Ecological Divergence and Speciation in Common Bottlenose Dolphins in the Western South Atlantic

Ana P. B. Costa¹*, Pedro F. Fruet²,₃,₄,₅, Eduardo R. Secchi³, Fábio G. Daura-Jorge⁶, Paulo C. Simões-Lopes⁶, Juliana C. Di Tullio²,₃,₄, and Patricia E. Rosel⁷

¹Department of Biology, University of Louisiana at Lafayette, 410 E. St. Mary Boulevard, Lafayette, Louisiana 70503, U.S.A.;
²Museu Oceanográfico “Prof. Eliézer C. Rios”, Universidade Federal do Rio Grande, R. Heitor Perdigão 10, Rio Grande, RS, 96200-580 Brazil;
³Laboratório de Ecologia e Conservação da Megafauna Marinha (EcoMega), Instituto de Oceanografía, Universidade Federal do Rio Grande, Av. Itália Km 8, Rio Grande, RS, 96203-900, Brazil;
⁴Kaosa, Rua Caçapava 486, Rio Grande, RS, 96206-010, Brazil
⁵Centro Nacional de Pesquisa e Conservação de Mamíferos Aquáticos – ICMBio/CMA, Rua Alexandre Herculano, 197, Gonzaga, Santos, SP, Brazil
⁶Laboratório de Mamíferos Aquáticos (LAMAQ), Departamento de Ecologia e Zoologia, Universidade Federal de Santa Catarina, PO Box 5102, Florianópolis, SC, 88040-970, Brazil;
⁷National Marine Fisheries Service, Southeast Fisheries Science Center, 646 Cajundome Boulevard, Lafayette, Louisiana 70506, U.S.A.

*Correspondence: Ana P. B. Costa, Department of Biology, University of Louisiana at Lafayette, Lafayette, LA, U.S.A
Email: anapbccosta@gmail.com
†Present address: Institute of Biodiversity, Animal Health, and Comparative Medicine, University of Glasgow, 310 Graham Kerr Building, Glasgow, G12 8QQ, United Kingdom.
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Abstract

Coastal and offshore ecotypes of common bottlenose dolphins have been recognized in the western South Atlantic, and it is possible that trophic niche divergence associated with social interactions is leading them to genetic and phenotypic differentiation. The significant morphological differentiation observed between these ecotypes suggests they represent two different subspecies. However, there is still a need to investigate whether there is congruence between morphological and genetic data to rule out the possibility of ecophenotypic variation accompanied by gene flow. Mitochondrial DNA (mtDNA) control region sequence data and 10 microsatellite loci collected from stranded and biopsied dolphins sampled in coastal and offshore waters of Brazil as well as 106 skulls for morphological analyses were used to determine whether the morphological differentiation was supported by genetic differentiation. There was congruence among the data sets, reinforcing the presence of two distinct ecotypes. The divergence may be relatively recent, however, given the moderate values of mtDNA nucleotide divergence ($d_A = 0.008$), presence of one shared mtDNA haplotype, and possibly low levels of gene flow (around 1% of migrants per generation). Results suggest the ecotypes may be in the process of speciation and reinforce they are best described as two different subspecies until the degree of nuclear genetic divergence is thoroughly evaluated: *Tursiops truncatus gephyreus* (coastal ecotype) and *T. t. truncatus* (offshore ecotype). The endemic distribution of *T. t. gephyreus* in the western South Atlantic and number of anthropogenic threats in the area reinforces the importance of protecting this ecotype and its habitat.

Keywords: dolphin, genetics, morphology, taxonomy, speciation, ecological specialization
**Introduction**

Marine environments have the potential for gene flow across large geographic distances since absolute barriers are uncommon in this habitat. Restriction of gene flow, however, is not always associated with geographic barriers, and speciation can occur in parapatry or sympatry (Rundle & Schluter, 2004; Rundle & Nosil, 2005, Berner *et al.*, 2009). Environmental conditions may serve as barriers to gene flow: ocean currents and water temperature can create biogeographic regions and limit the dispersal of species (Palumbi, 1994). For example, Teske *et al.* (2019) showed evidence of thermal-mediated genetic divergence among populations of a coastal fish (*Psammogobius knysnaensis*) inhabiting the South African coastline. This region is characterized by different temperature-defined marine bioregions over a small geographic scale and this thermal-gradient seems to be associated with phylogeographic breaks separating several coastal species in this region (Teske *et al.*, 2011).

There are also examples of behavioral barriers to gene flow in marine environments. Evidence of rapid ecologically-based divergence has been demonstrated for two ecotypes of European flounders (*Platichthys flesus*) in the Baltic Sea based on distinct spawning behavior associated to salinity tolerance (Momigliano *et al.*, 2017). Mate recognition can be another mechanism driving divergence between marine species. It has been hypothesized that distinct vocalization may be used by sympatric reef fish species (genus *Haemulon*) that spawn at night to find mates in the dark (Rocha *et al.*, 2008). Speciation in other reef fish species (e.g., gobies) at range boundaries or in sympatric areas can be influenced by assortative mating associated with coloration (Taylor & Hellberg, 2005). Further, prey quality, energetic demands and competition can influence animals’ feeding strategies and habitat selection (Spitz *et al.*, 2012). Differences in prey preference, foraging techniques, and social interactions may lead to habitat segregation, and the interaction of the individuals with their
environment can result in ecologically-based divergent selection (Schluter, 2001; Rundle & Nosil, 2005).

Such niche specialization can lead to the segregation of populations into ecotypes, which are defined as populations within a species that differ in multiple traits, including allele frequencies across loci, and are adapted to distinct ecological conditions that can act as barriers to gene flow (Lowry, 2012). It has been argued that ecotypes can be considered as an early stage of divergence in which genetic differences are “a result of adaptations to specific sets of environmental factors that define habitats” (Lowry, 2012). Divergent selection on traits in populations occupying contrasting environments or with distinct niches can result in reproductive isolation and ultimately may even lead to speciation (i.e., ecological speciation) if divergence is maintained through time (Schluter, 2001; Rundle & Nosil, 2005). Ecotypes that represent advanced stages of the differentiation process may coincide with distinct taxonomic units – subspecies or species (Gregor, 1944). The term subspecies can be defined as “a population, or collection of populations, that appears to be a separately evolving lineage with discontinuities resulting from geography, ecological specializations, or other forces that restrict gene flow to the point that the population or collection of populations is diagnosably distinct” (Taylor et al., 2017a). While subspecies can have some low ongoing gene flow, a species is “a separately evolving lineage composed of a population or collection of populations” that is reproductively isolated from other species (Taylor et al., 2017a). Some examples of marine speciation driven by ecological barriers (e.g., habitat segregation) can be cited between ecotypes of manta rays (e.g., Kashiwagi et al., 2012), teleost fish (e.g., Beheregaray & Levy, 2000), and marine mammals (e.g., Foote & Morin, 2016).

