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# Evidence of introgressive hybridization between *Stenella coeruleoalba* and *Delphinus delphis* in the Greek Seas

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ABSTRACT

Natural interspecific hybridization might be more important for the evolutionary history and speciation of animals than previously thought, considering several demographic and life history traits as well as habitat disturbance as factors that promote it. In this aspect, cetaceans comprise an interesting case in which the occurrence of sympatric species in mixed associations provides excellent opportunities for interspecific sexual interaction and the potential for hybridization. Here, we present evidence of natural hybridization for two cetacean species commonly occurring in the Greek Seas (*Stenella coeruleoalba* and *Delphinus delphis*), which naturally overlap in the Gulf of Corinth by analyzing highly resolving microsatellite DNA markers and mitochondrial DNA sequences in skin samples from 45 individuals of *S. coeruleoalba*, 12 *D. delphis* and three intermediate morphs. Employing several phylogenetic and population genetic approaches, we found 15 individuals that are potential hybrids including the three intermediate morphs, verifying the occurrence of natural hybridization between species of different genera. Their hybrids are fertile and able to reproduce not only with the other hybrids but also with each of the two-parental species. However, current evidence does not allow firm conclusions whether hybridization might constitute a step towards the generation of a new species and/or the swan song of an already existing species (*i.e.*, *D. delphis*). Given that the focal species form mixed pods in several areas of Mediterranean, this study is an excellent opportunity to understand the mechanisms leading to hybridization in the context of gene flow and urges for the evaluation of the genetic status of common dolphins in the Mediterranean.

## 1. Introduction

In the early years of the modern evolutionary synthesis, natural hybridization had been considered as a rare phenomenon with very little evolutionary significance. Nowadays, this ceased to be the case with the numerous studies of hybridization that have been conducted providing clues on the reproductive behavior, dispersal capabilities and phylogenetic relationships of species (Pyle and Randall, 1994). Even though the evolutionary significance of hybridization is a controversial issue (Schwenk et al., 2008), the study of the causes and consequences of natural hybridization in hybrid zones [areas where genetically distinct groups of individuals meet and mate, resulting in at least some offspring of mixed ancestry (Harrison, 1990)] offers opportunities to evaluate the effects of gene flow, natural selection and recombination in natural populations and provides insights into the phenotypic and genotypic changes during speciation (Mullen et al., 2008).

The frequent occurrence of interspecific hybridization in several groups of animals is indicative of its' key role in animal evolutionary history and speciation mostly by increasing their adaptability to environmental change (Mallet, 2005). Factors considered to promote interspecific hybridization include several demographic and life history traits (population sizes, body sizes, timing of reproduction, behavior, climatic conditions, parental care) as well as habitat disturbance (Crossman et al., 2016; Frantzis and Herzing, 2002; Jahner et al., 2012; May-McNally et al., 2015; Randler, 2006; Rubidge and Taylor, 2005; Scribner et al., 2001; Taylor, 2004; Yau and Taylor, 2013). In a recent review of cetaceans (whales, dolphins, and porpoises) it has been shown that almost 20% of the species hybridize (*i.e.*, Amaral et al.,

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2014; Baird et al., 1998; Glover et al., 2010; Spilliaert et al., 1991) both in the wild and in captivity (for a review see Crossman et al., 2016).

The confusing taxonomy and uncertain phylogenetic relationships observed in some groups of cetaceans have been attributed to incomplete lineage sorting and hybridization (Amaral et al., 2012) as a consequence of the rapid events that characterized their radiation. The relatively recent evolutionary radiation of cetaceans [*i.e.* last 10 million years, (McGowen et al., 2009)] combined with the apparently slow evolutionary rate (Hoelzel et al., 1991; Schlotterer et al., 1991) could justify the constant number of chromosomes (2n = 44) and the common karyotic arrangement in most cetaceans (Arnason and Benirschke, 1973; Árnason et al., 1978; Pause et al., 2006). This in turn suggests a lack of major differences in chromosomal rearrangements among species (Amaral et al., 2014) and karyological uniformity (Arnason, 1980) that might indicate a greater potential for cetaceans in respect to other mammals, to hybridize and generate viable and fertile offspring (Amaral et al., 2014). This becomes evident in oceanic cetaceans with a karyotype of 44 chromosomes, where hybridization is known to occur in half of the species (Crossman et al., 2016).

The large diversity of marine habitats in the Greek Seas supports eight commonly occurring and three occasional cetacean species (Frantzis, 2009; Frantzis et al., 2003). In this study we focus on two of the species, the striped dolphin (*Stenella coeruleoalba*) and the short-beaked common dolphin (*Delphinus delphis*). *Stenella coeruleoalba*, which is the most common cetacean in the region (Frantzis, 2009), is typically pelagic, inhabiting the deep waters of the continental shelf and it is observed close to shore only where deep water is found close to the coast (Frantzis, 2009; Gannier, 2005). According to the International Union for the Conservation of Nature and Natural Resources (IUCN) Red List of Threatened Animals, its Mediterranean population is listed as "Vulnerable" (Aguilar and Gaspari, 2012). In the Mediterranean, *D. delphis* occurs in both neritic and pelagic environments, occasionally together with *Tursiops truncatus* (common bottlenose dolphin) and *S. coeruleoalba*, respectively (Bearzi et al., 2003). In the Greek Seas, all known population units of *D. delphis* inhabit shallow (< 200 m) and coastal waters, except the one inhabiting the Gulf of Corinth (GOC) (Frantzis, 2009). The Mediterranean population of this species is listed by IUCN as "Endangered", since it experienced a 50% decline in abundance over the last three generations (for more details see Bearzi, 2003; Natoli et al., 2008).

*Stenella coeruleoalba* and *D. delphis* belong to two taxonomically problematic genera of delphinid cetaceans (Natoli et al., 2006), due to the lack of correspondence between their morphological and genetic differentiation. Previous studies on the genetic variation of *S. coeruleoalba* detected significant differentiation among the Mediterranean and North Atlantic and Pacific populations (Bourret et al., 2007; García-Martínez et al., 1999; Gaspari et al., 2007; Valsecchi et al., 2004). Although mtDNA data showed no population subdivision in the Mediterranean Sea (Garcia-Martinez et al., 1995), the use of microsatellite data in two recent studies, revealed population genetic structure within the Mediterranean basin [subdivision in the western Mediterranean population (Bourret et al., 2007) and in inshore and offshore populations in the Tyrrhenian Sea (Gaspari et al., 2007)]. On the other hand, the patterns of genetic differentiation at the population level of *D. delphis* in the Mediterranean showed a marked differentiation between the Ionian and Alboran Seas (Natoli et al., 2008), at a similar or even higher level to that observed between populations of species from different sides of the Atlantic Ocean (Natoli et al., 2006). These patterns were correlated to the different habitat preferences displayed by *D. delphis* in the western (open water) and eastern (shallow coastal habitat) Mediterranean, suggesting the exploitation of different resources as a significant factor reducing movement between these regions (Natoli et al., 2008). Finally, preliminary results indicate the isolation of the Black Sea population of *D. delphis* from the rest of the Mediterranean (Natoli et al., 2008).

The GOC is the only known body of water globally, where three sympatric dolphin species form permanent mixed-species groups: *D. delphis*, the purely pelagic *S. coeruleoalba* and *Grampus griseus* (Risso's dolphin) (Frantzis and Herzing, 2002) are usually found in the deep waters of the continental slope (Frantzis, 2009). Although the GOC is a semi-enclosed sea, it shows several characteristics of an open sea due to its deep waters and steep slopes along its coasts, the systematic occurrence of wind-driven upwelling currents and the entrance of waters from the Ionian Sea (Frantzis and Herzing, 2002). Sympatric *D. delphis* and *S. coeruleoalba* that form temporal mixed-species groups have been recorded in two more areas of the Mediterranean Sea: the Alboran Sea in western Mediterranean, and the Tyrrhenian Sea in central Mediterranean (García-Martínez et al., 1999). Additionally, recent unpublished observations, indicate that temporal mixed groups of these two species also occur in other Mediterranean areas, such as the Balearic Sea and the Sicilian Strait (Ana Cañadas and Mediterranean common dolphin specialists group, pers. comm. 2017).

The occurrence of sympatric cetaceans in mixed associations provides excellent opportunities for interspecific sexual interaction and the potential for hybridization (Bérubé, 2009). Despite the high number of hybridization events in cetaceans hold in captivity (Bérubé, 2009), wild cetacean hybrids are typically identified based solely on morphology without any prior knowledge of parental interactions. Therefore, hybrid identification in the field is problematic with the number of well-documented incidences being limited (for a review see Bérubé, 2009; Crossman et al., 2016). The collection of genetic data for the verification of the occurrence of alive wild hybrid dolphins is difficult and has associated welfare considerations (Hodgins et al., 2014), rendering the molecular confirmation of hybridization scarce (Amaral et al., 2014; Bérubé and Aguilar, 1998; Willis et al., 2004).

