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Genetic isolation and morphological divergence of Black Sea bottlenose dolphins

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ABSTRACT

The Black Sea is a semi-enclosed body of water that differs from the adjacent Mediterranean Sea in terms of its biodiversity, oceanographical and ecological characteristics. There is growing international concern about pollution in the Black Sea and other anthropogenic threats to its fauna. The bottlenose dolphin (*Tursiops truncatus*) is one of three species of cetaceans living in the Azov-Black Sea basin. Despite considerable research on bottlenose dolphins elsewhere, the extent of human impacts on the Black Sea populations is unknown. Previous attempts to award special conservation status to Black Sea cetaceans have failed specifically because policy makers have viewed their ecological and evolutionary uniqueness as equivocal. This study assessed divergence between Black Sea, Mediterranean Sea and Atlantic Ocean bottlenose dolphins for 26 cranial measurements ($n = 75$ adult bottlenose dolphin skulls) and mitochondrial DNA ($n = 99$ individuals). Black Sea bottlenose dolphins are smaller than those in the Mediterranean, and possess a uniquely shaped skull. As in a previous study, we found the Black Sea population to be genetically distinct, with relatively low levels of mtDNA diversity. Population genetic models suggest that Black Sea bottlenose dolphins have so little gene flow with the Mediterranean due to historical isolation that they should be managed separately.

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1. Introduction

Numerous environmental organizations and international protection agencies have expressed concern about increasing pollution and diminishing fish stocks in the Black Sea, and consequential impacts on its faunal assemblage. The Black Sea is a naturally isolated body of water in which three cetacean species are found: the common dolphin *Delphinus delphis ponticus*, the bottlenose dolphin *Tursiops truncatus ponticus*, and the harbour porpoise *Phocoena phocoena relicta*. The full

extent of human impacts on these populations, whether or not they are biologically unique, and their degree of isolation are all currently unknown. However, it is clear that overfishing and declining water quality (e.g., increased nutrient loading) have significantly reduced stocks of anchovy (*Eugraulis encrasicolus*), horse mackerel (*Trachurus mediterraneus*), sprat (*Sprattus sprattus*), and other fish that sustain Black Sea cetaceans (Kideys, 1994; Tuncer et al., 1998). Until the 1980s, dolphin fisheries in the Black Sea killed hundreds of thousands of cetaceans during peak years (e.g., Danilevsky and

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Tuyutyunnikov, 1968; Kleinenberg, 1978; Smith, 1982). Anthropogenic impacts since that time have continued to diminish population sizes (Birkun et al., 1992), including the capture of live Black Sea bottlenose dolphins for aquatic parks.

International conservation organizations have expressed their concerns for the health and status of Black Sea bottlenose dolphins, which are widely recognized as having the smallest population size of the three Black Sea cetaceans (e.g., Tomilin, 1967; Perrin, 1984; Yukhov et al., 1986; Buckland et al., 1992; Birkun, 2002; IWC, 2003; Simmonds, 2003). The Black Sea bottlenose dolphin has been listed as Data Deficient by IUCN since 1996, and a zero annual export quota was established by CITES for live specimens in 2002. The IWC Scientific Committee reviewed the status of these dolphins in 2003 and suggested that Black Sea bottlenose dolphins should be managed as a distinct entity for conservation purposes (IWC, 2003). Nonetheless, protection plans have not yet been implemented due to a lack of quantitative studies on this population. The Agreement on the Conservation of Cetaceans of the Black Sea, Mediterranean Sea and Contiguous Atlantic Area (ACCOBAMS), whose aim is to “reduce threat to cetaceans in Mediterranean and Black Sea waters”, has stated that the bottlenose dolphin is “at greater risk of declining and disappearing from the Agreement area” than other cetacean species (Notarbartolo di Sciara and Birkun, 2002), and that an absence of scientific studies on this population impedes the credibility of its proposed conservation measure (ACCOBAMS, 2004, Resolution 2.19). Hence, international agreements that could protect the Black Sea bottlenose dolphin first require demonstration that it is isolated from the adjacent Mediterranean, and that it is evolutionarily unique (CITES Decision 11.91, IUCN, 2000; IWC, 2003; Simmonds, 2003; Reeves et al., 2004).

Bottlenose dolphin morphology has not been quantitatively compared between the Black Sea and adjacent Mediterranean. However, Barabash-Nikiforov (1940, 1960) compared Black Sea specimens to published data from distant populations, leading him to suggest a separate Black Sea subspecies: *Tursiops truncatus ponticus*. Because Kleinenberg (1978) had opposing conclusions, the subspecies designation has not been widely recognized. Recent genetic studies that included 16 Black Sea bottlenose dolphins found relatively low mtDNA gene diversity in the Black Sea, and supported the hypothesis that Black Sea populations are isolated from the Mediterranean (Natoli et al., 2003, 2005). These results were particularly strong for microsatellite data. Tests of mtDNA differentiation between the Black Sea and adjacent areas of the Mediterranean Sea were not statistically significant using F_{st} , although coalescent modeling estimated low levels of gene flow between these populations, and therefore significant divergence. The goals of our study were to jointly analyze morphological and genetic data with sufficiently large sample sizes to test the hypothesis that Black Sea bottlenose dolphins have diverged from the Mediterranean, and to further determine whether this divergence is attributable to historical evolutionary processes or more recent anthropogenic impacts. The results of our study are intended to help clarify the taxonomic status of this population, and facilitate the implementation of conservation plans by agencies such as ACCOBAMS.

2. Materials and methods

Genetic material and morphological measurements were collected in cooperation with representatives from nine countries (see Acknowledgements).

