarina (4,700 km Southern Brazil) than to the geographically closer *S. fluviatilis* in the Amazon River.

It would be noteworthy to analyze individuals of *Sotalia* from intermediate locations along the Amazon River, relatively nearby to the Amazon estuary, to detect how far upriver *S. guianensis* occurs, and verify if there is sympatry in any region with *S. fluviatilis*. A fine molecular population genetics analysis would be important, since it would allow not only the detection of any possible hybridization in the area but also its polarity.

A second question of interest is related with the use of molecular procedures to determine possible illegal traffic of dolphin species. In the Amazon, different species of dolphins are accidentally and also purposefully killed as sources of “love charms”. The first and third authors have seen the sexual organs (especially from females) of dolphins commercialized in the “Pasaje Paquito” in the Belén market or in the “artesanal San Juan” both at Iquitos (Peru) and in markets of Pucallpa (Peru). In these cases, these tissues basically corresponded to the pink river dolphin (*Inia geoffrensis*). The first author also observed sexual organs (of both sexes) and eyes commercialized in the “Ver ao Peso” market at Belem do Pará, and in the markets of Manaus and Porto Velho in Brazil. Our results showed that the dolphin-derived products, illegally sold in the Brazilian market of Belem (“Ver ao Peso”), do not belong to *Inia geoffrensis*, as many times the witches said and they are basically from *S. guianensis*.

S. guianensis amulets were detected not only in Belém, but in Manaus and Porto Velho, despite the availability of pink river dolphins and riverine tucuxis in those areas as Cunha and Solé-Cava [21], Gravena *et al.* [49] and Sholl *et al.* [50] showed. This indirectly shows that the Belem market provides derived products of *S. guianensis* to other inner Amazonian markets. Gravena *et al.* [49] observed that 90% of the eyeballs sold at the Ver-o-peso market of Porto Velho (Madeira River) were in fact from domestic animals and not from river dolphins. Additionally, Pinedo [51] showed that *S. guianensis* has been intentionally caught in the Amazon estuary to be used as shark bait. Thus, this species is basically the species employed by witches in the Brazilian Amazon for love charms.

4.1. Gene Diversity

Cunha *et al.* [16] determined that the Brazilian populations of *S. guianensis* presented a strong subdivision ($F_{ST} = 0.628$), with at least three evolutionarily significant units, north, northeastern and south/southeastern. Our study represents the first genetic analysis of the northern Brazilian population of *S. guianensis*.

The mitochondrial gene diversity found at the Amazon River mouth was clearly higher than the levels of gene diversity found in other *S. guianensis* populations. Hollatz *et al.* [22] failed to find any variability in the control region (480 bp) in the 50 individuals that they sampled ($H_d = 0$; $p = 0$). Their samples came from two areas along the Brazilian coast—Sepetiba Bay with the largest population of *S. guianensis* so far estimated (around 2,000 individuals; [52]) and the neighboring Paraty embayment with a similar population size. An absence of mitochondrial gene diversity agrees quite well with previous studies reported by Cunha *et al.* [16], who studied seven samples, and Caballero *et al.* [17], who analyzed 14 exemplars, for the southeastern area of Rio de Janeiro. Hoelzel *et al.* [53] demonstrated that for Cetaceans mtDNA is expected to reflect population structure more rapidly than nuclear loci, due to its relatively lower effective population size (only females) and high substitution rate. This means that the population of *S. guianensis* of the estuary of the Amazon River is older and has higher effective numbers than the populations of this species in southern Brazil. Nevertheless, Hollatz *et al.* [22], analyzing 10 DNA microsatellites detected significant genetic heterogeneity between Sepetiba embayment ($n = 43$) and Paraty ($n = 15$), for the statistics F_{ST} ($= 0.040$, $p < 0.01$) and R_{ST} ($= 0.076$, $p < 0.01$), while both populations were fixed for the same mitochondrial haplotype.

