Vol. 671: 219–233, 2021
https://doi.org/10.3354/meps13761

MARINE ECOLOGY PROGRESS SERIES
Mar Ecol Prog Ser

Published August 5

Beyond genetic differences: epigenetic variation in common bottlenose dolphins *Tursiops truncatus* from contrasting marine ecosystems

Ana Carolina Correa Tatsch[1,2,*], Maíra Carneiro Proietti[2], Rebeca Zanini[3,4], Pedro F. Fruet[1,5,6], Eduardo R. Secchi[1]

[1]Laboratory of Ecology and Conservation of Marine Megafauna, Institute of Oceanography, Federal University of Rio Grande (FURG), Rio Grande, RS 96203-900, Brazil

[2]Marine Molecular Ecology Laboratory, Institute of Oceanography, Federal University of Rio Grande (FURG), Rio Grande, RS 96203-900, Brazil

[3]Drosophila Laboratory, Institute of Biosciences, Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, RS 91509-900, Brazil

[4]Integrative Biomedicine Laboratory, Nova Medical School, Universidade Nova de Lisboa, Lisbon 1150-082, Portugal

[5]Oceanographic Museum Prof. Eliézer C. Rios, Federal University of Rio Grande (FURG), Rio Grande, RS 96200-970, Brazil

[6]KAOSA, Rio Grande, RS 96205-290, Brazil

ABSTRACT: Recent genetic and morphological studies have indicated an incipient ecological divergence between 2 ecotypes of common bottlenose dolphin *Tursiops truncatus* in the Southwestern Atlantic. However, genetic variation is not the only molecular mechanism that alters the phenotype of these animals: epigenetics can also influence phenotypic plasticity, as well as the ecological adaptation and divergence of natural populations. Nevertheless, very little is known about the role that epigenetics plays in the population ecology of marine mammals. In this work, we tested whether there are differences in DNA methylation patterns between a coastal and an offshore ecotype of common bottlenose dolphin. Methylation patterns were analyzed using the methylation-sensitive amplified polymorphism technique on biopsy samples collected from animals of both ecotypes. We found consistent differences in DNA methylation patterns between coastal and offshore individuals. We also confirmed the genetic differences described in previous studies, indicating that the divergence between ecotypes has both genetic and epigenetic components. Our data show that it is possible to differentiate animals from the coastal and offshore ecotypes using DNA methylation markers, supporting the hypothesis that contrasting environments — which are decisive for the ecological divergence of these populations — lead to epigenetic modifications in common bottlenose dolphins.

KEY WORDS: Marine mammals · DNA methylation · Epigenetics · Cetaceans · Ecological divergence

1. INTRODUCTION

Odontocete cetaceans are high trophic level predators with high mobility and ecological flexibility, and most species occur in different oceanic environments (Ballance 2018, Forcada 2018). The common bottlenose dolphin *Tursiops truncatus* is a marine mammal that is widely distributed and able to occupy several habitats with very distinct characteristics, from coastal to offshore regions (Forcada 2018, Wells & Scott 2018). Depending on their habitats, these animals may present considerably differentiated morphologies, physiologies, and behaviors (Duffield et al. 1983, Mead & Potter 1995, Costa et al. 2015, Wells & Scott 2018). Several authors have sought to investigate these populational differences

*Corresponding author: ana.tatsch@furg.br

using genetic analyses, thus addressing the long-term adaptations to environments and pressures to which these animals may be subjected (e.g. Hoelzel et al. 1998, Parsons et al. 2006, Louis et al. 2014, Segura-García et al. 2018). However, phenotypic variability, which is critical for natural selection, depends on the interaction of genetic and environmental factors (Willmore et al. 2007, Johnson & Tricker 2010); therefore, rapid physiological, behavioral, and even morphological changes to the contrasting environments they inhabit can also be considered key factors for the survival of many odontocetes, including *T. truncatus*. Such environmental diversity is of fundamental ecological importance (Bossdorf et al. 2008, Liebl et al. 2013) and can be decisive for the adaptation process, which ultimately influences the demographic structure and population dynamics of cetaceans (Bilgmann et al. 2008, McGowen et al. 2014). Nevertheless, there is still little understanding of the role that different environments play for populations of marine mammals, beyond that already described and well established by population genetics. Genetic diversity alone does not completely elucidate aspects related to the adaptation — and phenotypic alterations — of a species in a given environmental condition, especially in the context of recent environmental changes (Kilvitis et al. 2014).

Several studies have focused on the role of epigenetic mechanisms in the occurrence of ecologically relevant phenotypes (Schrey et al. 2013, Kilvitis et al. 2014). These mechanisms can affect gene expression and cause lasting phenotypic variations without any change in the genome (Rakyan et al. 2003, Kucharski et al. 2008). Such epigenetic changes are based on molecular processes that can activate, reduce, or completely suppress the activity of certain genes, mediating the effect of the environment on the organism and resulting in phenotypic changes (Bossdorf et al. 2008). Thus, epigenetic differences may be used as molecular markers of environmental pressures, from individuals to populations (Herrera & Bazaga 2011, Liu et al. 2012, Massicotte & Angers 2012, Richards et al. 2012, Schrey et al. 2013). In natural populations, both genetics and epigenetics can represent crucial molecular pathways for the ecological flexibility and colonization of new environments (Smith & Ritchie 2013, Kilvitis et al. 2014). The relationship between epigenetics and genetics can be relatively dependent or completely independent, with both molecular mechanisms being responsible for the phenotypic plasticity and varying degrees of adaptation of natural populations (Richards 2006).

DNA methylation is one of the most common epigenetic mechanisms, and in mammals occurs primarily at CG sites (Feng et al. 2010, Li & Zhang 2014), which are usually grouped in gene regulatory regions (Bossdorf et al. 2008, Li & Zhang 2014). Cytosine methylation is essentially mediated by the enzyme families DNA methyltransferase, which can add a methyl group to a cytosine (i.e. DNA methylation), and ten-eleven translocation, which can actively remove methyl groups from DNA (i.e. demethylation) (Martin & Fry 2018). The effects of the environment on global DNA methylation specifically depend on the activity of these enzymes and the availability of S-adenosylmethionine, a substrate required for DNA methylation (Feil & Fraga 2012). However, the molecular steps that determine how the environment affects the methylation of specific genes are not yet completely clarified. A possible explanatory mechanism for these steps is the presence of transcription factors (triggered by environmental variables), which can prevent methyltransferases from accessing the gene being expressed, resulting in the hypomethylation of this gene (Martin & Fry 2016, Zhu et al. 2016). In turn, a reduced demand for gene expression leads to a decrease in transcription factors in a given gene, which facilitates the access of the methyltransferase and, thus, allows hypermethylation of the gene. Methylation in CG dinucleotides can regulate gene expression, leading to transcriptional repression of the methylated gene (Jablonka & Lamb 2006, Bossdorf et al. 2008, Li & Zhang 2014). Although the stability of DNA methylation varies throughout the genome and certain loci may be more susceptible to environmental influence than others (Angers et al. 2010), the effect of methylation on the transcription machinery can directly influence the phenotype of an organism (Rapp & Wendel 2005, Bock 2012), thereby promoting the adaptation of populations to environmental changes (Herrera & Bazaga 2011).

The epigenetic process is of fundamental importance in an ecological and evolutionary context, since environmental pressures can alter gene expression patterns in individuals within a single generation (i.e. ontogenetically) (Bossdorf et al. 2008, Jablonka & Raz 2009). By directly affecting phenotypic plasticity and thus providing faster adaptation to environmental pressures, epigenetic variation may even impact reproductive success, which could ultimately influence the natural selection process (Bossdorf et al. 2008). Hence, environmental changes or contrasts can lead to epigenetic changes (Bossdorf et al. 2008) that may be determinant for the appearance of mar-

ine ecotypes (Hofmann 2017, Beal et al. 2018). Given the high adaptive capacity and ecological flexibility of odontocetes, the occurrence of ecotypes of a particular species residing in environments with considerably different characteristics can be influenced by both genetic and epigenetic variation. Therefore, understanding the mechanisms that regulate gene expression is highly relevant to both comprehend the adaptation processes and to predict the fundamental factors for the survival of cetaceans exposed to significant environmental pressures.

In the Southwestern Atlantic (SWA), 2 ecotypes of common bottlenose dolphin have been recently recognized as being in the process of ecological divergence, which can lead to reproductive isolation: *T. truncatus truncatus* (offshore ecotype) and *T. truncatus gephyreus* (coastal ecotype) (Committee on Taxonomy 2020, Costa et al. 2021). The offshore ecotype occurs primarily beyond the continental shelf break (>150 m depth) and usually over 100 km away from the coast (Di Tullio et al. 2016). These animals display a dark gray coloration, falcate dorsal fin, and relatively shorter beak, traits that are morphologically distinct from the coastal ecotype, which displays a lighter gray coloration, smaller and triangular dorsal fin, and longer beak (Fruet et al. 2017b). The occurrence of the latter is restricted to coastal regions from southeastern Brazil (27° S) to central Argentina (43° S) (Simões-Lopes & Fabian 1999), and they are generally seen in shallow waters (<20 m) less than 3 km from the coast (Di Tullio et al. 2015, Laporta et al. 2017). The marked ecological divergence between these populations represents an opportunity to understand how distinct environments can shape epigenetic marks in the genome, as well as the adaptive potential of epigenetic variation in dolphin ecotypes.