Observations on dolphins in the GOC have reported individuals with unusual pigmentation patterns (intermediate morphs between *S. coeruleoalba* and *D. delphis*) (Frantzis and Herzing, 2002) that either constitute potential hybrids between the two species or represent another incident of the high variability of pigmentation patterns in *S. coeruleoalba* (Acquarone and Notarbartolo di Sciara, 1992). Aiming to test whether natural hybridization is the case and understand the evolutionary mechanisms that may be behind the origin of the intermediate morphs, we used data from highly resolving microsatellite DNA markers and mitochondrial DNA (mtDNA) sequences. Skin samples from several individuals of *S. coeruleoalba* and *D. delphis* from several locations of the Greek Seas with emphasis on the GOC as well as from the intermediate morphs from the GOC were examined and their genetic data were analyzed through several phylogenetic and population genetic approaches. Genetic intermediacy between the two parental forms, nuclear admixture and mitochondrial capture and unique variation would indicate that these morphs constitute the results of recent hybridization.

## 2. Material and methods

### 2.1. Samples and DNA extraction

From 1997 to 2013 sixty dolphin skin samples were collected and preserved in ethanol. Twenty-two originated from the GOC and thirty-eight from other areas of the Greek Seas (Fig. 1). In total 45 specimens of *S. coeruleoalba,* 12 specimens of *D. delphis* and 3 intermediate morphs were sampled. The samples from the three intermediate morphs and one *D. delphis* were collected from free-ranging dolphins in the GOC while they were bow-riding by the use of a pole to minimize disturbance. Sampling occurred in accordance with international guidelines and under a research permit from the Greek authorities. All other samples were collected from dead animals stranded along the coasts (Fig. 1 and Table S1). The skin samples were washed three times in 1 mL of 10 mM Tris-HCl (pH 8.0) on a rotary mixer for 24 h per wash to re-hydrate (Austin and Melville, 2006). Total genomic DNA was extracted using the DNA IQ System (Promega, USA).

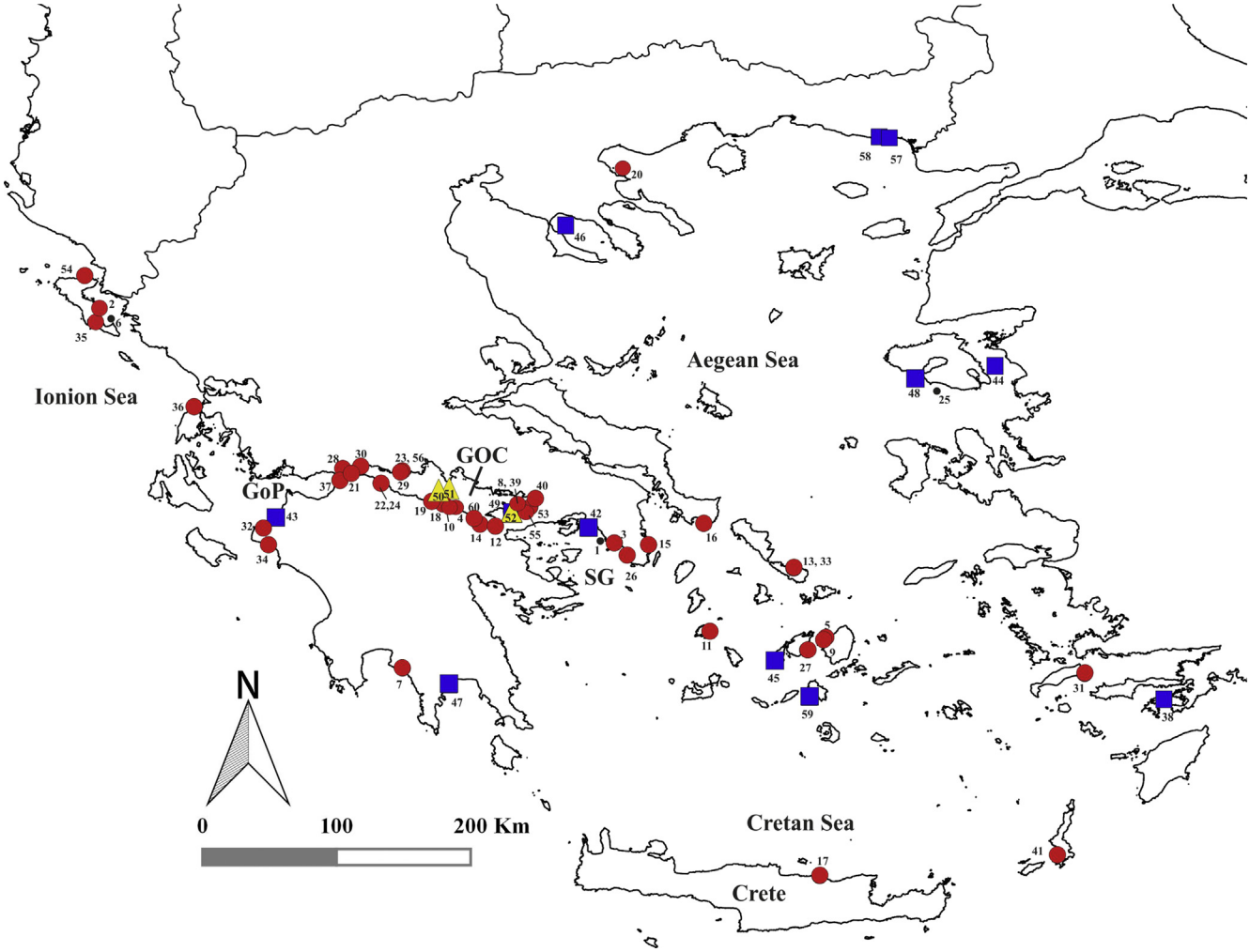


**Fig. 1.** Geographic distribution of the striped and short-beaked common dolphins collection sites. Red circles: *Stenella coeruleoalba* samples, Blue rectangles: *Delphinus delphis* samples, Yellow triangles: intermediate morph samples. Black circles: samples that were not used in the genetic analyses due to low quantity and quality of DNA. GoP: Gulf of Patras, GoS: Gulf of Saronikos, GOC: Gulf of Corinth.

### 2.2. DNA sequencing

A fragment of the cytochrome *b* gene (cyt *b*) of the mtDNA was amplified and sequenced using a pair of universal primers (L14724 and H15149) (Irwin et al., 1991). Amplification profile consisted of an initial cycle of denaturation at 94 °C for 5 min, and 35 cycles with the following thermal profile: 94 °C for 30 s, 52.9 °C for 30 s and 72 °C for 1 min. PCR products were purified with the Nucleospin PCR purification kit (Macherey-Nagel). Double stranded sequencing of the purified PCR products was performed using a Big-Dye Terminator Cycle Sequencing Kit (v.3.1) on an ABI 3730XL automated sequencer. All sequences obtained were edited using the software Codon Code Aligner (v. 3.7.1).

### 2.3. Microsatellite genotyping

Twelve microsatellite loci designed for short-beaked common dolphins (Coughlan et al., 2006), aduncus dolphins (*Tursiops aduncus*) (Krutzen et al., 2001) and other cetaceans species (Valsecchi and Amos, 1996) were used. Each locus was amplified separately (see Table S2 for locus-specific annealing temperature). PCR products were combined in two multi-loading schemes (Load1: Dde09, Dde59, Dde66, Dde84, MK5, Ev14 and Load2: Dde61, Dde65, Dde70, Dde72, MK3, Ev37) and genotyped on an ABI 3730 (Applied Biosystems) using GS-500 Liz (Applied Biosystems) as an internal size standard in each capillary. Genotypes were determined using STRand software v.2.4.109 (http://www.vgl.ucdavis.edu/STRand). To minimize the negative consequences of poor allele calling, binning was accomplished with Flexibin 2 (Amos et al., 2007) the output of which was manually evaluated.

### 2.4. Sex determination

Wherever possible the gender was determined morphologically, but also genetically, by using a multiplex PCR method in which both SRY (male determining factor) and cyt *b* (used as positive PCR control) genes were amplified. The PCR reactions were performed as in the case of cyt *b* using the SRY specific primers (SRY PMF 5′ CATTGTGTGGTC TCGTGATC 3′ and SRY PMR 5′ AGTCTCTGTGCCTCCTCGAA 3′) (Richard et al., 1994) and the primers of cyt *b* (L14724 and H15149). Visualization of the PCR products was performed with their electrophoresis on a 2% agarose gel. The cyt *b* product fluoresced under UV light at ~425 bp and the SRY (male) product at ~147 bp. Thus, males were identified when two bands were detected on the gel at ~425 bp and ~147 bp and females when only one band was present at ~425 bp. However, due to the high rate of false negatives (*i.e.* a male sample interpreted as female due to the lack of SRY amplification band) inherent in this method, sex determination should be treated with caution.

