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Population structure, phylogeography, and genetic diversity of the common bottlenose dolphin in the tropical and subtropical southwestern Atlantic Ocean

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We assessed the level of genetic variability and population structure of the common bottlenose dolphin (*Tursiops truncatus*) in the tropical and subtropical portions of the southwestern Atlantic Ocean and compared the results with previous morphological findings. We analyzed 109 samples of common bottlenose dolphins that were sequenced for control region mtDNA and genotyped for seven polymorphic microsatellite loci. The results suggested that the species in this region can be separated in two major biological units, northern and southern, with an area of parapatry at southern Brazil. The northern unit seems to occur in a wide range of depths, including

offshore waters, is consistent with the canonical morphology of *T. truncatus*, and can be divided into three management units: 1) Saint Paul's Rocks, 2) north and northeast of Brazil, and 3) Campos and Santos Basins (that extend at least to southernmost Brazil). The southern unit is coastal, occurring exclusively in very shallow waters (< 10 m) and estuaries, and is consistent with the previously described (putative) *Tursiops gephyreus*. Nevertheless, a formal decision on the taxonomic status of *T. gephyreus* should wait for a more geographically comprehensive and data-integrative study.

O objetivo deste estudo foi avaliar o nível de variabilidade genética e a estrutura populacional do golfinho-nariz-de-garrafa (*Tursiops truncatus*) das porções tropical e subtropical do Oceano Atlântico Sul Ocidental e comparar os resultados com análises morfológicas prévias. Para tanto, analisamos 109 amostras de golfinhos-nariz-de-garrafa, as quais foram sequenciadas para a região controle do DNAmt e genotipadas para sete loci de microssatélites polimórficos. Os resultados sugerem que a espécie nessa região pode ser separada em duas grandes unidades biológicas, norte e sul, com uma área de parapatria no sul do Brasil. A unidade norte parece ocorrer em uma faixa de profundidade ampla, incluindo águas oceânicas. Esta unidade é consistente com a morfologia canônica de *T. truncatus* e pode ser dividida em três unidades de manejo: i) Arquipélago de São Pedro e São Paulo; ii) Norte e Nordeste do Brasil; iii) Bacia de Campos e Santos (que se estende pelo menos até o extremo sul do Brasil). A unidade sul é costeira, ocorrendo exclusivamente em águas muito rasas (< 10 m) e estuários. Esta unidade é consistente com descrições anteriores para *Tursiops gephyreus* (putativo). Entretanto, uma decisão formal sobre o status taxonômico de *T. gephyreus* deve aguardar estudos mais integrativos e geograficamente abrangentes.

Key words: biological conservation, Brazilian coast, cetaceans, ecotypes, genetic diversity, microsatellites, mtDNA

The common bottlenose dolphin, *Tursiops truncatus* (Montagu 1821), has a cosmopolitan distribution, occurring in both tropical and temperate waters, as well as in coastal and oceanic habitats (Wells and Scott 1999; Reynolds et al. 2000). This broad distribution combined with wide morphological variation and high levels of structuring (e.g., Hoelzel et al. 1998; Parsons et al. 2002, 2006; Natoli et al. 2004; Caballero et al. 2012; Louis et al. 2014) resulted in the description of more than 20 different *Tursiops* species in the past (Walker 1981; Hersh and Duffield 1990; Ross and Cockcroft 1990; Wells and Scott 1999).

Currently, only *T. truncatus* and the Indo-Pacific bottlenose dolphin, *Tursiops aduncus* (Ehrenberg 1833), are recognized by the Committee on Taxonomy of the Society for Marine Mammalogy (Committee on Taxonomy 2017). However, molecular studies have suggested the existence of at least two more species: *Tursiops australis* in South Australia (Bilgmann et al. 2007; Möller and Harcourt 2008; Charlton-Robb et al. 2011) and an unnamed variation of *T. aduncus* on the coast of South Africa (Natoli et al. 2004). The distribution of common bottlenose dolphins in the southwestern Atlantic Ocean (SWA) encompasses coastal and oceanic waters from Pará state, in the northern Brazilian coast, to the province of Tierra del Fuego, Argentina (e.g., Laporta et al. 2016; Lodi et al. 2016; Milmann et al. 2017). More specifically in Brazilian waters, sightings, strandings, and incidental captures of common bottlenose dolphins have been reported across the entire coastline, without any evidence of an interrupted latitudinal distribution pattern (Lodi et al. 2016).

Studies on skull morphometrics suggested the existence of a distinct form of *T. truncatus* in the southern region of the SWA, proposed to be a subspecies (*Tursiops truncatus gephyreus*) by Costa et al. (2016) or a different species, *T. gephyreus*, first proposed by Lahille (1908) and recently resurrected by Wickert et al. (2016). It was proposed that *T. gephyreus* occurs from southern Brazil to the south (see Costa et al. 2016; Wickert et al. 2016) while *T. truncatus* is basically distributed from southern Brazil to the north. However, given the controversial status for the southern taxa and for simplicity, here we refer to this distinct morphotype as putative *T. gephyreus*, without assuming a species status. These morphological studies also revealed the existence of a large zone of co-occurrence between the two forms in southern Brazil (Costa et al. 2016; Wickert et al. 2016). One hypothesis suggests they are distinct units with a parapatric distribution, offshore (*T. truncatus truncatus*) and coastal (*T. truncatus gephyreus*—Costa et al. 2016), while the other suggests they are partially sympatric species (*T. truncatus* and *T. gephyreus*) based on the latitudinal overlap of the stranding records in southern Brazil (roughly from 25°30′S to 31°15′S) (Wickert et al. 2016). In fact, the existing information about these two distinct common bottlenose dolphins in the SWA is largely reliant on stranded individuals, which precludes a better comprehension of their spatial distribution. However, analysis of some previously photo-identified estuarine dolphins stranded in the southernmost areas of Brazil supports their identity as putative *T. gephyreus* (Wickert et al. 2016).

This coastal-estuarine population is suspected to be under a higher risk of extinction, mainly due to its incidental mortality in coastal gillnets (e.g., Fruet et al. 2012), habitat degradation (e.g., Daura-Jorge and Simões-Lopes 2011), and supposed small population size (Lodi et al. 2016 and references therein). On the other hand, very scanty information is available on the offshore populations of *T. truncatus* in southern Brazil, precluding any status assessment. In view of that, the putative *T. gephyreus* is currently classified as "vulnerable" (VU—criterion D1) in the southernmost Brazilian state (Rio Grande do Sul state—RS), whereas the offshore population of *T. truncatus* is considered as "data deficient" (DD), based on the International

Union for Conservation of Nature (IUCN) criteria (RS State Endangered Species Decree No. 51.797, of 8 September 2014).