2.1. Individual genotyping

Tissue samples were collected between 1993 and 2003 from 99 bottlenose dolphins from three basins (Fig. 1): the eastern Atlantic ($n = 25$ from the French and Portugal coasts), the Mediterranean Sea ($n = 31$ from the Liguria/Tyrrhenian Sea, Adriatic Sea, Aegean Sea and Israeli coast) and the Black Sea ($n = 43$). It is likely that very few of these samples represent familial groups, since they were obtained by different agencies through a variety of means (stranded, bycaught, captive) at different times (see Appendix A). Tissue was preserved in 20% dimethyl sulphoxide saturated with sodium chloride (DMSO) at -20°C . DNA was isolated using DNEasy extraction kits (Qiagen). The first 442 base pairs of the mitochondrial DNA (mtDNA) control region were amplified using polymerase chain reaction (PCR) with primers DLTurs-r (5'-CCT GAA GTA AGA ACC AGA TGT CTT ATA AA-3') and DLTurs-f (5'-CCA TTC CTC CTA AGA CTC AAG GAA G-3') developed in our laboratory. One μl of genomic DNA extraction was added to a 50 μl reaction mixture containing 5 μl 10 \times Ex Taq buffer (20 mM MgCl₂), 2 μl 10 μM of each primer, 5 μl 8 mM dNTPs and 0.25 μl Ex Taq DNA polymerase (TaKaRa). The PCR thermal profile was 2 min at 94°C , 35 cycles of {30 s at 94°C , 30 s at 60°C , 60 s at 72°C }, and a final 7 min at 72°C . PCR products were purified using GeneClean Turbo kits (Q.BIOgene), cycle sequenced using BigDye v.3.1 (ABI), and sequenced on an ABI 377 sequencer. We aligned sequences by eye using Sequencher 3.1 (Gene Codes Corp.).

2.2. Population genetic analysis

Using Arlequin v.2.0 (Schneider et al., 2001), genetic diversity was summarized as the proportion of polymorphic sites (S), haplotype diversity (equivalent to expected heterozygosity, adjusted for population size), and nucleotide diversity (π ; Nei, 1987). Preliminary analyses showed that the genealogical distances among haplotypes were at least somewhat related to their geographic distribution. Therefore, analysis of molecular variance (AMOVA) was used to estimate the degree of subdivision among the seven sites in Fig. 1 as Φ_{st} , using uncorrected distances among haplotypes (Excoffier et al., 1992). Other mutation models for Φ_{st} produced qualitatively similar results. Contingency tests of haplotype distributions (also referred to as “exact tests”) were also performed in Arlequin. (Waples and Gaggiotti (2006) found contingency tests to be a relatively powerful method for identifying gene pool boundaries.) Adjacent populations for which subdivision was not significant with either method were pooled and subsequently reanalyzed (see Section 3). The five populations inferred from these analyses consisted of Atlantic (ATL), western Mediterranean (WM), northeastern Mediterranean (NEM), southeastern Mediterranean (SEM) and Black Sea (BS) (Fig. 1).

Genetic patterns of isolation by distance (IBD) were visualized by plotting genetic similarity as a function of geographic

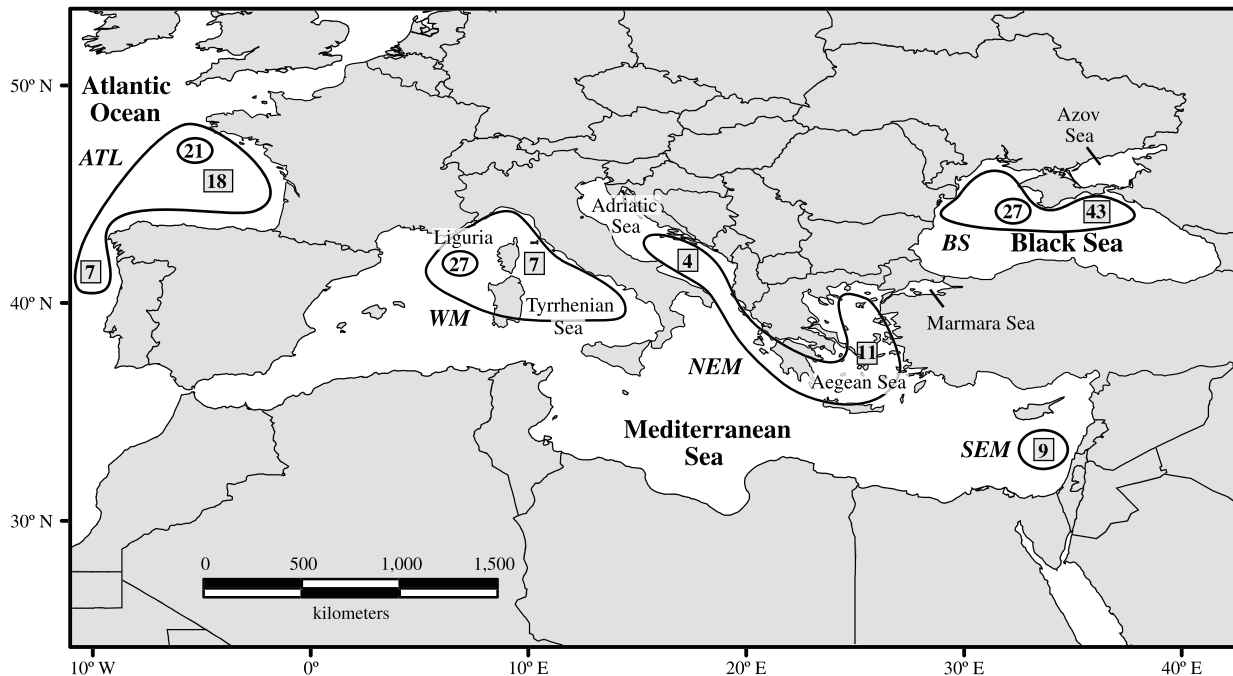


Fig. 1 – Sample sizes across the primary study area. Circled numbers represent the number of skulls measured from each basin. Shaded numbers in squares indicate the sample size at each site for genetic analyses. The large circles represent five designated populations: Atlantic (ATL), western Mediterranean (WM), northeastern Mediterranean (NEM), southeastern Mediterranean (SEM) and Black Sea (BS).

distance for each population pair. Geographic distances (in km) were calculated as Euclidean distances between the geographic centers of all individuals sampled in each population. A variety of genetic similarity or distance metrics have been used for IBD analyses, with one or both axes of the IBD scatterplot log-transformed in some cases. We chose the genetic similarity measure $\text{Log } \hat{M} = (1 - F_{st})/4F_{st}$ as recommended by Slatkin (1993), and calculated \hat{M} from both F_{st} and ϕ_{st} for comparison. Preliminary analyses showed that the IBD relationship was most linear and the least biased by outlying points when the variables were not log-transformed. Mantel tests for statistical significance of the IBD relationship were performed using the program “IBD” (Bohonak, 2002).

