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ABSTRACT

The bottlenose dolphin (*Tursiops truncatus*) has a worldwide distribution in contrasting habitats, high behavioral plasticity, and large genetic and morphological variation. In the Southwestern Atlantic Ocean (SWA), the bottlenose dolphin is distributed from Tropical zone until the Southernmost areas of Argentina. Nevertheless, most studies of population structure of the species in this ocean basin are restricted to a few locations, especially in Southern and Southeastern Brazilian coast. Indeed, morphological studies suggested the existence of another species, *T. gephyreus*, in the southern part of this area, in partial sympatry with *T. truncatus*. The goal of this study is to assess the levels of genetic variability and population structure of the bottlenose dolphins along the Tropical and Subtropical SWA and also compare the results with previous morphological identification. A total of 110 samples were analyzed in six areas of occurrence on the coast of Brazil, as well as specimens from French Guiana and Saint Paul's Rocks (ASPSP). After analyzing the mtDNA control region and seven microsatellite loci, we found significant population structure in both markers. The results indicate the existence of three geographically distinct genetic groups: ASPSP (comprising samples from ASPSP and the French Guiana), Northeast (from North and Northeast areas of Brazil) and Campos and Santos Basins (BC/BS, from the states of Rio de Janeiro, São Paulo and Santa Catarina - SC). Haplotype diversity and allelic richness of these groups were high as well as their genetic structure. Samples from the Rio Grande do Sul (RS) state, in the southernmost region of Brazil, comprise some individuals with high likelihood to be part of the BC/BS group and others to the Northeast group. Besides, several individuals of RS comprise a much differentiated genetic group in microsatellites and they have mtDNA haplotypes from a unique clade, where all individuals morphologically identified as *T. gephyreus* were placed. Combining these results with previous studies, we conclude that the bottlenose dolphin from the SWA consists of at least four management units: i) ASPSP; ii) North and Northeast of Brazil; iii) BC/BS (that seems to extends at least to RS); and iv) Bahía San Antonio, Argentina. Finally, from SC state, southern Brazil, to at least Uruguay it seems to exists a distinct genetic entity that is not the canonical *T. truncatus*, but partially sympatric to it, and that is also associated with the *T. gephyreus* morphology. However, the picture is currently not clear enough to allow a formal taxonomic proposition.

Introduction

Studies of population structure in cetaceans often found genetic differentiation between populations, despite the high potential dispersion of most species (*e.g.* Natoli *et al.* 2008, Ansmann *et al.* 2012, Cunha *et al*. 2014). This structure could be related both to environmental factors such as the influence of the temperature of marine and oceanic currents (*e.g*. Natoli *et al*. 2005), and to behavioral characteristics associated with the habitat (Louis *et al*. 2014).

The bottlenose dolphin, *Tursiops truncatus* (Montagu, 1821) has been also pointed as a species that often has high levels of structuring even among geographically close populations (*e.g.* Hoelzel *et al*. 1998, Parsons *et al*. 2002, 2006, Natoli *et al*. 2004, Caballero *et al*. 2012, Louis *et al*. 2014).

The species has a cosmopolitan distribution, occurring in temperate, tropical, coastal and oceanic waters (Wells and Scott 1999, Reynolds *et al.* 2000). In Brazil, *T. truncatus* is distributed from North to South, inhabiting different habitats such as coastal regions, lagoons, estuaries and inner seas, as well as pelagic and oceanic islands, showing high behavioral plasticity (Pinedo *et al.* 1992, Ott *et al.* 2009). This attribute combined with great morphological variation and the occupation of various habitats led the description of more than 20 different species in the past (Walker 1981, Hersh and Duffield 1990, Ross and Cockcroft 1990, Wells and Scott 1999).

Currently, only *T. truncatus* and *T. aduncus* (Ehrenberg, 1833), known as bottlenose dolphin and Indo-Pacific bottlenose dolphin, respectively, are recognized with species status by the Committee on Taxonomy of the Society for Marine Mammalogy (Committee on Taxonomy 2015). However, molecular studies suggest the existence of at least two more species – *T. australis* in South Australia (Bilgmann *et al*. 2007, Möller *et al*. 2008) and a variation of *T. aduncus* on the coast of South Africa (Natoli *et al*. 2004).

Despite its wide distribution and presence in areas of intense human activity, studies on the population genetic structure of the bottlenose dolphin are limited in terms of coverage of the ~8.000 km of Brazilian coastal zone (see Ott *et al.* in press). One mitochondrial DNA (mtDNA) study with three populations found that bottlenose dolphins populations have a strong genetic structure along the Brazilian coast, including an apparently isolated population around Saint Paul's Rocks (also known as São Pedro and São Paulo Archipelago – ASPSP) (an offshore island close to the equator) and two other populations in the southeastern (Rio de Janeiro/São Paulo) and southern (Rio Grande do Sul) Brazil (Ott *et al*., 2009). Population genetic structure was also found among bottlenose dolphins from southern Brazil (including Uruguay) and Argentina (San Antonio Bay) in both mtDNA and nuclear microsatellites (Fruet *et al.* 2014). This result indicated that bottlenose populations are under certain environmental conditions that can lead to habitat specialization resulting in differentiation between populations. Maternal (mtDNA) genetic differentiation has also been detected among neighboring communities (*sensu* Urian *et al.* 2009) of bottlenose dolphins in southern Brazil (Costa *et al*. 2015). Despite these recent advances related to genetic structure of bottlenose dolphins in the SWA, most of the studies are restricted to one molecular marker (mtDNA) or to a specific geographical area (usually southern Brazil), with no population structure analysis conducted covering the entire Brazilian coast.

Modern studies on skull morphometry of bottlenose dolphins from Southwestern Atlantic Ocean (SWA) suggested the existence of a distinct form of *T. truncatus* in the southern region of its distribution, which was suggested to be *T. t. gephyreus* (Barreto 2000) or *T. gephyreus*, first proposed by Lahille (1908) (Wickert 2013). These studies found a zone of simpatry between these two forms around the three southernmost

Brazilian states (PR, SC, and RS), with *T. truncatus* and *T. gephyreus* morphotypes found northern and southern of this zone, respectively.

In Brazil, the conservation threats to *T. truncatus* are related to the intensification of human activities near the coast. As a result of this particular threat, it was recorded a reduction in reproductive rate and an increase in mortality in coastal populations of the southernmost limits of its distribution (Viaud-Martínez *et al*. 2008, Fruet *et al*. 2012). The absence of genetic studies to other Brazilian areas makes difficult to understand the potential relationship among *T. truncatus* populations and their consequences for their conservation in the region. In fact, the bottlenose dolphin has suffered locally for at least four decades with the impact of fishing activity (Siciliano, 1994). Furthermore, the intensive use of coastal environments by bottlenose dolphins exposes these populations to various threats, including chemical contamination derived from agricultural and industrial activities and domestic effluent discharges (Yogui *et al.* 2010, Lemos *et al.* 2013). These factors can be related to recent cases of diseases with different origins and etiologies, diagnosed as lobomycosis or similar ('Lobomicose-like disease'), or even distinct from these (Daura-Jorge & Simões-Lopes 2011, Van Bressem *et al.* 2015). All these factors highlight the need to understand what are the effects of these threats on *T. truncatus* and its genetic diversity as a whole. In this context, it is necessary to determine what level of gene flow exists among their populations and whether they are significantly different, in order to stablish conservation strategies that correspond to the requirements of each population considered.

The differentiation among close populations of *T. truncatus* in distinct regions of the world could be related to its social system and fidelity to the birth site, as demonstrated by photo-identification studies (*e.g*. Parsons *et al*. 2006, Baird *et al*. 2009). The structure could also be explained by the existence of two distinct geographical forms suggested as

coastal (inshore) and oceanic individuals (offshore) (e.g. Louis *et al.* 2014). The identification of these two forms has been carried out through morphological, ecological and genetic analyzes on the coast of Scotland, North America, Black Sea and Mediterranean Sea (*e.g*. Duffield *et al*. 1983, Mead & Potter 1995, Hoelzel *et al*. 1998, Natoli *et al*. 2005). However, the results of Segura *et al.* (2006) based on the analyzes of mtDNA control region of *T. truncatus* from Gulf of Mexico, weakly corroborated the results from the morphology, behavior and ecology analyzes. But, the same authors suggested that these results could be "masking" a possible recent isolation for the species in the region, this because this study did not include nuclear markers.