### 2.5. Phylogenetic analyses on mtDNA data

Several cyt *b* sequences of *D. delphis*, and *S. coeruleoalba* were retrieved from GenBank and included in the phylogenetic analyses (see Table S3) along with the sequences generated in the present study. Moreover, several *D. capensis* sequences were also retrieved for comparison reasons, but see Cunha et al. (2015) in which *D. capensis* is considered invalid and those specimens must be considered as *D. delphis*. The cyt *b* sequence of *Tursiops truncatus* (KF570389; Moura et al., 2013b) was used as outgroup. All cytochrome *b* sequences were translated into amino acids prior to analysis in order to check for spurious gaps or stop codons. DNA sequences were aligned using MAFFT v.6 (Katoh et al., 2002) with auto strategy. Sequence divergences (uncorrected p-distance) were estimated in MEGA v.6.00 (Tamura et al., 2013).

The dataset was partitioned into the three codon positions. jModelTest v. 2.1.7 (Darriba et al., 2012) was employed in order to find the model that best fits the data for each partition according to the Bayesian Information Criterion (BIC; Schwarz, 1978). In more details, 7 substitution schemes were tested with the estimation of base frequencies (+F), gamma shape (+G) and invariable sites (+I), reaching a total of 56 models. The models including both G and I were ignored (Yang, 2006).

Maximum likelihood analyses were conducted with RAxML v. 8.1.21 (Stamatakis, 2014) using RAxMLGUI v.1.5 (Silvestro and Michalak, 2011) under the GTR + G model of evolution and parameters were estimated independently for each partition. The best ML tree was selected from 500 iterations and the confidence of the branches of the best ML tree was assessed based on 1000 thorough bootstrap replicates.

Bayesian Inference was performed in MrBayes v.3.2.6 (Ronquist et al., 2012). We ran eight concurrent chains (one cold and seven heated) for $5 * 10^7$ generations and recorded samples every 5000 generations. The first 25% of the samples were discarded as burn-in, and the remaining samples were used to summarize the posterior probability distributions of parameters ($\geq$95% indicate significant support) (Huelsenbeck and Ronquist, 2001). Results were analyzed in Tracer v1.6 (Drummond and Rambaut, 2007) to assess convergence and effective sample sizes (ESS) for all parameters. We checked if (i) the average standard deviation of split frequencies between chains failed below 0.01, (ii) the potential scale reduction factor (PSRF) of all the estimated parameters approached values of ~1, (iii) the plot of the generation versus the log probability of the data looks like "white noise" (the log likelihood values), and iv) the minimum value of minimum Estimated Sample Sizes (ESS) were larger than 100 (ESS values below 100 may indicate that the parameter is under-sampled). Support values of all phylogenetic analyses *i.e.* maximum likelihood bootstrap values and Bayesian posterior probability values were joined and mapped onto the Bayesian Inference tree (*i.e.*, the 50% majority-rule consensus tree calculated from the posterior distribution of trees, Fig. 2).

### 2.6. Population structure analyses on microsatellite data

One locus was excluded from further analyses (EV14) due to scoring problems during genotyping. Furthermore, samples that yielded genotypes for less than six loci (*i.e.,* individuals with genotypes for maximum five loci) were also excluded from further analyses providing a final dataset of 53 samples.

Comparative measures of genetic diversity *i.e.,* number of alleles ($N_a$), observed ($H_o$) and unbiased expected ($H_e$) (Nei, 1978) heterozygosity, were estimated using GENETIX v 4.05 (Belkhir et al., 2001). Departures from Hardy–Weinberg equilibrium (HWE) per locus were calculated using GENEPOP on the Web (http://genepop.curtin.edu.au/) (Raymond and Rousset, 1995; Rousset, 2008).

Relationships between individual multilocus genotypes were evaluated by two multivariate methods: Factorial Correspondence Analysis (FCA) and Principal Component Analysis (PCA). These methods do not depend on the mutational model of microsatellites, avoiding in this way the stochastic nature of microsatellites' mutation process (Estoup et al., 2002). The first method (FCA) was employed in GENETIX v 4.05 (Belkhir et al., 2001) to qualitatively explore the distribution of genotypes in the data using the frequencies of different alleles as the components of the visual representation of individual genotypes. The analyzed samples were visualized as groups of dots with different coloration for each species in a two-dimensional ruled surface with each dot representing one individual and its position in space defined by the individual's genotypic data. The second method (PCA) was implemented in the package ADEGENET v. 2.0.0 in the R environment v. 3.2.5 (Jombart, 2008). The allelic frequencies were scaled and missing data were replaced with the allele means using the function 'scaleGen'.

To assess population genetic structure within our reference samples of *D. delphis*, *S. coeruleoalba*, both model-based and non model-based methods were employed. First, we used STRUCTURE 2.3.4 (Pritchard et al., 2000) under the correlated allele frequency model allowing admixture, without location prior, and with a burn-in period of 500,000 followed by 1,000,000 iterations. Runs were conducted varying the number of clusters (*K*) from 1 to 5 with 10 replicate runs at each value of *K*. The inference of K was evaluated with two methods (a) the $\Delta K$ approach (Evanno et al., 2005) and (b) the posterior probabilities of each K as suggested by the developers in the software's documentation. The ten independent runs of the 'best' K were averaged in order to identify sets of highly similar runs, and separate distinct groups of runs that represent distinct modes in the space of possible solutions, generating consensus solution for each distinct mode to allow for label switching and testing of convergence. Both analyses (choosing of K and averaging) were performed with CLUMPAK server (Kopelman et al., 2015).

Second, multivariate Discriminant Analysis of Principal Components (DAPC) implemented in the ADEGENET package in the R environment (Jombart et al., 2010) was employed in order to infer population subdivision within the studied samples as a population genetics model-free method. The data were first transformed using Principal Components Analysis (PCA) and then Discriminant Analysis (DA) was performed on the retained principal components. The groups, as a requirement of DAPC, were identified using the *k-means* clustering algorithm which attempts to find the *k* that maximizes the between groups variation. The optimal number of clusters was identified based on the lowest BIC value (Jombart et al., 2010). The number of retained PCs was based on cross-validation, so as to include most sources of variation by retaining PCs associated with the highest mean success and most importantly lowest mean squared error. As a result, DAPC was run by retaining 100 PCs, for prior data transformation.

### 2.7. Hybridization analyses on microsatellite data

In order to examine whether natural hybridization is occurring between the two species, four different methodologies were used to detect potential hybrids by assigning samples to their respective stock and quantifying the level of introgression for each sampled individual. First, we used the approach implemented in NEWHYBRIDS v.1.1 (Anderson and Thompson, 2002) that computes the posterior probability of an individuals' assignment to various genotype frequency classes reflecting the level of certainty that an individual belongs to a certain hybrid category. There are six different categories: two pure parental populations corresponding to the two species that hybridize (*i.e.*, *D. delphis*, and *S. coeruleoalba* in our case), their F1 hybrids as well as the F2 hybrids and backcrosses of F1 hybrids with either parental population. The analysis was performed using the default genotype frequency classes with 100,000 iterations as a burn-in phase and 600,000 iterations post burn-in. Two independent runs with different starting points were performed for each of the two types of priors –'Jeffreys-like' and Uniform prior- available for both the mixing

