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INTERNATIONAL WHALING COMMISSION

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ABSTRACT

Despite our limited understanding about geographical variations, population connectivity and taxonomy of common bottlenose dolphins worldwide, coastal (or inshore) and offshore (or oceanic) ecotypes have been widely recognized in several oceanographic regions. In the Southwest Atlantic Ocean (SWA), however, there are scarce records of bottlenose dolphins differing in external morphology according to habitat preferences that resembles the coastal-offshore pattern observed in other parts of the world. The main aim of this study was to assess for the first time putative genetic differences between bottlenose dolphins sampled in coastal and offshore waters of the SWA. We used a combination of mtDNA control region sequences and microsatellite genotypes for population analyses, from which levels of genetic diversity were also estimated. Our results from both molecular marker types were congruent and revealed strong levels of structuring (microsatellites F_{ST} = 0.352, P<0.001; mtDNA F_{ST} = 0.1829, P<0.01; Φ_{ST} = 0.385, P<0.01) and contrasting genetic diversity between ecotypes, supporting patterns found in previous broad-scale studies elsewhere. Despite opportunity for gene flow in potential "contact zones", we found minimal current and historical connectivity between ecotypes, suggesting they are possibly following discrete evolutionary trajectories. Based on our findings, which seem to be in agreement with morphological differentiation recently described for bottlenose dolphins in our study area, we recommend recognizing the offshore bottlenose dolphin ecotype as an additional evolutionarily significant unit (ESU) in the SWA. Implications for the conservation of bottlenose dolphins in SWA are also discussed.

KEY WORD: Genetics, Biopsy sampling, Atlantic Ocean, South America

INTRODUCTION

The identification and characterization of intraspecific variation is extremely important in conservation biology because it addresses variability that is relevant for species persistence and evolutionary potential (*e.g.* Allendorf and Luikart, 2007). The identification of distinct population segments, however, can be a challenging task. This is particularly true for highly mobile and widely distributed species inhabiting the marine environment that lacks evident physical barriers to gene flow (Hoelzel, 2009; Palumbi, 1994). Some species might adapt to, and evolve in, different habitats or even in simpatry as a result of feeding specializations, forming so-called ecotypes, with limited or no contemporary gene flow between them (*e.g.* Foot *et al*., 2009; 2011; Louis *et al*., 2014a, 2014b; Natoli *et al*., 2004). Ecotypes may possess

unique adaptations and distinct evolutionary histories, and hence could be considered as separate Evolutionarily Significant Units (ESU) (Ryder, 1986), a practical concept widely used for prioritizing management actions within species (Moritz, 1999).

Inferring population structure through the use of molecular markers is a powerful tool for identifying distinct populations for management (Allendorf and Luikart, 2007). This also applies in studies of high mobile cetacean species, which have been extensively investigated in the last two decades and are known to present varying levels of populations structuring over large and small spatial scales (*e.g.* Möller *et al*., 2007; Natoli *et al*., 2004; Rosel *et al*., 2009). The molecular approach, when integrated with other phenotypic and/or ecological data, has proven to provide reliable information for cetacean taxonomic diagnosis and for understating the evolutionary and contemporary forces shaping genetic divergence (*e.g.* Caballero *et al*., 2007; Cunha *et al*., 2015; Louis *et al*., 2014b; Wang *et al*., 1999).

The common bottlenose dolphin (*Tursiops truncatus*) is a cosmopolitan cetacean species adapted to a wide range of environments. As a consequence of such plasticity, the species tends to vary geographically in a great number of biological traits. Despite our limited understanding about geographical variations, population connectivity and taxonomy of the species worldwide, coastal (or inshore) and offshore (or oceanic) ecotypes have been widely recognized in several oceanographic regions (Hoelzel *et al*., 1998; Mead and Potter, 1995; Perrin *et al*., 2011;Van Waerebeek *et al*., 1990). In the North Atlantic, for example, coastal and offshore ecotypes are notably distinct in their genetic profiles and several other morphological and biological aspects (*e.g.* Hersh and Duffield, 1990; Hoelzel *et al*., 1998; Mead and Potter, 1995). Broadly speaking, in the North Atlantic the coastal ecotype tends to be smaller, lighter gray, inhabits preferentially shallow waters close to coast and, forms small fragmented populations, while the offshore ecotype tends to be larger, darker, inhabits deeper waters, and forms larger populations of up to thousands of individuals connected over broad geographical scales (see Wells and Scott, 2009). Results of some regional studies investigating the ecotypes differentiation have reported marked differences in genetics, morphology and feeding habits between the ecotypes in the Northeastern Pacific (*e.g.* Perrin *et al*., 2011; Mead and Potter, 1995; Walker, 1981) and Northwestern Atlantic (WNA) (*e.g.* Hoelzel *et al*., 1998). In the Northeastern Atlantic, bottlenose dolphin ecotypes also form two clear genetically distinct groups and it is suggested that the coastal ecotype is currently isolated from the offshore ecotype, despite the lack of evident external morphological differences (Louis *et al*., 2014a, 2014b).