Differentiation among geographically close populations of *T. truncatus* could be related to the species' social system and site fidelity, as demonstrated by photo-identification studies (e.g., Parsons et al. 2006; Baird et al. 2009). Coastal (nearshore) and oceanic (offshore) forms of the species in the Atlantic Ocean have been described in several regions based on morphological, ecological, or genetic evidences (e.g., Duffield et al. 1983; Mead and Potter 1995; Hoelzel et al. 1998; Natoli et al. 2005; Louis et al. 2014; Costa et al. 2016; Fruet et al. 2017).

The first genetic study on the common bottlenose dolphin in the SWA (an unpublished thesis—Barreto 2000) suggested some distinction between *T. truncatus* and the putative *T. gephyreus*. However, it was very preliminary, since only 17 mitochondrial DNA (mtDNA) control region sequences were obtained, mostly from southern Brazil and from the putative *T. gephyreus* (called "southern form" by Barreto 2000), and only three sequences from *T. truncatus*, the "northern form" (see a review in Ott et al. 2016). Afterwards, an mtDNA study found that common bottlenose dolphin populations exhibited a strong genetic structure along the Brazilian coast, including an oceanic population around Saint Paul's Rocks (also known as São Pedro and São Paulo Archipelago), and two other populations in southeastern (Rio de Janeiro and São Paulo states) and southern Brazil (Rio Grande do Sul state—Ott et al. 2009). Significant genetic differentiation among common bottlenose dolphins from southern Brazil and Uruguay and those from Argentina (San Antonio Bay, SAB) in both mtDNA and nuclear microsatellites was used to classify them as two evolutionary significant units (ESUs—Fruet et al. 2014). These latter authors also suggested that there are five small management units (MUs) in coastal waters of southern Brazil and Uruguay, mainly related to the estuarine systems. Both ESU and MU concepts have been widely used in conservation biology, the former being genetically highly differentiated (usually isolated) populations that have high priory for conservation and the latter considered as populations whose degree of differentiation from others is sufficient so that they should be managed separately (see Funk et al. 2012 for further details). More recently, Fruet et al. (2017) analyzed genetic data (mtDNA and nuclear microsatellites) of common bottlenose dolphins with different external morphology (using pictures) collected in coastal and offshore waters in southern Brazil and Argentina. The authors found a high genetic differentiation between these two populations, referred by them as ecotypes, and suggest that the offshore ecotype should be considered as an additional ESU in the SWA.

Despite these recent advances on genetic structure of *T. truncatus* in the tropical and subtropical Atlantic Ocean off South America, these studies are still restricted geographically and rarely combine molecular with morphological data (the exceptions being the unpublished Barreto 2000 thesis and Fruet et al. 2017). In fact, a study examining the population structure of *Tursiops* within a wider geographic area and combining distinct genetic markers and osteological data has not been carried out along the eastern coast of South America.

This study aims to assess the level of genetic variability and population structure of common bottlenose dolphins, using both mitochondrial and nuclear data, in a broader geographical scale in the tropical and subtropical SWA. Moreover, since this is the first genetic study that included individuals that were morphologically identified as belonging to *T. truncatus* and the putative *T. gephyreus*, it also allows for investigating whether genetic evidence supports the hypothesis that these *Tursiops* represent different taxa. Finally, our data also contribute to a better understating of the relationship of the common bottlenose dolphins of the Brazilian coast in the context of the worldwide phylogeny of the genus (e.g., Moura et al. 2013).

Materials and Methods

Sample collection and DNA extraction.—Tissue samples from 45 stranded and 64 biopsied common bottlenose dolphins were obtained by a large collaboration network working along the Atlantic coast of South America from French Guiana to southern Brazil (Fig. 1; Supplementary Data SD1), including oceanic waters (Saint Paul's Rocks). The biopsies were collected only in four areas (from southern to northeastern Brazil): 1) in Campos and Santos Basins (CB–SB) (from 21°40′S to 27°00′S) during cetacean-sighting cruises conducted from September 2004 to February 2005, with an effort of 1,337 km surveyed in coastal and offshore waters; 2) in Barra Grande–Maraú (13°46′S; 38°49′W), Bahia state (northeastern Brazil), during dedicated surveys for humpback whales (*Megaptera novaeangliae*) in relative coastal waters (< 10 miles from the coast) from September to October 2009; 3) in Natal, Rio Grande do Norte state (northeastern Brazil), a single dolphin was sampled close to the mouth of the river Potengi (05°45′S; 35°11′W), within the first mile from the coast, in 6 January 2005; and 4) in Saint Paul's Rocks (00°56′S; 29°22′W), during a month of photo-identification effort of common bottlenose dolphins (from 10 January to 10 February 2005) around the archipelago (< 3.2 km from the shores of the archipelago and up to 200 m water depth) in offshore waters about 1,010 km away of the Brazilian coast (see Milmann et al. 2017 for a detailed map of the usual distribution of the resident common bottlenose dolphins in this area). The biopsy samples were collected from multiple groups over the course of several encounters, and all samples (even from stranded specimens) were checked for replicates (i.e., multiple sampling of the same individual) using their genotypes by MICRO-CHECKER (Oosterhout et al. 2004). The biopsy sampling procedure followed ASM guidelines (Sikes et al. 2016).

Sampling sites were grouped into four main geographic areas: 1) Saint Paul's Rocks (SPSPA, n = 18; an archipelago of ultramafic rocks that occur in in-situ outcrops located in offshore waters of northeast of Brazil; Fig. 1); 2) north–northeast (NE), with samples from the Brazilian states of Pará (PA, n = 1), Ceará (CE, n = 4), Rio Grande do Norte (RN, n = 4), and Bahia (BA, n = 6); 3) Campos and Santos Basins (CB–SB, n = 45);