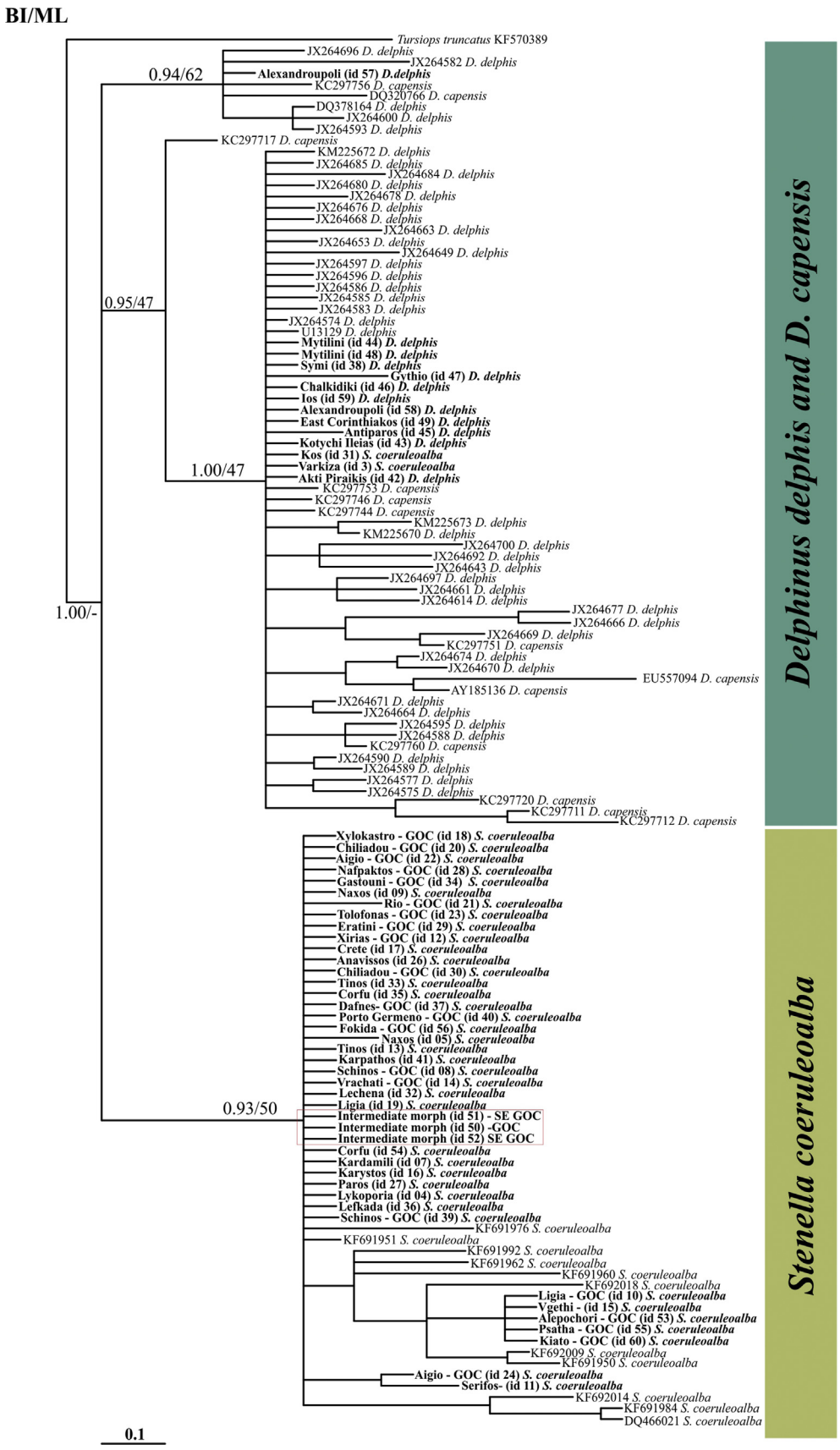


**Fig. 2.** Bayesian Inference (BI) tree reconstructed from the cyt *b* sequences. Numbers on branches indicate posterior probabilities and bootstrap supports (BI/ML).

proportions and the allele frequencies. Based on these results, 'pure' *S. coeruleoabla* and *D. delphis* individuals with pp. > 0.90, were considered as unambiguously belonging to each of the studied species and were used to generate pure parental and hybrid classes of the two species in HYBRIDLAB (Nielsen et al., 2006). The simulated dataset included 500 *Delphinus*-parental, 500 *Stenella*-parental, 500 F1 hybrids, 500 F2 hybrids, 500 backcrosses with *Delphinus*, and 500 backcrosses with *Stenella*.

The simulated data were then analyzed along with the real dataset in NEWHYBRIDS as above and in STRUCTURE, GENECLASS, and ADEGENET.

In STRUCTURE, we used the same parameters as described earlier with 50,000 burn-in and 150,000 iterations for ten replicate runs and setting the number of populations equal to two (K = 2). The membership coefficients of the simulated groups of genotypes resulting from this second STRUCTURE analysis were used as empirically derived assignment criteria for the real genotypes to the six categories.

Then, we used the Bayesian allocation method implemented in GENECLASS2 with the prior of Rannala and Mountain (1997) to optimally assign individuals ('real' genotypes) to one of six HYBRIDLAB's simulated categories (Piry et al., 2004).

Finally, Discriminant Analysis of Principal Components (DAPC) was performed, through ADEGENET in R, as an efficient descriptor of the simulated categories with real genotypes being used as supplementary individuals *i.e.,* observations which do not participate in constructing the model, and are projected onto the discriminant functions already defined in the DAPC analysis of the simulated data. In other words, the real data were transformed using the centering and scaling of the "training data" (the simulated categories), and then using the same discriminant coefficients the position of these individuals onto the discriminant functions was predicted deriving membership probabilities for each individual assignment to each category.

## 3. Results

### 3.1. Phylogenetic analyses on mtDNA data

A total of 394 base pairs (bp) of the mitochondrial cyt *b* from 56 individuals were obtained (4 specimens sample ids: 1, 2, 6 and 25 failed to amplify). These sequences were combined with 68 cyt *b* sequences retrieved from GenBank (43 *D. delphis*, 13 *D. capensis*, 11 *S. coeruleoalba,* and 1 *T. truncatus*, which was used as outgroup, Table S3). The alignment of 124 sequences revealed 56 haplotypes and contained 55 variable and 25 parsimony informative sites (60 and 25, respectively when the outgroup was also included). Pairwise genetic distances (p-distance) varied from 0 to 3.9%.

The best-fit nucleotide substitution model selected based on BIC was the HKY + G. Maximum Likelihood ($-\ln L = -2906.34$) and MrBayes analysis (arithmetic mean $-\ln L = 3321.74$) produced similar topologies (Fig. 2). Considering MrBayes analysis, the MCMC convergence diagnostics revealed that (a) the average standard deviation of split frequencies was 0.004, (b) the plot of the generation versus the log probability of the data (the log likelihood values) produced a "white noise" graph and (c) the average Potential Scale Reduction Factor (PSRF) was 1.00 for all parameters (maximum PSRF is 1.001), providing no clues of non-convergence and indicating stationarity, that is, there should be no tendency of increase or decrease over time. Two main clades were obtained through the phylogenetic analyses, which correspond to *S. coeruleoalba* and *D. delphis* & *D. capensis* [as was expected see Cunha et al. (2015)]. It is quite interesting that two specimens (sample ids 3 and 31), which based on morphology and nuclear DNA (see below), were recognized as *S. coeruleoalba*, possess mitochondrial haplotypes of *D. delphis*.

## 4. Data exploration, population structure and hybridization analyses on microsatellite data

### 4.1. Exploratory data analysis

The number of alleles among the two species (excluding the three samples with intermediate morphology) was ranging for *S. coeruleoalba* (n = 40) from 9 (Dde70, Ev37) to 17 (MK5, Dde61) and for *D. delphis* (n = 10) from 4 (Dde09, Dde84) to 8 (MK5, Dde61, Ev37). The mean number of alleles and the levels of heterozygosity were of the same magnitude between the two species ($H_o$ 0.6633 and 0.7133, respectively). Both species deviated from Hardy-Weinberg (HW) equilibrium with three out of eleven loci displaying heterozygote deficit in each species. None of the studied loci displayed heterozygote excess. Comparative measures of genetic diversity among the two species are depicted in Table S4.

The visual representation of FCA analysis depicted in **Fig. S1**, indicates a clear discrimination between the two-studied species with the morphologically intermediate individuals as well as few other individuals being positioned in the space between the two species (see Table S1 for their id codes). The first two factors of the FCA explained 11.06% of total inertia. PCA analysis yielded similar results with clear discrimination among the two species and with a small number of samples occupying similar space to that occupied by the morphological intermediates laying among the two species. Both analyses indicated the same samples as being intermediate to the two species. The results of the PCA analysis are presented graphically along the first and second axes in line with eigenvalues (PC1: 6.26%, PC2: 4.99%) in Fig. S2.

## 5. Population structure

According to the clustering analysis performed with STRUCTURE, the optimal number of populations that best described the genotyped data was K = 3 where *S. coeruleoalba* samples were assigned to two clusters with no geographical distinction. The majority of *S. coeruleoalba* samples were assigned to one of the two clusters with high membership coefficients (*i.e.,* q > 0.9), while few samples had mixed ancestry from the two *S. coeruleoalba* clusters (**Fig. S3A**). Samples of *D. delphis* were assigned to a third cluster with high membership coefficients except sample with id code 59 where q was 0.78. Furthermore, sample with id code 47 that based on morphology and mtDNA was described as *D. delphis* had most of its genome assigned to one of the two clusters of *S. coeruleoalba*. In respect to the three morphological intermediates, two were assigned with high membership coefficients (q > 0.9) to one of the two species clusters *i.e.* the sample 50 was assigned to *D. delphis* cluster, and the sample 52 was assigned to one of the two *S. coeruleoalba* clusters (the same as sample 47 mentioned above), while the sample 51 displayed mixed ancestry from all three clusters. It is worth mentioning that intermediate individuals (either genetically or both genetically and morphologically) of FCA and PCA analyses were either assigned to one of the two *S. coeruleoalba* clusters or were unassigned (q < 0.9). The only exception is the morphologically intermediate sample 50 mentioned above.