Molecular studies have demonstrated genetic differences between *T. truncatus* SWA ecotypes (e.g. Fruet et al. 2017b, de Oliveira et al. 2019, Costa et al. 2021), with coastal animals having lower levels of genetic diversity (Fruet et al. 2014, Costa et al. 2015) than the offshore ones (Castilho et al. 2015, Fruet et al. 2017b). Such genetic differences can also occur within an ecotype of these dolphins, since social organization, habitat use, and resource distribution can also affect the genetics of populations. The coastal ecotype animals that live in or near the Patos Lagoon Estuary (32° S), southern Brazil, are structured into 3 different social units (1 unit of residents of the estuarine environment and 2 units of residents of the adjacent coastal zone, of which one occupies the southern coast and the other the northern coast), which have shown low but significant genetic differentiation (Genoves et al. 2020). Despite recent advances in the understanding of the genetic differences between and within *T. truncatus* SWA ecotypes, to our knowledge no study has evaluated epigenetic markers, which can answer questions more directly related to the environment where the animals live and whose effects on DNA can occur over a short time scale. Thus, considering that epigenetic changes can be environmentally induced (Bossdorf et al. 2008, Richards et al. 2010), we hypothesized that dolphins living in ecologically distinct environments have different epigenetic marks. Therefore, in this work we investigated the occurrence of differences in DNA methylation patterns between the coastal and offshore ecotypes of the SWA common bottlenose dolphins.

2. MATERIALS AND METHODS

2.1. Sample collection and DNA extraction

Skin biopsy samples of common bottlenose dolphins were obtained from 2 natural populations (coastal, n = 19; offshore, n = 20). In estuarine and coastal waters, samples were collected between 2013 and 2017 during small boat-based surveys conducted at depths shallower than 10 m and less than 2 km from shore off southern Brazil. In offshore waters, samples were collected between 2011 and 2015 during ship-based surveys in water depths greater than 100 m (mean = 412 m) and at a minimal distance of 103 km from shore (mean = 143 km) (Fig. 1). Thus, this sampling scheme aimed to obtain a representative sample from each of the target ecotypes based on their known distributions. Biopsies were taken using modified darts specifically designed for small cetaceans (F. Larsen, Ceta-Dart) fired from 120 lb (~54.5 kg) draw weight crossbows. Only sub-adult and adult individuals were biopsied, as they have large body mass and a thick blubber layer (Fruet et al. 2017a). All samples were preserved in 20% dimethyl sulfoxide saturated with sodium chloride (Amos & Hoelzel 1991), and stored at −20°C. Genomic DNA was extracted using the PureLink™ Genomic DNA Mini Kit (Invitrogen) according to the manufacturer's protocols. DNA integrity was checked with 1% agarose gel electrophoresis. DNA concentration and purity were measured by spectrophotometry with a BioDrop device (BioDrop UV/VIS Spectrophotometer). DNA samples were stored at −20°C prior to use.

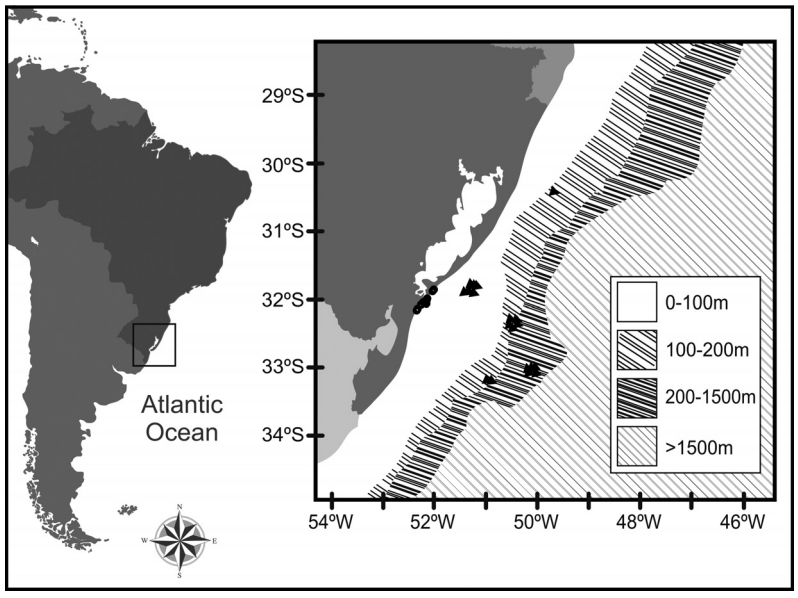

Fig. 1. Study area and sampling sites of *Tursiops truncatus* ecotypes in coastal (black circles) and offshore (black triangles) waters in the Southwestern Atlantic

2.2. Methylation-sensitive amplified polymorphism

Molecular analyses were performed using the fluorescence-labeled methylation-sensitive amplified polymorphism (MSAP) technique, adapted from Reyna-López et al. (1997) and Xu et al. (2005), which allows detecting polymorphisms in DNA methylation patterns. Similar to amplified fragment length polymorphism (AFLP), MSAP is based on use of the methylation-sensitive restriction enzymes *Hpa*II and *Msp*I. These enzymes recognize the same restriction site (the 5′-CCGG-3′ sequence), but have different sensitivities to cytosine methylation in this sequence (Roberts et al. 2007). Although *Hpa*II is sensitive to full methylation (i.e. in both DNA strands) in both cytosines, it cleaves only at the site where the external cytosine is hemimethylated. On the other hand, *Msp*I cleaves at sites whose internal cytosines are completely methylated. In addition, both enzymes cleave the 5′-CCGG-3′ sequence when completely unmethylated (Liu et al. 2012), while they do not cleave hypermethylated (i.e. methylation in the outer and inner cytosines) and completely methylated sites in the outer cytosine (i.e. on both strands of DNA). This cleavage pattern generates 2 profiles, when isochizomers are used along with the *Eco*RI enzyme: profile H, cleaved only by the enzyme pair *Eco*RI–*Hpa*II, and profile M, cleaved only by the enzyme pair *Eco*RI–*Msp*I. Together, these profiles allow the evaluation of the methylation state of CG sites in the individual's DNA (Herrera & Bazaga 2010), which are not only highly methylated in mammals (Jabbari & Bernardi 2004), but also widely found in mammalian promoter regions (Fatemi et al. 2005).

First, DNA samples were separately digested with the enzyme combinations *Eco*RI–*Hpa*II or *Eco*RI–*Msp*I. The first digestion reaction of each sample was performed in a 30 µl solution containing 500 ng genomic DNA, 0.6 µl *Eco*RI enzyme (20 U μl^{-1}), 6 µl Anza buffer, and distilled water. This mix was incubated at 37°C for 2 h. The enzyme was then inactivated by heating at 65°C for 15 min. The solution from the previous step was divided into 2 separate series, one for *Hpa*II, and one for *Msp*I. For each reaction, we used 15 µl of the solution from the previous step, 14.1 µl of distilled water, and 0.9 µl of either *Hpa*II or *Msp*I (10 U μl^{-1}). Samples were incubated overnight and enzymes were inactivated by heating at 65°C for 15 min. Subsequently, the ligation procedure of the adapters was prepared adding the following components to the 30 µl solution that resulted from the digestion reactions: 11.2 µl of distilled water, 6 µl 10× T4 ligase buffer with ATP, 6 µl *Eco*RI adaptor (10 pmol), 6 µl *Hpa*II/*Msp*I adaptor (10 pmol) (Table S1 in the Supplement at www.int-res.com/articles/suppl/m671p219_supp.pdf), and 0.3 µl T4 ligase (5 U μl^{-1}). The mixture was then incubated for 2 h at room temperature.

Preamplification PCRs were performed in 10 µl solutions containing 1 µl of ligation products, 0.4 µl of *Eco*RI+1 and *Hpa*II+1/*Msp*I+1 preamplification primers (10 pmol) (Table S1), 0.1 µl of Platinum *Taq* polymerase (5 U μl^{-1}), 0.25 µl of dNTPs (10 mM), 0.5 µl of $MgCl_2$ (50 mM), 1 µl of 10× PCR buffer, and 6.35 µl of ultrapure water. The PCR conditions were as follows: 94°C for 5 min; 30 cycles of 94°C for 30 s, 56°C for 1 min, and 72°C for 1 min; and extension at 72°C for 7 min prior to selective amplification. The PCR products from the preamplification step were diluted to 1:5 (v:v) with ultrapure water and stored at −20°C until use. For the selective PCR step, an initial screening of *Eco*RI+3 and *Msp*I+3/*Hpa*II+3 selective primer combinations was performed with 2 test samples of preselected DNA in order to find primer combinations yielding multiple strong and well-separated MSAP bands. Thus, 4 primer combinations (i.e.

*Eco*RI+AAC / *Hpa*II/*Msp*I+TAA, *Eco*RI+ACT / *Hpa*II/*Msp*I+TAT, *Eco*RI+AAC / *Hpa*II/*Msp*I+TAT, *Eco*RI+ACT / *Hpa*II/*Msp*I+TAA; Table S1) were used to amplify the preselective PCR products. Selective amplifications were performed in 15 µl of solution containing 1.5 µl of the diluted preamplification product, 0.6 µl *Eco*RI+3 primer (10 pmol), and 0.6 µl of *Hpa*II+3 or *Msp*I+3 primer labeled with fluorescent dye (10 pmol) (Table S1), 0.15 µl of Platinum *Taq* polymerase (5 U μl^{-1}), 0.375 µl of dNTPs (10 mM), 0.75 µl of $MgCl_2$ (50 mM), 1.5 µl of 10× PCR buffer, and 9.52 µl of ultrapure water. The PCR amplification reactions were performed using touchdown cycles, and the conditions were as follows: 94°C for 5 min; 13 touchdown cycles of 94°C for 30 s, 65°C (reducing each cycle by 0.7°C) for 30 s and 72°C for 1 min; 23 continued cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min; and extension at 72°C for 7 min. The amplification products were observed with 2% agarose gel electrophoresis. Finally, the MSAP profiles were fluorescently detected along with GeneScan 600-LIZ size standards with an ABI3730 Genetic Analyzer (Applied Biosystems).