This study aims to assess the levels of genetic variability and population structure of the bottlenose dolphin in more than ~6.300 km of coast in the Western Tropical and Subtropical South Atlantic Ocean (down to the southern Brazilian coast), including specimens of Saint Paul's Rocks and French Guiana. Therefore, microsatellite *loci* and sequences of the mtDNA control region were used to investigate the genetic population structure and the degree of genetic differentiation and gene flow between them. The wide-ranging geographical sampling of this study also includes biopsied and individuals identified as inshore and offshore, allowing the test of the hypothesis that environmental differences could shape the genetic differentiation in populations of *T. truncatus.* Finally, since this is the first genetic study using both mtDNA and nuclear markers in which the sample of individuals has been morphologically identified as belonging to the two proposed species (i.e. *T. truncatus* and *T. gephyreus*), it also allows to test the relationship between the morphological proposal and the genetic evidences.

Material and Methods

Sample collection and DNA extraction

Tissue samples were collected from 110 stranded or biopsied bottlenose dolphins, mainly from the Western Tropical and Subtropical South Atlantic (Figure 1), and through the collaboration with research groups along this coast (see Table 1 – Supplementary material).

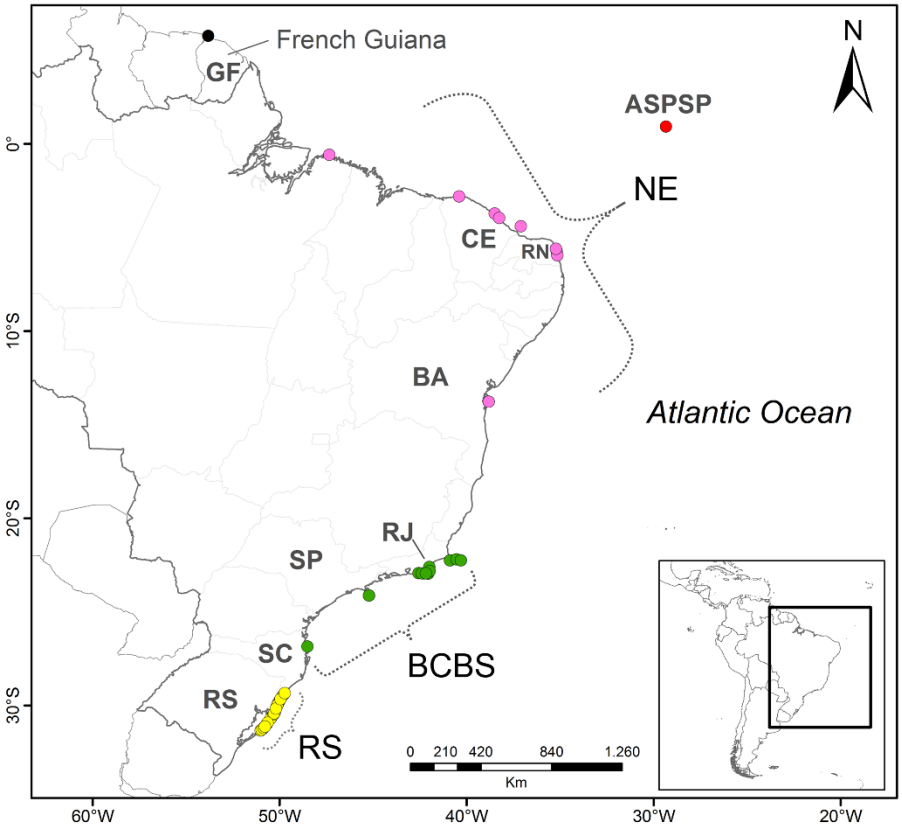

Figure 1. Map with the sampling localities of bottlenose dolphins. The colors and labels are for geographic areas considered in the analyses. Red: Saint Paul's Rocks (ASPSP), Black: French Guiana (GF), Violet: Northeast (NE), Green: Campos and Santos Basins (BC/BS) and Yellow: Rio Grande do Sul (RS).

The sampling sites were grouped into four main areas (for most of the analyzes), and named as: Northeast (NE), with samples of Brazilian states of Pará (PA, *n*=1), Ceará (CE, *n*=4), Rio Grande do Norte (RN *n*=4) and Bahia (BA, *n*=6) besides one sample from French Guiana (GF, *n*=1); Saint Paul's Rocks (ASPSP, *n*=19) (distant approximately 1010km from the Brazilian coast); Campos and Santos Basins (BC/BS includes the states of Rio de Janeiro (RJ), São Paulo (SP) and one biopsy sample from Santa Catarina (SC), which was considered as belonging to the Campos and Santos Basins by the fact that it

extends to that region) (*n*=45); and the northern coast of Rio Grande do Sul (RS, *n*=30). It is important to mention that 22 of the 30 samples from RS were also analysed in the study of cranial morphology of Wickert (2013) and were there identified as *T. gephyreus* or *T. truncatus*. Tissue samples were cryo-preserved at -20°C in ethanol 96% or dimethyl sulfoxide (DMSO) saturated with sodium chloride (Amos & Hoelzel 1991). The genomic DNA was extracted with phenol-chloroform protocol (Sambrook *et al.* 1989) adapted by Shaw *et al.* (2003).

Mitochondrial DNA amplification and analyzes

The following primers were used to amplify a 316 bp region of the mtDNA control region: L15926 THR (5´- TCA AAG CTT ACA CCA GTC TTG TAA ACC - 3´) (Kocher *et al.* 1989) and H16498 (5´- CCT GAA GTA GGA ACC AGA TG - 3´) (Rosel *et al.* 1994). Each PCR was conducted in a 20 µl reaction volume containing 20 ng of template DNA, 1X PCR buffer (10mM Tris-HCl pH 8.3, 50mM KCl); 0.2mM de dNTPs; 0.1mg/mL BSA; 3.5 mM $MgCl_2$; 0.2 µM of each primer; 1U of Taq DNA Polymerase (Gibco BRL®).

The following PCR conditions were used: one cycle of five min at 93°C; 30 cycles of 1 min at 93°C, 1 min at 51.5 °C and 1 min at 72°C; and one final extension cycle of 10 min at 72°C. The resulting PCR products were purified using shrimp alkaline phosphatase and exonuclease I (*Amersham Biosciences*) following the manufacturer's recommended protocol. All fragments were then sequenced from both ends on a MegaBACE™ 1000 capillary sequencer (*Amersham Biosciences*) using the DYEnamic ET Dye Terminator Cycle Sequencing Kit (*Amersham Biosciences*).

Sequences quality were visually checked with ChromasPro 1.7 (http://technelysium.com.au) and automatically aligned (with minor manual correction)

in ClustalW and MEGA 6 (Tamura *et al*. 2013), resulting in a 316 bp stretch of high-quality sequence that was obtained for all individuals.

Haplotype (h) and nucleotide diversities (π) were estimated for the whole sample set and for each sampled area separately using in Arlequin 3.5.1 (Excoffier & Lischer 2010) and DnaSP 5.10.1 (Librado & Rozas 2009). Analysis of Molecular Variance (AMOVA) between and within sampled areas and pairwise F-statistics between areas, using both F_{ST} (Weir & Cockerham 1984) and Φ_{ST} approaches were estimated with Arlequin. The Tajima's D and Fu's FS neutrality tests were also performed with Arlequin. All these analyses were performed with 10,000 permutations. Haplotype network was constructed using the median-joining approach (Bandelt *et al.* 1999) implemented in Network 4.6.11 (http://www.fluxus-engineering.com).