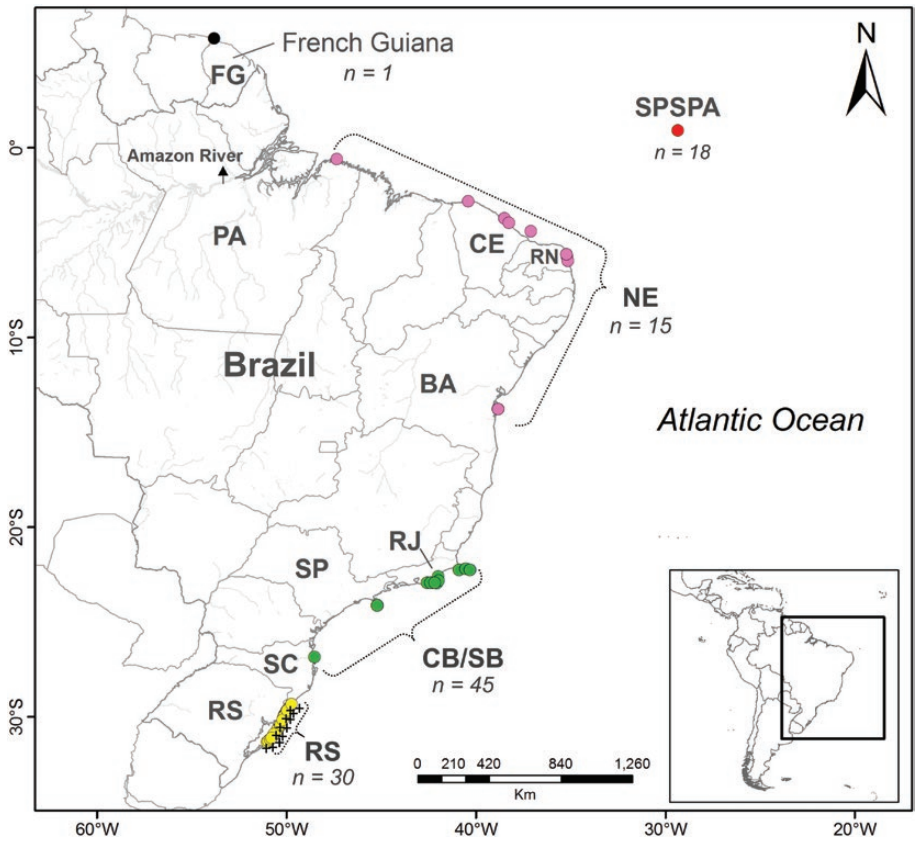

Fig. 1.——Map with the sampling localities of common bottlenose dolphins (*Tursiops truncatus*), including specimens of the putative *Tursiops gephyreus* (crosses), from the Southwestern Atlantic Ocean. The labels refer to geographic areas considered in the analyses: Saint Paul's Rocks (SPSPA), French Guiana (FG), north–northeast (NE), Campos and Santos Basins (CB–SB), and Rio Grande do Sul (RS). Inside the map are abbreviations of pertinent Brazilian states (from North to South: PA = Pará, CE = Ceará, RN = Rio Grande do Norte, BA = Bahia, RJ = Rio de Janeiro, SP = São Paulo and SC = Santa Catarina).

and 4) Rio Grande do Sul (RS, n = 30). We also included a sample from a single-stranded individual from French Guiana.

Of the 30 samples from RS, 22 had been previously identified by morphological characters as either *T. truncatus* (n = 9) or putative *T. gephyreus* (n = 13—Wickert et al. 2016). Unfortunately, the remaining eight skulls were not available for analysis. Tissue samples of all specimens were cryopreserved at −20°C in 96% ethanol with 20% dimethyl sulfoxide (DMSO) saturated with sodium chloride (Amos and Hoelzel 1991). The genomic DNA was extracted with phenol-chloroform protocol (Sambrook et al. 1989) as adapted by Shaw et al. (2003).

Mitochondrial DNA amplification and analyses.—The following primers were used to amplify a 316-base pair (bp) region of the mtDNA control region: L15926 THR (5′-TCAAAGCTTACACCAGTCTTGTAAACC-3′—Kocher et al. 1989) and H16498 (5′-CCTGAAGTAGGAACCAGATG-3′—Rosel et al. 1994). Each PCR was conducted in a 20 µl reaction volume containing 20 ng of template DNA; 1× PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl); 0.2 mM deoxynucleotides (dNTPs); 0.1 mg/ml BSA; 3.5 mM $MgCl_2$; 0.2 µM of each primer; 1 U of Taq DNA Polymerase Platinum (Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts). The following PCR conditions were used: one cycle of 5 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 Corp., Piscataway, New Jersey) following the manufacturer's recommended protocol. All fragments were then sequenced from both ends on a MegaBACE 1000 capillary sequencer using the DYEnamic ET Dye Terminator Cycle Sequencing Kit (Amersham Biosciences). Sequence quality was visually checked with ChromasPro 1.7 (http://technelysium.com.au) and automatically aligned (with minor manual correction) in CLUSTALW and MEGA 7 (Tamura et al. 2013), resulting in a consensus fragment of 316-bp portion for all individuals.

All laboratory analyses, excepting sequencing (Macrogen Inc., Seoul, South Korea), were carried out in Laboratory of Genomics and Molecular Biology of Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS).

Haplotype (*Hd*) and nucleotide diversities (π) were estimated for the entire sample set and for each sampled area separately using ARLEQUIN 3.5.1 (Excoffier and Lischer 2010). Analysis of molecular variance (AMOVA) between and within sampled areas and pairwise F-statistics between areas, using both F_{ST} (Weir and Cockerham 1984) and Φ_{ST} (using pairwise differences) approaches were estimated with ARLEQUIN. The Tajima's D and Fu's F_S neutrality tests were also performed

with ARLEQUIN. All these analyses were performed with 10,000 permutations. Significance levels (F_{ST}: $P < 0.01$; Φ_{ST}: $P < 0.001$) were corrected for multiple comparisons with the sequential Bonferroni method (Rice 1989; see Table 3) to test if there are deviations from Hardy–Weinberg equilibrium (HWE). A haplotype network was constructed using the median-joining approach (Epsilon = 0 and other parameters as default—Bandelt et al. 1999) implemented in NETWORK 4.6.11 (http://www.fluxus-engineering.com).

Our SWA sequences were aligned (as above) with the whole mtDNA genomes of Moura et al. (2013) (GenBank PopSet: 557468180), that presented the most comprehensive and well-resolved mtDNA phylogeny of the genus, and also with sequences from the northeast Atlantic (Louis et al. 2014) (GenBank PopSet: 572098934), since standard BLAST searches of our sequences against GenBank found several matches between them. Our objective here was not to resolve the phylogeny of the genus, since our and most sequences available are too short for this, but to evaluate how our SWA sequences compare with the other Atlantic sequences and to place our sequences in the general context of *Tursiops* spp. mtDNA global phylogeny as estimated with the mtDNA genomes (from Moura et al. 2013). To achieve this, we used a maximum parsimony (MP) method maintaining the whole mtDNA genomes in the alignment (16,363 positions) which created a backbone for the tree where the smaller sequences would be placed. The MP tree was estimated in MEGA version X (Kumar et al. 2018) using the Subtree-Pruning-Regrafting algorithm with search level 2 in which the initial trees were obtained by the random addition of sequences (10 replicates). Finally, we also aligned our sequences with those from the closer Wider Caribbean samples by Caballero et al. (2012) (GenBank PopSet: 359291922) using the same parameters for the MP tree.

Microsatellite DNA amplification and analyses.—We successfully amplified seven polymorphic loci previously developed for cetaceans: *KWM2b*, *KWM9b*, and *KWM12a* (Hoelzel et al. 1998), *EV37Mn* (Valsecchi and Amos 1996), *TexVet5* and *TexVet7* (Rooney et al. 1999), and *D08* (Shinohara et al. 1997).

The microsatellite PCR reactions were carried out following the protocol in Natoli et al. (2004). Each PCR was conducted in a 20 µl reaction volume containing 20 ng of genomic DNA; 1× PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl), 0.1 nM of dNTPs; 1.5 mM $MgCl_2$; 0.016 µM forward primer; 0.25 µM of reverse primer; 0.5 U of Taq DNA Polymerase Platinum (Invitrogen); 0.2 µM of a M13 primer (5′-CACGACGTTGTAAAACGAC-3′). Forward primers were 5′-tailed with the M13 sequence that was used in combination with a M13 primer marked with 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 nine 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. PCR reactions were done individually, but the genotyping reactions were made in multiplex: groups of three PCR reactions (total of 25 µl) were gathered together in a plate of 96 wells for sequencing (5 µl of one reaction with FAM, NED, and HEX were added to a 15 µl of H_2O).