Along the Southwest Atlantic Ocean (SWA), bottlenose dolphins occur in both coastal and offshore zones. In coastal regions they are commonly seen in shallow coastal waters (<20 m) within 3 km from the coast (*e.g.* Di Tullio *et al*., 2015; Laporta, 2009). They are predominantly distributed between southern Brazil (27°21'S) down to central Argentina (43°S), forming small populations associated with productive environments such as estuaries of river mouths and lagoons (see Lodi *et al. in press* and Laporta *et al*. *in press* for review). Sighting data suggest no movement of coastal bottlenose dolphins to deep waters (*i.e.* >20 m depth), though movements of some individuals of approximately 200 km along the southern coast occur frequently (Laporta, 2009). Recent studies using microsatellites and mitochondrial DNA analyses have shown remarkably low levels of genetic diversity and strong genetic differences among these coastal populations at both marker types (Costa *et al*., 2015; Fruet *et al*., 2014). At large geographical scale, it was suggested that bottlenose dolphins from Bahía San Antonio (BSA), Argentina and south Brazil-Uruguay (SBU), form two distinct ESUs with negligible contemporary gene flow between them. Additional sub-divisions were also found for the SBU ESU, consisting of multiples management units (Fruet *et al*., 2014). On the other hand, sightings of bottlenose dolphins in offshore waters in the SWA are reported mainly beyond the continental shelf-break (>150 m), and approximately 100 km from the coast (*e.g.* Di Tullio *et al*. *in press*). Despite occasional sightings of the offshore ecotype near coastal areas at the north extreme end of the main occurrence area of the coastal ecotype (see above), unconfirmed records of bottlenose dolphins in the mid continental shelf (between 15 and 100 m) are scarce in southernmost Brazil (E. Secchi, pers. obs.) and possibly Uruguay and northern Argentina, suggesting a potential coastal-oceanic hiatus on its distribution in this area. Despite little information being available for bottlenose dolphins in offshore waters, observational data and photographs taken in these waters during numerous surveys suggest that they present clear differences in external morphology and coloration patterns in relation to coastal bottlenose dolphins (Simões-Lopes, 1996; ECOMEGA, unpub. data), which is similar to the coastal-offshore pattern observed in other parts of the world (*e.g.* Hersh and Duffield, 1990; Van Waerebeek *et al*., 1990).

The main aim of this study was to assess putative genetic differences between coastal and offshore bottlenose dolphins sampled in the SWA. We used a combination of mtDNA control region sequences

and microsatellite genotypes for population analyses, from which levels of genetic diversity were also estimated. These data, in conjunct with previous information, allowed reassessing the population structure of common bottlenose dolphins in a broader geographical context in the SWA, with results leading to the proposal of a new ESU for the species in this region.

METHODS

Sample collection and stratification

The study area covers approximately 2,100km and 1,000km of linear distance in coastal and oceanic waters of the SWA, respectively. It extends from the state of Paraná, in southern Brazil, to Bahía San Antonio, in the Patagonian Argentina (25°18'S - 54°40'S) (Fig. 1). Along this region, biopsies were taken from common bottlenose dolphins using modified darts specifically designed for small cetaceans (F. Larsen, Ceta-Dart) fired from a 68kg draw weight crossbows.

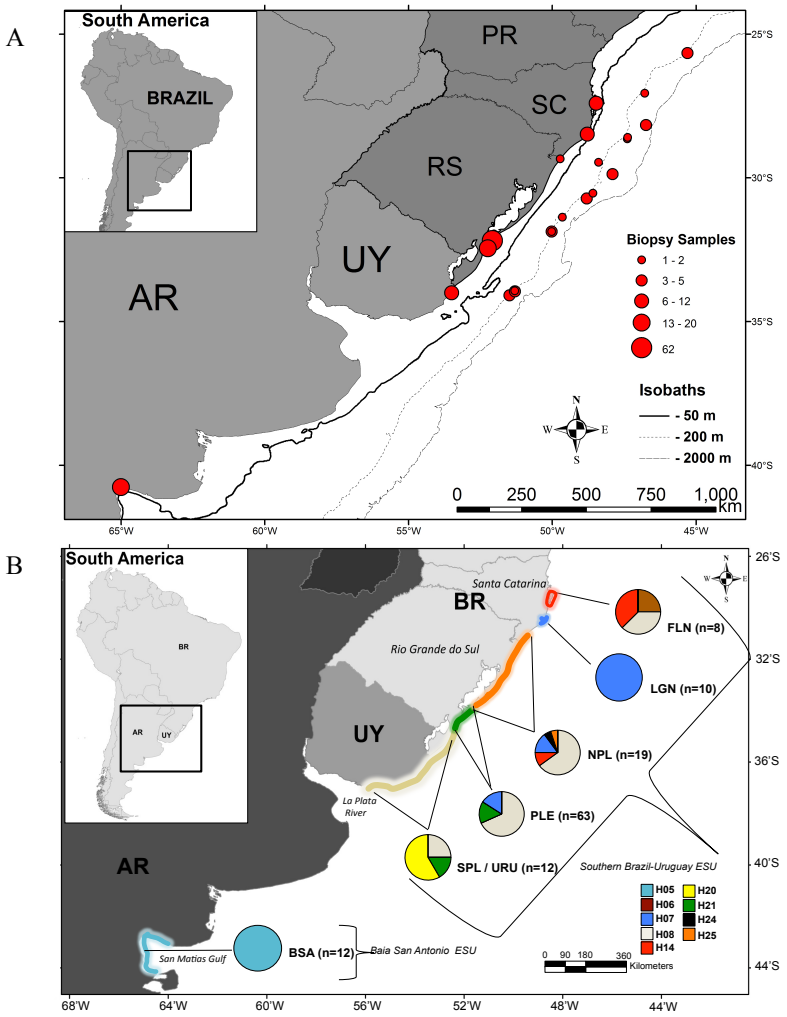

Figure 1. Study area in the Southwest Atlantic Ocean. (A) Sampling sites of common bottlenose dolphins (*Tursiops truncatus*) in coastal and offshore waters; (B) Figure modified from Fruet *et al*. (2014) showing the proposed evolutionarily significant units (ESUs) and management units (MUs) (colour counter lines) for the coastal ecotype, and the respective frequencies of mitochondrial control region haplotypes (pie charts). Arrows indicate the main sampling locations for each bottlenose dolphin community. The dashed rectangle highlights the area of heightened conservation concern. FLN= Florianópolis; LGN= Laguna; NPL= north Patos Lagoon; PLE= Patos Lagoon estuary; SLP/URU= south Patos Lagoon/Uruguay; BSA= Bahía San Antonio.