Marine mammals are highly mobile predators and exhibit a variety of habitat and prey preferences, and foraging techniques (see Heithaus & Dill, 2002). A classic example of a marine mammal species that has diverged into morphologically and genetically disparate 3
ecotypes due to specialized foraging behavior and niche preferences is the killer whale, *Orcinus orca* (Ford *et al.*, 1998; Pitman *et al.*, 2007; Foote *et al.*, 2009). In particular, the distinct ecotypes of the eastern North Pacific are believed to be in the process of speciation, possibly initiated by differential ecological pressures due to different foraging tactics followed by limited gene flow reinforced by strong social structure, and expansion of these new populations along distinct matrilineal lines (Foote & Morin, 2016).

The presence of different ecotypes (coastal and offshore) has also been recognized for the common bottlenose dolphin *Tursiops truncatus* (Montagu, 1821) in many parts of the world (Van Waerebeek *et al.*, 1990; Mead & Potter, 1995; Hoelzel *et al.*, 1998; Rosel *et al.*, 2009; Perrin *et al.*, 2011; Vollmer & Rosel, 2013; Louis *et al.*, 2014; Costa *et al.*, 2016; Fruet *et al.*, 2017). The coastal ecotype of common bottlenose dolphins is generally found in shallower, nearshore coastal waters, including bays, sounds and estuaries, and in some geographic regions it can be lighter colored than the offshore ecotype which is found in deeper, more pelagic waters (Hersh & Duffield, 1990; Van Waerebeek *et al.*, 1990; Sanino & Yañez, 2001; Torres *et al.*, 2003; Vollmer & Rosel, 2013; Fruet *et al.*; 2017, Félix *et al.*, 2018; Simões-Lopes *et al.*, 2019).

In the western South Atlantic (wSA), the taxonomic status of the two ecotypes has been debated (see Costa *et al.*, 2016; Wickert *et al.*, 2016). Lahille (1908) suggested the presence of a new species, *Tursiops gephyreus*, based on the cranial morphology of two specimens collected in the La Plata River, Argentina. More recently, two different hypotheses have emerged based on morphology. Cranial and skeletal morphological analyses conducted by Costa *et al.* (2016) revealed the presence of two well-differentiated and diagnosably distinct groups with morphological characteristics indicating distinct habitat preferences. These findings led the authors to suggest the presence of distinct ecotypes in the western South Atlantic. Ecotypes that are diagnosably distinct from each other by morphological
characters may be considered as subspecies (Clausen et al., 1941; Gregor, 1944). Therefore, these findings led Costa et al. (2016) to recognize the wSA ecotypes as the subspecies T. t. truncatus (offshore ecotype) and T. t. gephyreus (coastal ecotype, because it was considered morphologically similar to the previously described gephyreus-type by Lahille, 1908). Conversely, a concurrent morphological study (Wickert et al., 2016) elevated both forms to species based on six qualitative cranial characters and following a “diagnosable version of the Phylogenetic Species Concept” where species are defined “as the smallest aggregation of populations (sexual) or lineages (asexual) diagnosable by a unique combination of character states in comparable individuals” (Nixon & Wheeler, 1990). However, these morphological characters did not classify all the samples with 100% accuracy: four out of the six morphological characters they identified to visually distinguish between the ecotypes showed some degree of character overlap (see Wickert et al., 2016 – Results and Supporting Information S5); results that are more in line with a subspecies description (Martien et al., 2017). In addition, both studies used skulls collected from stranded animals, resulting in a lack of knowledge about their population of origin since ocean currents can disperse carcasses far from their original habitat (Peltier et al., 2012), and none has examined the level of genetic differentiation between these groups. A population genetic study was conducted by Fruet et al. (2017) using biopsied bottlenose dolphins collected in coastal and offshore waters of the western South Atlantic but the authors did not examine the congruence between the morphological and genetic findings.

Accurate species-delimitation, in other words defining whether groups represent different populations, subspecies or species, is essential for understanding at which stage of the speciation process these groups are found and for helping to better define species diversity, ecological interactions, and effective conservation and management strategies. As stated by Dayrat (2005), morphology-based taxonomy is the study of morphological diversity
and the potential species described should be considered as hypotheses to be tested using additional approaches. The use of ‘integrative taxonomy’, which involves the use of different sources of data (e.g., morphological, molecular, behavioral), has been growing in the literature as a strategy to more accurately delimit species and address issues that arise when using a single line of evidence alone, such as morphological data (Padial et al., 2010). The congruence of additional approaches with the morphological findings of potential species is considered more robust evidence supporting lineage divergence (Dayrat, 2005; Padial et al., 2010).

Here, we compared and integrated morphological and molecular genetic data to examine the level of evolutionary divergence between the ecotypes of bottlenose dolphins in the western South Atlantic (wSA). Additionally, we examined the genetic relationship of the two wSA ecotypes with the well-studied ecotypes described for the western North Atlantic (wNA) to test the hypothesis of genetic connectivity between the two oceanographic regions and place this study in a broader phylogeographic context in the western Atlantic Ocean. We also discuss on the potential speciation processes driving the divergence between the wSA ecotypes.

**Methods**

*Samples for genetic analyses*

We analyzed 253 samples of *T. truncatus* from the western South Atlantic, which included 161 biopsy and 92 stranding samples (55 soft tissues; 37 teeth) (Table S1, Figure 1). Skin biopsy samples (*n* = 161) were collected in 2007-2013 from photo-identified resident dolphins inhabiting the estuaries and adjacent waters of Laguna (*n* = 16) and Patos Lagoon (*n* = 83), southern Brazil, and from dolphins in Brazilian waters deeper than 100 m and at least 100 km from the coast (*n* = 62) using a biopsy dart system designed for small cetaceans (F.
Larsen, Ceta-Dart). These biopsies included some samples ($n = 120$) used by Fruet et al. (2017), with new samples ($n = 41$) collected in all locations. Tissues ($n = 55$) from stranded dolphins were also collected in 2005-2013. Two stranded individuals were photo-identified as resident dolphins from Laguna (coastal ecotype), 18 had skulls available and were identified to the ecotype level based on cranial morphology (see below), and the remaining 35 were considered of unknown origin since there was no information available that allowed their classification to ecotype. Further, to increase the sample size of specimens with both morphological and genetic data for the analysis of congruence, DNA was extracted from the teeth of 37 additional bottlenose dolphins that stranded in 1978-2012 along the southern Brazilian coast (Figure 1B). These samples were identified to the ecotype level by their cranial morphology. Skulls were also available from two previously biopsied animals after their death in subsequent years (Table S1). Therefore, a total of 57 out of the 253 samples had both morphological and genetic data available, but due to problems with DNA amplification of the tooth samples (see below) only 34 of these 57 were used in the analyses of congruence between the data sets. DNA extraction and molecular sexing methodologies are described in the Supporting Information. All maps in this study were generated using MARMAP (Pante & Simon-Bouhet, 2013) implemented in R v3.3.1 (R Core Team, 2016) and the ETOPO1 data set (Amante & Eakins, 2009).