Another interesting fact is that our results showed more gene diversity than that obtained by Cunha *et al.* [16] with 56 *Sotalia* specimens for the same marker herein studied (484 bp). They found 22 polymorphic sites and defined 16 haplotypes. Furthermore, they estimated a haplotype diversity ($H_d = 0.792 \pm 0.049$) and a nucleotide diversity ($\pi = 0.0052$) for the marine populations similar to the freshwater samples of *S. fluviatilis* ($H_d = 0.788 \pm 0.090$; $\pi = 0.0043$). Although, our samples were from a much more restricted geographical area than the samples of the study of Cunha *et al.* [16], we found 29 polymorphic sites, 33 haplotypes, $H_d = 0.917 \pm 0.020$ and $\pi = 0.00455$. This shows the importance of analyzing higher numbers of samples of determined geographical points to estimate the most accurate gene diversity within populations. One difference of our work with that of Cunha *et al.* [16] is that the gene diversity that we estimated for *S. fluviatilis*, although only three samples were studied, was considerably higher than the values estimated by these authors. In this sense, our results reinforced the results of Caballero *et al.* [17,54], who determined higher levels of gene diversity for *S. fluviatilis*.

than for *S. guianensis*. Additionally, our molecular gene diversity results, as well as those from Cunha *et al.* [16,19] and Caballero *et al.* [17,54], agree quite well with *Sotalia* populations, of both species, with considerable effective numbers. Da Silva and Best [3] found both species to be abundant in the freshwater river systems as well as in marine ecosystems. With regard to *S. guianensis*, there are some censuses estimates with elevated sizes. In Bahía Guanabara, a minimum of 278 individuals were determined, whereas in Cananéia estuary (Brazil) nearly 3,000 individuals were estimated [55] and around 2,000 exemplars in the Sepetiba Bay and a similar quantity in Paraty [52]. With regard to *S. fluviatilis*, Magnusson *et al.* [56] determined a minimal density of 1.1 individuals/km in the transect from Manaus to Tefé (Amazon River) while Vidal *et al.* [57] estimated nearly 410 individuals in 250 km² of the Amazon River at the Colombian, Peruvian and Brazilian frontier.

4.2. Historical Demographic Evolution of the *S. guianensis* Population at the Amazon River Mouth and Proliferation of Haplotype Lineages

Different time splits were proposed for the divergence between the *Sotalia* species. Cunha *et al.* [16] estimated the divergence between *S. fluviatilis* and *S. guianensis* to be 2.5%, for both the control region and the cytochrome b. The evolutionary rates of those markers have been estimated at between 0.5% and 1% per MYA for the control region of cetaceans [58] and 1%/MYA for cytochrome b [59]. Hence, the speciation event that separated both lineages probably happened between 5 and 2.5 MYA, during the Pliocene. These authors claimed that at that time, the Amazon River was already flowing along its present course, with its outlet to the Atlantic [60,61]. These authors also stated that for the last 4 MYA, several sea level oscillations occurred, as a consequence of glacial and interglacial periods. During the periods of sea level rise, river discharge was prevented, and freshwater inflow into the Amazon basin increased, causing the inundation of the Amazon basin with the highest marine transgression happening around 2.5 MYA [62]. It is possible that *Sotalia* colonized the Amazon basin during one of those transgression/inundation events. Other authors, such as Caballero *et al.* [17], calibrated a molecular clock for the mtDNA control region using the estimated divergence between *Sotalia* and *Phocoena phocoena* based on the fossil record (10–11 MYA) and a net divergence of 24% between them with the Tamura–Nei model, with the rate of nucleotide substitution estimated to range from 1.11×10^{-8} to 1.22×10^{-8} bp⁻¹ yr⁻¹. Therefore, they arrived at a faster substitution rate, and dated the divergence between *S. fluviatilis* and *S. guianensis* at 1 to 1.2 MYA, during the Pleistocene. This dating is also compatible with environmental oscillations in the Amazon basin [17]. McGowen *et al.* [63] obtained, using a relaxed molecular clock and cytochrome b data, a divergence time of 1.99 MYA (0.63–3.67 MYA) between both forms of *Sotalia*. Another value for the separation of both *Sotalia* species was obtained by Steeman *et al.* [64] and estimated to occur 3.5 MYA. It is noteworthy that, in spite of being a supermatrix analysis (of 15 mitochondrial and nuclear markers) using seven fossil calibration points and relaxed clock models, the divergence between the *Sotalia* species, in this last referred work, was based exclusively on the mtDNA cytochrome b gene. However, we preferred the estimation of Cunha *et al.* [40] because it considered the complete mitochondrial DNA molecule (2.3 MYA). Taking this last divergence split between *S. guianensis* and *S. fluviatilis*, we found that the population of *S. guianensis*, from the estuary of the Amazon River, suffered a population expansion that began around 1 MYA (Bayesian skyline plot with