Microsatellite DNA amplification and analyzes

We amplified seven polymorphic previously developed for cetaceans: KWM2b, KWM9b and KWM12a (Hoelzel *et al*. 1998), EV37mn (Valsecchi & Amos 1996), TexVet5, TexVet7 and D08 (Rooney *et al*. 1999, Shinohara *et al*. 199).

The microsatellite PCR reactions were carried out following Natoli *et al.* (2004) protocol. Each PCR was conducted in a 20 µl reaction volume containing 20 ng of genomic DNA; 1X PCR buffer (10mM Tris-HCl pH 8.3, 50mM KCl); 0.1 nm of dNTPs; 1.5 mM $MgCl_2$; 0.016µM primer forward; 0.25µM of primer reverse; 0.05 U of Platinum Taq DNA Polymerase (©Invitrogen); 0.2µM of fluorescence (FAM, HEX, NED) (Boutin-Ganache *et al.* 2001). The following PCR profile was used: one cycle of 2.5 min at 94°C; one cycle of 1 min at 60°C; a *touchdown* of 9 cycles of 1 min at 60°C (-1°C per cycle); one cycle of 1.5 min at 72°C; a second step of denaturation/amplification: 40 cycles of 30 s at 94°C; one cycle of 1 min at 50°C; one cycle of 1.5 min at 72°C; and a

final extension of 5 min at 72°C. The PCR products were genotyped on a MegaBACE™ 1000 capillary sequencer.

The allele size in base pairs was quantified with Genetic Profiler (*Amersham Biosciences*) and subsequently manually inspected and adjusted when necessary with the software Allelogram (Manaster 2002) and Micro-checker (Oosterhout *et al*. 2004).

Deviations from Hardy-Weinberg equilibrium (HWE) (Guo & Thompson 1992) linkage disequilibrium (LD), expected (He) and observed heterozygosity (Ho) were determined with Arlequin. AMOVA and F-statistics pairwise differentiation (F_{ST} and R_{ST}-like methods) between areas were also estimated with Arlequin. All these analyses using 10,000 permutations and significance levels $\alpha = 0.05$.

Genetic population structure was assessed by the Bayesian approach implemented in STRUCTURE 2.3.4 (Pritchard *et al*., 2000). We performed 10 independent runs for each K from K = 2 to K = 10, applying 1,000,000 Markov chain Monte Carlo iterations and a burn-in period of 1,000,000. The Admixture model for the ancestry individuals with LOCPRIOR information were used. The optimal number of clusters was determined using the Evanno method (Evanno *et al.* 2005) as implemented by structure harvester (Earl & Vonholdt 2012). Lastly, the Structure results were summarized in CLUMPP software (Jakobsson & Rosenberg, 2007) and represented graphically using the *distruct* program (Rosenberg, 2004).

The genetic distance among populations was inferred by estimation of the Shriver *et al.*'s (1995) D_{SW} distance and the neighbor-joining unrooted tree were estimated with Populations ver. 1.2.32 (Langella 1999). Support for tree nodes was assessed by bootstrapping across loci (1,000 iterations) and the resulting tree was displayed with FigTree 1.4.2. (http://tree.bio.ed.ac.uk/software/figtree).

The magnitude and direction of recent migration were estimated using a Bayesian method implemented in BayesAss3.0.3 (Wilson & Rannala 2003). BayesAss was performed using for 3 x 10^7 steps recorded every 1000, with the first $3x10^6$ discarded as burn-in. To reach the recommended acceptance rates between 20% and 60%, the values of parameters such as migration rates (ΔM), allele frequencies (ΔA) and inbreeding coefficient (ΔF) were adjusted to 0.1, 0.5 and 0.5, respectively. Trace files were examined for convergence in the Tracer 1.5 software (Rambaut & Drummond, 2007).

Results

mtDNA

The 316 bp mtDNA control region alignment of 109 bottlenose dolphins resulted in 30 segregating sites (92.2% transition and 8.8% transversion substitutions) in 32 haplotypes (Table S2 – Supporting Information). Haplotype diversity for the whole set (Hd = 0.85) and the areas were similar and moderately high, the same with the nucleotide diversity (π = 1.5%) (Table 1). The exception was the population of Saint Paul's Rocks, that only had two haplotypes, and therefore both genetic diversities were quite low.

The haplotype network loosely resembles a star (Figure 2), but with no central haplotype and with some haplotypes very divergent, like the French Guiana haplotype, and only four haplotypes are shared between areas (RS and BC/BS), the others are exclusive to each population. There are also some geographically well-structured clades, such one with individuals from NE and other from RS. It should be noted that this RS clade (identified by a rectangle in Figure 2) contains all and only sequences found in the individuals morphologically identified as *T. gephyreus* (Wickert 2013). However, a few

Table 1. Basic genetic statistics for the mtDNA control region of bottlenose dolphins of the Western Tropical and Subtropical South Atlantic. N = number of samples, S = variable sites, H = number of haplotypes, Hd = haplotype diversity, π = nucleotyde diversity (%), SD: standard deviation. ASPSP = Saint Paul's Rocks, NE = Northeast, BC/BS = Campos and Santos Basins and RS = Rio Grande do Sul. *P* = P-value. Areas as in Figure 1.

Areas	**N**	**S**	**H**	**Hd (SD)**	**π % (SD)**	**Tajima's D (*P*)**	**Fu's Fs (*P*)**
ASPSP	19	2	2	0.105 (0.092)	0.067 (0.059)	-1.51* (0.04)	0.59 (0.41)
NE	14	14	9	0.912 (0.059)	1.94 (0.302)	-0.23 (0.43)	-0.45 (0.40)
BC/BS	45	18	12	0.848 (0.028)	1.34 (0.174)	-0.42 (0.37)	-0.38 (0.48)
RS	30	20	12	0.862 (0.040)	1.948 (0.184)	-0.29 (0.43)	0.59 (0.63)
Total	109	30	32	0.855 (0.025)	1.56 (0.124)	-0.61 (0.32)	0.08 (0.48)

* *P*<0,05.

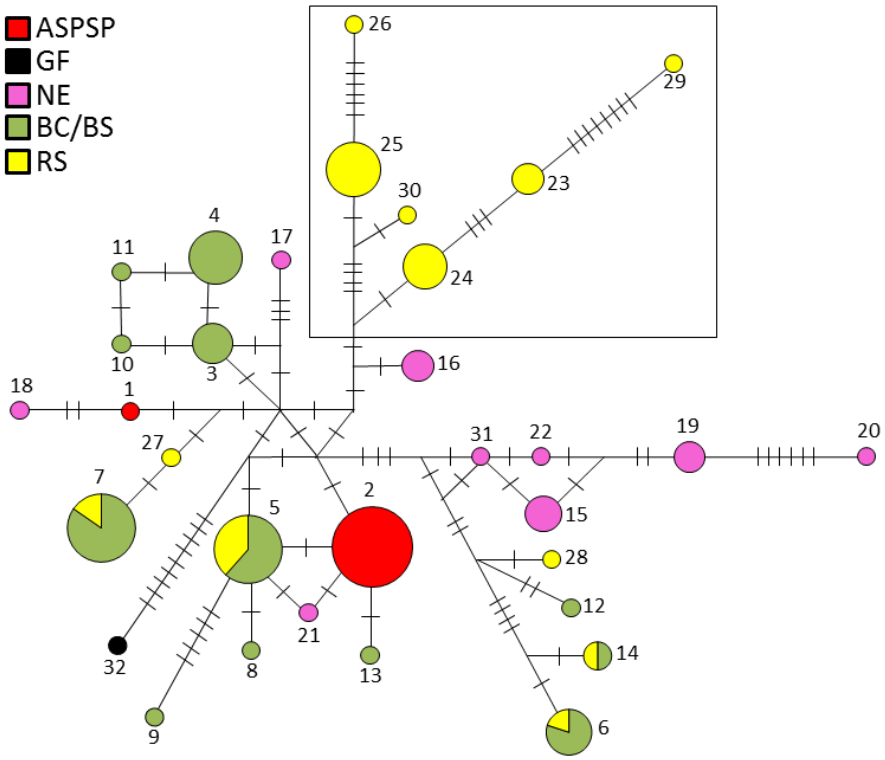

Figure 2. Median joining network of mtDNA control region sequences of bottlenose dolphins of the Western Tropical and Subtropical South Atlantic. The circles represent the haplotypes found and their sizes are proportional to the haplotype frequency across all 109 sampled individuals. The mutational steps are represented by the number of bars in the branches. The rectangle indicates the haplotypes of individuals morphologically identified as *T. gephyreus* (sensu Wickert 2013) (referred as *gephyreus* clade in this study). The colors refer to the sampling localities of individuals, following the legend and abbreviations in the Figure 1.