In the offshore waters 45 biopsies from 15 different groups were taken from bow-riding dolphins sighted during eight ship-based surveys carried out during spring and autumn between 2009 and 2012 on the

outer continental shelf (~150m) to the slope (1,500m) off southeast and southern Brazil (~23° S to ~34° S) (Di Tullio *et al*., *in press*). All samples were collected in waters depths greater than 146 m (mean=412m) and minimal distance of 103km from the coast (mean=143km). All bottlenose dolphin biopsies collected during these ship-based surveys were morphologically distinct from coastal dolphins (darker in coloration, falcate dorsal fin and with apparent shorter beak, Fig. 2) and thus were considered to belong to a putative offshore ecotype. There was no sampling effort in the offshore waters off Uruguay and Argentina.

Figure 2. Differences in external morphology and colouration between offshore and coastal ecotypes of bottlenose dolphin (*Tursiops truncatus*) in the Southwest Atlantic Ocean. (a) Offshore ecotype photographed in Bahía San Antonio, Argentina. Note the short beak and falcate dorsal fin. (b) Typical coastal resident bottlenose dolphin in Patos Lagoon estuary, southern Brazil. Note the light-grey colouration, triangular dorsal fin and relatively longer beak. (c) Differences in dorsal fin shape and colouration of sympatric offshore and coastal ecotypes of bottlenose dolphins in Bahía San Antonio, Argentina.

With the exception of three additional samples collected from dolphins regularly sighted in BSA that are morphologically distinct from their conspecifics, and resemble those of the putative offshore ecotype

(Bastida *et al*., 2007; Vermeulen and Cammareri, 2009 – see Fig. 2b), samples from coastal bottlenose dolphins (n=124) are the same used in a recent study that investigated the fine-scale genetic structuring of these dolphins in the SWA (Fruet *et al*., 2014). In brief, 120 biopsies were collected between 2004 and 2012 during small-boat based surveys conducted in coastal, shallow waters (<2km from shore, <10m deep) of south Brazil, Uruguay and Argentina. Biopsies were taken from individuals of six well-studied dolphin communities (four in Brazil, one in Uruguay and one in Argentina). This coastal bottlenose dolphins display a smaller and triangular dorsal fin and a relatively longer beak and a light grey colouration than offshore bottlenose dolphins (Fig. 2). Four samples of freshly stranded carcasses of photo-identified dolphins completed the final dataset (see Fruet *et al*. 2014 for more details). Fruet *et al*. (2014) proposed the existence of two distinct ESUs of coastal bottlenose dolphins in the SWA: one comprising a metapopulation of five communities along the Southern Brazil-Uruguay (SBU ESU) and another including the Bahía San Antonio dolphin community, Argentina (BSA ESU) (see Fig. 1 for details). Thus, the final data set of the coastal ecotype consisted of 15 samples from the BSA [12 previously analyzed by Fruet *et al.* (2012) plus three additional samples in this study] and 112 from the SBU ESUs (Table 1). Results of population structure analysis are given separately for each ESU. Indices of genetic diversity were extracted or calculated from the data set of Fruet *et al*. (2014) for comparison purposes.

Table 1. Summary of genetic diversity for coastal and offshore ecotypes of common bottlenose dolphins (*Tursiops truncatus*) in the Southwest Atlantic Ocean based on a fragment of the mtDNA control region and 16 microsatellite loci. Number between brackets indicates total sample size used for estimate genetic diversity separated by sex. For the calculation of P_A and A_R for the coastal ecotype we grouped the genetic data from all populations in Fruet *et al*. (2014), excluding three individuals sampled in coastal waters of BSA which were morphologically and genetically identified as part of the offshore ecotype. Other measures of genetic diversity are the same reported in Fruet *et al*. (2014).

	mtDNA					Microsatellites					
	Hap.	*s*	*Indels*	*h*	π	P_A	N_A	A_R	H_E	H_O	F_{IS}
OFFSHORE (20F:25M)	22	38	2	0.940 (0.016)	0.019 (0.010)	4.9	9.3	7.1	0.730	0.654	0.10*
COASTAL (61F:63M)	11	18	0	0.702 (0.034)	0.019 (0.010)	1.1	3.6	3.1	0.28	0.23	0.19*

Hap number of haplotypes*; S* polymorphic sites*; h* haplotype diversity; π nucleotide diversity*;* P_A number of private alleles; N_A mean number of alleles per locus; A_R mean allelic richness; H_E mean expected heterozygosity; H_O mean observed heterozygosity; F_{IS} inbreeding coefficient. *Significant multi-locus P value (P<0.001).

All samples used in this study (offshore and coastal) were preserved in 20% dimethyl sulphoxide (DMSO) saturated with sodium chloride (Amos and Hoelzel, 1991) or 98% ethanol, and followed identical laboratory procedures.

DNA extraction and molecular sexing

Samples were processed at the Molecular Ecology and Evolution Lab, Flinders University, South Australia. DNA was extracted following a salting-out protocol (Sunnucks and Hales, 1996) and molecular sexing was determined by the amplification of fragments of the SRY and ZFX genes through the polymerase chain reaction (PCR) using the protocol developed by Gilson *et al*. (1998).

mtDNA sequencing and haplotypes definition

We sequenced approximately 550 bp of the mtDNA control region (the same fragment used by Fruet *et al*. (2014) to investigate the population structure in coastal bottlenose dolphins in SWA) of 45 samples collected in the offshore waters plus three collected in BSA, Argentina. Sequencing was carried out on an ABI 3730 (Applied Biosystems) automated DNA sequencer according to manufacturer's instructions. Details for mtDNA PCR and sequencing procedures are found in Möller and Beheregaray (2001). To account for potential errors, a total of 10% of samples were re-sequenced. Sequences were edited using SEQUENCHER 3.0 (Gene Codes Corporation, Ann Arbor, MI). Alignment was ran together with the 124

sequences of coastal dolphins available for SBU and BSA ESUs (see Fruet *et al*., 2014) using the ClustalW algorithm in MEGA 5.05 (Tamura *et al*., 2011) and rechecked by eye. Haplotypes were defined using DNASP 5.0 (Librado and Rozas, 2009).