2.3. Phylogenetic analyses of haplotypes

Because the haplotypes were closely related and recently diverged, we estimated their evolutionary relationships using network parsimony reconstruction, implemented in TCS 1.13 (Clement et al., 2000). Network methods tend to perform better than traditional phylogenetic methods when few mutational steps separate recently diverged alleles, and ancestral alleles still persist in the species (reviewed by Posada and Crandall, 2001). To improve phylogenetic accuracy, we included 10 bottlenose dolphins from the Pacific Ocean (Curry, 1997) in the phylogenetic reconstruction, although these were not used in the population genetic analyses described above. Nested sets of relationships (i.e., nested clades) were inferred based on established rules (e.g. Templeton et al., 1987). Geographic associations were assessed for each clade using Geodis 2.5 (Templeton, 1998; Posada et al., 2000; Posada and Templeton, 2005). This approach calculates several geo-

graphic association metrics for each clade based on sampling locations for individual dolphins, and the null hypothesis of no geographic association was tested with 100,000 permutations in each case. For clades with statistical significance, evolutionary processes were inferred using the 2005 version of the GeoDis inference key, which suggests likely scenarios regarding gene flow and/or population history. This entire approach, known as nested clade phylogenetic analysis (NCPA) is summarized by Templeton (1998, 2001). The final inference key was the focus of criticisms by Knowles and Maddison (2002), which were defended by Templeton, 2004, 2005, 2007. A more recent critique by Panchal and Beaumont (2007) has suggested a higher rate of false positive inferences than previously suggested. In light of this ongoing debate, we consider the NCPA inferences to be working hypotheses, testable and better interpreted using the other frequency- and coalescent-based analyses presented here.

2.4. Estimation of gene flow, effective population sizes and coalescent time

The population genetic and phylogenetic analyses suggested historical separation between the Black Sea and the Mediterranean Sea. To estimate both historical and contemporary connectivity between these two areas, we used the “isolation with migration” (IM) model of Hey and Nielsen (2004). This coalescent-based approach estimates the following parameters: (1) the standard population genetic parameter θ_A for the ancestral population ($\theta = 4N_e\mu$, N_e = effective population size, μ = mutation rate); (2) the parameter θ for each of two populations that evolved from the ancestral population (in our analyses, θ_1 : Black Sea; θ_2 : Mediterranean Sea); (3) s = the

fraction of the ancestral population that founded the Black Sea; (4) migration rates between populations, m_1 (from the Mediterranean to the Black Sea) and m_2 (vice versa), scaled by mutation rate. These parameters can be converted to the average number of migrants per generation M_1 and M_2 . (5) The time t since the populations diverged, scaled by mutation rate. We analyzed multiple runs of the model to assure parameter convergence, and examined parameter autocorrelations during each run. Models settings were: >2,000,000 cycles with a burn-in of 200,000, upper and lower range of the population splitting parameter (s) of 0.1 and 0.5, inheritance scalar = 0.25 and HKY mutation model. The range of known mutation rates for marine mammals (0.5–7% per My, Hoelzel et al., 1991; Harlin et al., 2003) and a generation time of 22.4 years (Taylor et al., 2007) were used to calculate effective population sizes and divergence time. While this analysis can be run with one marker, results would be more robust based on multiloci (Hey and Nielsen, 2004).

2.5. Morphological measurements

We sampled 75 physically mature *T. truncatus* adults from the Black Sea (22 females, 5 males from fisheries bycatch), western Mediterranean (9 females, 9 males, 9 unknown) and eastern Atlantic (12 females, 6 males, 4 unknown) for body size and 26 cranial measurements as illustrated in Perrin (1975) (condylobasal length, length of rostrum, rostrum width at base, rostrum width at quarter-length, rostrum width at half-length, rostrum width at 3-quarter-length, width of premaxillaries at half-length, tip of rostrum to external nares, tip of rostrum to internal nares, preorbital width, postorbital width, zygomatic width, width of external nares, maximum width across premaxillaries, parietal width, braincase height, braincase length, length of temporal fossa, height of temporal fossa, length of orbit, length of antorbital process, width of internal nares, length of upper toothrow, length of lower toothrow, length of ramus, height of ramus; see Appendix B for a list of measured specimens). These individuals differed from those used for genetic analyses because teeth were not present on some museum specimens, and permission to sample teeth was not granted for others. Specimens for the morphological analyses could not be located from the northeastern Mediterranean or the southeastern Mediterranean. Measurements were taken on the right side of each skull to avoid redundancy and possible bias due to skull asymmetry (Ness, 1967; Arvy, 1977; Perrin et al., 1994). Physical maturity was judged from the fusion of vertebral epiphyses to the centra, and distal fusion of the maxillae and premaxillae (Perrin and Heyning, 1993; Heyning and Perrin, 1994).

Missing measurements (4.4% of the total data set) were estimated using the expectation maximization (EM) method (Dempster et al., 1977; Strauss et al., 2003) because specimens with missing data are not permissible in PCA and DFA analyses. Missing data are commonly estimated in multivariate morphometric studies with damaged specimens, such as this study (e.g., Rising, 2001; Strauss and Atanassov, 2006), and preliminary analyses with vs. without estimated values showed comparable levels of differentiation among populations. A multivariate analysis of variance (MANOVA) was used to test for morphological divergence among regions and be-

tween sexes. Significant results were interpreted with Scheffe post hoc tests. Males and females were pooled together for populations with no sexual dimorphism, and the MANOVA was rerun with the 26 cranial variables. One-way ANOVAs tested for differences among localities in total body length and skull length/body length. A principal component analysis (PCA) was used to standardize the variables; the PCA was performed on direct measurements using the correlation matrix (no factor rotation, extraction parameter: minimum eigenvalue = 0.8). The number of significant multivariate components (PCs) was chosen using the broken-stick method (Frontier, 1976; Jackson, 1993). A multivariate analysis of variance (MANOVA) was used to test whether each component differed among the three localities (Black Sea, Mediterranean Sea and eastern Atlantic). Two discriminant function analyses (DFA) were carried out to identify variables that best discriminate the three regions: a typical DFA based on direct measurements, and a size-free canonical discriminant analysis (Reis et al., 1990). Because DFA adjusts for covariance within groups, each analysis was performed on the covariance matrix of the input data (measurements or residuals) with a tolerance limit for the matrix inversion of 0.001 and forward stepwise variable selection (probability of 0.25 for variable extraction). To find the optimum number of principal components and variables that robustly discriminate populations, additional exploratory PCA and DFA analysis were performed using different types of factor rotation and extraction parameters using SYSTAT, v.10.2 (Systat Software Inc., 2002).