dolphins with haplotypes from this clade did not have morphological identification. Individuals from the NE area have a wide geographic distribution (Figure 1), but in general their haplotypes were grouped in a specific geographic clade, including the haplotype from one specimen collected further north in Brazil (haplotype 31), as well as five out of six individuals (haplotype 21 is the exception) collected further south of this area (Bahia state).The AMOVArevealed evidence for strong population differentiation, with more than 26% (P<0.05) of the genetic variability being partitioned among the studied areas (Table 2). The pairwise F_{ST} and Φ_{ST} were highly significant among all areas, and the population of ASPSP was the most genetically different (Table 3).

Tajima's D and Fu's F_S tests of selective neutrality yielded negative, but non-significant values (Table 1), indicating no strong evidence for recent population expansion for the species as a whole or even for separate areas, excepting for the ASPSP.

Table 2. AMOVA for the mtDNA control region (F_{ST} and Φ_{ST}) and for the microsatellite data (F_{ST} and R_{ST}) for bottlenose dolphins of the Western Tropical and Subtropical South Atlantic as a whole.

Source of variation	**mtDNA**		**Microsatellites**	
	F_{ST}	**Φ_{ST}**	**F_{ST}**	**R_{ST}**
between areas	26.82	28.08	9.47	12.41
within areas	73.18	71.92	90.53	87.59

All values were significant for P<0,05.

Table 3. Pairwise F statistics for mtDNA control region between areas: Φ_{ST} (above diagonal) and F_{ST} (below diagonal): Φ_{ST} (above diagonal) and F_{ST} (below diagonal). ASPSP = Saint Paul's Rocks, NE = Northeast, BC/BS = Campos and Santos Basins and RS = Rio Grande do Sul.

Areas	**ASPSP**	**NE**	**BC/BS**	**RS**
ASPSP	-	0.548	0.152	0.370
NE	0.529	-	0.323	0.260
BC/BS	0.444	0.120	-	0.222
RS	0.468	0.112	0.095	-

All values were significant for P<0,05.

Microsattelites

A total of 102 individuals were genotyped and all seven microsatellite loci were moderately polymorphic, with an average expected heterozygosity of 0.76 (SD = 0.057) and an average number of alleles per *locus* of 12 (Table 4). MICRO-CHECKER results suggested the presence of null alleles and *stuttering* in some areas, but since there was no consistency between *loci* and areas in these results, no *locus* was excluded from the analyses. Similarly, since there was no consistency between *loci* and areas in the linkage disequilibrium and deviations from Hardy–Weinberg equilibrium, indicating low levels of interactions between *loci* (D'Aoust-Messier and Lesbarrères 2015), no *locus* was excluded from the analyses because of this.

Table 4. Microsatellite genetic diversities of bottlenose dolphins of the Western Tropical and Subtropical South Atlantic. N = number of individuals analyzed, A = number of alleles, K = average number of alleles, E = exclusive alleles, Ho = observed heterozygosity, He = expected heterozygosity. ASPSP = Saint Paul's Rocks, NE = Northeast, BC/BS = Campos and Santos Basins and RS = Rio Grande do Sul.

Areas	N	A	K	E	Ho	He (SD)
ASPSP	19	6.86	6.04	1.14	0.63	0.76 (0.07)
NE	12	5.43	5.36	0.28	0.58	0.65 (0.28)
BC/BS	43	9.43	6.52	1.57	0.51	0.72 (0.22)
RS	27	7.57	5.89	0.71	0.36	0.64 (0.23)
Total	101	12	6.885	3.71	0.524	0.760 (0.17)

The AMOVA of the microsatellite data showed a significant ~10% between-populations genetic variability (Table 2). The pairwise F_{ST} and R_{ST} values between the four areas were moderate and all significant (P<0,05) (Table 5).

The mean likelihood value for ten independent runs in STRUCTURE peaked at K = 4 genetic groups (Figure 1 – Supporting Information), that in general correspond to the geographic areas used here (Figure 3), except for the RS area.

Table 5. Pairwise F statistics for microsatellite data between areas: R_{ST} (diagonal above) and F_{ST} (diagonal below). ASPSP = Saint Paul's Rocks, NE = Northeast, BC/BS = Campos and Santos Basins and RS = Rio Grande do Sul.

Areas	**ASPSP**	**NE**	**BC/BS**	**RS**
ASPSP	-	0.124	0.085	0.131
NE	0.113	-	0.158	0.141
BC/BS	0.084	0.099	-	0.118
RS	0.126	0.107	0.077	-

All values were significant for P<0,05.

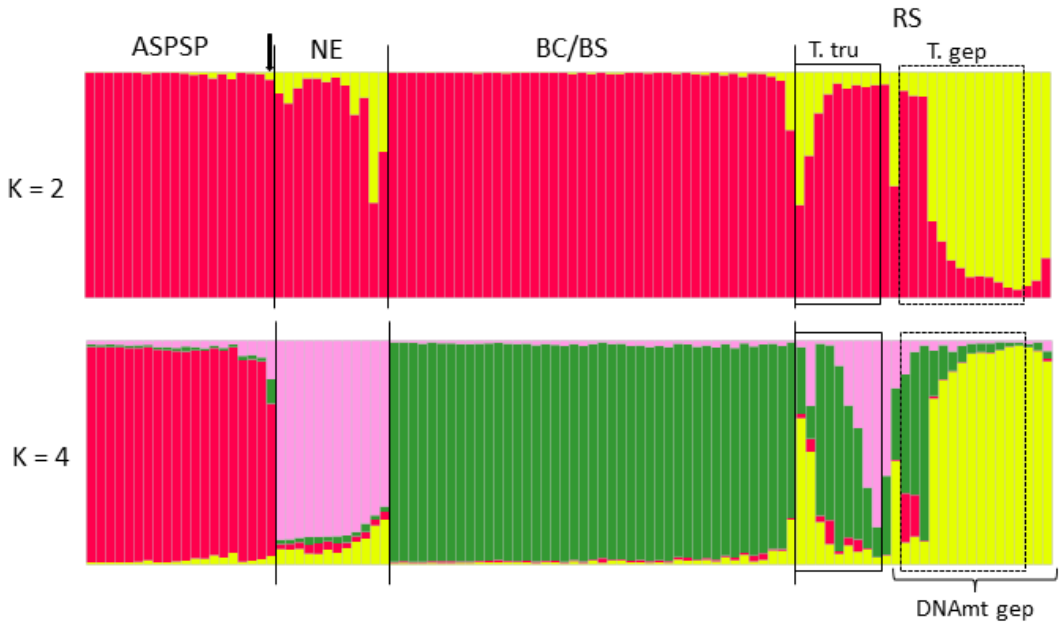

Figure 3. Bayesian analysis of the seven microsatellite loci showing the proportional membership (q) of each bottlenose dolphin of the Western Tropical and Subtropical South Atlantic in the genetic clusters inferred by STRUCTURE with K=2 and K= 4. Each individual is denoted by a vertical bar, and the length of each bar shows the probability of membership in each genetic cluster (represented by the colors). The arrow indicates the individual of French Guiana, considered as belonging to the area of Saint Paul's Rocks in the analyzes. The rectangle with a solid line represents the individuals morphologically identified as *T. truncatus* (T.tru) and the rectangle with dashed line the individuals identified as *T. gephyreus* (T.gep) (sensu Wickert 2013), and the caption with brackets indicates individuals with mtDNA belonging to the *T. gephyreus* clade (mtDNA gep, sensu Figure 2). The abbreviations according to Figure 1.