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).

Genetic diversity of each population was estimated based on average number of alleles across loci (*A*), average number of alleles across individuals (*K*), exclusive alleles (*E*), and observed heterozygosity (H_o) determined with ARLEQUIN. Deviations from HWE (Guo and Thompson 1992), and linkage disequilibrium (LD), expected (H_e) and observed heterozygosity (H_o) were also calculated with ARLEQUIN. AMOVA and *F*-statistics for all samples as a whole and for pairwise (a priori identified) populations (F_{ST}- and R_{ST}-like methods) were calculated with ARLEQUIN. Significance levels for departure from HWE, LD, and AMOVA were corrected for multiple comparisons (F_{ST}: $P < 0.001$; R_{ST}: $P < 0.0001$) with the sequential Bonferroni method (Rice 1989; see Table 5).

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 different values of *K* ranging from $K = 1$ to $K = 10$ genetic groups, applying 1,000,000 Markov chain Monte Carlo steps after a burn-in period of 1,000,000. We used the admixture and correlated allele frequencies models with sampling location as prior information to assist the clustering with the LOCPRIOR model (Hubisz et al. 2009), following the four areas defined previously.

The optimal number of clusters was determined using the Evanno method (Evanno et al. 2005) as implemented by STRUCTURE HARVESTER (Earl and vonHoldt 2012). Lastly, the STRUCTURE results were summarized in CLUMPP software (Jakobsson and Rosenberg 2007) and represented graphically using the DISTRUCT program (Rosenberg 2004).

Results

mtDNA control region.—The 316-bp alignment of 109 common bottlenose dolphin mtDNA control region sequences for the SWA dolphins only (these do not include other haplotypes from GenBank) resulted in 30 variable sites (17.75 transition [92.2%] and 1.50 transversion [8.8%] substitutions) in 32 unique haplotypes (Supplementary Data SD2). Genetic diversity for the entire data set combined was relatively high ($Hd = 0.86$, $\pi = 1.56\%$) as well as for each region (Table 1). The exception was the area of Saint Paul's Rocks, which presented only two haplotypes (from 18 samples) and very low genetic diversity ($Hd = 0.11$; $\pi = 0.07\%$). The single sequence from French Guiana was not included in the population analyses.

The haplotype network loosely resembles a star (Fig. 2), but with no central haplotype and with some very divergent ones,

Table 1.—Genetic diversity (*Hd* = haplotype diversity, π = nucleotide diversity [%]) estimated for 316 bp of the mitochondrial DNA (mtDNA) control region sequences of common bottlenose dolphin (*Tursiops truncatus*), including specimens of the putative *Tursiops gephyreus*, from the southwestern Atlantic Ocean. *n* = number of samples, *S* = variable sites, *H* = number of haplotypes, *SD* = standard deviation. Area abbreviations as in Fig. 1. *Value significant ($P < 0.05$) after Bonferroni correction.

Areas	*n*	*S*	*H*	*Hd* (*SD*)	π % (*SD*)	Tajima's *D* (*P*)	Fu's F_S (*P*)
SPSPA	18	2	2	0.11 (0.09)	0.07 (0.06)	−1.51* (0.04)	0.59 (0.41)
NE	15	14	9	0.91 (0.06)	1.94 (0.30)	−0.23 (0.43)	−0.45 (0.40)
CB/SB	45	18	12	0.85 (0.03)	1.34 (0.17)	−0.42 (0.37)	−0.38 (0.48)
RS	30	20	12	0.86 (0.04)	1.95 (0.18)	−0.29 (0.43)	0.59 (0.63)
Total	108	30	32	0.86 (0.03)	1.56 (0.12)	−0.61 (0.32)	0.08 (0.48)

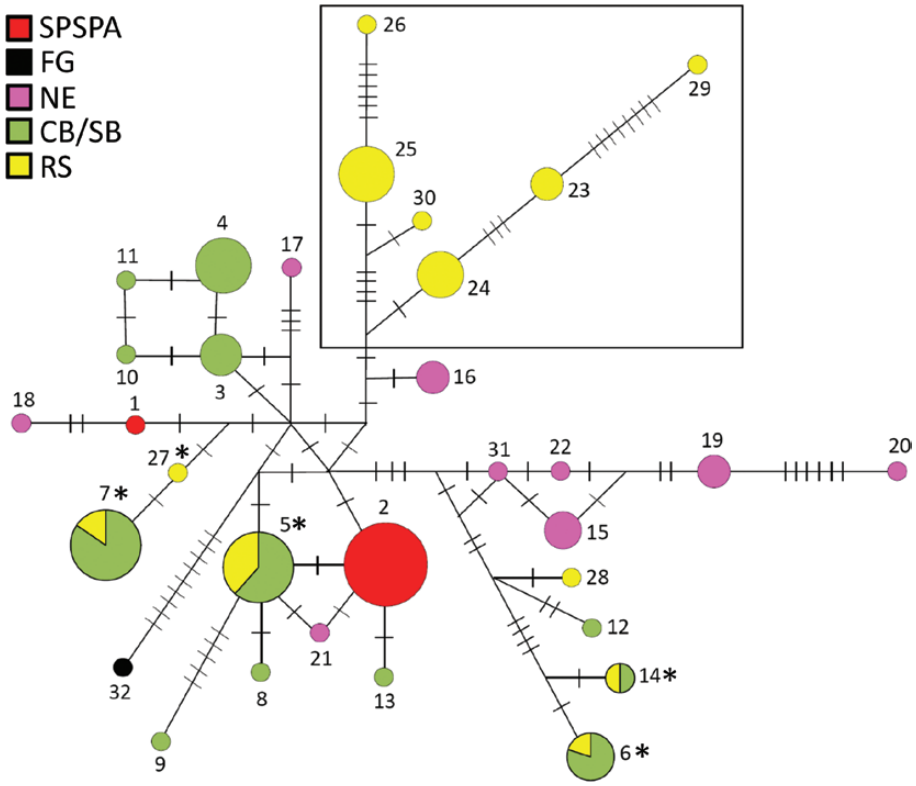

Fig. 2.——Median-joining network of mitochondrial DNA (mtDNA) control region sequences of common bottlenose dolphins (*Tursiops truncatus*), including specimens of the putative *Tursiops gephyreus* (crosses), from the southwestern Atlantic Ocean. The circles represent the haplotypes found and their sizes are proportional to the haplotype frequency across all 108 sampled individuals. The mutational steps are represented by the number of bars in the branches and the nodes represent unsampled haplotypes. The rectangle indicates the haplotypes found in putative *Tursiops gephyreus* (named as the *gephyreus* haplogroup) and * indicate haplotypes found in *T. truncatus*. The sampling localities of individuals follow the legend and abbreviations in the Fig. 1. See this figure online for the color version.