3. Results

3.1. Population genetic structure

Twenty-six unique mitochondrial haplotypes were found in 99 individuals, and six of these were shared among regions (Table 2, Fig. 2). Twenty-six of 442 sites were polymorphic, with two insertions/deletions, 21 transitions, and three sites with transitions and transversions. Haplotype diversity and nucleotide diversity were similar in the Atlantic, Liguria/Tyrrhenian and Adriatic populations ($H_e > 0.84$, $\pi \geq 0.01$) but lower in the Black Sea ($H_e = 0.58$, $\pi = 0.003$), the Israeli ($H_e = 0.42$, $\pi = 0.002$) and the Aegean populations ($H_e = 0.65$, $\pi = 0.016$). Only six haplotypes were found in 43 Black Sea individuals, and only three of these were restricted to the Black Sea.

Based on preliminary analyses, we pooled two sites in the eastern Atlantic (AMOVA, d.f. = 98, $p > 0.05$; exact test $p = 0.27$), and we pooled the Adriatic and Aegean sites into a “northeastern Mediterranean” population (AMOVA, d.f. = 98, $p > 0.05$; exact test $p = 0.49$). The Liguria/Tyrrhenian and the Israeli sites were renamed western Mediterranean (WM) and southeastern Mediterranean (SEM) populations respectively. Differentiation among the five populations defined above (also see Fig. 1) was highly significant and similar using different model of evolution (AMOVA, $\Phi_{st} = 0.48$, d.f. = 98, overall $p < 0.001$; see Table 1). Exact tests of haplotype distributions clearly showed that Black Sea bottlenose dolphins differ genetically from the Mediterranean and Atlantic (Table 1).

Scatterplots of genetic similarity vs. geographical distance was not consistent with a pattern of isolation by distance

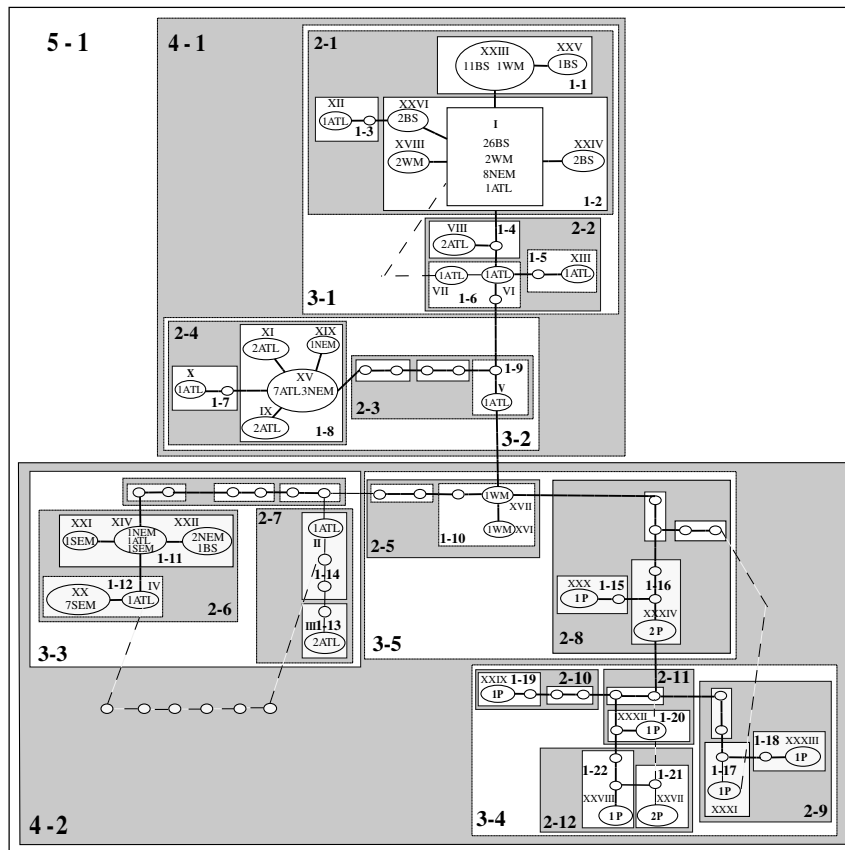


Fig. 2 – Haplotype network for mitochondrial DNA. Population codes are as in Fig. 1, with the addition of samples from the eastern Pacific (P). Oval size is proportional to the number of individuals, which precedes the population code. Each line represents one mutational step, and empty ovals are unsampled haplotypes. Four levels of nesting are enclosed in successively larger boxes. Three ambiguous loops (unresolved portions of the network) were resolved according to the criteria of Crandall et al. (1994) and Templeton et al. (1995).

Table 1 – Genetic differentiation estimated with Φ_{st} (above the diagonal) and exact tests (p values below the diagonal)

Locality	N	ATL	WM	NEM	SEM	BS	Pacific Ocean
ATL	25		0.254**	0.059	0.509**	0.521**	0.363**
WM	7	0.015 ± 0.006		0.084	0.809**	0.191*	0.468**
NEM	15	0.006 ± 0.01	0.029 ± 0.005		0.535**	0.324**	0.372**
SEM	9	<0.001	0.001 ± 0.001	<0.0001		0.895**	0.771**
BS	43	<0.0001	0.003 ± 0.002	<0.0001	<0.0001		0.745**
Pacific Ocean	10	<0.001	0.007 ± 0.002	<0.0001	0.001 ± 0.0007	<0.0001	

All exact test contrasts are significant at $p < 0.05$, and those ≤ 0.003 are significant after Bonferroni correction for 15 tests. * $\Phi_{st} > 0$ ($p < 0.05$), ** $\Phi_{st} > 0$ ($p \leq 0.003$). Populations are: Atlantic (ATL), western Mediterranean (WM), northeastern Mediterranean (NEM), southeastern Mediterranean (SEM) and Black Sea (BS). Sample sizes for each population are reported in the second column (N).