2.3. Data analysis

To verify possible differences between coastal and offshore ecotypes of bottlenose dolphins through the MSAP technique, we first used Peak Scanner (v3.0, Applied Biosystems) to detect fluorescent peaks and to calculate the size of *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I products. RawGeno v. 2.0.2 (Arrigo et al. 2012) was used for scoring MSAP fragments to filter low-quality peaks, and to transform the band profiles into a binary matrix using absence (0) and presence (1) data. Thus, DNA fragments obtained within the size range of 50–500 bp were recorded, and a peak height of 70 relative fluorescence units (RFU) was set as a threshold. We also used an alternative method with more conservative parameters to check if the reliability of our main results did not depend on the fragment identification method used; therefore, fragments <100 bp or >350 bp in length, and/or <100 RFU, were excluded from this verification. The MSAP matrices from the 4 primer combinations were then pooled to generate a single dataset. These data were analyzed using the 'msap' package (v. 1.1.9; Pérez-Figueroa 2013) in the R environment (R Core Team 2019). As a result, fragments present in both *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I profiles (1/1) were classified as unmethylated; the presence of only 1 profile represents a methylated state (*Eco*RI/*Hpa*II profile: 1/0, hemimethylated; or *Eco*RI/*Msp*I profile: 0/1, internal cytosine methylation); and fragments absent for both enzymes (0/0) were considered as missing data (uninformative state) because these loci can be hypermethylated or absent due to underlying genotypic differences (Schulz et al. 2013). Such profiles were used to classify all fragments as either methylation-susceptible loci (MSL; epigenetic markers) or non-methylated loci (NML; genetic markers), depending on whether the methylation state of the locus, across all samples, exceeded a predefined threshold of 5% error rate. The fragments that did not reach the error threshold, which were categorized as NML, were scored as AFLP dominant markers (presence or absence of bands). Therefore, MSL were used to assess epigenetic differences between ecotypes, and NML were used to evaluate genetic variation.

To test for epigenetic and genetic differentiations between coastal and offshore ecotypes, we performed analysis of molecular variance (AMOVA; Excoffier et al. 1992), using 10 000 permutations. We also used AMOVA to verify whether there are epigenetic differences among the 3 social units of bottlenose dolphins from the coastal ecotype. In addition, we assessed the level of epigenetic and genetic differentiation between ecotypes using principal coordinate analysis (PCoA). Moreover, Mantel tests were used to assess the correlation between the MSL and NML matrices, using 10 000 permutations. We also estimated epigenetic and genetic diversities using the Shannon diversity index (as described by Pérez-Figueroa 2013). These analyses were performed using the 'msap' package in R (Pérez-Figueroa 2013), and their fragment classifications (i.e. the MSL and NML binary matrices) were also used subsequently for Bayesian clustering analyses (see paragraph below). We also used Fisher's exact test to assess differences in locus-specific methylation status between ecotypes. Correction for multiple testing was done using the Benjamini-Hochberg procedure to control false discovery rate (Benjamini & Hochberg 1995). All loci with a probability of <0.01 were then selected, and the relationship among loci was estimated using Gower's coefficient of similarity (for more details, see Suarez-Bregua et al. 2020). These methylation status data were clustered by the UPGMA method and were used to generate a heatmap using the 'ComplexHeatmap' R package (v.2.0.0; Gu et al. 2016).

In order to determine whether epigenetic and genetic patterns reflect population structure according to ecotypes, we performed Bayesian clustering using STRUCTURE v.2.3.4 (Falush et al. 2007) to determine the number of clusters in our samples. The number of populations (K) was estimated by performing 20 in-

dependent replicates for each K, from K = 1 to K = 4, for both epigenetic and genetic profiles. Each analysis consisted of 1 000 000 Markov chain Monte Carlo iterations following a 100 000 burn-in period, assuming no *a priori* information on the population of origin, admixture model, and correlated allele frequency method. The selection of the most likely K value was done according to the following methods: Ln Pr(X|K) (Pritchard method; Pritchard et al. 2000), ΔK (Evanno method; Evanno et al. 2005), and MedMeaK, MaxMeaK, MedMedK, and MaxMedK estimators (Puechmaille 2016) using Structure Harvester (Earl & VonHoldt 2012) and StructureSelector (Li & Liu 2018). The summations and graphical presentations of STRCUTURE results were performed using CLUMPAK (Kopelman et al. 2015).

3. RESULTS

3.1. Epigenetic variation between ecotypes

Using the MSAP technique with 4 primer combinations, we obtained 1146 loci for the DNA 5′-CCGG-3′ sites, ranging from 270 to 323 loci per primer combination. As shown in Fig. 2, ~78% of the loci found were susceptible to methylation (MSL; 895 loci), with ~81% being polymorphic (729 loci). The frequencies of methylation status (i.e. unmethylated; hemimethylated; internal cytosine methylation; and full methylation or absence of target) were similar between the 2 ecotypes (Fig. 2). The consistency of DNA methylation status between coastal and offshore ecotypes and the high number of polymorphic MSL allowed us to test our hypothesis of differences in epigenetic fingerprints between the SWA common bottlenose dolphin ecotypes.

A large epigenetic difference was observed between coastal and offshore ecotypes (AMOVA, φ_{ST} = 0.12, p < 0.001; Table 1), which corroborates our postulated hypothesis. This result was checked using a more conservative method for identifying DNA fragments generated by MSAP (626 MSL, of which 83% were polymorphic), which confirmed the previous finding (φ_{ST} = 0.12, p < 0.001; Table S2). Moreover, PCoA showed a clear distinction in DNA methylation patterns, indicating a large epigenetic difference between the coastal and offshore ecotypes (Fig. 3). Bayesian clustering analysis using STRUCTURE also showed epigenetic structure between the 2 ecotypes (Fig. 4A); this was confirmed by all 6 estimators, which showed that K = 2 was the most likely K value for MSL data (Fig. S1, Table S3). In addition,

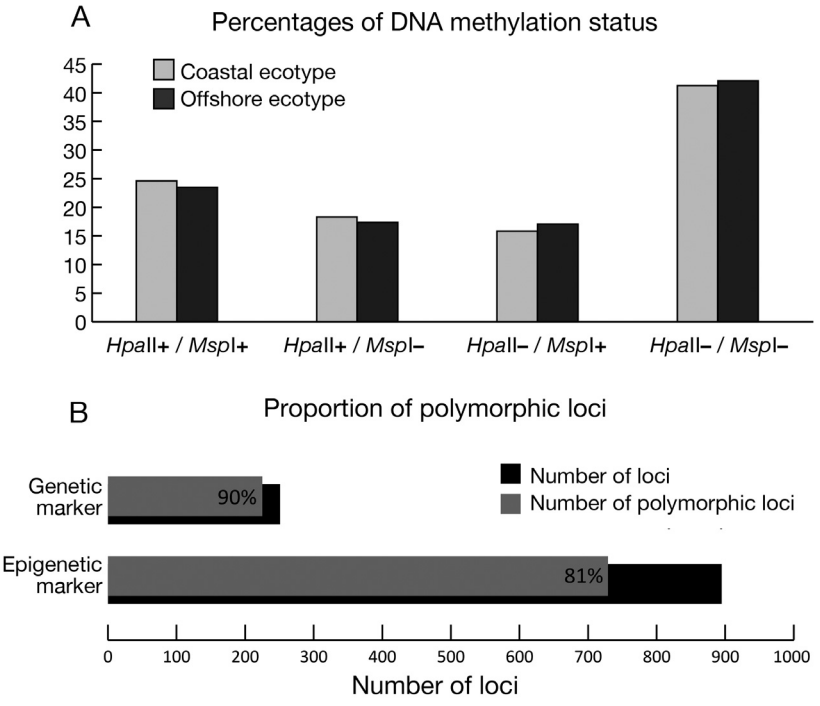

Fig. 2. (A) Descriptive data on DNA methylation status of common bottlenose dolphins in the Southwestern Atlantic (*Hpa*II+/*Msp*I+: no methylation; *Hpa*II+/*Msp*I–: hemimethylation of external cytosine; *Hpa*II–/*Msp*I+: full or hemimethylation of internal cytosine; *Hpa*II–/*Msp*I–: hypermethylation or mutation). (B) Total number of loci generated by the methylation-sensitive amplified polymorphism (MSAP) technique for epigenetic and genetic markers and their polymorphic loci

Table 1. Analysis of molecular variance between coastal and offshore ecotypes of the Southwestern Atlantic bottlenose dolphin, using epigenetic and genetic markers

Source of variation	df	SS	Variance (%)	φ_{ST}	p
Epigenetic markers					
Between ecotypes	1	475.6	17.55 (12%)	0.1161	<0.0001
Within ecotypes	37	4942	133.60 (88%)		
Total	38	5417			
Genetic markers					
Between ecotypes	1	26.9	0.85 (8%)	0.0769	<0.0001
Within ecotypes	37	379.2	10.25 (92%)		
Total	38	406.1			