The composition of the samples of RS is complex, some individuals in this area have a high level of assignment to a fourth genetic group (represented in yellow in Figure 3), others are very similar to individuals from BC/BS area, two dolphins presented high proportion of a component related to the NE population and finally, some have mixture of components. It is important to highlight that 10 of 13 individuals morphologically identified as *T. gephyreus* (rectangle with dashed line in Figure 3) have a high proportion of the yellow component (hereafter referred as the *gephyreus* component). On the other hand, individuals from RS morphologically identified as *T. truncatus* presented a diversified genetic background, some being more similar to NE or BC/BS individuals, but only two of them have a considerable presence of the *gephyreus* component (the two first columns in *T.tru*). Five individuals from RS do not have a morpholocial identification: three of them have a very high proportion of the *gephyreus* component (the last three individuals in the Fig. 3) and one (the first left of the T. gep group) presents ~50% proportion of this component. Interestingly, these four individuals are also the only ones (other than the specimens identified morphologically as *T. gephyreus*) that have mtDNA haplotypes from *T. gephyreus* clade (Figure 2). In the exploratory analysis with K = 2 (Figure 3), the two genetic groups formed are clearly (but not perfectly) associated with the *T. gephyreus* and *T. truncatus* morphotypes, not with the geographical areas or distances, suggesting a significant genetic differentiation between these two morphologies.

The NJ tree depicting the genetic distances between individuals shown a picture similar to the STRUCTURE analysis, where individuals from the same geographic area tend to group together (Figure 4). Again, all but three individuals with *T. gephyreus*

morphology (in blue) and with a high percentage of *gephyreus* component are in a very distinct branch (highlighted in gray), together with some unindentified individuals from other regions, but with no individual with *T. truncatus* morphology. Similarly, the three unidentified individuals from RS that also have the mtDNA haplotypes from the *T. gephyreus* clade (red branches) also grouped here.

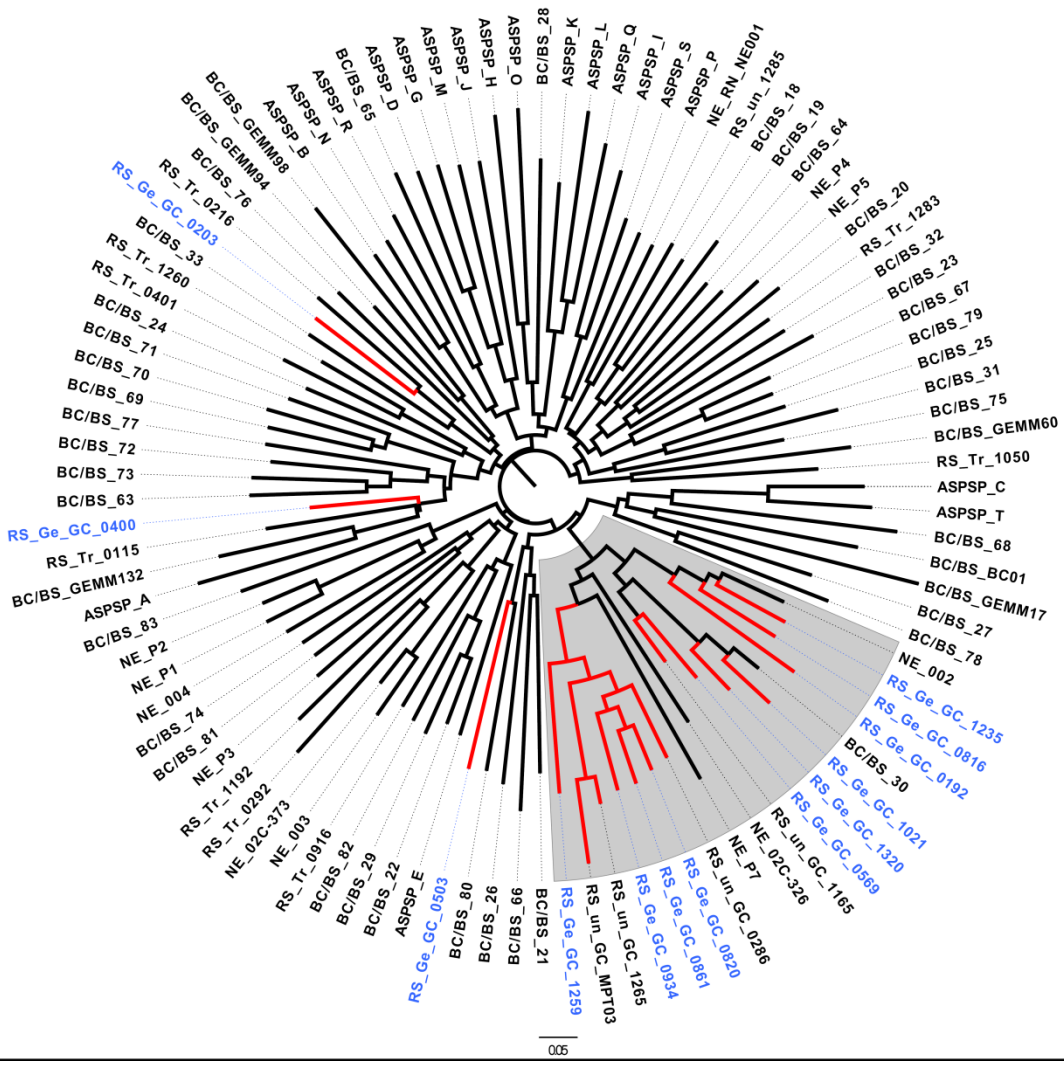

Figure 4. Unrooted neighbor-joining tree of the microsatellite D*SW* distances between the individuals of bottlenose dolphin of the Western Tropical and Subtropical South Atlantic. The individuals morphologically identified as *T. gephyreus* (sensu Wickert 2013) are in blue and in the red branches are those individuals with mtDNA haplotypes from the *T. gephyreus* clade. The group highlighted in grey has high percentage of the *gephyreus* component according to STRUCTURE. Area identification of the individuals are according Figure 1 and for the RS it was followed by the morphological identification (Ge = *T. gephyreus*, Tr = *T. truncatus*, Un = unknown) and those belong to the *gephyreus* clade (mtDNA) are also identified with GC letters.

The higher migration rates were from RS to BC/BS (M=0.069) and from ASPSP to NE (M=0.063) (Table 6) and the lower were from RS and BC/BS to ASPSP (M = 0.012 and 0.015 respectively), which could be correlated with the geographical distances between them.

Table 6. Migration rate (means of the posterior distributions of *m*) and respective values of their respective 95% CI (parenthesis) estimated by BayesAss for pairs of populations of bottlenose dolphins of the Western Tropical and Subtropical South Atlantic. ASPSP = Saint Paul's Rocks, NE = Northeast, BC/BS = Campos and Santos Basins and RS = Rio Grande do Sul.

From / To	ASPSP	NE	BC/BS	RS
ASPSP	-	0.063 (0.035)	0.024 (0.023)	0.026 (0.023)
NE	0.022 (0.021)	-	0.040 (0.041)	0.057 (0.035)
BC/BS	0.015 (0.013)	0.020 (0.018)	-	0.032 (0.022)
RS	0.012 (0.012)	0.041 (0.026)	0.069 (0.033)	-

Discussion

This is the first study to evaluate the genetic diversity and population differentiation of *T. truncatus* from the East coast of South America, from North to South of Brazil, including adjacent areas of Saint Paul's Rocks (ASPSP) and also one specimen from French Guiana. The mitochondrial genetic diversity found in the species in the region was high in comparison with other populations and even to other species (Table S3 – Supporting Information). However, observing the values for each sampling area separately, it is possible to recognize that ASPSP has the lowest nucleotide diversity for the species (0.067%). This result is expected, because ASPSP is the smallest studied population and it has a restricted distribution neighboring the archipelago (Oliveira *et al.* in press). Recent studies in Southern Brazil, Uruguay and Argentina found low or moderate levels of genetic variability (Fruet *et al*. 2014, Costa *et al*. 2015), probably due

to the existence of small communities with a high degree of residency and restricted gene flow between them as suggested by these authors as a potential explanation for their results.