like the French Guiana sequence. Shared haplotypes occurred only between RS and CB–SB (four cases); the other haplotypes are exclusive to each area. There is clade of five haplotypes that are present only in individuals from NE and another with six haplotypes found only in individuals from RS (identified by a rectangle in Fig. 2). This latter clade contains the haplotypes found in all the individuals morphologically identified as the putative *T. gephyreus* (*n* = 13) and none of those identified as *T. truncatus* (*n* = 9—Wickert et al. 2016). Four dolphins with haplotypes from this clade did not have morphological identification, since the skulls were not available for analysis. We named this clade associated with the putative *T. gephyreus*, the *gephyreus* haplogroup. BLAST searches of these six haplotypes against GenBank found no identical match. As the individuals associated with the *T. truncatus* morphology are distributed in other parts of the network, for simplicity we refer to the rest of the haplotype network as the *truncatus* haplogroup. Individuals from the NE area show a relatively wide geographic distribution (Fig. 1) and high genetic diversity (Table 1). Five of the nine haplotypes were grouped in an exclusive clade, including the haplotype from one specimen collected further north in Brazil (Pará state [PA], haplotype 31), as well as five out of six individuals (haplotype 21 is the exception) collected further to the south (Bahia state [BA]).

The AMOVA suggested strong population differentiation, with ~27% ($P < 0.05$) of the genetic variability being partitioned among the studied areas (Table 2). The pairwise F_{ST} and Φ_{ST} were highly significant between all areas; the SPSPA population was the most different genetically (Table 3). Tajima's *D* and Fu's F_S tests of selective neutrality were mostly negative,

Table 2.—Analysis of molecular variance (AMOVA) for the mitochondrial DNA (mtDNA) control region (F_{ST} and Φ_{ST}) and for the microsatellite data (F_{ST} and R_{ST}) for the whole sample of common bottlenose dolphins (*Tursiops truncatus*), including specimens of the putative *Tursiops gephyreus*. Groups used in this analysis: north/northeast (including samples of Brazilian states of Pará, Ceará, Rio Grande do Norte, and Bahia); Campos and Santos Basins (CB/SB); and Rio Grande do Sul. All values significant ($P < 0.01$) after Bonferroni correction.

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

Table 3.—Pairwise *F*-statistics for mitochondrial DNA (mtDNA) control region of common bottlenose dolphins (*Tursiops truncatus*), including specimens of the putative *Tursiops gephyreus*, between areas: Φ_{ST} (above diagonal) and F_{ST} (below diagonal). Area abbreviations as in Fig. 1. All values significant (*$P < 0.01$; **$P < 0.001$) after Bonferroni correction.

Areas	SPSPA	NE	CB–SB	RS
SPSPA		0.548**	0.152**	0.370**
NE	0.529**		0.323**	0.260**
CB–SB	0.444**	0.120**		0.222**
RS	0.468**	0.112*	0.095**	

Table 4.—Genetic parameters for the microsatellite data of common bottlenose dolphins (*Tursiops truncatus*), including specimens of the putative *Tursiops gephyreus*. *n* = number of individuals analyzed, *A* = number of alleles, *K* = average number of alleles, *E* = exclusive alleles, H_o = observed heterozygosity, H_e = expected heterozygosity. Area abbreviations as in Fig. 1. The single French Guiana individual was not included here.

Areas	*n*	*A*	*K*	*E*	H_o	H_e (*SD*)
SPSPA	18	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)
CB/SB	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	100	12	6.89	3.71	0.52	0.76 (0.17)

Table 5.—Pairwise *F*-statistics (microsatellite data) of common bottlenose dolphins (*Tursiops truncatus*), including specimens of the putative *Tursiops gephyreus*, between areas: R_{ST} (above diagonal) and F_{ST} (below diagonal). Area abbreviations as in Fig. 1. The single French Guiana individual was not included here. All values significant (*$P < 0.001$; **$P < 0.0001$) after Bonferroni correction.

Areas	SPSPA	NE	CB–SB	RS
SPSPA		0.124*	0.085*	0.131**
NE	0.113**		0.158*	0.141**
CB–SB	0.084**	0.099**		0.118**
RS	0.126**	0.107**	0.077**	

but nonsignificant (except for SPSPA), indicating no strong evidence for recent population size changes for the species as a whole or for each separate area (Table 1).

The maximum parsimony tree of *Tursiops* mtDNA diversity (Supplementary Data SD3) maintained the main topology found in the mtDNA genome phylogeny (figure 2 of Moura et al. 2013). Considering only the major relationships, our sequences are placed inside the *T. truncatus* clade (as defined in figure 2 of Moura et al. 2013), not being related to the very divergent *T. aduncus* and *T. australis* clades. Our sequences seem also not related to coastal western North Atlantic individuals (the WNAC haplotypes), which supports the great distinction of the latter group within *T. truncatus* (Moura et al. 2013; Louis et al. 2014). Within the *T. truncatus* clade, our haplotypes are mostly distributed in small sets inside some major *T. truncatus* clades (see also figure 2 of Moura et al. 2013). The six haplotypes from the *gephyreus* haplogroup grouped with haplotypes from northeast Atlantic coastal habitats (Clade B, Supplementary Data SD3). A larger set of the haplotypes associated with the *T. truncatus* morphology (mostly from the oceanic SPSPA and from CB–SB) is closely related to a large group of exclusively North Atlantic pelagic individuals (including several identical matches with our haplotypes), especially from eastern areas (Clade A, Supplementary Data SD3). Another set of *T. truncatus* haplotypes (that includes most of the NE haplotypes) grouped with the other group of North Atlantic pelagic individuals (Clade C, Supplementary Data SD3). None of our sequences, including those found in the putative *T. gephyreus*, grouped with the Wider Caribbean "inshore"–coastal clade found by Caballero et al. (2012; Supplementary Data SD4). Within their "worldwide distributed"–oceanic–pelagic clade, our sequences mostly were located in separate subgroups. Only four Wider Caribbean haplotypes were closer to some of our sequences, mostly those that were also closer (see Caballero et al. 2012) to those from Madeira and the Azores (Quérouil et al. 2007) and haplotypes described as WNA pelagic (WNAp, by Natoli et al. 2004), corroborating our findings using Louis et al. (2014) and Moura et al. (2013) sequences described above.

Microsatellites.—The 100 individuals were genotyped (nine stranded specimens were removed from the analyses because they only amplified few loci), and the seven microsatellite loci were highly polymorphic, with an average expected heterozygosity of 0.76 (*SD* = 0.057) and an average number of alleles per locus of 6.89 (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, no locus was excluded from the analyses.