The *k-means* algorithm run prior to DAPC analysis indicated that the optimal clustering solution, corresponding to the lowest BIC value, was for K = 2. As a result, a single discriminant function was retained. The densities of individuals on this discriminant function are plotted in **Fig. S3B** where the inferred groups are depicted with different colors. The first group has a unimodal density distribution and contains only *S. coeruleoalba* samples while the second group has bimodal density distribution containing *D. delphis* samples, one *S. coeruleoalba* sample (sample 9) as well as the three morphological intermediates. It is worth noting that in the second group, there are individuals with positions in between the extreme modes of the two groups which are consistent with the results of both FCA and PCA analyses, containing samples from both species as well as the morphologically intermediates. Given that in

**Table 1**
Hybridization analyses assignment. Individuals were assigned to each of the predefined categories [two pure parental (*Stenella*, *Delphinus*), F1, F2, BC with either parental] using as threshold the value 0.9 (posterior probabilities in all runs of NewHybrids and DAPC, probability scores in GeneClass and q-values in Structure). *Only according to Jeffreys prior, **only according to uniform prior. For more details see Table S5.

| Id code | NewHybrids (1st Run) | NewHybrids (2nd Run) | GeneClass | DAPC with Supp. Inds. | Structure |
|---|---|---|---|---|---|
| **3** | *Stenella* | *Stenella* | *Stenella* | *Stenella* | *Stenella* |
| **4** | *Stenella* | *Stenella* | *Stenella* | *Stenella* | *Stenella* |
| **5** | *Stenella* | *Stenella* | *Stenella* | *Stenella* | *Stenella* |
| **8** | *Stenella* | *Stenella* | *Stenella* | *Stenella* | *Stenella* |
| **10** | *Stenella* | *Stenella* | *Stenella* | *Stenella* | *Stenella* |
| **11** | *Stenella* | *Stenella* | *Stenella* | *Stenella* | *Stenella* |
| **12** | *Stenella* | *Stenella* | *Stenella* | *Stenella* | *Stenella* |
| **13** | *Stenella* | *Stenella* | *Stenella* | BC with *Stenella* | *Stenella* |
| **14** | *Stenella* | *Stenella* | *Stenella* | *Stenella* | *Stenella* |
| **16** | *Stenella* | *Stenella* | *Stenella* | *Stenella* | *Stenella* |
| **17** | *Stenella* | *Stenella* | *Stenella* | *Stenella* | *Stenella* |
| **18** | *Stenella* | *Stenella* | *Stenella* | *Stenella* | *Stenella* |
| **19** | *Stenella* | *Stenella* | *Stenella* | *Stenella* | *Stenella* |
| **20** | *Stenella* | *Stenella* | *Stenella* | *Stenella* | *Stenella* |
| **22** | *Stenella* | *Stenella* | *Stenella* | *Stenella* | *Stenella* |
| **23** | *Stenella* | *Stenella* | *Stenella* | *Stenella* | *Stenella* |
| **24** | *Stenella* | *Stenella* | *Stenella* | *Stenella* | *Stenella* |
| **26** | *Stenella* | – | *Stenella* | *Stenella* | *Stenella* |
| **27** | *Stenella* | *Stenella* | *Stenella* | *Stenella* | *Stenella* |
| **28** | *Stenella* | *Stenella* | *Stenella* | *Stenella* | *Stenella* |
| **29** | *Stenella* | *Stenella* | *Stenella* | *Stenella* | *Stenella* |
| **30** | *Stenella* | *Stenella* | *Stenella* | *Stenella* | *Stenella* |
| **32** | *Stenella* | *Stenella* | *Stenella* | *Stenella* | *Stenella* |
| **33** | *Stenella* | *Stenella* | *Stenella* | *Stenella* | *Stenella* |
| **35** | *Stenella* | *Stenella* | *Stenella* | *Stenella* | *Stenella* |
| **36** | *Stenella* | – | *Stenella* | *Stenella* | *Stenella* |
| **37** | *Stenella* | *Stenella* | *Stenella* | *Stenella* | *Stenella* |
| **41** | *Stenella* | *Stenella* | *Stenella* | *Stenella* | *Stenella* |
| **54** | *Stenella* | *Stenella* | *Stenella* | *Stenella* | *Stenella* |
| **56** | *Stenella* | *Stenella* | *Stenella* | *Stenella* | *Stenella* |
| **60** | *Stenella* | *Stenella* | *Stenella* | *Stenella* | *Stenella* |
| **42** | *Delphinus* | *Delphinus* | *Delphinus* | *Delphinus* | *Delphinus* |
| **43** | *Delphinus* | *Delphinus* | *Delphinus* | *Delphinus* | *Delphinus* |
| **44** | *Delphinus* | *Delphinus* | *Delphinus* | *Delphinus* | *Delphinus* |
| **45** | *Delphinus* | *Delphinus* | *Delphinus* | *Delphinus* | *Delphinus* |
| **48** | *Delphinus* | *Delphinus* | *Delphinus* | *Delphinus* | *Delphinus* |
| **49** | *Delphinus* | *Delphinus* | *Delphinus* | *Delphinus* | *Delphinus* |
| **58** | *Delphinus* | *Delphinus* | *Delphinus* | *Delphinus* | *Delphinus* |
| **50** | Hybrid (F2*) | – | BC with *Delphinus* or Hybrid (F1 or F2) | BC with *Delphinus* | Hybrid (F2) or BC with *Stenella* |
| **51** | Hybrid (F2*) | F2 | Hybrid (F1 or F2) or BC with *Stenella* | Hybrid (F1 or F2) | Hybrid (F2) or BC with *Stenella* |
| **52** | Hybrid (F2*) | F2 | Hybrid (F1 or F2) or BC with *Stenella* | BC with *Stenella* or Hybrid (F1 or F2) | Hybrid (F2) or BC with Stenella |
| **2** | Hybrid (F2*) | – | Hybrid (F1 or F2) or BC with *Stenella* | Hybrid (F1 or F2) | BC with *Stenella* or Hybrid (F2) |
| **7** | Hybrid (F2*) | BC with *Stenella* | BC with *Stenella* or Hybrid (F1 or F2) | BC with *Stenella* or *Stenella* | BC with *Stenella* or Hybrid (F2) |
| **9** | Hybrid (F2*) | – | BC with *Stenella* or Hybrid (F1 or F2) | Hybrid (F1 or F2) or BC with *Stenella* | BC with *Stenella* or Hybrid (F2) |
| **15** | Hybrid (F2*) | – | BC with *Stenella* or Hybrid (F1 or F2) | *Stenella* | BC with *Stenella* or Hybrid (F2) |
| **53** | Hybrid (F2*) | – | Hybrid (F1 or F2) or BC with *Stenella* | Hybrid (F1 or F2) | BC with *Stenella* or Hybrid (F2) |
| **47** | Hybrid (F2*) | – | BC with *Stenella* or Hybrid (F1 or F2) | BC with *Stenella* or Hybrid (F1 or F2) | BC with *Stenella* or Hybrid (F2) |
| **31** | – | – | BC with *Stenella* or Hybrid (F1 or F2) | BC with *Stenella* or Hybrid (F1) | BC with *Stenella* or *Stenella* |
| **34** | – | F2 | Hybrid (F1 or F2) or BC with *Stenella* | Hybrid (F1 or F2) or BC with *Stenella* | BC with *Stenella* or Hybrid (F2) |
| **21** | *Stenella*** | BC with *Stenella* | BC with *Stenella* or Hybrid (F1 or F2) | *Stenella* or BC with *Stenella* | BC with *Stenella* or Hybrid (F2) |
| **40** | – | – | BC with Stenella or Hybrid (F1 or F2) | *Stenella* | BC with *Stenella* |
| **57** | *Delphinus*** | F2 | BC with *Delphinus*or Hybrid (F1 or F2) | *Delphinus* | F2 or BC with *Delphinus* |
| **59** | – | F2 | Hybrid (F1 or F2) or BC with *Delphinus* | BC with *Delphinus* or Hybrid (F1) | Hybrid (F1 or F2) |

all analyses conducted so far, the id codes of the intermediate samples coincide, we refer to them (n = 12, samples 2, 7, 9, 15, 21, 31, 34, 40, 47, 53, 57, and 59) hereafter as genetically intermediates and treat them as a separate group (Table 1).

## 6. Hybridization

Both independent runs of NEWHYBRIDS for each prior yielded identical results (Fig. S4). Furthermore, the two priors employed in the analysis did not have a large influence on the results. However, 'Jeffreys-like' prior provided a clearer inference on the hybrid category of several individuals, probably due to the presence of many alleles at very low frequencies in both of the species as suggested by the software developers (NewHybrids documentation, http://ib.berkeley.edu/labs/slatkin/eriq/software/new_hybs_doc1_1Beta3.pdf). According to this prior the morphologically intermediate individuals were assigned with high posterior probability (> 0.9) to the F2 hybrid category while their assignment to any of the given categories was not possible when a uniform prior was employed (< 0.6). There were few more individuals that were either assigned to the F2 category or remained unassigned. Those actually correspond to the genetically intermediate individuals of previous analyses *i.e.* FCA, PCA and DAPC. A detailed description of each individual's posterior probability to each category is appended in Table 1.