The results of the present study demonstrated significant genetic structure, mainly in nuclear markers, in bottlenose dolphins along the Brazilian coast. Two of the four major genetic groups (Figure 3) seem to represent geographically distinct populations: one including individuals from ASPSP and French Guiana and the other with individuals from Northeast of Brazil (NE and perhaps few individuals from the southern area). The genetic group formed mainly by Campos and Santos Basins individuals represent a group that extends until the Rio Grande do Sul state, where this group probably occurs in sympatry with the other biological unit, the *T. gephyreus* morphotype (see below). However, the discontinuous distribution of part of our sampling area could have influenced, in an undetermined way, the degree of population structure detected across the Brazilian coast.

Nevertheless, the strong genetic differentiation of ASPSP population in comparison to the remaining areas, could be explained by its well-known oceanic habitat (all the specimens were biopsied roughly 1,010 km from the Brazilian coast in depths between 1000 m and 2000 m, while the other biopsy samples were obtained probably from coastal individuals in depths between 30 and 50 m). It is important to highlight that previous studies on Pará/Maranhão states and even Campos and Santos Basins (BC/BS) revealed the presence of coastal and oceanic individuals in all basins (Ramos *et al*. 2010). Moreover, there were sightings of bottlenose dolphins in offshore waters in BC/BS and also in RS (Siciliano *et al*. 2006, Ott *et al*. 2013). Therefore, our molecular results indicate the existence of at least one known genetically distinct oceanic population of *Tursiops*, ASPSP, but for the rest of the distribution studied here we do not have information to

decide on the existence of other genetic components (whether with microsatellite without mitochondrial clades) corresponding to the oceanic habitat, as found in other regions (Louis *et al.* 2014).

The structure found between NE and BC/BS areas are likely not related to the distance from the coast, since most of samples from these populations were probably from individuals that live in coastal habitats. The most likely explanation for the genetic differentiation between the NE and the BC/BS is the latitudinal separation of these populations caused by the upwelling zone present in the BC/BS. Many studies have demonstrated that the distribution of dolphin populations is influenced by environmental factors such as prey distribution (Heithaus & Dill 2002) and habitat structure (Lusseau *et al.* 2003).

Regarding the BC/BS, this region has peculiarities in its habitat as the mixture of tropical (median temperatures and high salinity) and coastal waters (high temperatures and low salinity) (Siciliano *et al.* 2006). There is also an upwelling event that occurs with great intensity in spring and summer seasons between latitudes 21º and 23ºS, controlled by the North and East winds when the South Atlantic Central Water (low temperature and low salinity) penetrates in the inner continental shelf causing decrease in ocean temperature (Siciliano *et al*. 2006). Between BC/BS there is also the most important coral reef area of the South Atlantic Ocean, the Abrolhos bank (ca. 18o S) (e,g, Bruce et al., 2012). These conditions may contribute to a regional difference in productivity, reflected in the abundance and distribution of prey, and consequently in the structuring the BC/BS population in relation to the NE population. It is noteworthy that the distribution of many other small cetaceans (e.g. *Stenella* spp.) seems to be influenced by the oceanographic features existent to the north and south of this central region of the Brazilian coast (Amaral *et al*. 2015). Genetic structuring between habitats with different productivity

have been reported for the bottlenose dolphins from the Black Sea region and from the Northeast Atlantic and Scotland (Natoli *et al.* 2005).

The genetic structure found in the RS is complex (Figure 3). The presence of individuals with a high proportion of genetic components associated with populations further north (BC/BS and NE) or a mixture of these, suggests some level of gene flow between these three areas. This hypothesis is consistent with the results from F_{ST} distances, in both mtDNA and microsatellite data (Tables 3 and 5), which are, in general, low between RS - BC/BS and RS - NE, as well with the relatively high migration rates between these three areas (Table 6). However, a migratory connection between RS and NE areas, due to the large geographical distance, is less likely and their similarity may requires additional explanation.

The presence in the RS of a distinctive and quite divergent genetic component (the *gephyreus* component, Figure 3) could be explained by two hypotheses, that were not mutually exclusive: 1) the existence of two distinct populations in the area (as evidenced in K = 2 in STRUCTURE analysis, Figure 3) which may be associated with the distance to the coast (since there was recent oceanic sightings, *e.g*. Ott *et al*. 2013); or 2) the existence of two geographical populations (but morphologically distinct) that are sympatric here, as suggested by Wickert (2013).

The hypothesis of inshore and offshore populations in this area could not be directly tested with our data, since the samples of RS were from stranded animals and were not accompanied by any direct information about the habitat used by these specimens in life, with one exception. One of the specimens (GEMARS 1259), which is highly associated with *gephyreus* component (and was morphologically identified as *T. gephyreus* and its mtDNA belongs to the *T. gephyreus* clade) was photo-identified in life and recognized as a highly resident individual from the Tramandaí Estuary (ca. 30ºS), on

the northern coast of RS, where it used to interact frequently with the artisanal fishermen over the years (from 1992 until its death in 2005) (Van Bressem *et al*. 2007, Moreno *et al.* 2008). In the result of STRUCTURE with K = 2 (Fig. 3), individuals with no *gephyreus* component are in the same group of ASPSP individuals, the only known offshore population. The fact that the individuals from the remaining sampled areas (that are not from offshore individuals) also have the same component of ASPSP (in K = 2) suggests that the *gephyreus* component differs from the others by distinct factors than the coastal or ocean habitat. In a much broader study for the North Atlantic, Louis *et al*. (2014) found a clear separation between the mtDNA sequences from pelagic (offshore) and coastal stocks (inshore). When we compare their sequences with ours (results not shown), our haplotypes from the *T. truncatus* clade (*i.e.* non-*gephyreus*) are grouped (including a few matches, *i.e.* identical haplotypes) with their pelagic sequences, while our haplotypes from the *T. gephyreus* clade (although more distant and with no identity) are a little closer to the haplotypes from the coastal habitat. These results indicate that currently it is difficult to directly relate the significant differentiation among the studied populations with the habitat (coastal and oceanic). It is important to note that, as in Louis *et al*. (2014), none of our sequences are grouped with the clade of individuals from the coastal region of the Northwest Atlantic, which confirms the great distinction of the latter group within *T. truncatus* (Moura *et al*. 2013). Therefore, currently there are insufficient data to test the hypothesis of inshore and offshore genetic groups of *Tursiops* in RS.

On the other hand, it is possible to test the hypothesis of an association between the morphology and genetic entities in the region, since this is the first genetic study with morphologically identified individuals (based on skull anatomy and morphometrics - Wickert 2013). We showed that there is a complete association between individuals with *T. gephyreus* morphology and a unique and divergent mitochondrial clade, as well as a

large association of these individuals with a biparental genetic component, which is also quite distinct (the *gephyreus* component). Therefore, these results suggest the existence of a real biological unit, which is different from the canonical *T. truncatus* taxon, at least in the northern coast of RS, and that is associated with the morphological proposal of *T. gephyreus* taxon (Barreto 2000, Wickert 2013). However, we found three specimens with mtDNA and morphology associated with *T. gephyreus*, but with a low proportion of the *gephyreus* component in nuclear markers, as well as two individuals with morphology and haplotype associated with *T. truncatus*, but with a relatively high proportion of component *gephyreus* in nuclear markers, suggesting the existence of some level of gene flow between these biological units, likely mediated by males.