(Mantel tests, $p > 0.05$ for \hat{M} derived from F_{st} and for \hat{M} derived from Φ_{st} ; see Fig. 3). This was due primarily to the Israeli dolphins, which were more similar to those from the Atlantic than to the closest population in the Aegean Sea. Excluding the Israeli samples in the western Mediterranean, the IBD plot showed a significant negative slope for \hat{M} derived from F_{st} (Mantel test, $p < 0.001$; Fig. 3) but not for \hat{M} derived from Φ_{st} ($p > 0.05$). However, the western Mediterranean samples were clear outliers in both analyses. These results suggest that genetic differentiation of Black Sea bottlenose dolphins is due to their geographic isolation, although more detailed conclusions regarding the Israeli population and the exact

location of geographic barriers require additional fine-scale sampling.

3.2. Population history and population parameter estimates

The mtDNA haplotype network showed moderate to high levels of divergence among populations (Fig. 2). For mtDNA, the Black Sea haplotypes include common ancestral allele I (also found in the Mediterranean), and four closely related alleles (Fig. 2). Ancestral haplotypes found in the Mediterranean and Black Sea in this portion of the network could be due to

Table 2 – Haplotype affiliations across the five putative populations and 10 samples from the Pacific used for comparison

Haplotype	ATL	WM	NEM	SEM	BS	Pacific Ocean
I	1	2	8		26	
II	1					
III	2					
IV	1					
V	1					
VI	1					
VII	1					
VIII	2					
IX	2					
X	1					
XI	2					
XII	1					
XIII	1					
XIV	1		1	1		
XV	7		3			
XVI		1				
XVII		1				
XVIII		2				
XIX			1			
XX				7		
XXI				1		
XXII			2		1	
XXIII		1			11	
XXIV					2	
XXV					1	
XXVI					2	
XXVII						2
XXVIII						1
XXIX						1
XXX						1
XXXI						1
XXXII						1
XXXIII						1
XXXIV						2
N	25	7	15	9	43	10

Population abbreviations are as in Table 1. Italicized rows represent shared haplotypes among locations.

incomplete lineage sorting (i.e., relatively recent divergence between these areas) or gene flow. However, distantly related

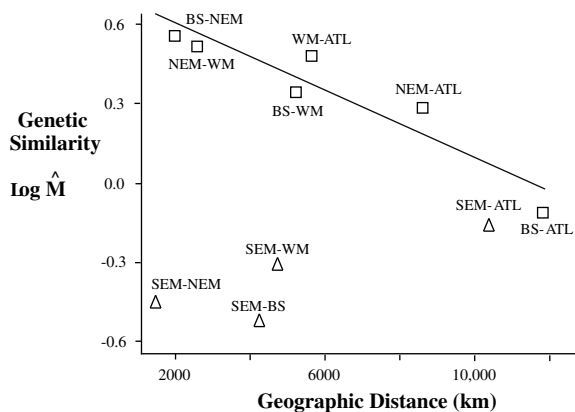


Fig. 3 – “Isolation by distance” plot of genetic similarity vs. geographic distance for all pairs of populations. Triangles represent comparisons with the southeastern Mediterranean population. The association is statistically significant with the southeastern Mediterranean population excluded ($p < 0.001$).

allele XXII was found in two northeastern Mediterranean dolphins and one from the Black Sea. This allele is most parsimoniously interpreted as a rare recent migrant into the Black Sea. Overall, phylogeographic patterns show that the Black Sea and the Mediterranean Sea have been isolated enough in the recent evolutionary past to facilitate significant genetic divergence.

Six clades possessed statistically significant nonrandom geographic associations (2-1; 2-6; 3-1; 3-3; 4-1; 4-2). The inference key suggested limited gene flow and isolation by distance for clades 2-6 and 4-1, due primarily to partial range overlap among subclades (see Appendix C). For example, within clade 4-1, ancestral clade 3-2 is restricted to the Atlantic and northeast Mediterranean. Its descendent 3-1 has geographical overlap in these areas, but then extends into the Western Mediterranean and Black Sea with clade 2-2 restricted to Atlantic haplotypes (Fig. 2). Clade 3-1 was interpreted as allopatric fragmentation between the Atlantic clade 2-2 and the more broadly distributed clade 2-1. Because clade 3-1 is necessarily younger than 4-1, this fragmentation postdates the previous pattern of IBD. The presence of allele XII in an Atlantic specimen is most parsimoniously interpreted as a rare recent migrant back to the Atlantic. Interpre-

tations of evolutionary process were not possible for clades 2-1, 3-3 and 4-2 due to insufficient resolution. The NCPA conclusions were generally consistent with the IBD analyses that excluded the Israeli dolphins (see above). Because significant differentiation exists at three successively older levels that involve the Mediterranean Sea and the Atlantic Ocean (2-6, 3-1, 4-1), isolation of the Mediterranean from the Atlantic is not a recent phenomenon.

Fig. 4 shows the posterior distributions for five of the seven parameters estimated with the IM model of divergence between the Black Sea and the Mediterranean Sea populations. These distributions are robust, since multiple independent long runs had identical distributions, and parameter autocor-

relations decreased rapidly at the beginning of each run. Probability distributions for θ_1 and θ_2 had clear maxima with maximum likelihood (ML) estimates of $\theta_1 = 0.45$ (95Lo – 95Hi: 0.21–6.88) for the Black Sea, and $\theta_2 = 21.16$ (11.56–53.58) for the Mediterranean (Fig. 4a and b). Parameters surrounding the ancestral population (θ_A) and the splitting parameter s were poorly resolved and could not be reliably estimated. The parameters θ_1 and θ_2 can each be translated to recent effective population size N_e if the per generation mutation rate μ is known. Assuming a mutation rate of $\mu = 0.5\%$ per site per million years (Hoelzel et al., 1991) and a generation time estimate of 22.4 years (Taylor et al., 2007), the effective size for the Black Sea population is estimated to be $N_1 = 2,273$ individuals