After Bonferroni corrections, LD was detected only in RS for the pairs *KWM2b*, *KWM9b* and *EV37Mn*, *KWM2b*. Deviation from HWE was detected in *EV37Mn*, *KWM9b* in SPSPA, and *D08*, *EV37Mn*, *KWM9b* in CB–SB, and in all loci except for *Tv7* in the RS area. The RS area corresponded to an admixed area between at least two biological entities (*T. truncatus* and the putative *T. gephyreus*, see results below). Since there was no consistency between loci and areas in LD and deviation from HWE, indicating low levels of interactions between loci, no locus was excluded from the analyses (e.g., D'Aoust-Messier and Lesbarrères 2015).

The AMOVA results based on microsatellite data showed a low (~10%) but significant between-population genetic variation (Table 2). The pairwise F_{ST} and R_{ST} values between the

four areas were moderate and all significant ($P < 0.001$ and $P < 0.0001$; Table 5). The single French Guianan individual was not included in these population analyses.

The mean likelihood value (2285.72 ± 4.41) for 10 independent runs in STRUCTURE peaked at $K = 4$ genetic clusters (Supplementary Data SD5A); three of them in general correspond to the geographic areas used here (Fig. 3, $K = 4$), except for the RS area where at least three genetic clusters were present. The Evanno's Delta K value peaked at $K = 3$, showing a similar pattern to the $K = 4$ scenario, but grouping SPSPA and NE (Supplementary Data SD5B). In this analysis, the individual from French Guiana grouped with the SPSPA population. The genetic composition of the samples from RS is complex: 14 of the 27 individuals in this area shared > 50% proportional membership with a fourth genetic cluster (Fig. 3, yellow in the online version and white in the printed version), six individuals shared > 50% proportional membership with the genetic cluster characteristic of the CB–SB area (Fig. 3, green in the online version and medium gray in the printed version), three dolphins shared > 50% proportional membership with the cluster characteristic of the NE population (Fig. 3, pink in the online version and light gray in the printed version), and four individuals shared < 50% proportional with several of the other genetic clusters. Ten out of 13 individuals morphologically identified as putative *T. gephyreus* (rectangle with dashed line in Fig. 3) shared > 70% proportional membership with the fourth genetic component (Fig. 3, yellow in the online version and white in the printed version, hereafter referred as the *gephyreus* component), while the other three shared at least 50% proportional membership with the CB–SB (Fig. 3, green in the online version and medium gray in the printed version) cluster. On the other hand, individuals morphologically identified as *T. truncatus* presented a diversified genetic background, some being more similar to NE or CB–SB individuals, and two presenting a considerable proportional membership with the *gephyreus* component (the first two left columns in "T. tru" in Fig. 3). Five genotyped individuals from RS did not have morphological identification: three of them had > 90% proportional membership of the *gephyreus* component (the last three individuals on the right), one (the first left of the "T. gep group") showed ~50% proportional membership of this component, and one (the first right of the "T. tru group") had a very low proportion of this component. The first four of those individuals are also the only ones (other than the specimens identified morphologically as putative *T. gephyreus*) that had mtDNA haplotypes from the *gephyreus* haplogroup (Fig. 2). In an exploratory analysis with $K = 2$ (Fig. 3), the two genetic groups formed seemed associated (although not perfectly) with the putative *T. gephyreus* and *T. truncatus*, rather than with the geographical distances or areas, suggesting some genetic differentiation between these two independent of geography.

Discussion

SWA population structure.—The results of the present study indicate significant geographic structure, especially for the

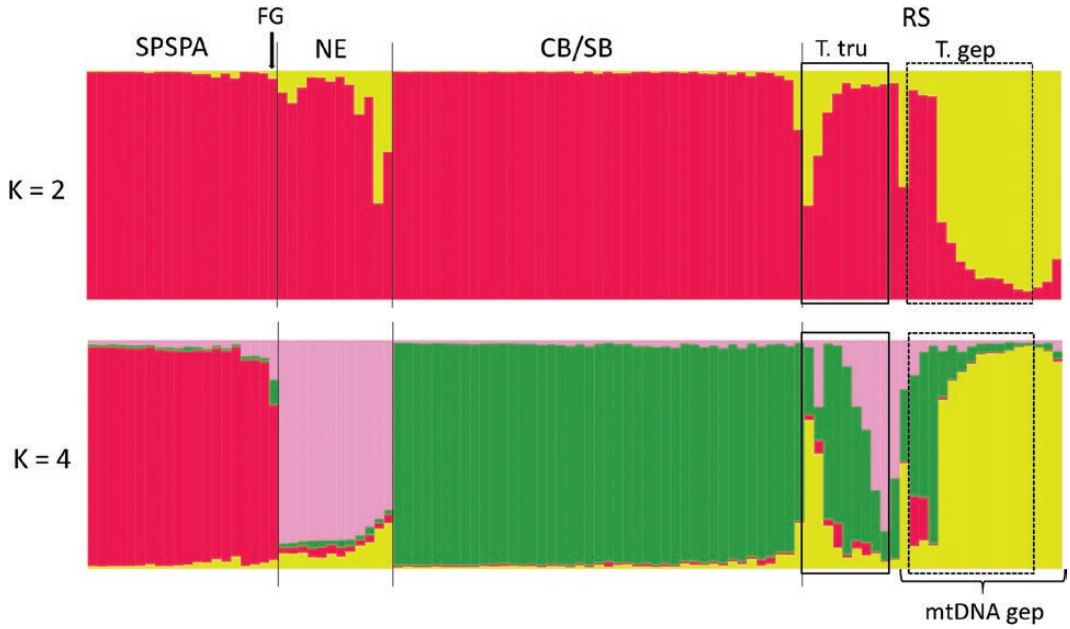

Fig. 3.——STRUCTURE Bayesian analyses of microsatellite variation of common bottlenose dolphins (*Tursiops truncatus*), including specimens of the putative *Tursiops gephyreus*, from the southwestern Atlantic Ocean considering $K = 2$ and $K = 4$. The sampling localities of individuals follow the legend and abbreviations in the Fig. 1. Each individual is denoted by a vertical bar, and the length of the color shows the proportional membership (q) in each genetic cluster (represented by different colors in online version or grayscale in printed version). The arrow indicates the individual from French Guiana. The rectangle with a solid line indicates the individuals morphologically identified as *Tursiops truncatus* (T. tru) and the rectangle with dashed line indicates the individuals identified as putative *T. gephyreus* (T. gep), and the open bracket indicates individuals with mitochondrial DNA (mtDNA) belonging to the *gephyreus* haplogroup (mtDNA gep, sensu Fig. 2). Area abbreviations as in Fig. 1. See this figure online for color version.

nuclear markers, in common bottlenose dolphins in the SWA, including Saint Paul's Rocks (SPSPA). One genetic group (Fig. 3, $K = 4$) comprises individuals from SPSPA and French Guiana; a second group comprises individuals from the north–northeast of Brazil (NE); a third genetic group is formed by all individuals from the Campos and Santos Basins (CB–SB) and extends at least as far south as the Rio Grande do Sul state, where this group probably co-occurs with another genetic cluster, comprising the putative *T. gephyreus* (see below).