Although the mtDNA sequences of the study of Fruet *et al.* (2014) are not available they do not have morphological information, it is possible to compare part of their results with ours, also because two individuals were sequence in both studies. They also found two distinct haplotype groups in the network (their Fig. 5) separated by four substitutions, which now we could demonstrate to correspond to *T. gephyreus* and *T. truncatus* clades described here. The former can be identified by the haplotype found in the population of Laguna (SC) and the latter by the haplotype found in the population of San Antonio Bay in Argentina. Moreover, in the unpublished study of Barreto (2000), part of the mtDNA control region (338 bp) of 17 specimens was sequenced (none of them analyzed here), including individuals from the two morphological forms. The author also found a clade completely associated with *T. gephyreus* morphology, formed by individuals found in RS, and one specimen from Argentina. Therefore, the existence of at least one separated genetic matrilineal lineage associated with a divergent morphology in RS (and perhaps further south) seems to be a fairly consistent result.

However, two points of Fruet *et al.* (2014) study suggested that this scenario may be more complex than indicated above. First, the hypothesis suggested by the morphological studies,of a basically North-South distribution of the forms *T. truncatus* and *T. gephyreus*, respectively, it is not consistent with the presence of a single haplotype of the *T. truncatus* clade in all individuals from the southernmost population (Bahía San Antonio, Argentina) (haplotype H 05 in the Figure 5 in Fruet *et al.* 2014). Besides, individuals from the above area were morphologically identified as *T. gephyreus* (Wickert 2013). Finally, the genetic structure results based on microsatellite data (Figure 3 in Fruet *et al.* 2014) do not corroborate our results related to the existence of a as very distinct *gephyreus* genetic component. The only sampling area in common between the two studies is their NPL (North of Patos Lagoon) and our RS area, but there is no evidence in their results of two distinct components in the region, as it was clearly found in the present study. Moreover, in Fruet *et al.* (2014) there was no clear association between the two groups of mtDNA haplotypes with two distinct nuclear genetic components, as we observed as a pattern in our study. It should be noted that Fruet *et al.* (2014) used a much larger number of microsatellite loci than we used here.

Therefore, combining all the information available (especially in this study and Fruet *et al.* 2014), we conclude that the species of bottlenose dolphins in the Southwestern Atlantic Ocean appears to be composed by at least four geographically distinct and well-structured management units: i) Saint Paul's Rocks; ii) Northeast of Brazil; iii) Campos and Santos Basins; and iv) San Antonio Bay, Argentina. Fruet *et al*. (2014) suggested the individuals from southern Brazil and Uruguay comprised a single unit in (which they considered as an Evolutionarily Significant Unit), but we suggest the situation is more complex, individuals the RS and perhaps even to Uruguay (without the *gephyreus* component) comprise a single unit with individuals further north, until the BC/BS.

Around the RS region, there is another unit (even partially sympatric with the other further North), that comprises individuals with the *gephyreus* genetic component and morphology.

Finally, although the results clearly point to the existence of a distinct biological entity in the Southwestern Atlantic Ocean, which is not the canonical *T. truncatus*, some aspects of this scenario are not clear enough to make any formal taxonomic proposal for the species in the region. In order to resolve this issue, it would be necessary a comprehensive and integrative study that covers the entire distribution of the species in SWA (including biopsy samples from oceanic specimens (offshore)), using both mtDNA as well as a large number of biparental markers (microsatellite or other markers), and if it is possible including the morphological description of the individuals.

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Supporting Information

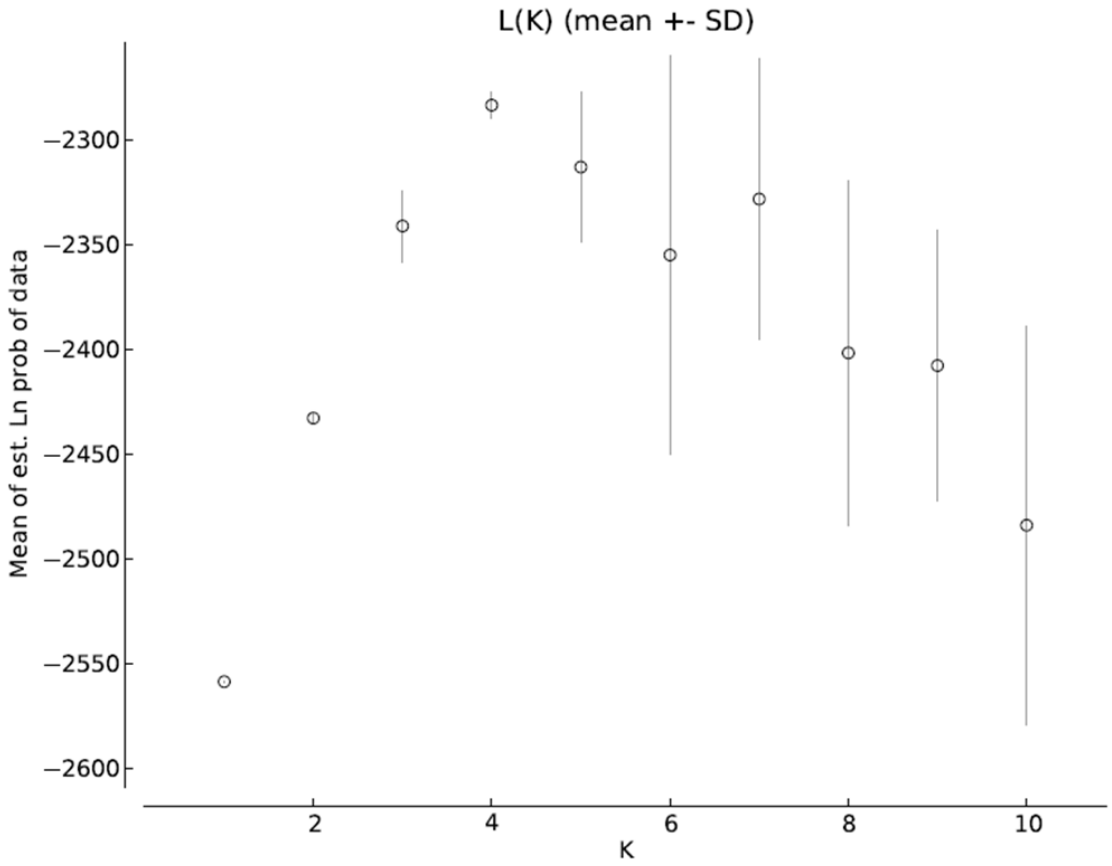

Figure 1. Log likelihood values as a function of the number genetically differentiated populations of bottlenose dolphins of the Western Tropical and Subtropical South Atlantic inferred from Bayesian STRUCTURE analysis using seven microsatellite *loci*.

Table S1. List of samples of bottlenose dolphins and respective collaborator group. N = number of samples.

Locality	Research Group	Type of sample	N
Saint Paul's Rocks (ASPSP)	GEMARS[1]	Biopsy	19
Les Hattes, French Guiana	Benoit Simon-Bouhet (CEBC/CNRS)[2]	Stranding	1
Marapanin, PA/North	GEMAM[3]	Biopsy	1
Aracati, Cruz, Fortim, Aquiraz, CE/Northeast	AQUASIS[4]	Biopsy Stranding	1 3
Natal, RN/ Northeast	GEMARS ECOMAR-PPC[5]	Biopsy Stranding	1 3
Barra Grande, BA/ Northeast	GEMARS	Biopsy	6
Campos and Santos Basins (BC/BS)	GEMM-Lagos[6]	Biopsy Stranding	38 7
Northern coast of Rio Grande do Sul, RS	GEMARS	Stranding	30

[1] Grupo de Estudos de Mamíferos Aquáticos do Rio Grande do Sul
[2] Kwata NGO, Cayenne, French Guiana & Institut Pasteur de la Guyane, Cayenne, French Guiana
[3] Grupo de Estudos de Mamíferos Aquáticos da Amazônia
[4] Associação de Pesquisa e Preservação de Ecossistemas Aquáticos
[5] ECOMAR - Projeto Pequenos Cetáceos – Rio Grande do Norte
[6] Grupo de Estudos de Mamíferos Marinhos da Região dos Lagos

Table S2. Mitochondrial DNA haplotype list: list of individuals that belong to each haplotype, haplotype frequency and sampling locality. PE: Pernambuco state, SP: São Paulo state, RJ: Rio de Janeiro state and RS: Rio Grande do Sul state.