When the simulated data were analyzed along with the real data in NEWHYBRIDS (using the same settings and threshold, 0.9), all or the majority of the simulated parental and F1 samples was assigned to their respective category while a small number of samples were left

unassigned (q < 0.9), [*Delphinus*-parental: all (n = 500) were assigned to their respective category, *Stenella*-parental: 480 were assigned to their respective category and 20 were left unassigned, F1: 408 were assigned to their respective category while 92 were left unassigned]. Although the majority of the simulated F2 samples (n = 278) were assigned to their respective category, a large number was left unassigned (n = 187), and few were assigned to F1 (n = 4) or to either backcross categories (20 to *Stenella*-backcross and 11 to *Delphinus*-backcross category). The majority of the simulated *Delphinus*-backcross samples (n = 298) were assigned to their respective category with the rest being left unassigned (n = 199) and only few assigned to F1 category (n = 3). Finally, the majority of the simulated *Stenella*-backcross samples (n = 307) were assigned to their respective category with the rest being left unassigned (n = 181) and only few assigned either to *Stenella*-parental category (n = 10) or to F1 category (n = 2). Regarding the real genotypes, all *D. delphis* (that were used for simulating the 500 parental samples of the species), were assigned to *Delphinus*-parental category, 28 of *S. coeruleoalba* were assigned to *Stenella*-parental category and 3 were left unassigned and of the intermediates (either morphological and genetical or solely genetical), 5 were assigned to F2, 2 were assigned to backcrosses with *S. coeruleoalba* and 8 were left unassigned (Table 1).

The distribution of membership coefficients (q-values) of the simulated groups of genotypes resulting from our second STRUCTURE analysis are plotted in Fig. 3, where the amount of overlap was assessed. Their distribution was used as empirically derived assignment criteria for the real genotypes to the six genotype categories. The distinction between F1 and F2 categories was not possible since their distributions were significantly overlapping. According to the results one morphological intermediate sample (sample id 50) was assigned to F1 or F2 hybrid category while the remaining two (sample ids 51 and 52) were assigned to F2 or backcross with *Stenella* parental population hybrid category with the latter being more probable (rhombs in Fig. 3). The remaining genetically intermediate samples, comprised of one sample (sample id 57) assigned to F2 or backcross with *Delphinus* parental population with the latter being more probable, one sample (sample id 59) assigned to F1 or F2 hybrid category, eight samples (sample ids 53, 34, 47, 9, 15, 2, 7, 21) being assigned either to F2 or backcross with *Stenella* parental population hybrid category with the latter being more probable, and two samples (sample ids 40, 31) being assigned to backcross with *Stenella* parental population hybrid category.

It is worth noting that the q-values of the last two samples were at the extreme end of the q-values distribution of backcrosses with *Stenella* parental population which slightly overlaps with the q-values distribution of *Stenella* parental population. Samples from the two-studied species were assigned to their corresponding category (*i.e.,* parental simulated categories of the two species).

According to GENECLASS2 results, individuals of the two species (excluding morphological and genetical intermediates) were assigned to their respective simulated reference populations, with high relative scores (Table S5). In more details, for the majority of *S. coeruleoalba* individuals the most likely population was the simulated *Stenella* population with scores greater than 90%. Six individuals with lower scores [sample ids: 13 (89.5%), 26 (76.9%), 33 (89.4%), 35 (66.6%), 36 (79.2%), and:37 (86%)] had a score of 9, 22, 10.4, 32.6, 16.4, and 13.5%, respectively of being a *Stenella* backcross reaching a cumulative relative score of at least 98% of having either *Stenella* population or *Stenella* backcross population as the most likely one. All *D. delphis* individuals had simulated *Delphinus* as their most likely population of assignment with a score greater than 92.8%. Regarding the totality of the intermediate samples (*i.e.,* the three morphological and the genetic intermediates), the most likely population was one of the four simulated hybrid populations. This was also true for the second and third most likely population up to a cumulative score of 99%. Only two individuals had one of their likely populations at the cumulative score of 99% being a non-hybrid population. In more details samples with ids 31 and 40 had a relative score of 11.5% and 6.4% respectively for the third most likely population of being the simulated *Stenella* population. Relative scores of each individual to the six simulated populations are depicted in Tables 1 and S5.

The results obtained with DAPC analysis using the 'real' genotypes as supplementary data, projected them onto their respective groups *i.e.,* assigned them to their respective simulated populations (Fig. 4). Individuals of *S. coeruleoalba* were assigned to the simulated parental group of *Stenella* with posterior probability (pp) of one. There were only two individuals deviating from this pattern; sample with id 13, which was assigned to the group of backcrossed hybrids with *Stenella* (pp 0.98), and sample with id 36, which was assigned to the simulated parental group of *Stenella* (pp 0.46) and to the group of backcrossed hybrids with *Stenella* (pp 0.54). All individuals of *D. delphis* were assigned to the simulated parental group of *Delphinus* with pp = 1.00. Furthermore, one morphological hybrid was assigned to the group of

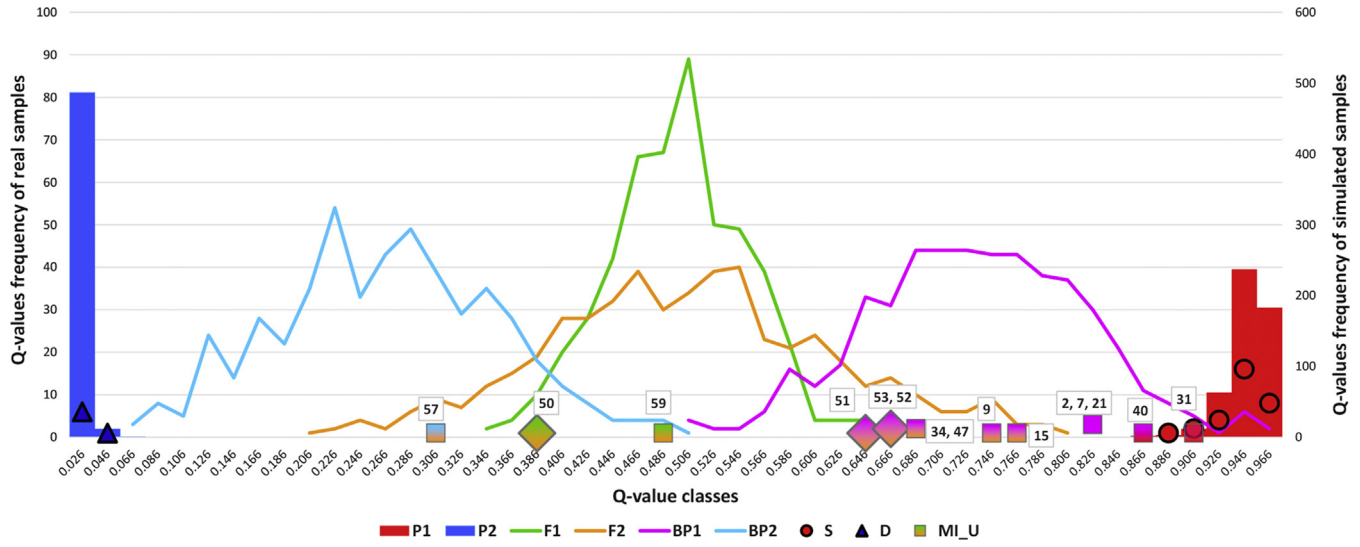


**Fig. 3.** Q-values distribution for original and simulated genotypes according to the second run of STRUCTURE. **P1:** simulated *Stenella* parental population, **P2:** simulated *Delphinus* parental population, **F1:** simulated F1 hybrids, **F2:** simulated F2 hybrids, **BP1:** simulated backcrosses with *Stenella* parental population, **BP2:** simulated backcrosses with *Delphinus* parental population, **S:** *S. coeruleoalba* samples (red circles), **D:** *D. delphis* samples (blue triangles), **MI_U:** morphologically intermediate samples (rhombs) and genetically intermediate samples (squares). Numbers indicate sample IDs for all MI_U samples.