We also used 72 published mtDNA control region haplotypes from genetically identified coastal ($n = 22$) and offshore ($n = 50$) bottlenose dolphins from the western North Atlantic (wNA) available in GenBank (Table S2) and nuclear microsatellite genotypes of 37 bottlenose dolphins biopsied in offshore waters of the wNA (Figure S1) to compare the signatures of dolphins of the wSA with those from wNA.

**Microsatellite genotyping and analyses**
Microsatellite genotyping was performed for the 216 soft tissues collected in the wSA and the 37 individuals biopsied in offshore waters of the wNA using 10 microsatellite loci amplified in multiplexes (multiplexes 1 and 2 in Table S3) with a Qiagen Type-it Microsatellite PCR kit following Rosel et al. (2017a). We also attempted to genotype 7 loci (Table S3) from a tooth of a specimen with a coastal skull but an offshore haplotype (see results). Genotyping was performed on an ABI 3130 Genetic Analyzer with Genescan Liz-500 size standard and scored using GeneMapper v5 (Applied Biosystems). Positive and no-DNA controls were included in all genotyping amplifications. Individuals were kept in the analyses when at least 8 loci were successfully amplified (wSA: 190 out of the 216; wNA: 37 out of the 37). Genotyping error rate was estimated by randomly selecting 19 individuals of the wSA and four of the wNA and re-genotyping at all 10 loci.

We initially identified duplicate samples using the genotypic information and the software MSTools (Park, 2001), and looked for congruence in the sex and mtDNA haplotype of the potential duplicates. We then genotyped these potential duplicate samples with 11 additional loci (multiplexes 3 and 4 in Table S3) to increase power in confirming the detection of duplicates before removal from the data set. One sample of each pair of duplicates identified using 21 loci was removed from further analyses (Table S1). Genotyping errors due to null alleles, allelic dropout, and incorrect scoring of stutter peaks were checked using MICRO-CHECKER v2.2.3 (Van Oosterhout et al., 2004) with 10,000 iterations. Each locus was tested for departure from Hardy-Weinberg equilibrium (HWE) (Guo & Thompson, 1992) and linkage disequilibrium using the Fisher’s exact tests in GENEPOP v4.6 (Rousset, 2008) using 10,000 dememorizations, 1,000 batches, and 10,000 iterations per batch. Both tests were applied to the full final data set and to the ecotype groups expected based on skull morphology or sample origin (i.e., photo-identification or biopsy sampling location). The sequential Bonferroni technique (Holm, 1979) was applied to correct for multiple tests. Loci
that exhibited homozygote excess were re-genotyped at a lower temperature (45°C) to check for the presence of null alleles.

Evidence for more than one genetic cluster in the wSA was investigated using the Bayesian clustering programs TESS v2.3.1 (Durand et al., 2009) and STRUCTURE v2.3.4 (Pritchard et al., 2010) and 147 samples of known origin (biopsy samples from coastal and offshore waters; stranding samples identified to ecotype by skull morphology or photo-identification) after the removal of duplicates (see results). The two approaches were used to look for congruence between results and ensure reliability in the determination of the wSA clusters. STRUCTURE was also used to assign 21 stranding samples of unknown origin to a cluster by activating the USEPOPINFO option with one run of \( K = 2 \) (best number of clusters, see results) and all the other prior settings. See Supporting Information for parameters used.

The STRUCTURE and TESS results (using the same individuals) were compared to reach a consensus in defining the best number of wSA clusters.

For each identified wSA cluster, inbreeding coefficient \( (F_{IS}) \), and mean observed \( (H_O) \) and expected \( (H_E) \) heterozygosities, as well as pairwise \( F_{ST} \) (Weir & Cockerham, 1984) between the clusters (with 10,000 permutations), were estimated using ARLEQUIN v3.5.1.2 (Excoffier & Lischer, 2010). Mean allelic richness (AR) was calculated using FSTAT v2.9.3 (Goudet, 1995) and the total numbers of alleles (NA) and private alleles per wSA cluster were identified with Convert (Glaubitz, 2004). The presence and directionality of contemporary gene flow between the wSA clusters was estimated using the microsatellite data set (10 loci) and the program BAYESASS v3.0.4 (Wilson & Rannala, 2003). See Supporting Information for the parameter settings.

Mean pairwise relatedness values \( (r) \) were estimated in COANCESTRY v1.0.1.8 (Wang, 2011) using the Queller & Goodnight (1989) index to identify closely related individuals. To exclude the possibility that kinship may be overestimating population
structure (Bilgmann et al., 2014), the clustering analyses and further nuclear statistical analyses were repeated by excluding one sample of each pair of individuals within each cluster with relatedness values, $r \geq 0.5$.

*Mitochondrial DNA sequencing and analyses (wSA)*

A 353 base pair (bp) portion of the mtDNA control region was successfully amplified and sequenced for 230 samples, which included all 216 soft tissue samples and 14 tooth samples (23 tooth samples failed to amplify due to DNA degradation) of the western South Atlantic (wSA). Primers and PCR conditions are described in the Supporting Information.

A total of 208 individual sequences of the wSA were used for the mtDNA data analyses after removal of 22 duplicates. Most of the samples ($n = 168$) were classified to an ecotype based on the nuclear clustering analyses. However, for samples we were able to sequence but not genotype for more than 8 loci ($n = 27$), ecotypic classification was defined according to cranial morphology or photo-identification. Further, stranding samples of unknown origin (and without skull available for morphological classification), which were sequenced but not genotyped ($n = 13$), were designated “unknown ecotype” and were only used in the mtDNA network analysis and in the Random Forest analysis for assignment probability to an ecotype (see Supporting Information and Results). Noteworthy, eight out of the 208 samples exhibited heteroplasmic (hpl) haplotypes (Vollmer et al., 2011) and they were only used in the Random Forest analysis (see below) due to software limitations in dealing with ambiguous bases.