The genetic differentiation of the common bottlenose dolphins of Saint Paul's Rocks from other Brazilian populations is not unexpected since they comprise an oceanic and resident population about 1,010 km distant from the Brazilian coast (Ott et al. 2009; Milmann et al. 2017). Also, the most frequent haplotype in SPSPA is identical to haplotypes from oceanic northeast Atlantic areas, including individuals from the archipelagos of Madeira and the Azores. This suggests that the Saint Paul's Rocks population is part of or was (relatively) recently colonized from migrants from a large oceanic North Atlantic population (see Quérouil et al. 2007). The absence of matches between the two SPSPA haplotypes and any other individuals from the coastal regions, especially those from the geographically closest NE area, suggests that oceanic SPSPA dolphins are genetically isolated from the other SWA areas studied here. On the other hand, the inclusion of the French Guiana individual in the SPSPA group by the nuclear markers raises two hypotheses: it could be a vagrant individual from the SPSPA area, although this would not explain its highly divergent mtDNA haplotype, or both areas may be part of a larger unknown population. Since there is no genetic information from common bottlenose dolphins from other offshore regions in the tropical SWA, Saint Paul's Rocks should be regarded as the only known genetically distinct offshore population of *T. truncatus* in this area.

The absence of haplotype sharing and the relatively large genetic distances between the NE and the other SWA areas suggest the NE area may be isolated from other SWA areas, although our sample size for this area is not large. It is worth mentioning that, despite their relative geographic proximity, the Pará state (PA, Brazil) and French Guiana are separated by the immense freshwater discharge of the Amazon River (Anthony et al. 2014). To the south, the maintenance of genetic isolation between NE and CB–SB areas is likely due to their distance (1,200 km), reinforced by the upwelling zone present in the CB–SB (Valentin 2001). This upwelling event occurs with great intensity in spring and summer seasons between latitudes 21° and 23°S, and is controlled by the north and east winds when the South Atlantic Central Water (low temperature and low salinity) penetrates the inner continental shelf causing a decrease in ocean temperature (Siciliano et al. 2006). Between NE and CB–SB there is also the most important coral reef area of the South Atlantic Ocean, the Abrolhos Bank. The reefs begin approximately 25 km off the coast and go up to 70 km, scattered across the platform (e.g., Bruce et al. 2012).

Diverse environmental conditions in this area may contribute to a regional difference in productivity (Gonzalez-Silvera et al. 2004), reflected in the abundance and distribution of prey. The distribution of other small cetaceans seems to be influenced by the oceanographic features that exist to the north and south of this central region of the Brazilian coast (Moreno et al. 2005; Amaral et al. 2015). Other studies have demonstrated that the distribution and genetic structuring of dolphin populations are influenced by environmental factors such as prey distribution (Heithaus and Dill 2002; Natoli et al. 2005) and habitat structure (Lusseau et al. 2003). However, the high population structure between these two areas may be a sampling effect from the absence of samples between the NE and CB–SB areas (Fig. 1).

Common bottlenose dolphins in southern Brazil.—Considering the three genetic components found in RS area, the presence of a component mostly associated with the NE area (Fig. 3, pink in the online version and light gray in the printed version, $K = 4$) is difficult to explain, particularly for the three individuals in which this component represents >50% of the proportional membership. A migratory connection between the two areas is not likely as these areas are very distant and these three individuals have a haplotype (labeled 5, Fig. 2) that is common in RS and CB–SB but is absent in NE. On the other hand, the presence of many individuals (about 30%) with a high proportion of the genetic component (Fig. 3, green in the online version and medium gray in the printed version, $K = 4$) mostly found in the CB–SB area supports the hypothesis that this population extends at least to part of the RS area, which is also supported by the very high sharing of mtDNA haplotypes between these areas. Most of the individuals in RS (that share ancestry with CB–SB) also presented the *T. truncatus* morphology.

The third main genetic component (Fig. 3, yellow in the online version and white in the printed version, $K = 4$), which was found (with proportions higher than 50%) only in individuals from this area in our study, is quite distinct and is mostly associated with individuals with the putative *T. gephyreus*. There is also a complete association between individuals with *T. gephyreus* morphology and a unique mitochondrial haplogroup. These results showed that the putative *T. gephyreus* is also genetically distinct from the canonical *T. truncatus* taxon in southern Brazil. However, we found three specimens with mtDNA and morphology associated with the putative *T. gephyreus*, but presenting a low proportion of the *gephyreus* component in nuclear markers. Moreover, two individuals in RS had a morphology and haplotype associated with *T. truncatus*, but had a relatively high proportion of the *gephyreus* component in nuclear markers. These findings suggest the existence of some level of gene flow between these biological units, or they could also partly be due to introgression from past hybridization events, most likely mediated by males, since the association between the morphology and the mtDNA clades seems complete, suggesting no female gene flow.

A previous study on mtDNA from coastal bottlenose dolphins from the southern part of the SWA found nine haplotypes separated into two distinct groups of haplotypes (see figure 5 in Fruet et al. 2014). Although their mtDNA sequences were not publically available and they did not present morphological information for their samples (they analyzed biopsies samples), we obtained from the authors the sequences of the two most

common haplotypes (H7 and H8) in each of the haplogroups. These haplotypes were identical to our haplotypes 25 and 24, respectively (Fig. 2), both belonging to the *gephyreus* haplogroup. In addition, two individuals from their study were also sequenced here and both presented *T. gephyreus* morphology (haplotypes 24 and 25). Therefore, it appears all their mtDNA sequences were from the *gephyreus* haplogroup, including those collected south of our study area, that is, southernmost part of Brazil, Uruguay, and from SAB, northern Argentina. These results agree with Wickert et al. (2016; 280 skulls examined) who found that all stranded individuals (with only a few exceptions) from southern RS, Uruguay, and Argentina (that also include individuals from SAB) are morphologically characterized as *T. gephyreus*.

Our findings coupled with Fruet et al. (2014) corroborated the hypothesis that the putative *T. gephyreus* has a very coastal (< 10 m depth) and estuarine distribution (Costa et al. 2016; Wickert et al. 2016; see also Di Tullio et al. 2015), since all of the samples in Fruet et al. (2014), except two from strandings from unknown origin, were taken in very shallow coastal waters (< 10 m depth). Additional support for this hypothesis is found in our study, since three of our samples, including two stranded individuals, were obtained from residents of estuaries in the RS region based on long-term photo-identification studies (Moreno et al. 2008). These individuals were morphologically identified as *T. gephyreus* (Wickert et al. 2016), exhibited mtDNA from the *gephyreus* haplogroup, and two of them had a high proportion of the nuclear *gephyreus* component; no microsatellite data were available for the third individual. The hypothesis that *T. truncatus* contains an oceanic genetic component is indirectly supported by the result of STRUCTURE with $K = 2$ (Fig. 3), in which most individuals with this genetic component grouped with the SPSPA and CB–SB individuals; the former is clearly an oceanic population and the latter have been sampled in depths > 20 m.