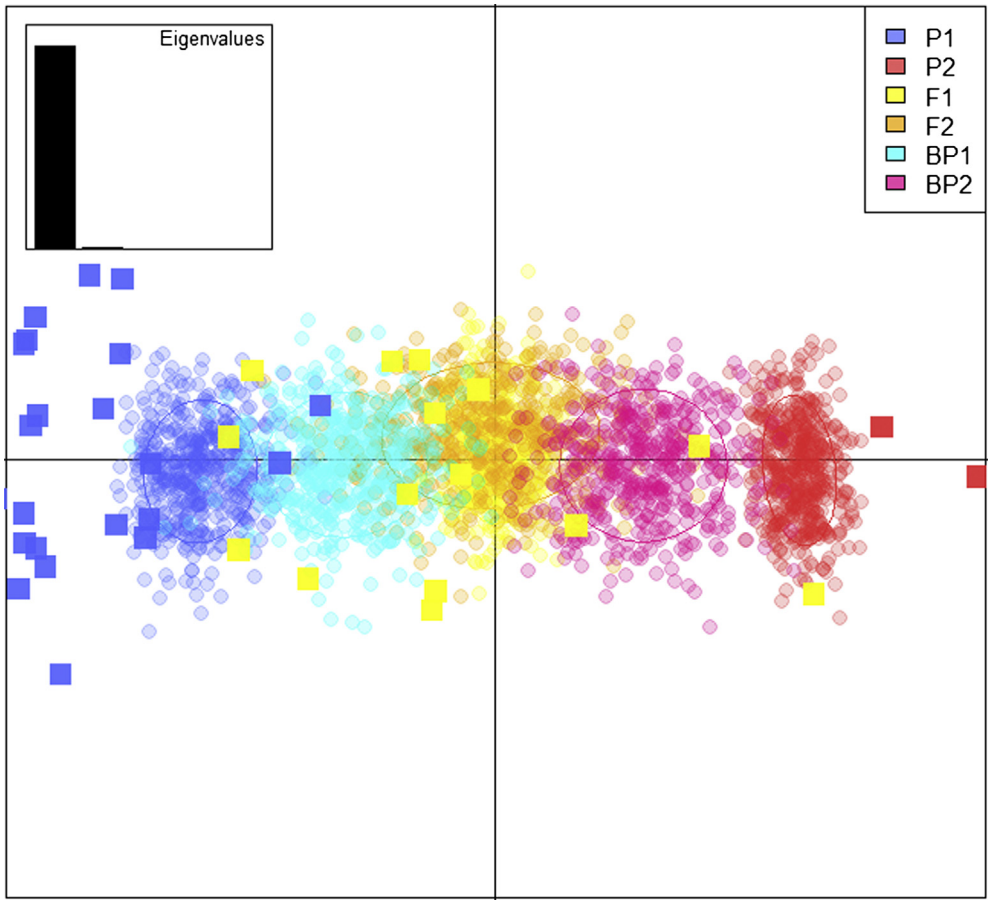


**Fig. 4.** DAPC of simulated data with real samples used as supplementary individuals. Circles and ellipses correspond to the analysis of the simulated data: **P1:** simulated *Stenella* parental population, **P2:** simulated *Delphinus* parental population, **F1:** simulated F1 hybrids, **F2:** simulated F2 hybrids, **BP1:** simulated backcrosses with *Stenella* parental population, **BP2:** simulated backcrosses with *Delphinus* parental population. Squares indicate the real data used as supplementary individuals: **Blue:** *S. coeruleoalba*, **Red:** *D. delphis*, **Yellow:** morphologically and genetically intermediate individuals. [Inset represents the eigenvalues of the three first discriminant functions (DAs).] (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

backcrossed hybrids with *Delphinus* (sample id 50, pp 0.93) while the remaining two (*i.e.* samples 51 and 52) had their posterior probabilities spread over three hybrid groups, *i.e.,* F1, F2, and backcrosses with *Stenella* (Table 1). Finally, the genetically intermediate individuals were assigned either to the parental group of *Stenella* (sample ids 15, 21, 40), or *Delphinus* (sample id 57) or had their posterior probabilities spread over two (sample id 7 *Stenella* parental pp 0.30, backcross with *Stenella* pp 0.70) or three groups (sample id 59 F1 pp 0.26, F2 pp 0.09, and backcross with *Delphinus* pp 0.66). The occurrence of individuals of different hybrid categories (*i.e.,* F1, F2 and backcrosses with either parental species) indicates that there is no reproductive isolation between hybrids and their parental species.

### 6.1. Sex determination

From the total number of specimens (n = 60), 54 provided positive results in sex determination. For six specimens [one *D. delphis* (sample id: 42) and five *S. coeruleoalba* (sample ids: 1, 2, 6, 25, and 60)], it was impossible to amplify either cyt *b* or cyt *b* and SRY and their sex could not be determined genetically due to their poor DNA quality. Among the 54 specimens, 23 were females (five *D. delphis,* seventeen *S. coeruleoalba*, and one intermediate morph) and 23 were males (six *D. delphis* and seventeen *S. coeruleoalba*) (Table S1). For eight specimens, the results of amplification were not clear and their sex remained genetically ambiguous. Five of them looked alike females (sample ids: 7, 16, 53, 50, 52) and three looked alike males (sample ids: 9, 31, and 36) (Table S1). For three of these animals (sample ids: 16, 50, and 52) there was no prior morphological information. For the specimens with sample ids 9 and 31 the morphology supports the unclear indication of genetic data, while for the rest three (sample ids: 7, 36, and 53) the morphology supports the opposite sex (Table S1). Wherever both morphological examination and unambiguous genetic analysis for gender determination were possible, no disagreements were observed between the two methods.

## 7. Discussion

In Cetaceans, both wild and captive, hybridization is a phenomenon that has often been documented in many species (Crossman et al., 2016), indicative of the incompleteness of pre- and post-mating barriers to interbreeding, that raises questions on the maintenance of species integrity in the face of interspecific (and often intergeneric) gene flow. This was also evident in the present study since the occurrence of individuals belonging to different hybrid categories (*i.e.,* F1, F2 and backcrosses with either parental species) suggests no reproductive isolation between hybrids and their parental species. Hybridization is more frequently observed between species pairs that share a greater number of behavioral and morphological traits (e.g. vocalization frequency and body size) than pairs that share less (Crossman et al., 2016). Therefore, in species on sympatry, the divergent selection on those features is deemed a key player in reducing the hybridization and preventing the fall down of parental species, while the high degree of shared traits renders the recognition of hybrids in the wild extremely difficult.

In our study, the discrimination of both species was possible with the use of the two types of molecular markers employed in this study (*i.e.,* mtDNA and microsatellites). Although there are indications of within species population subdivision (e.g., deviations from HW equilibrium), the number of analyzed samples is not adequate to indicate the patterns of population structure for the two species in the Greek Seas.

A geographic isolation of *S. coeruleoalba* and *D. delphis* in the GOC has been proposed due to the facts that GOC is open for cetacean exchange only to the west [cetacean intrusions from the east (Saronic

Gulf) are virtually non-existent (Frantzis and Herzing, 2002)], and both species are absent at the western quarter of the Gulf and the adjacent Gulf of Patras (Bearzi et al., 2011; Frantzis, 2009; Frantzis et al., 2003) (Fig. 1). This is also verified in a more recent study (Bearzi et al., 2016) and it is presumed to have led to the genetic differentiation of the populations of dolphins inhabiting GOC (Bearzi et al., 2016; Moura et al., 2013a). The results of this study cannot support such a genetic differentiation, but this can be due to the small sample size and/or to the possibility of relatively recent isolation for *S. coeruleoalba* and even more recent for *D. delphis* in the GOC. Nevertheless, the two dolphin species display common characteristics in the GOC in respect to their behavior and ecology, although they are known to have different ecological and dietary needs in other seas (Aguilar, 2000; Bearzi, 2003). In more details, *D. delphis* that inhabit the GOC have adapted their behavior, habitat preference and likely their dietary needs (the two species forage together in pelagic waters) to coexist with *S. coeruleoalba* when compared to those inhabiting the neighboring coastal and shallow areas of the Ionian Sea (Bearzi et al., 2005).

Despite the fact that at high levels of genetic differentiation, as the interspecific differentiation in our case, a high number of loci is required for accurate hybrid identification [*i.e.*, 48–50 loci in NewHybrids and STRUCTURE, (Fitzpatrick, 2012; Vaha and Primmer, 2006)], and that STRUCTURE can over-estimate the amount of admixture resulting in the misclassification of nonhybrid individuals as hybrid (Bohling et al., 2013), our conclusions are based on multilateral analysis approaches both model and non model-based, providing robust evidence. In addition, morphological evidence (individuals of intermediate morph) was consistent with the results of all hybridization analysis methods, placing them in-between the two species, and certifying their hybrid origin.