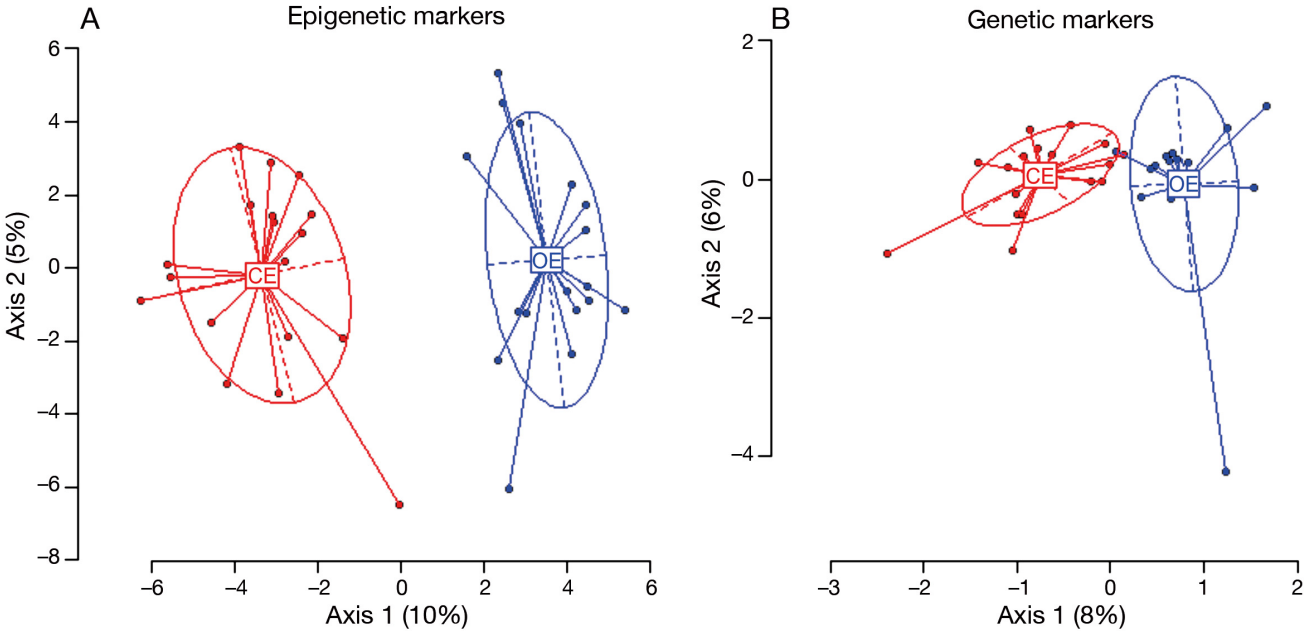

Fig. 3. Principal coordinate analyses (PCoA) using (A) epigenetic and (B) genetic markers, to compare coastal (CE) and offshore (OE) ecotypes of common bottlenose dolphins from the Southwestern Atlantic. The first two axes are shown with the percentage of variance explained. The individuals are grouped by the populations, as represented by their centroids and dispersion ellipses

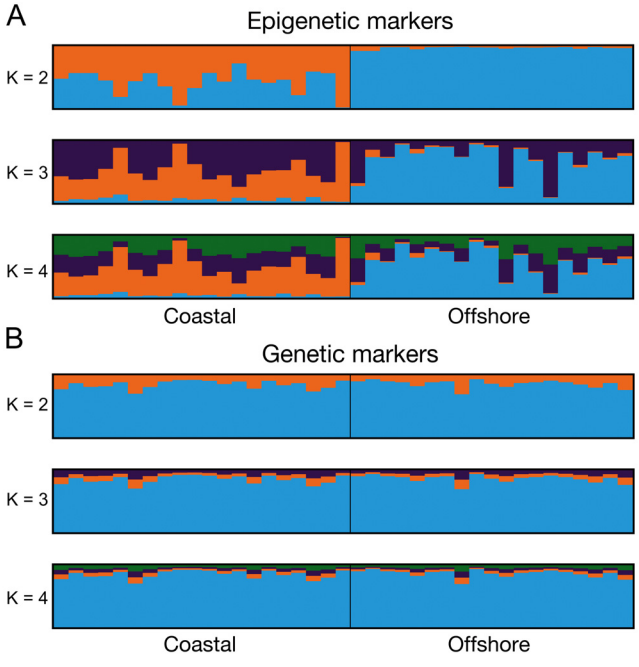

Fig. 4. Bayesian assignment probabilities using STRUCTURE software for K = 2, K = 3, and K = 4. (A) Epigenetic and (B) genetic structure between coastal and offshore ecotypes of common bottlenose dolphins from the Southwestern Atlantic, as estimated through methylation-sensitive amplified polymorphism (MSAP) loci. Each individual is represented as a vertical bar, and the length of the color shows the membership proportion to a cluster

Fisher's exact test indicated that there were significant differences between ecotypes in 318 methylation-susceptible loci (36% of total MSL and 44% of polymorphic MSL; Table S4). After Benjamini–Hochberg correction for multiple testing, we identified 205 differentially methylated loci (i.e. adjusted $p < 0.05$; Table S4) and 127 highly differentially methylated loci (i.e. adjusted $p < 0.01$; Fig. 5). In line with what was observed in the previous analyses (i.e. AMOVA, PCoA, and Bayesian clustering analysis), we saw a clear distinction between animals from coastal and offshore waters in the hierarchical clustering heatmap (Fig. 5). In other words, all of our data confirm that the coastal and offshore ecotypes of *Tursiops truncatus* exhibit markedly distinct methylation profiles.

Additionally, we verified the degree of epigenetic diversity of the samples. The Shannon index showed an important epigenetic diversity when considering all samples grouped (~0.52, Table 2); this diversity became slightly higher when considering each ecotype separately, with both presenting a diversity index of ~0.54. In view of this high epigenetic diversity observed in the studied populations, we sought to determine whether environmental and social structure differences within the ecotypes could help explain such epigenetic diversity. Thus, we examined the DNA methylation patterns in the 3 social units of the coastal ecotype, which occur in estuarine areas

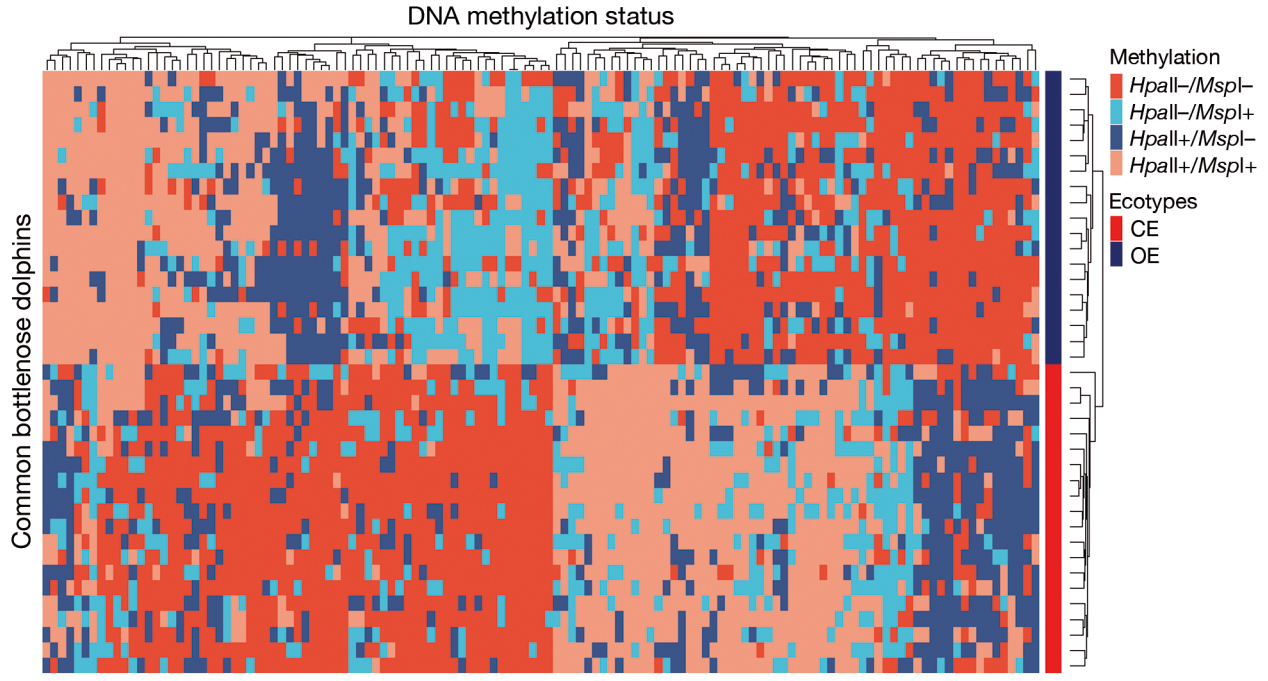

Fig. 5. Heatmap of 127 highly differentially methylated loci of common bottlenose dolphins from the Southwestern Atlantic (CE: coastal ecotype; OE: offshore ecotype) identified by Fisher's test ($p < 0.01$, after Benjamini–Hochberg correction). Different methylation status: *Hpa*II+/*Msp*I+: no methylation; *Hpa*II+/*Msp*I–: hemimethylation of external cytosine; *Hpa*II–/*Msp*I+: full or hemimethylation of internal cytosine; *Hpa*II–/*Msp*I–: hypermethylation or mutation

Table 2. Shannon index of phenotypic diversity of coastal and offshore ecotypes of the Southwestern Atlantic bottlenose dolphin, based on epigenetic and genetic markers. Methylation diversity refers to methylation-susceptible loci; genetic diversity refers to non-methylated loci. Shannon index of phenotypic diversity derived from the Shannon-Weaver index

Ecotypes	Methylation diversity Shannon index ± SD	Genetic diversity Shannon index ± SD
Coastal	0.536 ± 0.129	0.216 ± 0.059
Offshore	0.540 ± 0.125	0.223 ± 0.049
Both ecotypes	0.524 ± 0.144	0.189 ± 0.081

and adjacent coastal regions, but found no significant differences among the units (Table 3). Methylation patterns among the coastal ecotype units were barely distinguishable.