A median joining network of 29 mtDNA haplotypes was constructed in Network v5.0.0.3 (Bandelt et al., 1999) with default parameters to examine the relationships among the haplotypes found in the wSA. Haplotype (Nei & Tajima, 1981) and nucleotide (Nei, 1987) diversities, and genetic differentiation ($F_{ST}$, $\Phi_{ST}$) between the wSA ecotypes (conducted with and without closely related individuals) were estimated in ARLEQUIN. Net between-group
nucleotide divergence ($d_A$; Nei, 1987) was estimated using the STRATAG package (Archer et al., 2017a) in R v3.3.1. The best model of evolution to calculate the divergences was identified using jModelTest v2.1.6 (Posada, 2008) and Bayesian Information Criterion (BIC) on CIPRES Science Gateway (Miller et al., 2010) – Tamura-Nei (Tamura & Nei, 1993) with invariant sites.

Finally, percent diagnosable (PD) based on a Random Forest methodology (Archer et al., 2017b) was used to produce classification models to examine whether there is subspecies or species level diagnosability between the wSA ecotypes using 195 mtDNA sequences (without the unknown ecotype samples). In brief, this method develops a classification model, based on multiple classification trees, that maximizes the probability of correct classification using all variable sites in the mtDNA sequence alignment (see more details in Archer et al., 2017b). We followed the 95% diagnosability threshold (Taylor et al., 2017b) for the subspecies level due to the fact that although gene flow has been restricted between the subspecies, low levels of gene flow may still occur, what can result in some small level of overlap between the groups, and 100% for the species level, since species are expected to be 100% diagnosable from one another (see Archer et al., 2017b). See Supporting Information for specifications of the run.

**MtDNA and microsatellite analyses for the wSA and wNA combined**

The 208 mtDNA control region sequences of the western South Atlantic (wSA) were aligned with 72 control region haplotypes of the western North Atlantic (wNA) using CLUSTALW implemented in Geneious v9.1.8 (Biomatters) and default parameters, producing a 354 bp alignment. Phylogenetic relationships among *T. truncatus* haplotypes of the western Atlantic (wSA: 29; wNA: 21) were investigated using a maximum likelihood tree constructed in IQ-TREE web server (Trifinopoulos et al., 2016) with Ultrafast bootstrap (UFBoot) analysis, 1,000 bootstrap replicates and all other default parameters.
Lagenorhynchus acutus, Steno bredanensis and the holotype of T. aduncus were used for outgroups (Table S2). The best evolutionary model for DNA substitution was selected using jModelTest and BIC on the CIPRES portal – Hasegawa-Kishino-Yano (Hasegawa et al., 1985) with invariant sites and a gamma distribution. We also constructed a median joining network of 50 mtDNA haplotypes in Network (Bandelt et al., 1999) and default parameters to examine the relationships among the haplotypes found in the wSA and wNA. Lastly, the TESS and STRUCTURE analyses were repeated with 10 microsatellite loci and 168 wSA samples and 37 wNA offshore samples following the methodologies described above.

Morphological data and statistical analyses

A principal component analysis (PCA) was performed on 100 out of 106 physically mature skulls available in this study, including 83 previously examined in Costa et al. (2016), using 21 cranial measurements (Table S1). The samples were assigned to an ecotype following the qualitative characters defined in Costa et al. (2016) to visually identify the ecotypes based on skull morphology (coastal: 75; offshore: 25). Our goal was to examine the distribution of the individuals on the orthogonal axes and visually identify possible clusters along the PCA axes based on the a priori classifications. A Random Forest analysis (R package randomForest; Liaw & Wiener, 2002) was performed using the morphometric data set to quantify the accuracy of the a priori classifications. The Random Forest arguments were set as mtry = 8, ntree = 10,000, and sampsize = 12 (half of the smallest sample size; used to correct for unbalanced models due to differences in sample sizes). The PCA and Random Forest were conducted in R v3.3.1. A total of 28 out of the 100 specimens used in the morphological multivariate analyses also had tissue available for the molecular analyses described above. Using visual inspection of the skull, we also classified to the ecotype six additional specimens (coastal: 5; offshore: 1) that had some missing cranial measurements.
(\textit{i.e.}, were not included in the multivariate analyses above) but also had tissue available for molecular analyses.

\textbf{Results}

\textit{Quality control – genetic data}

The genotyping data set comprised 190 samples from the western South Atlantic (wSA) that were successfully amplified for at least eight microsatellite loci. However, a total of 25 pairs of duplicates (including individuals with more than one duplicate) were identified and, after removal of 22 duplicate samples (including a sample of unknown location; see Table S1), the final wSA nuclear data set comprised 168 samples (coastal: 107; offshore: 61; see results below). The genotyping of the DNA extracted from the tooth (UFSC1077) failed for all loci. The genotyping error rate was 0.006 (three scoring differences in 506 alleles). The mtDNA control region was successfully amplified for 230 samples; the final sample size after removal of the 22 duplicates was 208 (coastal: 131; offshore: 64; unknown: 13; see results below) of which 97 were males, 96 were females, and 15 of unknown sex (see Table 1).

Neither significant departure from HWE nor linkage disequilibrium was observed after Bonferroni correction when dividing the data set into the ecotype groups expected based on skull morphology or sample origin. MICRO-CHECKER detected possible null alleles and incorrect scoring of stutter peaks for locus Ttr61 in the coastal cluster. Re-genotyping a subset of homozygotes at a significantly lower annealing temperature confirmed the original calls, suggesting null alleles were not present and the locus was retained. High relatedness values were only observed within the coastal wSA cluster and no significant change in the clustering results was observed after the removal of 74 related samples (Figure S2); therefore we kept all the samples in the subsequent analyses.