Microsatellite genotyping
DNA extractions from which the mtDNA fragment could be successfully amplified (n=48) were then used for subsequent microsatellite loci amplification. Samples were genotyped at 16 microsatellite loci (same used by Fruet *et al*. (2014) for coastal bottlenose dolphins) with GenScan 500 LIZ 3130 internal size standard. Procedures for microsatellite PCR and genotyping are found in Möller and Beheregaray (2004) and Amaral *et al*. (2012). For microsatellites, bins for each locus were determined and genotypes scored in GENE MAPPER 4.0 (Applied Biosystems). Rare alleles (*i.e.* frequency<5%) or alleles that fell in between two bins were re-genotyped. Micro-Checker 2.2.3 (Van Oosterhout *et al*., 2004) was used to check for potential scoring errors, the presence of null alleles, stuttering, and large allelic drop out. Genotyping error rates were estimated by re-genotyping eight randomly selected samples, representing ~17% of the total sample size (n=48). We used GENALEX 6.5 (Peakall and Smouse, 2012) to find potential matches between genotypes. Samples matching at all genotypes or those mismatching at only few alleles (1–2) were double-checked for potential scoring errors. Samples sharing identical genotypes, mtDNA haplotype and sex were considered as re-sampled individuals and we retained only one of each of those identified pairs.

Clustering analysis
We used STRUCTURE 2.3 (Pritchard *et al*., 2000) to run a Bayesian model–based clustering in order to infer population structure based on 16 microsatellites for a final data set of 172 samples (48 from this study plus 124 from Fruet *et al*. 2014). This model calculates the log likelihood value of the data to determine the most likely number of clusters (K). Membership coefficient (q) at cluster and individual levels are also estimated providing valuable information on the similarity between individuals based on shared ancestry, reducing Hardy–Weinberg disequilibrium effects between loci within clusters when no *a priori* information about the populations is used. We assumed correlated allele frequencies (Falush *et al*., 2003) and an admixture model setting no *a priori* information (Hubisz *et al*., 2009). Simulations were performed using 200,000 burn-in period and 10^6 repetitions of Markov Chain Monte Carlo (MCMC) assuming values of K varying between 1-4 (two coastal ESUs, one putative offshore population plus one). As recommended by Gilbert *et al*. (2012), we performed 20 independent runs in order to limit the influence of stochasticity and to increase the precision of the parameter estimates. Results were checked using STRUCTURE HARVESTER (Earl and vonHoldt, 2012).

Genetic diversity and population structure within and between STRUCTURE clusters
Genetic diversity was assessed within and between clusters inferred by STRUCTURE. For mtDNA, genetic diversity was assessed by estimating haplotype (*h*) and nucleotide diversities (π) (Nei, 1987) using ARLEQUIN 3.5.1.2 (Excoffier and Lischer, 2010). For microsatellites, genetic diversity was expressed as the number of alleles (N_A), expected (H_E) and observed (H_O) heterozygosity, and inbreeding coefficient (F_{IS}), and was calculated using GenoDive 2.0 (Meirmans and Van Tienderen, 2004). Departures from Hardy–Weinberg equilibrium and linkage disequilibrium were tested using the Fisher's exact test and a Markov chain method with 1,000 iterations in GENEPOP on the web (Raymond and Rousset, 1995). Corrected allelic richness (A_R) per population was estimated in FSTAT 2.9.3.2 (Goudet, 2002). All statistical tests followed sequential Bonferroni correction to address the chance of increased Type I error associated with multiple tests (Rice 1989). Conventional pairwise F-statistics tests (Weir and Cockerham, 1984; F_{ST} and Φ_{ST} for mtDNA, and only F_{ST} for microsatellites) were performed to assess population structure between inferred clusters using ARLEQUIN 3.5.1.2 (Excoffier and Lischer, 2010). For Φ_{ST} we used the Tamura and Nei (1993) model with a gamma correction of 0.5. Significance was tested based on 10,000 permutations. Additionally, we used GenAlEx 6.5 (Peakall and Smouse, 2012) to run a principal coordinate analysis (PCoA) based on the allele frequencies of the 16 microsatellites to visually interpret genetic similarities between individuals without the constraint of forcing them into a set of clustering sub-divisions. A median-joining network implemented in the program PopArt (Leigh and Bryant, 2015) was constructed for the visualization of the genealogical relationships among the mtDNA haplotypes.

RESULTS

Microsatellites genotyping and mtDNA sequencing were identical in replicated samples from the offshore dataset. We found neither completes genotyping matches or pair of samples matching at few loci in the new 48 samples analysed in the present study. Thus, the final new data set consisted of 25 males and 20 females for offshore samples and two males and one female for the three dolphins sampled along the coast of BSA (Table 1). Examination of microsatellite genotypic data across loci revealed significant deviations from Hardy-Weinberg expectations (HWE) for offshore samples after Bonferroni correction for multiple tests. The inbreeding coefficient calculated over all loci was also significant for the offshore ecotype (F_{IS}=0.10, P<0.01). No locus pair was in linkage disequilibrium.

Inferred clusters

Results of the STRUCTURE Bayesian-clustering analyses showed a strong pattern of population structure with best estimate for K=2 when applying the Evano method for the 172 dolphins analysed (coastal + offshore). Assignment probabilities for all individuals to their respective clusters were above 0.99 and 0.98, respectively. All individuals collected in offshore waters (n=45) were placed in cluster A, and all, except three dolphins (possible migrants from cluster A to B, which were collected in coastal waters of BSA and show morphological characteristics of the offshore type), collected in coastal waters (n = 124) were placed in cluster B (Fig. 3). One individual assigned to cluster A (sampled in offshore waters) showed strong admixture with B. Previous analyses had showed strong genetic differentiation among SBU and BSA bottlenose dolphins when running STRUCTURE separately for the same sub-set of samples of coastal dolphins. Therefore, the following results of population structure and genetic diversity are presented considering offshore and coastal dolphins as different populations, with further proposed sub-division for the coastal ecotype (see methods).