the mean) or around 0.4 MYA (Bayesian skyline plot with median and MJN) and that practically continued until present day. The MNJ also showed near of 1 MYA among the most differentiated haplotypes and many time splits were around 0.3–0.4 MYA and also since 0.02–0.2 MYA.

These temporal split between *S. fluviatilis* and *S. guianensis* coincides with the beginning of the Pleistocene (2.4–1.8 MYA; [65]). The temperature was around $4\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$ lower than today and the precipitation levels were 500–1000 mm less than today. At 2,500 meters above sea level (masl), the temperature was $10\text{ }^{\circ}\text{C}$ lower than today [66]. This also coincides with the last phase of formation of the central Andes and the beginning of very dry ambient temperature in the Andean mountains. The entire Andean chain between Cajamarca and Huancavelica (Peru) appeared by volcanic activity during this period together with other Andean tectonics effects [67,68]. An important marine introgression occurred during the Interensenedian (2.5 MYA), [61,68]. Additionally, the Amazon River only attained its present conformation by the beginning of the Pleistocene in this period [68,69]. This marine introgression and the current Amazon River conformation could be decisive phenomena to understand the colonization of the Amazon River by the ancestors of the current *S. fluviatilis* and the beginning to the haplotype lineage diversification for the marine *Sotalia* at the Amazon River mouth. Other dolphin species with difficult taxonomic assignment at the generic level, such as *Tursiops* spp. and *Stenella* spp., were all inferred to have diversified in the late Pliocene and in the beginning of the Pleistocene [40], as it could occur with the different *Sotalia* lineages herein studied, showing that these climatic and geological changes had an overall repercussion on the marine dolphin diversification.

Another clear fact is that *Sotalia* penetrated into the Amazon River for the current mouth because the connection of the Caribbean Sea with the Paleo-Orinoco River was present until the uplifting of the northern Andes cordillera around 8 MYA [60,70] and the molecular *Sotalia* diversification was clearly posterior.

A peak of mitochondrial diversification in *S. guianensis* was around 1.0 MYA. This coincides with the first part of the Pre-Pastonian glacial period (1.30–0.8 MYA). This period was also a time for haplotype diversification and separation for many terrestrial South American mammalian species, such as the Pampas cat [71], the jaguarundi [72], and the foxes of the genus *Pseudoalopex* [73]. For instance, around 1.3 MYA, the Buenos Aires's fauna transformed into a typical semi-arid Patagonian fauna, represented by the guanaco, *Lestodelphys* and *Lyncodon*, which means that the climate was considerably colder and drier and this was related with global climatic changes in the seas, which could have influenced haplotype lineage fragmentation within *S. guianensis*. Another, period of mitochondrial haplotype diversification (0.4–0.2 MYA) coincides with the very important population expansion detected using the Bayesian skyline plot with the median and with the MJN. This agrees quite well with the Mindel-Kansas glacial period (Elster glacial period for Scandinavia, Bonaerense period for Argentina and Kamasiense I for the Eastern Africa; 0.3–0.5 MYA). During the Bonaerense period, there was an increase of the diversity of many mammals (as Muridae and deer) in the Buenos Aires province. There was a major climatic stability to a hotter and warmer climate that could have helped in the geographic expansion of haplotypes in the *S. guianensis* population of the area of the estuary of the Amazon River. The last important mitochondrial diversification period for the population studied of *S. guianensis* was around 0.2–0.02 MYA, which coincides with the beginning of the fourth (and last) glacial period during the Pleistocene. Van der Hammen [66] determined the beginning of this period around 0.13 MYA by the pollen deposits at the Fuquene and Bogota lagoons