3.2. Genetic variation between ecotypes

We obtained 251 variable NML loci, of which 225 were polymorphic (90%; Fig. 2B). First, we examined the differences in these genetic markers between the coastal and offshore ecotypes through AMOVA, and found significant differences between these 2 groups ($\varphi_{ST} = 0.08$, $p < 0.001$; Table 1). PCoA showed that the

Table 3. Analysis of molecular variance among 3 social units of the coastal ecotype of the Southwestern Atlantic bottlenose dolphin using epigenetic markers. In the pairwise comparison, φ_{ST} values are given below the diagonal; p-values are shown above the diagonal. EU: estuarine social unit; SU: southern social unit; NU: northern social unit

Source of variation	df	SS	Variance	φ_{ST}	p		Pairwise φ_{ST} EU	SU	NU
Epigenetic markers									
Among units	2	219.2	−1.44	−0.0123	0.8667	EU	–	0.991	0.580
Within units	16	1892	118.2			SU	−0.042	–	0.313
Total	18	2111				NU	−0.003	0.010	–

2 groups have distinct genetic profiles with minimal overlap between them (Fig. 3). In addition, the Bayesian clustering analysis with genetic marker data, based on the results from Evanno ΔK and Pritchard methods (Fig. S1, Table S3), showed that the genetic structure is better represented by 2 clusters (i.e. K = 2; Fig. 4B). However, when CLUMPAK was used to combine the clusters generated by the 20 independent replicates per K analyzed through STRUCTURE, visual inspection of the graph did not allow identification of the 2 genetic clusters as easily as the epigenetic clusters (Fig. 4). Nevertheless, the results provided by STRUCTURE confirmed the overall genetic structure patterns. Therefore, all of our results show a moderate but significant difference in the genetic profile between the ecotypes.

Considerable genetic diversity, calculated by the Shannon index using NML loci data, was observed for all samples grouped (~0.19, Table 2). The level of genetic diversity increased slightly when the Shannon index was calculated for each ecotype separately (~0.22, Table 2), with the genetic diversity of the offshore population being subtly superior to that of the coastal population. Finally, we observed a weak but significant correlation between epigenetic and genetic variation (Mantel test; r = 0.234, p = 0.009), demonstrating a low level of dependence between these 2 molecular mechanisms in *T. truncatus*.

4. DISCUSSION

In this study, we evaluated the epigenetic patterns in 2 ecotypes, one coastal and one offshore, of SWA common bottlenose dolphins, based on comparisons of differentially methylated loci. Our data demonstrated striking epigenetic differences between the ecotypes. Moreover, in line with previous genetic evaluations (Fruet et al. 2017b, Costa et al. 2021), we identified significant genetic variations between these populations; however, they were less marked than the differences found in the previous studies. We also observed a substantial epigenetic diversity in these ecotypes; the same pattern was seen for genetic diversity, which confirms what was found for these populations in previous genetic studies (Fruet et al. 2017b, Costa et al. 2021). In general, the obtained results are consistent with the hypothesis that epigenetics is involved in the ecological flexibility of *Tursiops truncatus*. The clearly distinct environments to which these populations are constantly exposed may demand long-term changes in the gene expressions of these animals. In other words, the phenotypic differences observed between these ecotypes (Fruet et al. 2017b, Costa et al. 2021) possibly have both genetic and epigenetic origins.

All multivariate analyses that were used to evaluate the epigenetic structure of *T. truncatus* populations clearly separated the coastal and offshore ecotypes. Together, these analyses suggest that the environmental features are sufficiently contrasting to generate robust differences in DNA methylation patterns between the ecotypes of this species in the SWA. On the other hand, no significant differences were observed in the degree of epigenetic divergence among the 3 social units (north, south, and estuary) of the coastal ecotype, which have significant genetic differentiation (Genoves et al. 2020). Although the animals of these 3 units have distinct social structures, estuarine dolphins frequently use the adjacent marine coast, leading to an overlap of their habitat usage (Genoves et al. 2020). These indistinguishable methylation patterns among the coastal ecotype units may indicate that differences in environment and habitat use were not large enough to cause alterations in the epigenetic fingerprints of these animals, reinforcing that the epigenetic contrast observed between ecotypes may be due to more strongly distinct environmental characteristics.

The coastal and offshore ecotypes are separated from each other, and such isolation may have favored the development of local adaptations to very distinct environments. Taking into account the considerable contrast found between the methylation profiles of these ecotypes, methylation in regulatory regions possibly alters the expression of genes that respond to such environmental demands. The animals in the estuary and adjacent marine coast have a smaller home range and live predominantly in more sheltered and turbid, lower salinity (5–20 g kg^{-1}; mean: 11.5 g kg^{-1}; Haraguchi et al. 2015), and shallower (up to 20 m; Vieira et al. 2008) waters than the offshore animals. The offshore environment along the outer continental shelf and slope is characterized by more exposed conditions, as well as higher transparency and salinity (33.5–37 g kg^{-1}; de Carvalho-Borges et al. 2018), and greater depth (offshore common bottlenose dolphins occur mainly at depths between 500 and 1000 m; Di Tullio et al. 2016). Furthermore, these 2 areas are exposed to different pollutant loads, since the estuary receives and is affected by substantial discharges of contaminants of continental origin, from domestic and industrial sewage to agricultural pesticides (Niencheski et al. 1994, Seeliger & Costa 1997, Righetti et al. 2019), which do not occur in the continental slope ecosystem. The presence of vessels

is much more frequent in the coastal ecotype environment than in the pelagic zone, which can, in turn, impact the coastal marine soundscapes (Hildebrand 2009, Merchant et al. 2014). Therefore, the significant epigenetic differences shown in this study may be reflecting the local adaptations that have demanded alterations in the gene expression of the animals of each ecotype.

Epigenetic variations do not depend solely on the physicochemical environment to which organisms are exposed. One of the primary sources of epigenetic variation among organisms is differences in diet (e.g. Feil 2006, Li et al. 2011, Soubry 2015, García-Fernández et al. 2017), and distinct habitats may provide access to different prey resources. Common bottlenose dolphins of the coastal ecotype consume mainly demersal and demersal-pelagic fishes of the Sciaenidae family from the coastal zone, with the white croaker *Micropogonias furnieri* as the most frequent prey (Secchi et al. 2017). Although the diet of the offshore ecotype is poorly known, the scarce evidence available indicates a higher consumption of epipelagic fishes and squids (Pereira et al. 2020). Stable isotope analyses have confirmed the dietary differences between the coastal and oceanic common bottlenose dolphins (Botta et al. 2012, Pereira et al. 2020). Consequently, part of the epigenetic difference found between these ecotypes may be related to the consumption of distinct prey. In addition, the feeding behavior and foraging strategies of each ecotype (e.g. Botta et al. 2012, Bezamat et al. 2019, Pereira et al. 2020) may expose these animals to different environmental pressures, which could ultimately also alter their long-term gene expression.

Epigenetic differences such as those found in the present study have been observed in populations of numerous groups, from plants to vertebrates (including mammals), in which it is suggested that natural epigenetic variations may influence the phenotype of an individual via the regulation of gene expression, and facilitate the adaptation of species to their respective environments (e.g. Herrera & Bazaga 2010, Massicotte et al. 2011, Richards et al. 2012, Schrey et al. 2012, Liu et al. 2015). The effects of different environments on DNA methylation in natural populations have already been observed in marine organisms such as polychaetes, bivalves, and tunicates (e.g. Marsh & Pasqualone 2014, Zhang et al. 2018, Hawes et al. 2019; see Hofmann 2017 for a review). Watson et al. (2018) reported that populations of a bivalve species (*Perna perna*) living in contrasting environments (protected bay and open coast) had different patterns of DNA methylation, suggesting that the distinct conditions generated by local topography shaped the epigenetic profiles of these populations. These interactions between epigenetic processes and environmental factors can cause changes in the development of the organism (Díaz-Freije et al. 2014, García-Fernández et al. 2017), including sexual maturation (Morán & Pérez-Figueroa 2011), impact the ecological niche of a population, and even facilitate ecological divergence in a species (Boffelli & Martin 2012, Flatscher et al. 2012, Smith et al. 2016).

Additionally, we found moderate genetic differences between the 2 ecotypes. However, differences were much more pronounced in a previous study that showed a marked genetic divergence in the mitochondrial DNA control region and nuclear microsatellite data used to compare these same populations (Fruet et al. 2017b). Such differentiation is expected because of the consistent parapatry of these ecotypes, which present both negligible gene flow that indicates significant reproductive isolation (Fruet et al. 2017b, de Oliveira et al. 2019) and divergent selection (Costa et al. 2021). Given the significant genetic and, now, epigenetic differentiations found between these ecotypes, it is apparent that both molecular mechanisms are operating simultaneously (but not necessarily in the same proportion) in response to the adaptive challenges posed by their local environments. The proportion of genetic and epigenetic differences between divergent populations may depend not only on the environmental contrasts and reproductive isolation, but also on the time these populations have been under such conditions. It is likely that changes in methylation may provide an initial ecological advantage to organisms, since epigenetic mechanisms can lead to faster adaptive response to environmental demands when compared to responses that depend on changes in allele frequencies in the population (Furusawa & Kaneko 2013, Klironomos et al. 2013, Kronholm & Collins 2016). Thus, after epigenetic changes facilitate ecological flexibility and colonization of new environments, genetic changes can occur over a longer time frame (Ardura et al. 2017).