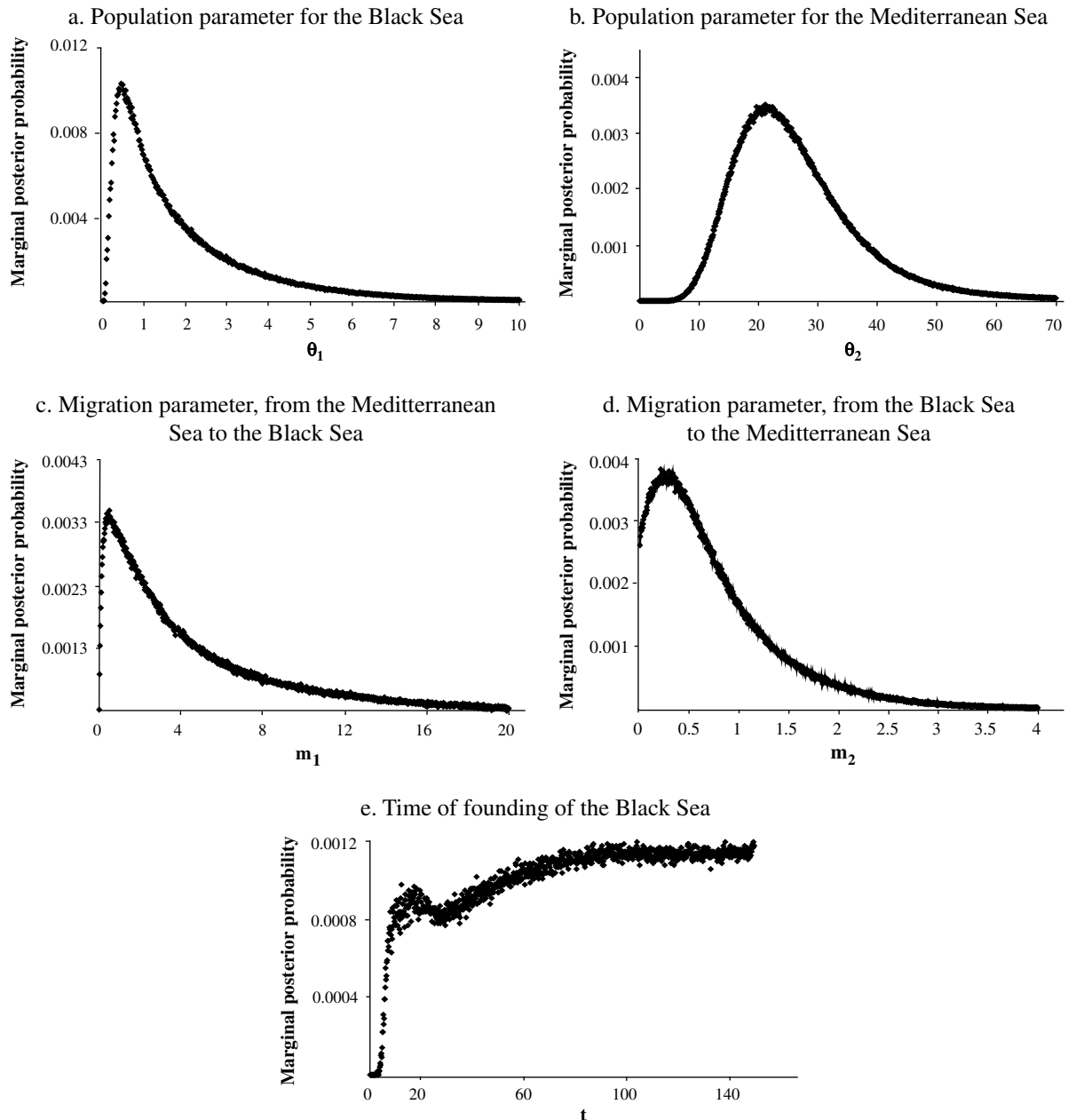


Fig. 4 – Marginal posterior probability distributions for model parameters (scaled by the neutral mutation rate). (a and b) Population parameters for the Black Sea (θ_1) and the Mediterranean Sea (θ_2). (c and d) Migration rates between the Black Sea and the Mediterranean Sea (m_1 , m_2). (e) Time parameter (t) since population divergence began.

(95Lo – 95Hi: 1061–34,745 individuals). The Mediterranean population was estimated to be considerably larger, with $N_2 = 106,860$ individuals (58,379–270,584 individuals). These population size estimates are likely to be high since we used a conservative mutation rate of 0.5% per My. If this is increased to 7% per My (Harlin et al., 2003), the maximum likelihood estimate for effective population size decreases to $N_1 = 162$ individuals for the Black Sea population (95Lo – 95Hi: 75–2482 individuals) and $N_2 = 7,669$ individuals for the Mediterranean population (4170–19,327). If the actual mutation rate is even higher (see Ho et al., 2007) then the effective population size of bottlenose dolphins in the Black Sea could be even smaller.

Migration parameter m_1 (from the Mediterranean to the Black Sea) had the highest probabilities for the zero interval and the maximum likelihood estimate of m_2 (Black Sea to the Mediterranean) was 2.22 individuals per generation or 1.1 female per generation as found in Natoli et al. (2005) (Fig. 4c and d). The Black Sea gene pool has thus been isolated for some time, supporting the NCPA conclusion that the Black Sea population has had low gene flow since it began to diverge from the Mediterranean population.

The likelihood surface for divergence time between the Black Sea and the Mediterranean population had a very low probability for $t = 0$, but leveled off and became flat for high values (Fig. 4e). Some amount of historic gene flow with the North Atlantic may have prevented the precise estimation of t , as well as the ancestral population parameter size. Ultimately, the distinction between noise and model bias will require the analysis of other genetic markers in parallel (see Carstens and Knowles, 2007).

3.3. Morphology

No sexual dimorphism was found in the Atlantic, Mediterranean Sea or Black Sea (MANOVA, d.f. = 62, all p values in post hoc test ≥ 0.05), so males and females were pooled in each population for subsequent analysis. Bottlenose dolphins differed significantly among the Atlantic Ocean, Black Sea, and Mediterranean Sea in terms of total body length (ANOVA, d.f. = 59, $p < 0.001$). Bottlenose dolphins were smallest in the Black Sea, with body lengths of 194–244 cm, in contrast to 220–315 cm and 246–320 cm for the Mediterranean and Atlantic respectively. Twenty-five skull measurements differed significantly among regions (MANOVA, d.f. = 74, $p < 0.001$), and Black Sea specimens had the lowest mean value in every case (confirmed with post hoc tests). Only brain case width was not significant ($p \geq 0.08$). Black Sea individuals had a mean skull length of 452.3 mm (maximum length = 503 mm), compared to mean lengths of 520.3 mm and 537.4 mm in the Mediterranean and the Atlantic Ocean. Although the Black Sea had the smallest specimens in terms of skull length and body length, the ratio of skull length to body length was proportionally larger in the Black Sea (20.6% vs. 19.2% and 17.8% for the Mediterranean and Atlantic respectively; ANOVA, d.f. = 54, $p < 0.001$).