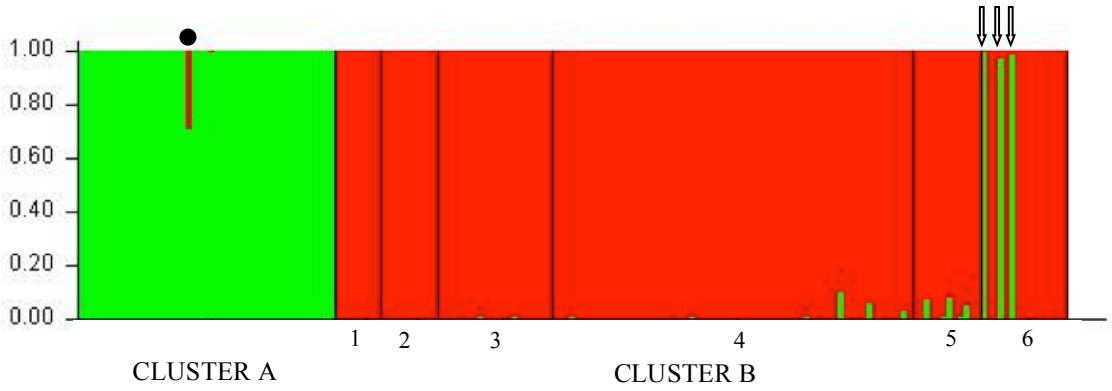

Figure 3. STRUCTURE bar plot of the likelihood (Y-axis) of each individual's (X-axis) assignment to a particular genetic cluster with best estimate for k=2 populations when applying the Evano method (Evanno *et al*., 2005). Vertical black lines in Cluster B separate sampled coastal bottlenose dolphin communities. Cluster A (green vertical lines) contains all common bottlenose dolphins (*Tursiops truncatus*) collected in offshore waters in the SWA, while cluster B (red vertical lines) holds coastal dolphins from SBU and BSA ESUs (see Fruet *et al*., 2014 for details). Black circle in cluster A indicates an admixed individual. Each arrow in cluster B indicates the three biopsied dolphins in Baia San Antonio, Argentina, who morphologically resemble offshore bottlenose dolphins and are likely to represent migrants to the coastal population. Black lines separate sampled coastal bottlenose dolphin communities as presented in Fruet *et al.* (2014): (1) Florianópolis, (2) Laguna, (3) north of Patos Lagoon, (4) Patos Lagoon estuary, (5) south of Patos Lagoon/Uruguay and (6) Bahía San Antonio.

Population structure

The results of principal coordinate analysis (PCoA) based on the analysis of 16 microsatellite loci (Fig. 4) confirmed the patterns of genetic structure revealed by STRUCTURE, with all offshore dolphins grouped towards one side of the ordination plot and the first and second axis explaining 56.7% and 18% of variation, respectively. PCoA analysis also assigned the three new samples of individuals collected in BSA ESU to the offshore ecotype. The same individual identified in STRUCTURE with strong sign of admixture was placed between clusters. Additional sub-division was also marked among coastal samples, with BSA and SBU grouping closer to each other than to offshore samples, but with a clear separation between them.

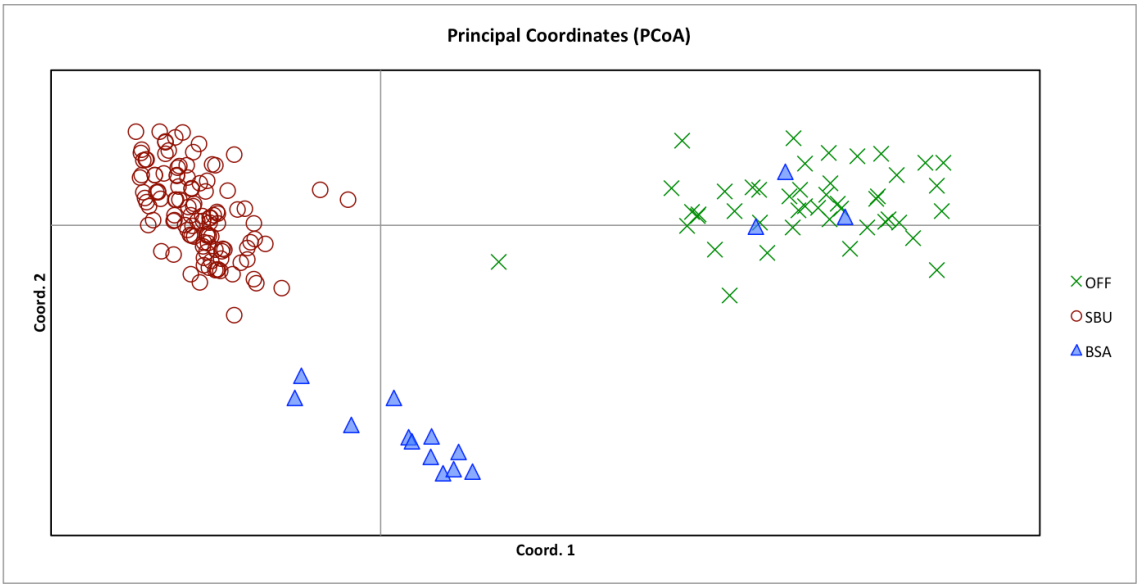

Figure 4. Scatterplot of PCoA scores of genetic similarity among common bottlenose dolphins (*Tursiops truncatus*) from the Southwest Atlantic Ocean based on the allele frequencies of 16 microsatellite loci. OFF (green x), samples from dolphins collected in offshore waters; SBU (open red circle) and BSA (blue triangles) represent dolphins from coastal southern-Brazil-Uruguay and Bahía San Antonio Evolutionarily Significant Units, respectively, which were previously proposed by Fruet *et al*. (2014).