Epigenetic alterations in response to a new environment or environmental changes can occur in a single generation (i.e. ontogenetically), whereas the process of adaptation via DNA mutations depends on the differential reproduction of individuals with favorable genetic variants, requiring successive generations to occur (i.e. phylogenetically) (Massicotte et al. 2011, Ardura et al. 2017). Genetic variations obtained through natural selection, or other evolutionary mechanisms, may take considerably longer to

produce divergences (physiological, behavioral, and morphological) in populations in response to environmental pressures (Rando & Verstrepen 2007, Flatscher et al. 2012). This can be especially true for long-lived animals, such as common bottlenose dolphins (Wells & Scott 1999). Recent studies have demonstrated that there may be a sequential mechanism for phenotypic responses to environmental factors, with genetic divergence being preceded by an initial epigenetic divergence, which serves as a substrate for the gradual genomic alterations among isolated populations (e.g. Flatscher et al. 2012, Smith & Ritchie 2013). Through DNA sequence and methylation analyses, Skinner et al. (2014) showed that the phylogenetic distances of 5 species of Darwin's finches are more compatible with their epigenetic variations (which continue to accumulate even over long periods of time) than with changes in their genome. Therefore, these 2 molecular mechanisms are crucial for adaptations and phenotypic variations seen in natural populations (Smith & Ritchie 2013).

Similarly, the 2 SWA common bottlenose dolphin ecotypes present consistent phenotypic differences and adaptive divergence. In a recent study, Costa et al. (2021) reported a significant differentiation between these ecotypes based on morphological traits and microsatellite markers; however, there was a lower differentiation for mtDNA. Such data suggest that these populations are undergoing recent ecological divergence, which may be leading to speciation (Costa et al. 2021). Although there are significant genetic differences between these ecotypes (Fruet et al. 2017b, Costa et al. 2021), the levels of epigenetic variation reported in the present study may provide an additional explanation for their phenotypic differences. Since the ecological divergence is recent, DNA methylation likely still plays a fundamental role in the adaptation of these animals, complementing the already established, and probably increasing, genetic differentiation between ecotypes. Indeed, phenotypic plasticity and its epigenetic basis are determinant for the colonization and persistence of populations in new environments, which may result, over time, in reproductive isolation and consequently ecological speciation (Smith & Ritchie 2013). If the colonized environment is subject to divergent selective pressures, populations may begin the speciation process; thus, epigenetic variations may be initially necessary for the persistence of animals, and only then does allelic selection occur and animals diverge genetically from the original population (Pál & Miklós 1999, Flatscher et al. 2012, Smith et al. 2016). In other words, epigenetic modifications (and their consequent phenotypic changes) can play a key role in the early stages of ecological speciation by facilitating the process of adaptation of organisms to new environments — thereby allowing the colonization of contrasting habitats — which favors ecological divergence and reproductive isolation, potentially leading to genetic assimilation and accommodation (Smith & Ritchie 2013). Thus, epigenetic alterations may also contribute to the considerable phenotypic variation observed between the SWA ecotypes of common bottlenose dolphin.

In this study, we reported evidence on the role of epigenetics in the ecology of divergent populations of common bottlenose dolphins. Here, the MSAP technique was used to provide epigenetic fingerprints for 2 groups of *T. truncatus*; however, our study is not without limitations inherent to the protocol used. Estimation of epigenetic variation using methylation-susceptible loci allowed a comparison between DNA methylation patterns of both SWA ecotypes; nevertheless, the use of non-methylated loci to estimate genetic variation cannot be deemed as accurate as the epigenetic marker. Our data on genetic diversity and differences between the ecotypes, however, qualitatively corroborate previous studies (Fruet et al. 2017b, Costa et al. 2021). In addition, although the obtained results represent a significant advance in the understanding of the population epigenetics of these marine mammals, the technique that we used only allows identifying patterns in DNA methylation, without access to the specific genes that are differentially methylated. Hence, it was not possible to address the specific functions of each methylated gene, which is an issue that should be prioritized in future studies.

The colonization of coastal and estuarine regions by marine mammals required significant ecological adaptations to the new habitat. Similarly, recent human-driven changes in these environments, which have been accelerating over the last century, continue to exert pressure on these animals. Some examples are changes in diet and reduction of prey due to overfishing (e.g. Secchi et al. 2017), anthropogenic impacts on behavior and physiology, and the presence of contaminants to which they are exposed. Diet, stress, and pollutants are well-known factors with potential to alter DNA methylation, affecting gene expression and, thus, the phenotype of animals. Epigenetic variations may therefore have been determinant for not only the colonization success of the coastal habitat by offshore counterparts, but also for the permanence of the common bottlenose dolphins in highly dynamic and changing environments. The

present study provided a first step to access the epigenetic status of natural populations of this and other marine mammals. To our knowledge, this is the first study to evaluate epigenetic diversity in a cetacean species. Based on DNA methylation data, we were able to clearly distinguish animals from coastal and offshore ecotypes, supporting the hypothesis that these contrasting environments can lead to epigenetic divergence in *T. truncatus*. Further studies with other marine mammals, subject to different types of environmental contrasts, may help clarify the role that epigenetics plays in the adaptation and ecological divergence of natural populations.

Acknowledgements. We are thankful for the financial support of the Organization for the Conservation of South American Aquatic Mammals – YAQU PACHA e.V., Nuremberg Zoo, Porto do Rio Grande RS, and Chevron Brazil Upstream Frade Ltda (number CW702315). PETROBRAS, through the Brazilian Inter-Ministerial Commission for the Resources of the Sea (CIRM), supplied diesel for all surveys onboard the FURG's RV 'Atlântico Sul' that allowed sampling oceanic dolphins. We thank all researchers and students who helped collect data at sea, and the crew of RV 'Atlântico Sul,' especially Rodrigo Genoves, who also helped sample coastal dolphins. We also thank Dr. Vera Lúcia da Silva Valente Gaiesky for technical training and advice. This study was partially funded by the Brazilian Long-Term Ecological Research Program (PELD) from the National Council for Scientific and Technological Development (CNPq) (Proc.441492/2016-9 and 442206/2020-8), and the Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (Proc. 16/2551-0000102-2). The Coordination for the Improvement of Higher Education Personnel (CAPES) provided access to the Portal de Periódicos and financial support through the Programa de Excelência Acadêmica – PROEX. CNPq also provided fellowships to E.R.S. (PQ 310597/2018-8), M.C.P. (PQ 312470/2018-5), and A.C.C.T. (GD 142397/2016-4), and an international fellowship to P.F.F. (SWE 201567/2011-3). This article is part of A.C.C.T.'s PhD thesis in Biological Oceanography under the supervision of E.R.S. and M.C.P., and is a contribution of the Research Group Ecologia e Conservação da Megafauna Marinha – EcoMega FURG/CNPq.

LITERATURE CITED

Amos W, Hoelzel AR (1991) Long-term preservation of whale skin for DNA analysis. In: Hoelzel AR (Ed) Genetic ecology of whales and dolphins. Report of the International Whaling Commission. Special issue 13. International Whaling Commission, Cambridge, p 99–103

Angers B, Castonguay E, Massicotte R (2010) Environmentally induced phenotypes and DNA methylation: how to deal with unpredictable conditions until the next generation and after. Mol Ecol 19:1283–1295

Ardura A, Zaiko A, Morán P, Planes S, Garcia-Vazquez E (2017) Epigenetic signatures of invasive status in populations of marine invertebrates. Sci Rep 7:42193

Arrigo N, Holderegger R, Alvarez N (2012) Automated scoring of AFLPs using RawGeno v 2.0, a free R CRAN library. In: Pompanon F, Bonin A (eds) Data production and analysis in population genomics. Methods in molecular biology (methods and protocols), Vol 888. Humana Press, Totowa, NJ, p 155–175

Ballance LT (2018) Cetacean ecology. In: Würsig B, Thewissen JGM, Kovacs KM (eds) Encyclopedia of marine mammals, 3rd edn. Academic Press, San Diego, CA, p 172–180

Beal A, Rodriguez-Casariego J, Rivera-Casas C, Suarez-Ulloa V, Eirin-Lopez JM (2018) Environmental epigenomics and its applications in marine organisms. In: Oleksiak MF, Rajora OP (eds) Population genomics: marine organisms. Springer Nature, Cham, p 325–359

Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc B 57:289–300

Bezamat C, Simões-Lopes PC, Castilho PV, Daura-Jorge FG (2019) The influence of cooperative foraging with fishermen on the dynamics of a bottlenose dolphin population. Mar Mamm Sci 35:825–842

Bilgmann K, Möller LM, Harcourt RG, Gales R, Beheregaray LB (2008) Common dolphins subject to fisheries impacts in Southern Australia are genetically differentiated: implications for conservation. Anim Conserv 11:518–528

Bock C (2012) Analysing and interpreting DNA methylation data. Nat Rev Genet 13:705–719