All individuals with intermediate morphological characteristics of the two species (observed only in the GOC) were of admixed ancestry in respect to their nuclear DNA as indicated by all types of hybridization analyses, having a mtDNA of *S. coeruleoalba*. Furthermore, there were few more individuals, sampled from the majority of the studied areas, that were found to have admixed ancestries, *i.e.*, they were characterized as hybrids (NewHybrids, STRUCTURE second run, GeneClass and DAPC with supplementary individuals) or could not be assigned with high posterior probabilities to any of the categories (parental or hybrids) in NewHybrids. Their position in all ordination analyses was at the space in between the two species with slight exceptions where samples were positioned at a close proximity to one of the two species. It is worth noticing that according to the first STRUCTURE run, those individuals (morphological intermediate and genetic hybrids) were either assigned to the second *S. coeruleoalba* cluster or were left unassigned ($q < 0.9$) with only exception that of morphologically intermediate sample 50. The geographic distribution of those potential hybrids is not restricted. On the contrary, they are found in most studied sites. This is a result that we cannot easily explain, considering the current apparent geographic isolation of both species of dolphins inhabiting the GOC and the lack of observations of mixed-species groups between *S. coeruleoalba* and *D. delphis* in the remaining sampled areas of the Greek Seas (Frantzis, 2009). It might imply that either hybridization took place in a much larger scale in the past or hybrids from the GOC had the possibility to migrate out of the GOC. All of these are highly speculative hypotheses and definitive answers cannot be provided at this stage. A much larger sample size than what has actually being acquired and information from a greater number of molecular markers might deemed necessary in order to shed more light on this issue in future studies. According to STRUCTURE's second run analysis most individuals that are characterized as putative hybrids are assigned to backcrosses with *S. coeruleoalba* hybrid category. This legitimates the assumption that those probably constitute individuals that rose after many generations of backcrossing that in turn renders their discrimination from pure parental individuals rather difficult even if a great number of diagnostic markers was employed (Boecklen and Howard, 1997). Therefore, there is a high probability that individual assignment to either pure parental or backcrosses could have been the other way around. However, according to the analysis of simulated and real genotypes in NEWHYBRIDS (2nd run), there were only simulated backcrosses with *Stenella* that were misassigned to the *Stenella*-parental simulated samples and not the other way around.

The results of the present study, in combination to the fact that morphological hybrids carry *S. coeruleoalba* mtDNA corroborate to a hypothesis of introgressive hybridization between the two species in the GOC, where males of *D. delphis* mate and produce fertile hybrids with females of *S. coeruleoalba*. This is further supported by the fact that *S. coeruleoalba* are amongst the more abundant cetaceans in the Mediterranean Sea (Aguilar, 2000), including the waters of Greece (Bearzi et al., 2016; Frantzis et al., 2003) and appear either in single or mixed species groups in the GOC (Bearzi et al., 2016; Frantzis and Herzing, 2002). There were two specimens (sample ids 3 and 31) that have been morphologically assigned to *S. coeruleoalba*, but they contained mtDNA of *D. delphis*. This result combined with the fact that the microsatellite genotypes have shown that both specimens cluster with *Stenella* and backcross to *Stenella*, respectively, might indicate that introgressive hybridization is bidirectional. Furthermore, both species have experienced dramatic population declines during the last half century, that possibly still go on, with *D. delphis* being affected the most in the Mediterranean (Reeves and Notarbartolo di Sciara, 2006).

The once abundant Mediterranean populations of *D. delphis* have been dramatically declining since the 1960s with a reduction of more than 50% in population size over a three-generations period (*i.e.,* the past 30–45 years) leading to their current endangered status (Bearzi et al., 2003). The species progressively disappeared from the Adriatic, Balearic and Ligurian Seas and Provencal Basin and significantly declined in the eastern Ionian Sea (Bearzi et al., 2003; Reeves and Notarbartolo di Sciara, 2006), while it is relatively abundant only in the westernmost portion of the basin *i.e.,* in the Alboran Sea. On the other hand, the Mediterranean subpopulation of *S. coeruleoalba* falls into the Vulnerable category of IUCN Red List of Threatened Species, primarily because of past mortality events (Aguilar and Gaspari, 2012) that led to a 30% reduction in the last three generations (Reeves and Notarbartolo di Sciara, 2006).

Observations made by Frantzis and Herzing (2002) suggest that when numbers of Mediterranean *D. delphis* decline, the animals tend to associate with *S. coeruleoalba* fact attributed to their tendency of staying in large groups. This obviously affects their interspecific hybridization potential. The mating-like behavior displayed by cetaceans, has been hypothesized as being a form of social play (Brown and Norris, 1956; Herzing and Johnson, 1997), also observed in other mammalian taxa such as primates, that could be solely for "entertainment" or for the establishment of dominance hierarchy between individuals (Vasey, 1995). Another explanation ascribes a learning role to these ongoing mating attempts that enhances the probability of successful matings, especially for males (Mann, 2006). Males that practice successful mating, even with females of another species, might have a greater reproductive success during the breeding season in respect to males that lack such an experience and thus a greater probability and number of produced offspring in that season. This is also indirectly supported by observations where males are frequently seen mating with animals of different age/sex classes (Herzing, 1997; Mann, 2006) even when females are not in estrous (Shane et al., 1986).

Mixed species groups aggregations while preserving all benefits and consequences of single species groups they also introduce the potential of interspecific hybridization. This does not come at no cost given the risks of hybridization for the fitness of hybrid offspring and their parents, leading in some cases, to limitations in the distribution or persistence of the hybridizing species. This is quite common in the absence or low abundance of potential conspecific mates, as in the case of rare or depleted species (Crossman et al., 2016). This effect becomes even more pronounced in cases where hybrids fitness is either not negatively

affected or is greater than in parental species allowing for backcrosses that may outcompete individuals of a parental species (Crossman et al., 2016; Mallet, 2007). Currently, *D. delphis* in the GOC constitutes a geographically distinct conservation unit, with probably limited (if any) demographic and genetic exchanges with other populations. Their small population size (22 animals estimated during the 2011–2015 period; Bearzi et al., 2016), limited distribution, as well as hybridization (present study) with a 60-fold larger subpopulation of striped dolphins (Bearzi et al., 2016), renders their extinction as highly probable. Even in the case that hybrids are not better fit in comparison to the parental species, again the low abundance of *D. delphis* species could lead to backcrosses with only or primarily *S. coeruleoalba*. This can jeopardize the genetic integrity of the GOC populations of the species and if hybridization concerns all areas where hybrids have been detected, the existence of the species in the Greek Seas. Present data do not provide strong indications on whether this is a case of hybrid speciation or not.

In populations that experience large-scale declines, hybridization and introgression might occur when a rare species interbreeds with a common species due to the scarcity of conspecific mates [e.g., Dall's porpoises (*Phocoenoides dalli*) and harbor porpoises (*Phocoena phocoena*): (Willis et al., 2004), *Arctocephalus* spp.: (Lancaster et al., 2006), Grevy's zebra (*Equus grevyi*) and plains zebra (*Equus burchelli*): (Cordingley et al., 2009), European mink and polecat: (Cabria et al., 2011), giant sable and roan antelopes: (Pinto et al., 2016)] often referred as the Hubb's principle or "desperation hypothesis" (Hubbs, 1985). This is of high biodiversity conservation concern rendering the early detection of hybrids when managing small populations extremely necessary.

Another consideration on the occurrence of interspecific hybridization might be the reproductive impairment induced by organochlorine pollutants and/or polychlorinated biphenyls (PCBs) both of which have been found at high levels in Mediterranean striped dolphins and presumably in other Mediterranean dolphin species, that could be held responsible for alterations in the reproductive strategy of both species (Reeves and Notarbartolo di Sciara, 2006).

## 8. Conclusions

Naturally occurring hybridization in *S. coeruleoalba* had only been observed with its congeneric species so far *i.e., S. longirostris* × *S. coeruleoalba* giving rise to *S. clymene* (Amaral et al., 2014). In this study, we verify the occurrence of natural introgressive hybridization between two species of different genera with hybrids being fertile and able to reproduce not only with the other hybrids but also with each of the two-parental species (*i.e.* occurrence of not only F1 hybrids between the two species but also F2 and backcrosses). Current evidence does not allow firm conclusions whether hybridization might constitute a step towards the generation of a new species and/or the swan song of the isolated population of *D. delphis* in the GOC.

The case described in this study fulfills the requirements for hybridization to occur where heterospecific mates are genetically and physiologically compatible, the two species have been observed to be behaviorally predisposed to mate and form mixed species assemblages [*i.e.*, in mixed-species sightings (Bearzi et al., 2016; Frantzis and Herzing, 2002)] in an area where natural range overlap occurs for *S. coeruleoalba* and *D. delphis*, and is further confirmed by the presence of individuals of intermediate morphology and genome. The hybrid classes detected in the present study indicate that hybrids of the two species are viable and are able to reproduce, with their offspring often being the result of backcrosses to one of the parental species which is also the most abundant *S. coeruleoalba*. This legitimates the assumption that there is no reproductive isolation between hybrids and their parental species. Furthermore, it is safe to conclude that both sexes are fertile since hybrids contain individuals of both sexes.

This study adds to an ever-increasing amount of recent research that indicates hybridization as a common and important part of animal evolution. This in turn urges the evaluation of the status of *D. delphis* in the Mediterranean in a comprehensive manner that will require the estimation of their distribution and abundance throughout the basin, identifying critical habitats and characterizing threats as proposed by the Agreement on the Conservation of Cetaceans of the Black Sea, Mediterranean Sea and Contiguous Atlantic Areas with a priority in the eastern Mediterranean (ACCOBAMS, 2002). Furthermore, those studies will benefit from the association of the observed patterns with food availability (like in the study of Giannoulaki et al., 2016), and especially the observed patterns of genetic diversity in space and time.

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2018.09.007.

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