Haplotype	Individuals	Frequency	Locality
1	ASPSP_A	1	Saint Paul's Rocks (PE)
2	ASPSP_B, ASPSP_C, ASPSP_D, ASPSP_E, ASPSP_G, ASPSP_H, ASPSP_I, ASPSP_J, ASPSP_K, ASPSP_L, ASPSP_M, ASPSP_N, ASPSP_O, ASPSP_P, ASPSP_Q, ASPSP_R, ASPSP_S, ASPSP_T	18	Saint Paul's Rocks (PE)
3	BC/BS018, BC/BS023, BC/BS028, GEMM94	4	Campos and Santos Basins (SP and RJ), Arraial do Cabo (RJ)
4	BC/BS019, BC/BS024, BC/BS030, BC/BS063 , BC/BS066, BC/BS067, BC/BS078, BC/BS079, BC/BS080, BC/BS082, BC/BS083	11	Campos and Santos Basins (SP and RJ)
5	BC/BS020, BC/BS025, BC/BS065, BC/BS070, BC/BS073, BC/BS01, GEMM132, GEMM98, GEMARS1192, GEMARS1260, GEMARS1285, GEMARS292, GEMARS916	13	Campos and Santos Basins (SP, RJ and SC) Northern coast of Rio Grande do Sul (RS)
6	BC/BS021, BC/BS022, BC/BS029, BC/BS033, GEMARS216	5	Campos and Santos Basins (SP and RJ) Northern coast of Rio Grande do Sul (RS)
7	BC/BS026, BC/BS031, BC/BS032, BC/BS064, BC/BS068, BC/BS069, BC/BS072, BC/BS074, BC/BS076, BC/BS077, GEMM60, GEMARS115, GEMARS401	13	Campos and Santos Basins (SP and RJ) Northern coast of Rio Grande do Sul (RS)
8	BC/BS027	1	Campos and Santos Basins (SP and RJ)
9	BC/BS071	1	Campos and Santos Basins (SP and RJ)
10	BC/BS075	1	Campos and Santos Basins (SP and RJ)
11	BC/BS081	1	Campos and Santos Basins (SP and RJ)
12	GEMM120	1	Campos and Santos Basins (SP and RJ)
13	GEMM17	1	Campos and Santos Basins (SP and RJ)
14	GEMM57, GEMARS1050	2	Campos and Santos Basins (SP and RJ) Northern coast of Rio Grande do Sul (RS)
15	NE001, NE002, Ne004, P1	4	Northern coast of Rio Grande do Norte (RN), Northern coast of Rio Grande do Norte (RN), Barra Grande (BA)/Northeast
16	NE003, 02c325	2	Northern coast of Rio Grande do Norte (RN) Pontal de Cima, Fortim (CE)/ Northeast
17	02c373	1	Barro Preto, Aquiraz (CE)/ Northeast
18	02c375	1	Northeast
19	P2, P3	2	Barra Grande (BA)/ Northeast
20	P4	1	Barra Grande (BA)/ Northeast
21	P5	1	Barra Grande (BA)/ Northeast
22	P7	1	Barra Grande (BA)/ Northeast

23	GEMARS1021, GEMARS1094	2	Northern coast of Rio Grande do Sul
24	GEMARS1165, GEMARS1259, GEMARS286, GEMARS400, GEMARS934	5	Northern coast of Rio Grande do Sul
25	GEMARS1235, GEMARS192, GEMARS203, GEMARS333, GEMARS503, GEMARS569, GEMARS816, GEMARS861, MPT003	9	Northern coast of Rio Grande do Sul
26	GEMARS1265	1	Northern coast of Rio Grande do Sul
27	GEMARS1283	1	Northern coast of Rio Grande do Sul
28	GEMARS1477	1	Northern coast of Rio Grande do Sul
29	GEMARS820	1	Northern coast of Rio Grande do Sul
30	GEMARS1320	1	Northern coast of Rio Grande do Sul
31	GEMAM441	1	Marapanim (Pará)/ Northeast
32	Guiana	1	Les Hattes/ French Guiana

Table S3. The mtDNA control region genetic diversity for different cetacean species, N: number of samples; H: total number of haplotypes found in each study; h: haplotype diversity and π: nucleotyde diversity.

Population	**N**	**H**	**h**	**π**
Tursiops truncatus (ASPSP this study)	**19**	**2**	**0.105**	**0.0006**
Tursiops truncatus (Northeast this study)	**14**	**9**	**0.912**	**0.019**
Tursiops truncatus (BC/BS this study)	**45**	**12**	**0.848**	**0.013**
Tursiops truncatus (RS this study)	**30**	**12**	**0.862**	**0.019**
Tursiops truncatus (total - this study)	**109**	**32**	**0.85**	**0.015**
Tursiops truncatus south Brazil, Uruguay and Argentina (Fruet *et al.* 2014)	124	9	0.71	0.009
Tursiops truncatus south Brazil (Costa *et al.* 2015)	32	8	0.71	0.0168
Tursiops truncatus coastal - Choros Island, Chile (Sanino *et al.* 2005)	8	2	-	0.0069
Tursiops truncatus oceanic - Chile (Sanino *et al.* 2005)	8	6	-	0.0200
Tursiops truncatus coastal - Peru (Sanino *et al.* 2005)	3	2	-	0.0021
Tursiops truncatus oceanic - Peru (Sanino *et al.* 2005)	12	12	-	0.0179
Tursiops truncatus coastal - Northwestern Atlantic (Hoelzel *et al.* 1998)	29	6	-	0.006
Tursiops truncatus oceanic - Northwestern Atlantic (Hoelzel *et al.* 1998)	26	12	-	0.027
Tursiops truncatus north Bahamas (Parsons *et al.* 2006)	56	11	0.76	0.006
Tursiops truncatus coastal – Caribe (Caballero *et al.* 2012)	112	22	0.57	0.009
Tursiops truncatus Gulf of México (Sellas *et al.* 2005)	56	7	0.79	0.009
Tursiops truncatus coastal - Northeastern Atlantic (Natoli *et al.* 2004)	9	2	0.42	0.016
Tursiops truncatus coastal - (north) Northeast Atlantic (Louis *et al.* 2014)	76	5	0.66	0.006
Tursiops truncatus coastal - (south) Northeast Atlantic (Louis *et al.* 2014)	115	4	0.49	0.001
Tursiops truncatus pelagic - Northeast Atlantic (Louis *et al.* 2014)	101	38	0.92	0.014
Tursiops truncatus Azores (Quérouil *et al.* 2007)	83	29	0.95	0.015
Tursiops truncatus Madeira (Quéroil *et al.* 2007)	24	14	0.92	0.012
Tursiops truncatus Portugal (Quéroil *et al.* 2007)	7	5	0.85	0.014
Tursiops truncatus pelagic - Mediterranean Sea (Louis *et al.* 2014)	51	15	0.90	0.013
Tursiops truncatus coastal - Gulf of California (Segura *et al.* 2006)	32	11	0.86	0.011
Tursiops truncatus oceanic - Golfo da California (Segura *et al.* 2006)	51	20	0.94	0.013
Tursiops truncatus Hawaii (Martien *et al.* 2011)	130	25	0.88	0.022
Tursiops truncatus South Africa (Natoli *et al.* 2004)	38	5	0.29	0.008
Tursiops truncatus pelagic - China (Natoli *et al.* 2004)	17	12	0.92	0.024
Tursiops aduncus China (Natoli *et al.* 2004)	19	11	0.88	0.015
Pontoporia blainvillei (Cunha *et al*. 2014)	162	30	0.86	0.009
Delphinus sp. (Stockin *et al.* 2014)	84	65	0.99	0.017
Phocoena phocoena Pacific Ocean (Rosel *et al.* 1995)	-	-	0.90	0.0137
Phocoena phocoena Pacific Ocean (Rosel *et al.* 1995)	-	-	0.89	0.0081
Eubalaena australis (Patenaude *et al.* 2007)	136	34	0.91	0.0271
Megaptera novaengliae (Félix *et al.* 2012)	182	41	0.92	0.019