\textit{Genetic analyses (wSA)
Results of TESS and STRUCTURE were congruent for the samples of known origin: the samples of the western South Atlantic (wSA) were divided into the groups expected based on skull morphology and/or sample origin (i.e., photo-identification or biopsy sampling location). For TESS, the DIC curve initially decreased sharply and began to level off at $K_{\text{max}} = 4$ (Figure S3-A). The bar plots in TESS (Figure 2) indicated at most three clusters ($K = 3$) with most of the individuals (94.6%) assigned to two distinct clusters corresponding to the wSA coastal and offshore ecotypes (cut-off $\geq 0.5$). The most likely number of clusters identified in STRUCTURE using the Evanno method was $K = 2$, whereas $\text{LnP}(D)$ suggested $K = 3$ (Figure S4-A). Comparisons between the two clustering analyses demonstrated congruence of 100% for $K = 2$ and of 76% for $K = 3$ (Figure 2). The plots of $K=3$ indicated the subdivision of the wSA offshore cluster in two. However, there was no consistency in the assignment of offshore individuals to a third cluster when comparing both TESS ($n = 8$ samples) and STRUCTURE ($n = 27$ samples) results (Table S4). Further results (i.e., mtDNA haplotypes, geographic distribution, sex information, genetic connectivity with the western North Atlantic samples) did not reveal any pattern that could logically explain the subdivision of the wSA offshore group. We also did not detect any significant level of relatedness within the offshore data set. Therefore, considering the results obtained for both clustering analyses, the lack of a biological explanation for the presence of a third cluster of a small number of wSA offshore samples, and the fact that in many cases $\text{LnP}(D)$ overestimates population structure, while $\Delta K$ more accurately detects the uppermost hierarchical level of genetic structure (Evanno et al., 2005), $K = 2$ was considered the most likely number of clusters in the wSA at the highest hierarchical level, resulting in 87 individuals assigned to the coastal cluster and 60 to the offshore cluster.

Using the USEPOPINFO option in STRUCTURE, 20 individuals of unknown origin were strongly assigned (assignment probabilities $> 0.97$) to the coastal cluster, creating a final
coastal data set of 107 genotyped individuals, and one sample was strongly assigned to the offshore cluster, forming a final offshore data set of 61 genotyped individuals (assignment probability = 1.0).

The 353 bp control region alignment for the 195 individuals assigned to an ecotype revealed 37 haplotypes (including eight hpl) defined by 44 polymorphic sites, with 11 (including four hpl) exclusively found in samples considered as coastal \((n = 131)\) and 25 (including another four hpl) exclusively found in samples considered as offshore \((n = 64)\).

Only one haplotype (OTtr34) was shared between the ecotypes (Figure 3). It was found in five samples classified as the offshore ecotype and one stranding sample (UFSC1077) assigned to the coastal ecotype by skull morphology. No fixed nucleotide differences were observed between the ecotypes. The 13 stranding samples designated “unknown ecotype” exhibited four previously described haplotypes: three exclusively found in coastal samples and one that matched the haplotype shared between the wSA ecotypes (Figure 3). All the “unknown ecotype” samples \((n = 12)\) that exhibited the “coastal” haplotype were predicted (based on the mtDNA Random Forest analysis) to belong to the coastal ecotype (assignment probabilities > 99.5%), whereas the single “unknown ecotype” sample with the shared haplotype was predicted to belong to the offshore ecotype (assignment probabilities > 99.35%).

Allelic diversity and heterozygosity values were lower for the coastal (which also exhibited two monomorphic loci: Ttr54 and Ttr58) than the offshore nuclear cluster. The same was observed for the genetic diversity patterns for the mtDNA (Table S5). A significant positive inbreeding coefficient (after Bonferroni correction) was only observed in the coastal cluster when the closely related individuals were included in the analysis (Table S5).

Significant genetic differentiation was observed between the ecotypes for both markers with and without closely related individuals included (Table 2). Nei’s \(d_A\) was 0.008
and diagnosability \(PD = 98.44\%\) (Table S6), both values indicative of subspecies level
distinction (Taylor et al., 2017b). Recent gene flow rates were extremely low in both
directions between the coastal and offshore ecotypes (Table 2).

**Genetic comparisons between wSA and wNA ecotypes**

The control region alignment revealed that 30 of the 37 haplotypes identified in the
western South Atlantic (wSA) were exclusively found in the wSA samples (SWATtr and hpl),
while seven (OTtr) were shared with offshore common bottlenose dolphins of the western
North Atlantic (wNA) (new haplotypes were deposited in GenBank: accession numbers
MK105857-MK105886). The shared haplotype observed in the wSA was also seen in wNA
offshore dolphins. No haplotypes were shared with the coastal wNA samples. The wNA
coastal dolphins formed a separate group in the haplotype network and phylogenetic tree,
whereas both coastal and offshore samples of the wSA grouped together with the wNA
offshore ecotype (Figures 4 and 5).

TESS and STRUCTURE runs incorporating wSA dolphins and wNA offshore
samples returned a similar number of clusters (Figure 6). The DIC curve decreased sharply
and slowed after \(K_{\text{max}} = 5\) (Figure S3-B) and TESS bar plots indicated at most four clusters,
with 97.1% of the individuals assigned among three distinct clusters (cut-off \(\geq 0.5\)). The most
likely number of clusters identified in STRUCTURE using the Evanno method was \(K = 2\),
whereas \(\text{LnP}(D)\) suggested \(K = 4\) (Figure S4-B). For \(K = 2\), all the wSA coastal samples were
clustered together, while all the offshore samples from both the wSA and wNA formed a
second cluster for the western Atlantic (wATL) (all assignment probabilities \(> 93\%\)). At \(K = 3\) there was also a strong geographic component to the clusters (i.e., wSA coastal vs. wSA
offshore vs. wNA offshore), whereas at \(K = 4\), TESS and STRUCTURE subdivided the
offshore samples into additional clusters (assignment probabilities \(\geq 50\%\)), which did not
show any discernable geographic pattern (e.g., wSA vs. wNA). Comparisons between the two
analyses demonstrated congruence in the individual assignments of 100% for $K = 2, 87\%$ for $K = 3$, and 75.5% for $K = 4$ (Table S4). Considering the lack of any obvious biological explanation for the subdivision of the offshore samples into three clusters (as seen in $K = 4$), $K = 3$ was considered the most likely number of clusters in the wATL (wSA coastal, wSA offshore, wNA offshore) with evidence for a small number of admixed individuals between the two offshore clusters, particularly a few wSA offshore animals with some affinity to the wNA offshore group.

**Morphological analyses**

The 100 specimens from the western South Atlantic (wSA) were distributed in two well-defined clusters along the PCA plot, showing congruence with the ecotype classifications based on morphological characters and previous observations (see Costa et al. 2016 for more details). The first two principal components explained 75.8% of the variance (Figure 7). Random Forest showed congruence of 98.7% with the PCA results in the grouping classification. One individual (UFSC1281), a priori classified as coastal, was assigned to the offshore ecotype by Random Forest with low scores (60.7%). This individual is placed closer to the coastal than offshore cluster in the PCA plot (Figure 7), and therefore it was still classified as belonging to the coastal ecotype. The six individuals visually assigned to an ecotype based on morphological characters were classified as five coastal and one offshore.