Boffelli D, Martin DIK (2012) Epigenetic inheritance: a contributor to species differentiation? DNA Cell Biol 31 (Suppl 1):S11–S16

Bossdorf O, Richards CL, Pigliucci M (2008) Epigenetics for ecologists. Ecol Lett 11:106–115

Botta S, Hohn AA, Macko SA, Secchi ER (2012) Isotopic variation in delphinids from the subtropical western South Atlantic. J Mar Biol Assoc UK 92:1689–1698

Castilho CS, Bertuol F, Fruet P, Genoves RC (2015) Insights about the genetic diversity and population structure of an offshore group of common bottlenose dolphins (*Tursiops truncatus*) in the Mid-Atlantic. Genet Mol Res 14:3387–3399

Committee on Taxonomy (2020) List of marine mammal species and subspecies. www.marinemammalscience.org/science-and-publications/list-marine-mammal-species-subspecies/

Costa APB, Fruet P, Daura-Jorge FG, Simões-Lopes PC, Ott PH, Valiati VH, De Oliveira LR (2015) Bottlenose dolphin communities from the southern Brazilian coast: Do they exchange genes or are they just neighbours? Mar Freshw Res 66:1201–1210

Costa APB, Fruet PF, Secchi ER, Daura-Jorge FG, Simões-Lopes PC, Di Tullio JC, Rosel PE (2021) Ecological divergence and speciation in common bottlenose dolphins in the western South Atlantic. J Evol Biol 34:16–32

de Carvalho-Borges M, Orselli IBM, de Carvalho Ferreira ML, Kerr R (2018) Seawater acidification and anthropogenic carbon distribution on the continental shelf and slope of the western South Atlantic Ocean. J Mar Syst 187:62–81

de Oliveira LR, Fraga LD, Ott PH, Siciliano S and others (2019) Population structure, phylogeography, and genetic diversity of the common bottlenose dolphin in the tropical and subtropical southwestern Atlantic Ocean. J Mammal 100:564–577

Di Tullio JC, Fruet PF, Secchi ER (2015) Identifying critical areas to reduce bycatch of coastal common bottlenose dolphins *Tursiops truncatus* in artisanal fisheries of the subtropical western South Atlantic. Endang Species Res 29:35–50

Di Tullio JC, Gandra TBR, Zerbini AN, Secchi ER (2016) Diversity and distribution patterns of cetaceans in the subtropical Southwestern Atlantic outer continental shelf and slope. PLOS ONE 11:e015584

Díaz-Freije E, Gestal C, Castellanos-Martínez S, Morán P (2014) The role of DNA methylation on *Octopus vulgaris* development and their perspectives. Front Physiol 5:62

Duffield DA, Ridgway SH, Cornell LH (1983) Hematology distinguishes coastal and offshore forms of dolphins (*Tursiops*). Can J Zool 61:930–933

Earl DA, VonHoldt BM (2012) STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conserv Genet Resour 4:359–361

Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol Ecol 14:2611–2620

Excoffier L, Smouse PE, Quattro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131:479–491

Falush D, Stephens M, Pritchard JK (2007) Inference of population structure using multilocus genotype data: dominant markers and null alleles. Mol Ecol Notes 7:574–578

Fatemi M, Pao MM, Jeong S, Gal-Yam EN, Egger G, Weisenberger DJ, Jones PA (2005) Footprinting of mammalian promoters: use of a CpG DNA methyltransferase revealing nucleosome positions at a single molecule level. Nucleic Acids Res 33:e176

Feil R (2006) Environmental and nutritional effects on the epigenetic regulation of genes. Mutat Res 600:46–57

Feil R, Fraga MF (2012) Epigenetics and the environment: emerging patterns and implications. Nat Rev Genet 13:97–109

Feng S, Cokus SJ, Zhang X, Chen PY and others (2010) Conservation and divergence of methylation patterning in plants and animals. Proc Natl Acad Sci USA 107: 8689–8694

Flatscher R, Frajman B, Schönswetter P, Paun O (2012) Environmental heterogeneity and phenotypic divergence: Can heritable epigenetic variation aid speciation? Genet Res Int 2012:698421

Forcada J (2018) Distribution. In: Würsig B, Kovacs KM, Thewissen JGM (eds) Encyclopedia of marine mammals, 3rd edn. Academic Press, San Diego, CA, p 259–262

Fruet PF, Secchi ER, Daura-Jorge F, Vermeulen E and others (2014) Remarkably low genetic diversity and strong population structure in common bottlenose dolphins (*Tursiops truncatus*) from coastal waters of the Southwestern Atlantic Ocean. Conserv Genet 15:879–895

Fruet PF, Dalla Rosa L, Genoves RC, Valiati VH, De Freitas TRO, Möller LM (2017a) Biopsy darting of common bottlenose dolphins (*Tursiops truncatus*) in southern Brazil: evaluating effectiveness, short-term responses and wound healing. Lat Am J Aquat Mamm 11:121

Fruet PF, Secchi ER, Di Tullio JC, Simões-Lopes PC and others (2017b) Genetic divergence between two phenotypically distinct bottlenose dolphin ecotypes suggests separate evolutionary trajectories. Ecol Evol 7:9131–9143

Furusawa C, Kaneko K (2013) Epigenetic feedback regulation accelerates adaptation and evolution. PLOS ONE 8:e61251

García-Fernández P, García-Souto D, Almansa E, Morán P, Gestal C (2017) Epigenetic DNA methylation mediating *Octopus vulgaris* early development: effect of essential fatty acids enriched diet. Front Physiol 8:292

Genoves RC, Fruet PF, Botta S, Beheregaray LB, Möller LM, Secchi ER (2020) Fine-scale genetic structure in Lahille's bottlenose dolphins (*Tursiops truncatus gephyreus*) is associated with social structure and feeding ecology. Mar Biol 167:34

Gu Z, Eils R, Schlesner M (2016) Complex heatmaps reveal patterns and correlations in multidimensional genomic data. Bioinformatics 32:2847–2849

Haraguchi L, Carstensen J, Abreu PC, Odebrecht C (2015) Long-term changes of the phytoplankton community and biomass in the subtropical shallow Patos Lagoon Estuary, Brazil. Estuar Coast Shelf Sci 162:76–87

Hawes NA, Amadorou A, Tremblay LA, Pochon X, Dunphy B, Fidler AE, Smith KF (2019) Epigenetic patterns associated with an ascidian invasion: a comparison of closely related clades in their native and introduced ranges. Sci Rep 9:14275

Herrera CM, Bazaga P (2010) Epigenetic differentiation and relationship to adaptive genetic divergence in discrete populations of the violet *Viola cazorlensis.* New Phytol 187:867–876

Herrera CM, Bazaga P (2011) Untangling individual variation in natural populations: ecological, genetic and epigenetic correlates of long-term inequality in herbivory. Mol Ecol 20:1675–1688

Hildebrand JA (2009) Anthropogenic and natural sources of ambient noise in the ocean. Mar Ecol Prog Ser 395:5–20

Hoelzel AR, Potter CW, Best PB (1998) Genetic differentiation between parapatric 'nearshore' and 'offshore' populations of the bottlenose dolphin. Proc R Soc B 265: 1177–1183

Hofmann GE (2017) Ecological epigenetics in marine metazoans. Front Mar Sci 4:4

Jabbari K, Bernardi G (2004) Cytosine methylation and CpG, TpG (CpA) and TpA frequencies. Gene 333:143–149

Jablonka E, Lamb MJ (2006) The evolution of information in the major transitions. J Theor Biol 239:236–246

Jablonka E, Raz G (2009) Transgenerational epigenetic inheritance: prevalence, mechanisms, and implications for the study of heredity and evolution. Q Rev Biol 84: 131–176

Johnson LJ, Tricker PJ (2010) Epigenomic plasticity within populations: its evolutionary significance and potential. Heredity 105:113–121

Kilvitis HJ, Alvarez M, Foust CM, Schrey AW, Robertson M, Richards CL (2014) Ecological epigenetics. In: Landry CR, Aubin-Horth N (eds) Ecological genomics: ecology and the evolution of genes and genomes. Springer Science, Dordrecht, p 191–210

Klironomos FD, Berg J, Collins S (2013) How epigenetic mutations can affect genetic evolution: model and mechanism. BioEssays 35:571–578

Kopelman NM, Mayzel J, Jakobsson M, Rosenberg NA, Mayrose I (2015) Clumpak: a program for identifying clustering modes and packaging population structure inferences across K. Mol Ecol Resour 15:1179–1191

Kronholm I, Collins S (2016) Epigenetic mutations can both help and hinder adaptive evolution. Mol Ecol 25: 1856–1868

Kucharski R, Maleszka J, Foret S, Maleszka R (2008) Nutritional control of reproductive status in honeybees via DNA methylation. Science 319:1827–1830

Laporta P, Martins CCA, Lodi L, Domit C, Vermeulen E, Di

Tullio JC (2017) Report of the Working Group on Habitat Use of *Tursiops truncatus* in the Southwest Atlantic Ocean. Lat Am J Aquat Mamm 11:47–61

Li CCY, Cropley JE, Cowley MJ, Preiss T, Martin DIK, Suter CM (2011) A sustained dietary change increases epigenetic variation in isogenic mice. PLOS Genet 7:e1001380

Li E, Zhang Y (2014) DNA methylation in mammals. Cold Spring Harb Perspect Biol 6:a019133

Li YL, Liu JX (2018) StructureSelector: a web-based software to select and visualize the optimal number of clusters using multiple methods. Mol Ecol Resour 18:176–177