Geographic structuring between ecotypes was also evident and highly significant in the pairwise microsatellite F_{ST} population comparisons (F_{ST} =0.352, P<0.001). High genetic differentiation was also observed when comparing each of the coastal ESU with the offshore ecotype, but F_{ST} was nearly twice as higher for offshore-SBU as for offshore-BSA comparisons (Table 2). Both Φ_{ST} and F_{ST} pairwise comparisons for mtDNA data confirmed the pattern of population structure indicated by the nuclear DNA analysis, with coastal bottlenose dolphins highly and significantly differentiated from those inhabiting offshore waters (F_{ST} =0.1829, P<0.01; Φ_{ST} = 0.385, P<0.01). Results were similar for both Φ_{ST} and F_{ST}, but in general Φ_{ST} had greater values differentiating populations. Levels of genetic differentiation between offshore and coastal ESUs were contrasting. The highest levels of differentiation were found between SBU-offshore and between BSA-offshore when considering F_{ST} and Φ_{ST}, respectively (Table 3).

Table 2. Pairwise comparisons of genetic differentiation between coastal and offshore ecotypes of common bottlenose dolphins (*Tursiops truncatus*) in the Southwest Atlantic Ocean based on 16 microsatellite loci. Pairwise comparisons between the offshore population and the two proposed Evolutionarily Significant Units (ESUs) for the coastal ecotype (Fruet *et al*., 2014) are also shown. Differentiation is expressed as F_{ST}. SBU-ESU = Southern Brazil-Uruguay/ BSA ESU= Bahía San Antonio; *P<0.01.

	OFFSHORE	COASTAL	SBU-ESU	BSA-ESU
OFFSHORE	0.000			
COASTAL	0.352*	0.000		
SBU-ESU	0.390*	-	0.000	
BSA-ESU	0.215*	-	0.372*	0.000

Table 3. Pairwise comparisons of genetic differentiation between coastal and offshore ecotypes of

common bottlenose dolphins (*Tursiops truncatus*) in the Southwest Atlantic Ocean based on 457bp of the mtDNA control region. Pairwise comparisons between the offshore population and the two proposed Evolutionarily Significant Units (ESUs) for the coastal ecotype (Fruet et al. 2014) are also shown. Differentiation is expressed as Φ_{ST} (above diagonal) and F_{ST} (below diagonal). SBU-ESU = Southern Brazil-Uruguay/ BSA ESU= Bahia San Antonio; *P<0.01.

	OFFSHORE	COASTAL	SBU-ESU	BSA-ESU
OFFSHORE	0.000	0.385*	0.403*	0.272*
COASTAL	0.183*	0.000	-	-
SBU-ESU	0.223*	-	0.000	0.262*
BSA-ESU	0.295*	-	0.444*	0.000

Genetic diversity

Overall genetic diversity at both nuclear and mtDNA contrasted among ecotypes (Table 1). For microsatellites, mean number of alleles per locus was 3.6 in coastal and 9.3 in the offshore dolphins. Allelic richness, a measure that takes sample size into account, was twice as higher for offshore than for coastal bottlenose dolphins. Mean observed heterozygosity showed a similar pattern of variation and was lower than the expected for both ecotypes. Offshore dolphins displayed high average number of private alleles per locus, but few in high frequency (*i.e.* >10% – data not shown). Mitochondrial control region sequences of the 457bp aligned for the 172 samples revealed 33 haplotypes defined by 44 polymorphic sites and two indels (Table 1). Indels were exclusively found for offshore dolphins.

There was no haplotype sharing between ecotypes. Haplotype frequencies were highly variable, with offshore dolphins revealing several single haplotypes whereas coastal dolphins displayed few haplotypes at high frequencies (Fig. 5). Very low nucleotide and moderate haplotype diversity were found for the coastal ecotype (Table 1). The median-joining network showed three main haplogroups enclosing: (A) only dolphins collected in offshore waters (n=41); (B) all coastal samples plus four offshore dolphins (n=128), which two of them grouped very close to the most common coastal haplotype; and (C) two offshore dolphins plus the three individuals resembling the offshore ecotype sampled in coastal waters of Argentina (n=5) (Fig. 5). Offshore dolphins displayed highly divergent haplotypes, with a minimum of seven mutational steps separating offshore haplogroups. Twenty-four mutational steps separated the two most distant haplotypes identified for dolphins collected in offshore waters.