Congruence was observed between the mtDNA and morphological results, with one exception. In brief, 28 of 34 samples had both a coastal morphotype and mtDNA haplotype only found in dolphins of coastal waters, five exhibited the offshore morphotype and haplotypes found in dolphins collected in offshore waters, and one single sample (UFSC1077) was identified as coastal based on skull morphology but its tooth DNA sequencing (successfully extracted three times and amplified and sequenced two times for each
extraction) revealed a haplotype (OTtr34) originally found in offshore dolphins of both wSA and wNA (see information for 28 out of the 34 samples in Figure 7).

Discussion

Ecological divergence between the wSA ecotypes

Ecological factors may be the driving force in the evolutionary divergence between the ecotypes of the western South Atlantic (wSA). The two wSA ecotypes exhibit differences in morphological traits that have been attributed to differential prey and habitat preferences (Costa et al., 2016). The congruence seen here between the morphological and genetic data confirms the presence of two distinct ecological groups in the wSA – namely coastal and offshore ecotypes – with significant level of evolutionary divergence. The correspondence between habitat (based on biopsy location) and genetic differentiation further support the initial suggestion by Costa et al. (2016) that the ecotypes have a parapatric distribution.

Evidence for habitat-driven population structure was also supported by previous molecular analyses (Fruet et al., 2017) and by the observation of differential habitat distribution between the ecotypes (Simões-Lopes et al., 2019).

The coastal ecotype appears to be restricted to shallower waters (< 20 m) within ~3 km of the coast between latitudes -23° and -43° (Di Tullio et al., 2015; Costa et al., 2016; Fruet et al., 2017; Simões-Lopes et al., 2019), usually forming small associated groups (less than 100 individuals) with high site-fidelity to estuaries, enclosed bays, and river mouths (Simões-Lopes et al., 1998; Vermeulen & Cammareri, 2009a; Fruet et al., 2011; Daura-Jorge et al., 2013; Giacomo & Ott, 2016), and employing habitat-specific learned foraging techniques (Simões-Lopes et al., 1998). The offshore ecotype has a larger home range, is usually distributed along the coast in deeper waters (> 30 m), although there are records of these dolphins closer to the coast (Simões-Lopes et al., 2019; Tardín et al., 2019), which may
be influenced by the presence of upwelling (Tardín et al., 2019), and they usually form
groups up to hundreds of individuals (Di Tullio et al., 2016; Fruet et al., 2017; Simões-Lopes
et al., 2019).

Populations occupying different environments or exploiting different resources in
sympatry or parapatry can experience contrasting natural selection pressures on traits, which
will become advantageous in one environment but not in the other (Schluter, 2001; Rundle &
Nosil, 2005). This ecological differentiation can lead to reproductive isolation and ultimately
result in ecological speciation (Schluter, 2001; Rundle & Nosil, 2005), with a reduced
probability of mating between such ecologically differentiated groups possibly arising due to
individuals’ preference to mate within their native habitat (i.e., habitat preferences), the
selection of mates on the basis of phenotypic traits (i.e., mate choice), or migrants presenting
lower growth, reproduction and survival rates in a different environment than their natal
habitat because of a less-adapted phenotype (i.e., selection against migrants) (Hendry et al.,
2007; Schluter & Conte, 2009).

For the western South Atlantic, there are records of a small area of overlap for the two
ecotypes in shallower waters (Vermeulen & Cammareri, 2009b; Fruet et al., 2017), so mating
between them could conceivably occur. However, sightings of co-occurrence of the ecotypes
are uncommon (Simões-Lopes et al., 2019). The genetic data suggested low migration rates
between the wSA ecotypes (around 1% per generation based on microsatellite data) and
stronger differentiation was found between common bottlenose dolphins occupying adjacent
but ecologically distinct habitats (i.e., wSA coastal vs. wSA offshore) than between dolphins
occupying distant but ecologically similar habitats (i.e., wSA offshore vs. wNA offshore). The
single haplotype we found to be shared between the two ecotypes in the western South
Atlantic was also shared with dolphins from offshore waters of the western North Atlantic
(wNA). Seven additional haplotypes (out of the 37 found in the wSA samples) were shared
among offshore dolphins of the wSA and wNA. The nuclear data also suggested some degree of admixture between the offshore samples of the two regions, to the exclusion of the wSA coastal samples, suggesting there may be some genetic interconnection between the offshore dolphins of both ocean basins, although whether this is historical or ongoing is unknown. Taken together these findings indicate that distinct habitat choices might be leading the ecotypes to more frequently mate with individuals inhabiting either their natal area or similar environmental conditions. Therefore, habitat preferences and low dispersal rates may be the potential primary drivers of the reproductive isolation between these ecotypes.

Examples of ecological specialization as the driving force of speciation have been cited before for other marine species (Rocha et al., 2005; Kashiwagi et al., 2012; Foote & Morin, 2016), and the levels of genetic and morphological divergence observed between the wSA common bottlenose dolphin ecotypes in this study suggest they may provide another example of ecological speciation in the marine environment.

The wSA ecotypes and their relationship to the wNA ecotypes

Similar to the results in Fruet et al. (2017), the offshore ecotype was more genetically diverse in both the nuclear and mitochondrial DNA than the coastal ecotype, which seems to be a worldwide characteristic (Natoli et al., 2004; Louis et al., 2014). In the western South Atlantic, we observed only one shared haplotype between the wSA ecotypes. It was an offshore-type haplotype found in five offshore individuals and one stranded dolphin with a skull characteristic of the coastal ecotype. In contrast, Fruet et al. (2017) found no shared haplotypes between biopsies collected in coastal and offshore waters of the wSA. Including samples from stranded animals and, more importantly, combining genetic and morphological data from those samples may have increased the power to detect animals with mixed histories. If only morphological data, or only genetic data, were available for the stranding sample (UFSC1077), we would not have detected it as unusual. This result raises the possibility of
further shared haplotypes in the stranding samples of unknown origin ($n = 13$) for which there is only mtDNA sequence data available. Random Forest analysis using the mtDNA variable sites of these “unknown ecotype” samples allowed us to predict their ecotype based on classification probabilities; however the Random Forest analysis is only looking at maternal data (mtDNA) so it will not be able to detect the presence of possible “hybrids” of the two ecotypes based on nuclear data, and higher assignment probability of the mtDNA haplotype is expected to the ecotype where the haplotype in question is found in higher frequency. Therefore, we conclude that although we can use a quantifiable probability to classify “unknown ecotype” samples, it is impossible to reliably classify these 13 samples to an ecotype using only mtDNA sequence, reinforcing the need to use multiple lines of evidence when working with stranding data.