Principal component analyses with and without rotation were qualitatively similar and resulted in identical biological interpretations. Consequently, we only present the results without rotation. PC1, PC2 and PC3 explained 80.1%, 4.6%

and 3.5% of the total variance respectively, and PC1 and PC3 differed significantly among the three seas (MANOVA, d.f. = 74, $p < 0.001$, Fig. 5a). Correlations between the skull measurements and PC1 were large and positive (loadings > 0.60) with the exception of braincase width (0.12). We therefore interpreted PC1 as a multivariate measure of overall skull size, with high values representing larger skulls. PC2 loaded positively and highly on skull width only (parietal width = 0.95, |all other loadings| < 0.30). PC3 loaded positively on width of the external nares and length of orbit (0.35 and 0.42 respectively), and negatively on length variables including skull length and rostrum length (-0.16 and -0.30). Therefore, PC3 was interpreted as the shape of the orbit and external nares, with high values characterizing dolphins with large orbits and wide external nares, relative to their skull length. Because Black Sea dolphins displayed low PC1 values (mean = -1.18) and PC3 values near zero (mean = 0.17), this population has relatively small skulls and a typical shape in terms of PC3.

Discriminant function analysis (DFA) of *T. truncatus* skull morphometry easily discriminated the Atlantic, Mediterranean, and Black Sea populations (Fig. 5b). Only 13 of 31 vari-

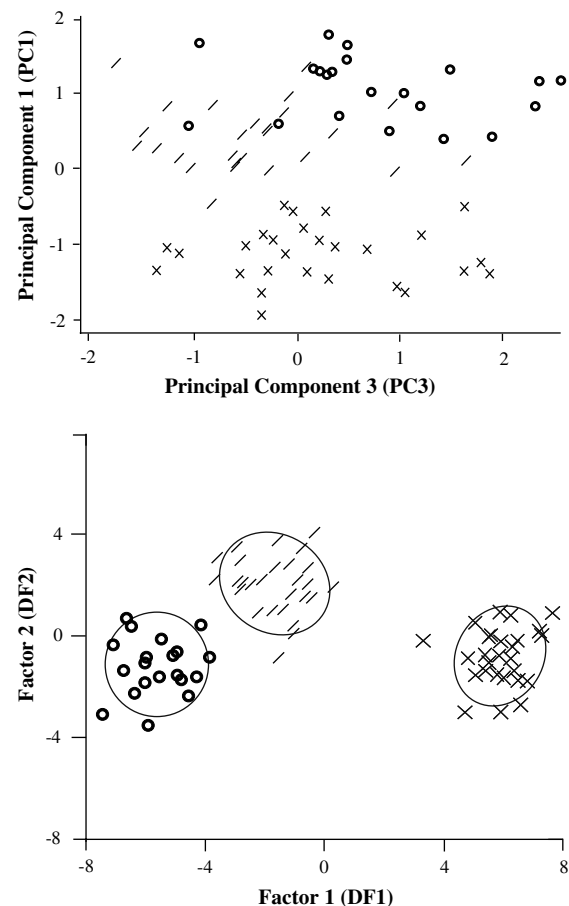


Fig. 5 – Results from multivariate morphological analyses. (a) Scatterplot of principal components 1 and 3. (b) Scatterplot of canonical factors DF1 and DF2. Each point represents an individual from the Black Sea (crosses), Mediterranean Sea (oblique lines) or Atlantic Ocean (circles).

ables were needed for discrimination, with 100% classification success (length of rostrum, rostrum width at base, rostrum width at half-length, tip of rostrum to internal nares, postorbital width, width of external nares, maximum width across premaxillaries, parietal width, braincase height, braincase length, length of upper toothrow, length of ramus, height of ramus; Viaud, 2005). In contrast, size-free discriminant analysis resulted in only 50–70% classification success using various combinations of 13 or more variables. Canonical factors DF1 and DF2 explained 23.1% and 1.5% of the total variance in the raw data respectively, and can both be interpreted in terms of skull shape. DF1 loaded positively on postorbital width (0.11) and maximum width of premaxillaries (0.12), and negatively on rostrum width at half-length (−0.21) and ramus length (−0.11). DF2 loaded positively on rostrum length (0.13) and ramus height (0.18), and negatively on rostrum width at the base (−0.13) and the width of the external nares (−0.10). Because Black Sea bottlenose dolphins displayed high DF1 (mean = 5.90) and moderately low DF2 values (mean = −0.54), this population is characterized by large postorbital width and premaxillaries, and by a rostrum that is short relative to the skull length, narrow at the half-length, and relatively wide at the base.

4. Discussion

This study demonstrates that Black Sea bottlenose dolphins are genetically and morphologically differentiated from Mediterranean and Atlantic specimens.

4.1. Gene flow and population history

Haplotype diversity and nucleotide diversity found in this study for Atlantic and Mediterranean *T. truncatus* are similar to those in previous *Tursiops* studies (Curry, 1997; Natoli et al., 2003). Genetic diversity is also lower in the Black Sea than in the Mediterranean and Atlantic, with the exception of the southeastern Mediterranean population.