Liebl AL, Schrey AW, Richards CL, Martin LB (2013) Patterns of DNA methylation throughout a range expansion of an introduced songbird. Integr Comp Biol 53:351–358

Liu S, Sun K, Jiang T, Ho JP, Liu B, Feng J (2012) Natural epigenetic variation in the female great roundleaf bat (*Hipposideros armiger*) populations. Mol Genet Genomics 287:643–650

Liu S, Sun K, Jiang T, Feng J (2015) Natural epigenetic variation in bats and its role in evolution. J Exp Biol 218: 100–106

Louis M, Fontaine MC, Spitz J, Schlund E and others (2014) Ecological opportunities and specializations shaped genetic divergence in a highly mobile marine top predator. Proc R Soc B 281:20141558

Marsh AG, Pasqualone AA (2014) DNA methylation and temperature stress in an Antarctic polychaete, *Spiophanes tcherniai*. Front Physiol 5:173

Martin EM, Fry RC (2016) A cross-study analysis of prenatal exposures to environmental contaminants and the epigenome: support for stress-responsive transcription factor occupancy as a mediator of gene-specific CpG methylation patterning. Environ Epigenet 2:dvv011

Martin EM, Fry RC (2018) Environmental influences on the epigenome: exposure- associated DNA methylation in human populations. Annu Rev Public Health 39:309–333

Massicotte R, Angers B (2012) General-purpose genotype or how epigenetics extend the flexibility of a genotype. Genet Res Int 2012:317175

Massicotte R, Whitelaw E, Angers B (2011) DNA methylation: a source of random variation in natural populations. Epigenetics 6:421–427

McGowen MR, Gatesy J, Wildman DE (2014) Molecular evolution tracks macroevolutionary transitions in Cetacea. Trends Ecol Evol 29:336–346

Mead JG, Potter CW (1995) Recognizing two populations of the bottlenose dolphin (*Tursiops truncatus*) of the Atlantic coast of North America: morphologic and ecologic considerations. Int Biol Res Inst Rep 5:31–44

Merchant ND, Pirotta E, Barton TR, Thompson PM (2014) Monitoring ship noise to assess the impact of coastal developments on marine mammals. Mar Pollut Bull 78:85–95

Morán P, Pérez-Figueroa A (2011) Methylation changes associated with early maturation stages in the Atlantic salmon. BMC Genet 12:86

Niencheski LF, Windom HL, Smith R (1994) Distribution of particulate trace metal in Patos Lagoon estuary (Brazil). Mar Pollut Bull 28:96–102

Pál C, Miklós I (1999) Epigenetic inheritance, genetic assimilation and speciation. J Theor Biol 200:19–37

Parsons KM, Durban JW, Claridge DE, Herzing DL, Balcomb KC, Noble LR (2006) Population genetic structure of coastal bottlenose dolphins (*Tursiops truncatus*) in the northern Bahamas. Mar Mamm Sci 22:276–298

Pereira LB, Botta S, Teixeira CR, Fruet P, Simões-Lopes PC, Daura-Jorge FG (2020) Feeding ecology of two subspecies of bottlenose dolphin: a tooth tale. Aquat Ecol 54:941–955

Pérez-Figueroa A (2013) Msap: a tool for the statistical analysis of methylation-sensitive amplified polymorphism data. Mol Ecol Resour 13:522–527

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics 155:945–959

Puechmaille SJ (2016) The program STRUCTURE does not reliably recover the correct population structure when sampling is uneven: subsampling and new estimators alleviate the problem. Mol Ecol Resour 16:608–627

R Core Team (2019) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna

Rakyan VK, Chong S, Champ ME, Cuthbert PC, Morgan HD, Luu KVK, Whitelaw E (2003) Transgenerational inheritance of epigenetic states at the murine AxinFu allele occurs after maternal and paternal transmission. Proc Natl Acad Sci USA 100:2538–2543

Rando OJ, Verstrepen KJ (2007) Timescales of genetic and epigenetic inheritance. Cell 128:655–668

Rapp RA, Wendel JF (2005) Epigenetics and plant evolution. New Phytol 168:81–91

Reyna-López GE, Simpson J, Ruiz-Herrera J (1997) Differences in DNA methylation patterns are detectable during the dimorphic transition of fungi by amplification of restriction polymorphisms. Mol Gen Genet 253:703–710

Richards EJ (2006) Inherited epigenetic variation — revisiting soft inheritance. Nat Rev Genet 7:395–401

Richards CL, Bossdorf O, Verhoeven KJF (2010) Understanding natural epigenetic variation. New Phytol 187: 562–564

Richards CL, Schrey AW, Pigliucci M (2012) Invasion of diverse habitats by few Japanese knotweed genotypes is correlated with epigenetic differentiation. Ecol Lett 15:1016–1025

Righetti BPH, Mattos JJ, Siebert MN, Daura-Jorge FG and others (2019) Biochemical and molecular biomarkers in integument biopsies of free-ranging coastal bottlenose dolphins from southern Brazil. Chemosphere 225:139–149

Roberts RJ, Vincze T, Posfai J, Macelis D (2007) REBASE — enzymes and genes for DNA restriction and modification. Nucleic Acids Res 35:D269–D270

Schrey AW, Richards CL, Meller V, Sollars V, Ruden DM (2012) The role of epigenetics in evolution: the extended synthesis. Genet Res Int 2012:286164

Schrey AW, Alvarez M, Foust CM, Kilvitis HJ and others (2013) Ecological epigenetics: beyond MS-AFLP. Integr Comp Biol 53:340–350

Schulz B, Eckstein RL, Durka W (2013) Scoring and analysis of methylation-sensitive amplification polymorphisms for epigenetic population studies. Mol Ecol Resour 13:642–653

Secchi ER, Botta S, Wiegand MM, Lopez LA, Fruet PF, Genoves RC, Di Tullio JC (2017) Long-term and gender-related variation in the feeding ecology of common bottlenose dolphins inhabiting a subtropical estuary and the adjacent marine coast in the western South Atlantic. Mar Biol Res 13:121–134

Seeliger U, Costa CSB (1997) Natural and human impact. In: Seeliger U, Odebrecht C, Castello JP (eds) Subtropical convergence environments: the coast and sea in the Southwestern Atlantic. Springer, Berlin, p 197–203

Segura-García I, Rojo-Arreola L, Rocha-Olivares A, Heckel G, Gallo-Reynoso JP, Hoelzel R (2018) Eco-evolutionary processes generating diversity among bottlenose dolphin, *Tursiops truncatus*, populations off Baja California, Mexico. Evol Biol 45:223–236

Simões-Lopes PC, Fabian ME (1999) Residence patterns and site fidelity in bottlenose dolphins, *Tursiops truncatus* (Montagu) (Cetacea, Delphinidae) off Southern Brazil. Rev Bras Zool 16:1017–1024

Skinner MK, Gurerrero-Bosagna C, Muksitul Haque M, Nilsson EE, Koop JAH, Knutie SA, Clayton DH (2014) Epigenetics and the evolution of Darwin's finches. Genome Biol Evol 6:1972–1989

Smith G, Ritchie MG (2013) How might epigenetics contribute to ecological speciation? Curr Zool 59:686–696

Smith TA, Martin MD, Nguyen M, Mendelson TC (2016) Epigenetic divergence as a potential first step in darter speciation. Mol Ecol 25:1883–1894

Soubry A (2015) Epigenetic inheritance and evolution: a paternal perspective on dietary influences. Prog Biophys Mol Biol 118:79–85

Suarez-Bregua P, Pérez-Figueroa A, Hernández-Urcera J, Morán P, Rotllant J (2020) Temperature-independent genome-wide DNA methylation profile in turbot post-embryonic development. J Therm Biol 88:102483

Vieira JP, Garcia AM, Grimm AM (2008) Evidences of El Niño effects on the mullet fishery of the Patos Lagoon estuary. Braz Arch Biol Technol 51:433–440

Watson RGA, Baldanzi S, Pérez-Figueroa A, Gouws G, Porri F (2018) Morphological and epigenetic variation in mussels from contrasting environments. Mar Biol 165:50

Wells RS, Scott MD (1999) Bottlenose dolphin *Tursiops truncatus* (Montagu, 1821). In: Ridgway SH, Harrison R (eds) Handbook of marine mammals. Academic Press, San Diego, CA, p 137–182

Wells RS, Scott MD (2018) Common bottlenose dolphin, *Tursiops truncatus*. In: Würsig B, Thewissen JGM, Kovacs KM (eds) Encyclopedia of marine mammals, 3rd edn. Elsevier, Academic Press, San Diego, CA, p 118–125

Willmore KE, Young NM, Richtsmeier JT (2007) Phenotypic variability: its components, measurement and underlying developmental processes. Evol Biol 34:99–120

Xu Q, Sun D, Zhang Y (2005) F-MSAP: a practical system to detect methylation in chicken genome. Chin Sci Bull 50:2039–2044

Zhang X, Li Q, Kong L, Yu H (2018) Epigenetic variation of wild populations of the Pacific oyster *Crassostrea gigas* determined by methylation-sensitive amplified polymorphism analysis. Fish Sci 84:61–70

Zhu H, Wang G, Qian J (2016) Transcription factors as readers and effectors of DNA methylation. Nat Rev Genet 17:551–565

Editorial responsibility: Philippe Borsa, Montpellier, France
Reviewed by: A. Pérez-Figueroa and 2 anonymous referees

Submitted: August 22, 2020
Accepted: May 19, 2021
Proofs received from author(s): July 20, 2021