Further, as previously stated a total of eight offshore-type haplotypes (including the shared haplotype between the wSA ecotypes) were also found in offshore dolphins of the western North Atlantic (wNA). Louis et al. (2014) also detected control region haplotypes shared between coastal and offshore ecotypes in the eastern North Atlantic (eNA) and offshore individuals from the western North Atlantic. As in this current study, there were no haplotypes shared with the wNA coastal dolphins. Evidence for genetic connectivity between wNA offshore dolphins and common bottlenose dolphins of other oceanographic regions has been observed elsewhere (Natoli et al., 2004; Quérouil et al., 2007; Tezanos-Pinto et al., 2009). Moura et al. (2013) suggested that climate changes during the Late Pleistocene may have allowed oceanic bottlenose dolphins to colonize coastal habitats, resulting in an opportunity for divergence between coastal and offshore bottlenose dolphin ecotypes. As pointed out by Louis et al. (2014), low levels of genetic diversity, as seen for the western South Atlantic (wSA) coastal ecotype (Fruet et al., 2017; this study), may be due to founder events. The absence of shared haplotypes between the wSA ecotypes and the wNA coastal
ecotype supports the hypothesis of independent founder events. Further, while the phylogenetic analysis supported separation of the wNA coastal dolphins from all the others, it could not distinguish among the wSA coastal, wSA offshore and wNA offshore dolphins. The inability to differentiate among these three groups may be due to low power associated with this short control region fragment; the use of longer sequence data, i.e., whole mitochondrial genomes, may improve the phylogenetic resolution of these taxa. Evidence of speciation between the two ecotypes in the wNA has been previously suggested (Kingston & Rosel, 2004) and should be further investigated.

**Taxonomic and conservation implications**

Statistical analysis of morphological divergence has revealed that the wSA ecotypes may be considered at least different subspecies (Costa *et al.*, 2016), a conclusion accepted by the Society for Marine Mammalogy’s Committee on Taxonomy (2018). In this current study, we detected morphological diagnosability of 98.7% between the ecotypes using 100 samples (coastal: 75; offshore: 25) and a Random Forest analysis. Nevertheless, Wickert *et al.* (2016) suggested the observed morphological differentiation is strong enough to warrant species status for the two ecotypes following the Phylogenetic Species Concept.

Application of the Phylogenetic Species Concept can significantly increase the number of described species, particularly when very few characters or small sample sizes are used (Walsh, 2000; Agapow *et al.*, 2004). The erroneous split of a species can result in new taxa, each with smaller ranges and population sizes than the original species. This can potentially increase the number of endangered species and result in negative consequences for conservation strategies and the study of biodiversity where there are often limited resources (Agapow *et al.*, 2004). The use of additional lines of evidence can help to reinforce the findings based on the Phylogenetic Species Concept and improve species classifications.
be “treated as tentative” (Agapow et al., 2004) and tested using additional lines of evidence since it may lead to some problematic classifications due to (1) possible subjectivity in deciding whether the level of morphological differentiation is congruent with species-level divergence; (2) a large number of individuals is needed to demonstrate that the morphological qualitative characters are fixed differences between the groups (Agapow et al., 2004; Dayrat, 2005; Padial et al., 2010). Therefore, in order to evaluate whether the level of differentiation seen between the two ecotypes in the wSA is sufficient to raise them to species status, we followed the subspecies and species concepts defined in Taylor et al. (2017a) and made use of the integrative taxonomy framework, which uses different sources of data to test the level of diagnosability between the groups under study.

We also made use of metrics using mtDNA control region sequence data, net between-group nucleotide divergence (Nei’s $d_A$) and Percent Diagnosable ($PD$), since they have been suggested as useful tools to distinguish cetacean populations, subspecies, and species (Rosel et al., 2017b; Taylor et al., 2017b). The mtDNA control region has been commonly used in taxonomic studies with cetacean taxa, however, as pointed out by Rosel et al. (2017c), there has been a lack of consistency in how subspecies and species were defined based on this data type. Rosel et al. (2017b) used mtDNA control region sequence data from well-accepted pairs of populations, subspecies and species of cetaceans to compare several different metrics and observed that Nei’s $d_A$ and Percent Diagnosable performed best in discriminating each taxonomic group and provided highly accurate thresholds of classification, which, coupled with additional lines of evidence (e.g., nuclear markers), can “improve taxonomic investigations in cetaceans”. Moderate values for Nei’s $d_A$ (0.008) and diagnosability ($PD$) around 98% were observed between the two wSA ecotypes, both of which are in line with the thresholds considered informative for subspecies descriptions (0.004 < $d_A$ < 0.02; 95% < $PD$ < 100%; see Taylor et al., 2017b). We found one shared haplotype, no fixed substitutions
separating the mtDNA clusters, and no clear phylogenetic distinction between the wSA ecotypes. The low level of differentiation and shared haplotype may be indicative of a relatively recent divergence and incomplete lineage sorting in the mtDNA genome or a low level of genetic exchange (approximate 1% per generation) as suggested by the microsatellite data. Previous studies have also indicated possible low levels of gene flow between the wSA ecotypes. Using microsatellite data, Fruet et al. (2017) and Oliveira et al. (2019) both provided evidence for some admixed individuals. However, the number of loci in these studies was relatively low and the very low allelic diversity of the coastal ecotype increases the likelihood of shared common alleles that could create the appearance of admixture. The level of admixture identified by Oliveira et al. (2019) prevented the authors from recommending any formal taxonomic proposal for raising the subspecies *T. t. gephyreus* to the species level.

Taken together, these results suggest the wSA ecotypes are in the process of ecological divergence leading to speciation, although it may be incomplete since we cannot currently rule out the possibility of some gene flow. The results support the description of the wSA ecotypes as the subspecies *Tursiops truncatus gephyreus* (wSA coastal ecotype) and *T. t. truncatus* (offshore ecotype, which includes the wSA and wNA offshore dolphins) (Costa et al., 2016). Interestingly, the low level of mtDNA divergence contrasts sharply with the large amount of morphological differentiation observed between the wSA ecotypes. Further studies with considerably higher number of nuclear genetic markers, a possibility provided by next-generation sequencing methods, will be able to more comprehensively evaluate the genetic drivers of divergence and levels of male-mediated gene flow. Integrating nuclear data with the morphological and mitochondrial data provided here will allow a complete and thorough evaluation of the taxonomy of these ecotypes and whether they may represent species, particularly when placed in a larger geographic context.
The western South Atlantic subspecies represent incipient evolutionary lineages and we urge that these two subspecies be managed independently and preserved for conservation, morphological diversity, and evolutionary purposes. *T. t. gephyreus* exhibits low levels of genetic variability and this subspecies appears to be restricted to the coastal waters of southern Brazil, Uruguay, and northern Argentina (Fruet et al., 2017; Oliveira et al., 2019; this study), although further work is needed to identify the northernmost distribution along the Brazilian coast. These coastal areas are affected by several anthropogenic stressors (*e.g.*, overfishing, bycatch, contamination, habitat degradation) that seem to be impacting the dolphins’ survival (Daura-Jorge & Simões-Lopes, 2011; Fruet et al., 2012; Fruet et al., 2016), with some records of population decline (see Vermeulen et al., 2017).