in the Cundiboyacense highlands in Colombia and it coincided with the beginning of the Lujanense period in Argentina. For instance, since 80,000–70,000 YA, the cold was extreme during the early Pleni-glacial, which was the first extreme cold period in the fourth glaciation. Absy *et al.* [74], Van der Hammen [66] and Van der Hammen and Absy [75] analyzed the vegetation in Carajas (Eastern Amazon) covering a time period starting approximately 65,000–51,000 YA and determined that this current area of the Amazon forest was a savannah and probably formed by an extension of the early Pleni-glacial period. Liu and Colinvaux [76] and Colinvaux and Liu [77] analyzed Ecuadorian Andean valleys and the Amazon and concluded that the vegetation limit drastically decreased by 27,000–34,000 YA and, in addition, the temperature decreased by 4.5 °C during the middle and the upper Pleni-glacial periods. For example, 30,000 YA, was one of the drier and colder moments of the middle Pleni-glacial period. The Bogota lagoon was dried in that period [66]. Afterwards, another period of extreme cold occurred around 23,000 to 20,000 YA (Dryas I) as MacNeish [78] showed in the Pikimachay Cove through his pollen and soil acidity study. The Last Glacial Maximum (LGM) occurred between 19,000 to 16,500 YA. It was the moment where the extension of the snow reached the maximum in the Central Andes. These changes of very cold and then warm periods could provoke different conditions for diversification of mitochondrial haplotypes in the species analyzed and, probably, the expansion of *S. guianensis* for the coasts of South America. The study of Hollatz *et al.* [22] based on microsatellite data found no evidence of a population bottleneck in Sepetiba or Paraty Bay in the southern distribution of *S. guianensis* in Brazil. These authors commented that geological data also indicated that the formation of Sepetiba embayment would have occurred during the Holocene postglacial period, around 6,000 YA [79]. The partial closure of the bay leading to the formation of a highly productive estuarine ecosystem would have provided a suitable habitat for the establishment of this dolphin species. They hypothesized that not enough time has elapsed to allow mtDNA control region sequences to accumulate gene diversity. Additionally, the microsatellite results obtained by them indicated a significant genetic structure between two localities 60 km apart from each other, which suggested that dolphins from both bays might not be currently interbreeding or if so, only rarely. Therefore, if we compare our results to that obtained by Hollatz *et al.* [22], the *S. guianensis* population from the estuary of the Amazon River is a very old population compared with those from the southern Brazilian coast and, probably, it could be one of the sources from which the southern populations originated. It could be important to carry out a microsatellite analysis among different populations of *S. guianensis* in the estuary of the Amazon River to determine if the genetic structure in that area is as significant as it was between the two *S. guianensis* populations in Southern Brazil. It would be interesting to note if the genetic structure of *S. guianensis* in the estuary of the Amazon River is a consequence of ecological or geological barriers among the populations, if it is a consequence of the social dynamics inside the groups or if it is predominantly governed by habitat type and resource specializations as it has been determined for other species of Cetaceans [80–83].

Thus, this is the first genetic study made on the population of *S. guianensis* at the Amazon River mouth including a considerable number of individuals analyzed. Future studies must include DNA microsatellite analyses and define potential genetic structure differences among different *S. guianensis* populations residing in the mouth of the Amazon River. We suggest that future studies of *S. guianensis* take into account that *S. guianensis* seems to be the main dolphin affected by bycatch with gillnets deployed from artisan fishing boats. It may prove to be interesting to not only sample *S. guianensis*

near the delta of the Amazon River, but to do so across multiple years, applying methods of estimation of effective numbers with allele frequency changes methods [84,85]. It would also be of great value to determine if and where a hybrid zone exists between the *S. fluviatilis* population and the *S. guianensis* population in the Amazon River. It is interesting to note the high potential to obtain samples of wild fauna in South-America in Indian and afro-descendent markets (witches, wizards, *etc.*) and how wild fauna products destined to be employed as charms could be extremely useful to reconstruct the evolutionary history and genetics conservation parameters of many wild species, such as in the case of *S. guianensis* presented herein.

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Conflicts of Interest

The authors declare no conflict of interest.

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