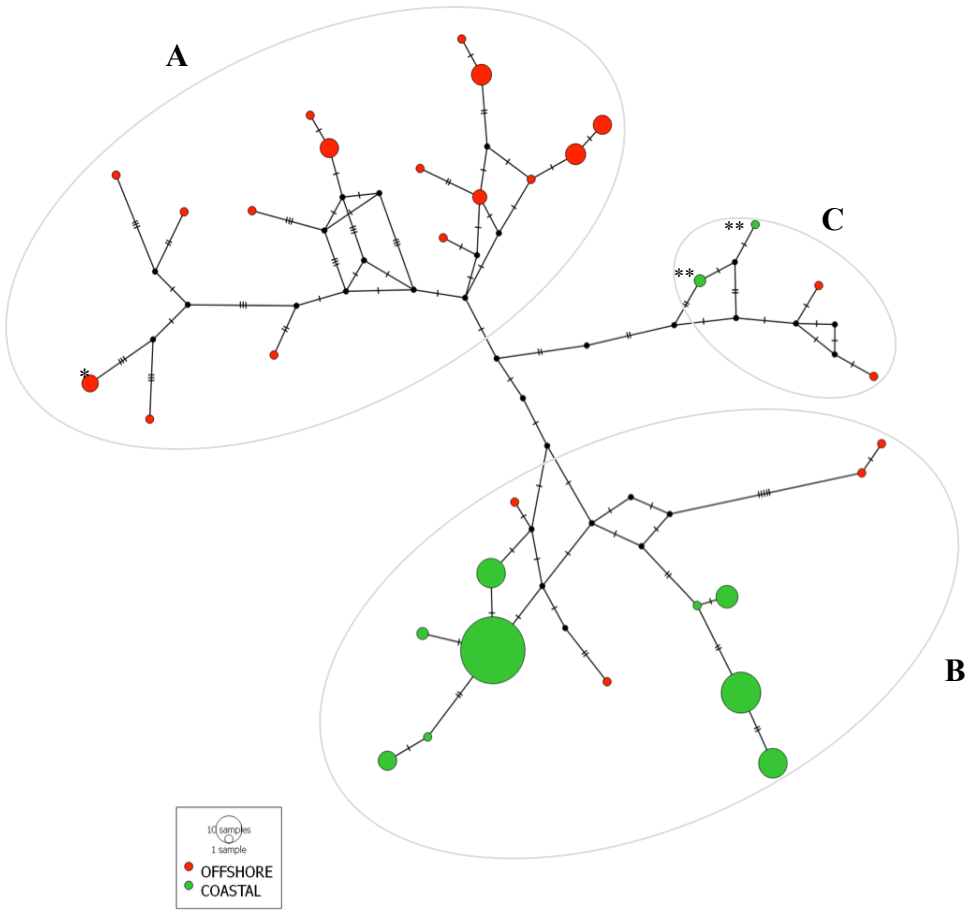

Figure 5. Median-joining network of haplotypes identified from the analysis of a fragment of the mtDNA control region (473bp) in coastal and offshore ecotypes of common bottlenose dolphins (*Tursiops truncatus*) from the Southwest Atlantic Ocean. Light gray ellipses separate the three main groups of haplotypes. Different colors denote dolphins collected in offshore and coastal waters. Dashes represent extinct or unsampled haplotypes. *Haplotype of the individual identified with strong sign of admixture in nuclear DNA (see results for STRUCTURE and PCoA analyses for microsatellites). **Haplotypes of individuals (n=3) resembling the offshore ecotype but sampled in coastal waters of Bahía San Antonio, Argentina.

DISCUSSION

To our knowledge, this is the first study that explicitly tested for genetic differentiation between bottlenose dolphins sampled in distinct habitats (coastal *versus* offshore) in the SWA, and estimated genetic diversity for offshore dolphins from this region. We found strong levels of structuring and contrasting genetic diversity between the two bottlenose dolphin ecotypes. Results were concordant for mitochondrial and microsatellite DNA markers, supporting patterns found in previous broad-scale studies that used similar markers (as described below). Results from the Bayesian clustering method implemented in STRUCTURE (with no *a priori* information) and the PCoA analysis were highly congruent, suggesting that the strong genetic differentiation is not linked to analytical artefacts potentially produced by significant inbreeding coefficients. The observed deviation from HWE in the offshore samples did not seem to be due null alleles, closely related individuals or allelic dropout, and therefore may reflect the existence of further genetic structuring (Wahlund's effect) in the offshore ecotype. For the coastal ecotype, significant deviation of HWE may be due to a combination of further sub-structuring (coastal ESU's and multiple management units identified) as well as inbreeding in one of the populations (see Fruet *et al*., 2014).

Genetic Diversity

The overall genetic diversity was high at both marker types in the offshore dolphins of the SWA. Particularly, mtDNA haplotype and nucleotide diversities (h=0.940 π=0.019; n=101) was higher than that reported for the offshore ecotype in a worldwide perspective (h=0.880 π=0.028; Tezanos-Pinto *et al*., 2009) and slightly higher than reported for pelagic Northeast Atlantic (h=0.929 π=0.014; n=101) and Mediterranean (h=0.902 π=0.013; n=51) bottlenose dolphins, despite the use of a shorter fragment and a lower sample size in our study (see Louis *et al*., 2014a for details). Similarly, genetic variation was abundant over the 16 microsatellite loci, mirroring the overall pattern reported for the offshore ecotype of bottlenose dolphins (*e.g.* Hoezel *et al*., 1998; Louis *et al*., 2014a) and for other pelagic small cetacean species worldwide (*e.g. Delphinus delphis* Mirimin *et al.,* 2009; *Lagenorhynchus acutus* Banguera-Hinestroza *et al*., 2014). Comparatively within our study area, we found that the offshore ecotype had higher values of all measures of genetic diversity than the coastal ecotype, with levels of genetic diversity being up to two times or higher for the offshore dolphins. Such differences in genetic diversity are likely reflecting their contrasting demography, as neutral markers such as mtDNA and microsatellites can have a strong relationship with population size. High levels of genetic diversity typically represent a large panmictic population of thousands of individuals, as it was reported to the offshore bottlenose dolphins in the Northeast Atlantic, which displayed high gene flow and no population structure (Quérouil *et al*., 2007; Louis *et al*., 2014a). This seems to be in agreement with reports of sighting data from systematic ship surveys conducted across the outer continental shelf and slope of southeast and southern Brazilian coast. Despite no abundance estimates are yet available, the species was frequent sighted across the offshore sampling area and in large groups (mean=37 individuals; SE=8; Di Tullio *et al*. *in press*). On the other hand, remarkably low levels of genetic diversity for the coastal ecotype likely reflects small population sizes of possibly a few hundreds individuals (Fruet *et al*., 2014; Fruet *et al*., *in press*). Mark-recapture data from long-term studies of coastal populations along the SWA indicate critical small population sizes (populations not exceeding 90 individuals) and high site fidelity of individuals (*e.g.* Daura-Jorge *et al*., 2013; Fruet *et al*., 2015; Laporta *et al*., in press; Vermeulen and Cammareri 2009b).

Population Structure

We found strong signals of population structure between coastal and offshore ecotypes of bottlenose dolphins in the SWA that is consistent with current habitat usage preferences. Ecotypes displayed a great number of private alleles and did not share mtDNA haplotypes, suggesting current and long-term genetic isolation. This is surprising given the absence of geographical barriers to gene flow in the broad geographical sampling area examined in the present study, which encompasses a few zones with high potential for gene flow between the ecotypes (*i.e.* zones where offshore ecotypes are often seen close to the shore). In Bahía San Antonio, for example, Vermeulen and Cammareri (2009) reported on three morphologically distinct individuals that were always seen together interacting in a regular basis with the small coastal dolphin population of this area. We biopsy-sampled these three individuals and included them in our analysis together with the BSA population. Both microsatellites and mtDNA analyses clustered their genetic profile with the offshore ecotype, suggesting they are possible emigrants of the offshore population. The evidence of genetic isolation between offshore and coastal ecotypes living in simpatry in BSA shed light on the complex mechanisms that could be associated in shaping genetic structure of bottlenose dolphins.