**Data Availability**

Haplotypes found in this study were deposited in GenBank under the accession numbers MK105857-MK105886. Microsatellite and morphological data sets can be found in the Figshare repository under the DOI: 10.6084/m9.figshare.9963212

**References**


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Rosel, P. E., Taylor, B. L., Hancock-Hanser, B. L., Morin, P. A., Archer, F. I., Lang, A. R., ... & Leslie, M. S. (2017c). A review of molecular genetic markers and analytical approaches that have been used for delimiting marine mammal subspecies and species. *Marine Mammal Science, 33*, 56-75.


Table 1: Sample sizes (a) for the microsatellite and mitochondrial DNA (mtDNA) data sets, indicating initial number of samples available, the number that failed (see main text), the number of duplicates, and the final sample size for each data type; (b) number of samples in common across datasets. Values in bold indicate the final total number of samples available for that data set. s: soft tissue samples; t: tooth samples.

(a) | Initial data set | Failed | Duplicates removed | Final data set |
---|----------------|--------|--------------------|---------------|
**microsatellites** | 216 s, 1 t | 26 s, 1 t | 22 s | 168 s |
**mtDNA** | 216 s, 37 t | 23 t | 22 s | 194 s, 14 t |

(b) | microsatellites | mtDNA | skulls |
---|----------------|-------|--------|
**microsatellites** | 168 | | |
**mtDNA** | 168 | 208 | |
**skulls** | 2 | 34 | 106 |
Table 2: Mean recent migration rates and respective 95% confidence intervals (CI) between the wSA clusters identified by STRUCTURE, inferred using microsatellite data and BAYEASS. Genetic differentiation ($F_{ST}$ and $\phi_{ST}$) between the wSA clusters inferred using microsatellite data and mitochondrial DNA data ($p$-values < 0.0001 for all tests). The migration rates were estimated as the proportion of individuals that migrate from one cluster to the other per generation. The analyses were performed with and without the closely related coastal samples (see text). NA: Not Applicable. Total sample size per ecotype for nuclear data: offshore ($n = 61$); coastal (with related samples: $n = 107$; without related samples: $n = 33$). Total sample size per ecotype for mtDNA data: offshore ($n = 64$); coastal (with related samples: $n = 131$; without related samples: $n = 57$).

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<th>Migration rates between clusters</th>
<th>Genetic differentiation between clusters</th>
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<td>With closely related coastal samples</td>
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<td>From/To</td>
<td>Coastal (95% CI)</td>
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<td>Coastal</td>
<td>0.997 (0.991 – 1.0)</td>
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<td>Offshore</td>
<td>0.003 (0.0 – 0.009)</td>
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<td>Without closely related coastal samples</td>
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<td>From/To</td>
<td>Coastal (95% CI)</td>
</tr>
<tr>
<td>Coastal</td>
<td>0.99 (0.972 – 1.0)</td>
</tr>
<tr>
<td>Offshore</td>
<td>0.01 (0.0 – 0.028)</td>
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Figure 1 Map of the western South Atlantic study area showing sampling locations of (A) biopsy and (B) stranding samples used in the genetic analyses. Samples are identified by color according to the origin (see text): coastal waters/morphology (green), offshore waters/morphology (blue), and unknown origin (orange).

Figure 2 Bayesian assignment probabilities of common bottlenose dolphins in the western South Atlantic based on 10 nuclear microsatellite loci and inferred using (A) TESS and (B) STRUCTURE for $K = 2$ and $K = 3$. Each column represents one individual with colors representing the membership proportion to each of the clusters: wSA coastal cluster (green), wSA offshore cluster (blue), unknown offshore (third) cluster (gray).

Figure 3 Median joining network of haplotypes of common bottlenose dolphins of the western South Atlantic. Haplotypes color coded as coastal ecotype (green), offshore ecotype (blue), “unknown ecotype” (orange). The size of the circles is proportional to the haplotype frequency in each group. Small red dots indicate either extinct or unsampled haplotypes. Small red numbers represent mutational steps.

Figure 4 Median joining network of haplotypes of common bottlenose dolphins of the western Atlantic. Haplotypes color-coded as western South Atlantic coastal ecotype (green), western South Atlantic offshore ecotype (blue), western North Atlantic coastal ecotype (red) and western North Atlantic offshore ecotype (purple). The size of the circles is proportional to the haplotype frequency in each group. Haplotypes from the western North Atlantic coastal ecotype were retrieved from GenBank and therefore there is only one individual per haplotype. Small black dots indicate either extinct or unsampled haplotypes. Small red numbers represent mutational steps.
Figure 5 Phylogenetic tree for common bottlenose dolphins of the western Atlantic Ocean based on maximum likelihood methodology using 354 bp of mtDNA control region sequence. Values above nodes represent bootstrap values (cut-off > 50%). Ttr: wNA coastal haplotypes; OTtr: wNA offshore haplotypes; SWATtr: wSA haplotypes. The haplotype names are colored following descriptions in Figure 4. The shared haplotype between ecotypes is colored in black.

Figure 6 Bayesian assignment probabilities of common bottlenose dolphins in the western Atlantic Ocean based on 10 nuclear microsatellite loci and inferred using (A) TESS and (B) STRUCTURE for $K = 2$, $K = 3$, and $K = 4$. Each column represents one individual. The colors represent the membership proportion to each of the clusters: wSA coastal cluster (green), wSA offshore cluster (blue), wNA offshore cluster (purple), unknown offshore (fourth) cluster (gray).

Figure 7 Scatter plot of the principal component 1 (PC1) and 2 (PC2) scores from the principal component analysis of 21 cranial measurements and 100 common bottlenose dolphins of the western South Atlantic. Black shapes represent the specimens with only morphological data available (circle: coastal morphotype; triangle: offshore morphotype), whereas colored shapes represent the specimens with both morphological and genetic data available (green: coastal haplotype; blue: offshore haplotype). The sample UFSC1077 (see text) is represented by a blue circle. The sample UFSC1281 (see text) is represented by “*”. Ellipses represent 95% confidence.
Patos Lagoon

Laguna

183 km