However, the three genetic components found in the RS area (Fig. 3, pink, green, and yellow in the online version, and light gray, medium gray, and white, respectively, in printed version, $K = 4$) did not fully agree with the recently published study by Fruet et al. (2017) with mtDNA and microsatellite loci, who only found two ecotypes (offshore and inshore) for southern Brazil, with minimal current and historical connectivity between ecotypes. We suspect that differences in sampling methods could explain these distinct results, since Fruet et al. (2017) biopsied animals from two opposite areas (estuarine-coastal versus outer continental shelf-slope) and in specific seasons (spring and autumn in offshore waters), whereas stranded animals collected over a long time period, from which our study was based in this area, may include animals from a broader water depth and different seasons. Therefore, our data suggest that a more complex scenario of *Tursiops* occurs along the southern Brazilian coast, and additional studies should be implemented in the region, mainly increasing the sample size and comparing phenotypic (external morphology and osteology) and genetic information.

Considering the presently available information, the common bottlenose dolphins in the tropical and subtropical Atlantic Ocean of South America appear to be divided into two major morphological, genetic, and geographical units: a northern unit, inhabiting a wide depth range, and a southern unit, exclusively coastal, with an area of parapatry in southern Brazil. Some authors (e.g., Costa et al. 2016) suggested that the southern, coastal taxon should be considered a different subspecies (*T. truncatus gephyreus*), or only management or evolutionarily units (Fruet et al. 2017), while others (Wickert et al. 2016) argued that the latter should be afforded full species status (*T. gephyreus*). In this context, the *gephyreus* mtDNA sequences grouped well inside the major *T. truncatus* clade (Clade B, Supplementary Data SD3), not as deeply divergent clades as found between *T. truncatus* and the other recently proposed species in the genus (*T. aduncus* and *T. australis*, Supplementary Data SD3 and figure 2 of Moura et al. 2013). In addition, at the nuclear level the association between the morphology and the genetic components is not complete. Therefore, we think that a formal decision about this taxonomic category requires further research, including a larger and richer data set, integrating information on the precise sampling location, morphology, and a large number of nuclear markers.

A comparable situation involving the taxonomic status of the Neotropical dolphins of the genus *Sotalia* has recently been resolved. Two currently recognized species inhabiting coastal marine waters of Central and South America (*Sotalia guianensis*) and freshwater ecosystems (*Sotalia fluviatilis*) of the Amazon basin are accepted. For a long time, it was argued that subtle differences between the "forms" simply represented phenotypic variation and therefore the two "forms" came to be regarded as conspecific ecotypes. In the 2000s, studies of skull morphology (Monteiro-Filho et al. 2002) and genetic markers from the Brazilian coast (Cunha et al. 2005) and further independent samples from populations along most of the distributional range of the two subspecies, including locations along the Amazon River and some of its tributaries as well as coastal locations in Nicaragua, Colombia, Venezuela, French Guiana, and Brazil, provided definitive evidence for the separation of the genus into two species (Caballero et al. 2007). However, in contrast to our *T. truncatus* versus *T. gephyreus* scenario, the mtDNA sequences from the two *Sotalia* species formed two well-divergent clades (Cunha et al. 2005; Caballero et al. 2007).

Regardless of the taxonomic issue above, in the SWA, we suggest that the canonical *T. truncatus* could be divided into three MUs: 1) Saint Paul's Rocks; 2) north and northeast of Brazil; and 3) Campos and Santos Basins, extending at least to the southernmost part of Brazil. Concerning the putative *T. gephyreus* , Fruet et al. (2014) suggested that these coastal populations could be separated into two ESUs, Argentina (SAB) and RS–Uruguay, and that the latter should be further divided into five MUs. We could not test these proposals here since our samples are from only one of the MUs (their North of Patos Lagoon area). Nevertheless, it is largely recognized that these coastal populations are facing increasing threats at a local

scale (e.g., Fruet et al. 2012; Di Tullio et al. 2015; Van Bressem et al. 2015) and deserve further effective protection.

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Supplementary Data

Supplementary data are available at *Journal of Mammalogy* online.

Supplementary Data SD1.—List of samples of common bottlenose dolphins (*Tursiops truncatus*), including specimens of the putative *Tursiops gephyreus,* and respective collaborator groups. *n* = number of samples.

Supplementary Data SD2.—Mitochondrial DNA haplotype list of samples of common bottlenose dolphins (*Tursiops truncatus*), including specimens of the putative *Tursiops gephyreus*: list of individuals that belong to each haplotype, haplotype frequency, and sampling locality. Abbreviations of Brazilian states: PA = Pará, CE = Ceará, RN = Rio Grande do Norte, PE = Pernambuco, BA = Bahia, RJ = Rio de Janeiro, SP = São Paulo, SC = Santa Catarina, and RS = Rio Grande do Sul.

Supplementary Data SD3.—Maximum parsimony tree of mtDNA from our haplotypes (labeled as Haplo1 to 32), the sequences from Louis et al. (2014; labeled as Ttrunc1 to 55 as in their results), and the whole mtDNA genomes of Moura et al. (2013; labeled as in their figure 2). Our haplotype labels are followed by the sampling area abbreviation as in Fig. 2 and are identified with an inverted triangle or circle, colored as in Fig. 2, except for shared haplotypes that are in black. The six yellow circles are the haplotypes from the *gephyreus* haplogroup. Clades A, B, and C are described in the main text. Tree length is 1,667, consistency index is 0.745051 (0.716855), the retention index is 0.969599 (0.969599), and the composite index is 0.722401 (0.695063) for all sites and parsimony-informative sites (in parentheses). The tree is drawn to scale, with branch lengths calculated using the average pathway method and are in the units of the number of changes over the whole sequence. The analysis involved 162 nucleotide sequences. There was a total of 16,363 positions in the final data set. Tree is rooted in the *Tursiops australis* clade based on Moura et al. (2013).

Supplementary Data SD4.—Maximum parsimony tree of mtDNA from our haplotypes (labeled as Haplo1 to 32) and the sequences from Caballero et al. (2012; labeled as TtruCARxxx as in their results). Haplotypes are identified with a red or a yellow circle, respectively. Caballero et al.'s (2012) coastal form clade is identified. Tree length is 128, consistency index is 0.484375 (0.415929), the retention index is 0.852349 (0.852349), and the composite index is 0.412857 (0.354517) for all sites and parsimony-informative sites (in parentheses). The tree is drawn to scale, with branch lengths calculated using the average pathway method and are in the units of the number of changes over the whole sequence. There was a total of 265 positions in the final data set.

Supplementary Data SD5.—A) Log likelihood values as a function of the number genetically differentiated populations, and B) Delta *K* likelihood values as a function of the number of genetically differentiated populations of common bottlenose dolphins (*Tursiops truncatus*), including specimens of the putative *Tursiops gephyreus,* of the Western Tropical and Subtropical South Atlantic inferred from Bayesian STRUCTURE analysis using seven microsatellite loci.

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