Several hypotheses have been proposed in order to explain processes driving high genetic diversification in species living in environments where there are no geographical barriers to gene flow. For the well-studied killer whales (*Orcinus orca*), for example, feeding strategies are believed to have played a crucial role in shaping genetic structuring in sympatric and parapatric populations (*e.g.* Foot *et al*., 2011). For bottlenose dolphins, despite several hypotheses proposed (e.g. habitat preferences, philopatry to natal area, vertical transmition of social learning, feeding specialization), there is only one study that explicitly tested for forces driving ecotype differentiation and population structure (Louis *et al*., 2014b). This study suggested that coastal populations in North Atlantic were founded by pelagic dolphins after the Last Glacial Maximum, perhaps due to emerging opportunities to explore vacant ecological niches. The occupation of these coastal zones would have followed successive events of feeding specialization and natal philopatry, leading to fine-scale population structuring and a reduction in genetic diversity (*e.g.* Hoelzel *et al*., 1998; Louis *et al*., 2014b; Natoli *et al*., 2004; Tezanos-Pinto *et al*., 2009). This process of diversification is a plausible scenario for bottlenose dolphins in the SWA, which presented similar genetic signals to those found in the North Atlantic (*i.e.* ecotypes with contrasting levels of genetic diversity and following independent evolutionary trajectories). However, this hypothesis should be explicitly tested exploring the historical demography of ecotypes through coalescent-based analysis in combination with

other ecological and biological data.

In the Northeastern Atlantic (NEA) and wider Caribbean, as well as in the Pacific Ocean, there was no complete lineage sorting despite high genetic differentiation between ecotypes in nuclear and mtDNA markers (Caballero *et al*., 2011; Louis *et al*., 2014a; Lowther-Thieleking *et al*., 2015; Segura *et al*. 2006). In the Northwestern Atlantic (NWA), however, current gene flow seems to be trivial between ecotypes, with the coastal haplotypes forming an evolutionary separate lineage (Natoli *et al*., 2004; Tezanos-Pinto *et al*., 2009), similar to what we have found in the present study. In the NWA, the coastal ecotype is highly differentiated in ecology (distribution, feeding ecology and parasite loads), morphology, and genetics (*e.g.* Hersh and Duffield, 1990, Mead and Potter, 1990, Mead and Potter, 1995, Rosel *et al*., 2009), with restricted distribution to this oceanographic region (Natoli *et al*., 2004). It was further suggested that the coastal ecotype might in fact represent a different species from the offshore ecotype inhabiting the ocean region (see Kingston and Rosel, 2004). For the SWA, little information is available distinguishing both ecotypes. The presence of coastal and offshore ecotypes have been preliminary suggested based on color pattern (Simões-Lopes, 1996), feeding ecology (Botta *et al*., 2012) and genetics (Costa *et al*., 2015), and only recently a detailed study based on skull and skeletal morphology of stranded dolphins have demonstrated the presence of two distinct ecotypes living in parapatry (Costa *et al*., *in press*). In addition, the great morphological differentiation between the ecotypes led the later authors to suggest that these groups are distinct subspecies, being the coastal ecotype restricted to the southern coast of the Southwest Atlantic Ocean. The previous study, however, did not examine the potential genetic differentiation between the ecotypes. Our data did not genetically examine the same samples used in Costa *et al*. (in press), but there is an overlap in the sampling areas. Therefore, if the parapatric distribution suggested is correct, and considering our sampling areas, the results presented here seem to be in agreement with the ecotypes described by Costa *et al*. (in press). Ongoing analyses testing both nuclear and mitochondrial markers as well as morphology are exploring the congruence between the genetic and morphological data in attempt to clarify the taxonomic status of bottlenose dolphins in the SWA (Costa *et al*., *unpub. data*).

Implications for Conservation

Our results from maternal and bi-parental molecular markers were congruent and showed that coastal and offshore bottlenose dolphin ecotypes in the SWA are genetically distinct and possible following discrete evolutionary trajectories. Sighting data from the literature indicates that coastal bottlenose dolphins are restricted to shallow waters near the southern coast of the continent (above 25-27^{o}S), where the offshore ecotype preferentially inhabits deeper waters albeit some incursions to coastal areas can occur occasionally in the north limit of the distribution of the coastal ecotype. Despite opportunity for gene flow in this possible "contact zones" our results suggest negligible interbreeding between ecotypes, even in an area where dolphins of both ecotypes were observed to associate (Vermeulen and Cammareri, 2009). Based on our findings, which seem to be in agreement with the morphological differentiation described by Costa *et al*. (*in press*), we recommend recognizing the offshore bottlenose dolphin ecotype as an additional evolutionarily significant unit (ESU) in the SWA. The recognition of this ESU is very important because it prioritizes specific conservation strategies for the offshore ecotype (or subspecies *Tursiops truncatus truncatus* as suggested by Costa *et al*., *in press*) that might differ from those recommended to the two ESUs reported for coastal bottlenose dolphins (or the two ESUs suggested for the coastal subspecies *T. t. gephyreus*). Studies should therefore consider the offshore ESU separately for abundance estimates, monitoring and population assessments. Nevertheless, it is important to point out that the genetic isolation observed in the coastal ESUs (Fruet *et al*., 2014) increases the risk of inbreeding depression and extinction of the coastal ecotype. This ecotype is restricted to a relatively small area and is currently genetically depauperated, with small population sizes and evidence of increasing threats from several anthropogenic activities (Fruet *et al*., 2014; see Fruet *et al*. *in press* a and b for review) and local population declines (Coscarella *et al*., 2012; Vermeulen and Bräger, 2015). Thus, conservation measures to enhance the long-term viability of this possible endemic subspecies need to be prioritized.

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