The genetic data also allow some inferences regarding connectivity and history of the Black Sea. If the Black Sea population was large, completely isolated and ancient, one would expect to see a diverse network of unique Black Sea haplotypes and long branch lengths (comparable to those seen throughout the Mediterranean). In contrast, high gene flow between the Black and Mediterranean Seas either now or in the recent past would lead to multiple shared haplotypes, and no significant population structure. A more complex pattern exists: low genetic diversity in the Black Sea coupled with significant differentiation and some shared haplotypes. A literal interpretation of the theta estimates would suggest that averaged across the genealogy, the Black Sea effective population size is 2% of that found in the Mediterranean. This could be due to (1) historically smaller population sizes in the Black Sea, and/or (2) largely comparable population sizes, but severe bottlenecks at some point in the Black Sea's history. The inclusion of sequence data from other genes in future studies would increase precision in the coalescent-based analyses, and help to distinguish current vs. past population parameters. Nonetheless, the hypothesis that the Black Sea consis-

tently sustains fewer dolphins than the adjacent Mediterranean seems plausible, given the connectivity of the Mediterranean to the Atlantic. However, it is unclear whether the Black Sea has very recently undergone a bottleneck severe enough to cause dramatic losses in genetic diversity. On recent surveys in a restricted portion of the Black Sea, Birkun et al. (2004) obtained an uncorrected abundance estimate of 4193 (2527–6965 – 95% CI) individuals, leading to the inference that the entire Black Sea contains at least several thousand individuals, and perhaps tens of thousands. After accounting for an effective population size likely to be 11–80% of the census population (Nunney and Elam, 1994; Frankham, 1995), our upper estimate of N_e qualitatively support the numbers reported by Birkun et al. Our lower estimate of $N_e = 162$ would seem remarkable in this context, but not unprecedented for marine vertebrates with large census population sizes (e.g., Hauser et al., 2002).

Microsatellite markers do not display the skewed frequencies that one would expect from recent, severe bottlenecks in the Black Sea (Natoli et al., 2005), although we note that Natoli et al.'s sample size of $n = 16$ may be too low to detect bottlenecks unless they were extremely severe. Nonetheless, the available data suggests that population sizes in the Black Sea are low enough to raise concerns for conservation and management, especially considering that migration rates into the Black Sea are estimated to be only 1–2 individuals per generation. Overall, the simplest conclusion based on all available data is that the Black Sea population has been relatively isolated since its colonization, and that this population may have been historically smaller than the population in the northeastern Mediterranean. It is highly probable that this isolation is now being exacerbated by more recent anthropogenic impacts, such as maritime traffic (Öztürk and Öztürk, 1996, 1997, 2002).

These impacts are not restricted to bottlenose dolphins, as a companion study on the Black Sea harbour porpoise *Phocoena phocoena* found evidence for very recent decreases in population size (Viaud-Martinez et al., 2007). As with *T. truncatus*, Black Sea and northern Aegean harbour porpoises are morphologically and genetically unique, suggesting that their nominal subspecies designation is valid. For these reasons, the ACCOBAMS scientific committee recently recommended that the IUCN classify Black Sea/northern Aegean Sea *P. phocoena* as an endangered sub-species in its Red List (ACCOBAMS, 2006, Report of the SC4).

4.2. Morphological evolution of Black Sea bottlenose dolphins

We encountered no Black Sea bottlenose dolphins larger than 244 cm, and such specimens were rarely found in previous studies (e.g., Kleinenberg, 1978: 4/50 > 270 cm; Barabash-Nikiforov, 1940: 3/1450 > 300 cm). The smaller body size and relatively large skull of the Black Sea population could be interpreted as an adaptation to coastal environments. Black Sea bottlenose dolphins are confined primarily to the littoral zone of the western-central coast of Turkey, and the north-western Black Sea where the continental shelf extends 250 km offshore (Kleinenberg, 1978; Sorokin, 1983; Öztürk and Öztürk, 2002). Because the Black Sea is anoxic and

contains high concentrations of hydrogen sulfide below 200 m (Sorokin, 1983), deep diving is unlikely to occur. A smaller body may be adaptive for maneuverability in shallow waters, and Black Sea bottlenose dolphins are known to feed on reef fishes such as Black Sea whiting (*Merlangius merlangus euxinus*), croaker (*Sciaena cirrhosa*) and scorpionfish (*Scorpaena porcus*), and benthic fishes including flounder (*Bothus maeoticus*) (Kleinenberg, 1978). Similar morphological adaptations have been suggested for coastal bottlenose dolphins from the western Atlantic (Hersh and Duffield, 1990; Mead and Potter, 1995) and coastal spinner dolphins in the Gulf of Thailand, the Pacific and southeast Asia (Perrin et al., 1989, 1999). The unique Black Sea *T. truncatus* skull shape may also represent an adaptation for feeding (sensu Perrin, 1975), since strong, robust rostra are characteristic of coastal, benthic-feeding cetacean ecotypes (e.g. Walker, 1981; Perrin, 1984). Archer (1996) also suggested that wider premaxillaries would strengthen the lateral halves of the rostrum and prevent it from bending. Like for harbour porpoises (Viaud-Martinez et al., 2007), little is known about developmental differences among populations of bottlenose dolphins that could account for the overall smaller size and skull shape differences of those in the Black Sea.

4.3. Implications for conservation and management

Conservation and management efforts have become increasingly focused on taxa below the species level (e.g., stocks, evolutionary significant units) in order to better accommodate locally adapted gene pools and protect important ecological and evolutionary processes (e.g. Sherwin et al., 2000; McKay et al., 2005). In the case of the Black Sea bottlenose dolphin, conservation proposals are hampered by the unresolved taxonomic status and the lack of general information on the population status. For example, proposal CoP12Prop3 to CITES to transfer Black Sea bottlenose dolphin from Appendix II (not necessarily threatened with extinction) to Appendix I (threatened with extinction) was not approved in 2002. More recently, ACCOBAMS requested scientific arguments in favor of the establishment of marine protected areas to minimize threats to cetaceans and provide tools to enforce protection of cetaceans in the Black Sea region (ACCOBAMS, 2004, Resolution 2.14). Together with Natoli et al. (2003, 2005), we have demonstrated morphological, genetic, and likely ecological divergence between Black Sea and Mediterranean Sea bottlenose dolphins, sufficient to grant them separate protection and management status. In all likelihood, increased boat traffic in the Marmara Sea will further isolate the Black Sea cetaceans, and anthropogenic impacts such as pollution, diminishing food resources, diseases and physical injuries will continue. Our results support the classification of Black Sea bottlenose dolphins as a separate subspecies, and the original proposal of *Tursiops truncatus ponticus* Barabash-Nikiforov (1940) would seem to be most appropriate. Our data suggest that the Black Sea population would fit both Mayr's (1942) and Reeves et al.'s (2004) definitions of subspecies, since both morphology and genetics suggest that it is on an independent evolutionary trajectory. Thus, we recommend that urgent actions be taken to assure sustainability of the unique Black Sea population.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.biocon.2008.04.004](https://doi.org/10.1016/j.biocon.2008.04.004).

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