Mammalian Biology 76 (2011) 339–344

Contents lists available at ScienceDirect

Mammalian Biology

journal homepage: www.elsevier.de/mambio

Original Investigation

High genetic diversity and possible evidence of a recent bottleneck in Adriatic bottlenose dolphins (*Tursiops truncatus*)

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ARTICLE INFO

Article history:
Received 15 February 2010
Accepted 9 July 2010

Keywords:
Tursiops truncatus
Cetacea
Microsatellite loci
Bottleneck
Adriatic Sea

ABSTRACT

The bottlenose dolphin (*Tursiops truncatus*) is the only resident marine mammal species in the Croatian part of the Adriatic Sea, with an estimated number at around 220 individuals. It is an endangered and legally protected species in Croatia, and its demographic history is poorly known. This study investigates the level of genetic diversity in the bottlenose dolphin population from the Croatian part of the Adriatic Sea and a possibility of recent population size contraction, since there are indications that there has been intensive eradication operations in the mid 20th century that might have caused reductions in the effective population size and might have resulted in a loss of genetic variation. Thirty samples were genotyped at 12 dinucleotide microsatellite loci. The mean allelic richness (6.835 ± 0.705) and mean expected heterozygosity (0.692 ± 0.05) revealed high level of genetic diversity. Bottleneck analysis gave ambiguous evidence for a recent population decline in the investigated bottlenose dolphin population. The *M* ratio test, with two sets of parameter values, suggested a recent bottleneck; whereas the analysis by the Bottleneck program under three mutation models (TPM, SMM and IAM) showed no evidence for a genetic bottleneck. We take a more conservative approach to the interpretation of these results by accepting the evidence of a recent bottleneck. We suggest maintaining the current level of bottlenose dolphin protection in the area and careful monitoring of the population in the future.

Introduction

The bottlenose dolphin, *Tursiops truncatus* (Montagu, 1821) is a cetacean species found primarily in coastal and inshore regions of tropical and temperate waters of the world (Jefferson et al. 1993). It is the most common cetacean species found worldwide, with great capacity for adaptation. No estimates of worldwide population sizes exist and no accurate estimates of population trends are available (Baird et al. 1993). Furthermore, little is known about the numbers of bottlenose dolphins in the Mediterranean Sea, but it is unlikely to exceed the low 10,000s (Bearzi et al. 2009; Bearzi and Fortuna 2006).

The Adriatic Sea is an elongated semi-enclosed basin situated in the northern part of the Mediterranean Sea (Fig. 1). It is 150–200 km wide and about 800 km long. It is connected with the Ionian Sea through the Strait of Otranto, which is 85–100 km wide and about 800 m deep. Two cetacean species were considered regular inhabitants in the northern Adriatic Sea until the 1970s: the common dolphin (*Delphinus delphis*) and the bottlenose dolphin (*Tursiops truncatus*). Common dolphins have, however, progressively disappeared from the northern Adriatic and are considered now rare in the region (Gomerčić and Huber 1989; Gomercic et al. 1998a; Bearzi et al. 2004). The bottlenose dolphins were, alongside common dolphins, the main targets of extermination campaigns in the 19th century onwards when culling was promoted as a means of mitigating conflict with fisheries. Although not scientifically based, claims of numbers of dolphins by different authors are indicative of the perceptions of high dolphin abundance off the eastern Adriatic coast until the late 1950s. However, the main culling campaign was launched in Croatia in 1949, with the intent of eradicating dolphins from the Adriatic Sea. At least until the early 1960s, the animosity of eastern Adriatic fisherman towards dolphins was tremendous. In subsequent years the perception of dolphins as competitors and game trophies progressively changed and there is no record of

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1616-5047/$ – see front matter
doi:10.1016/j.mambio.2010.07.002

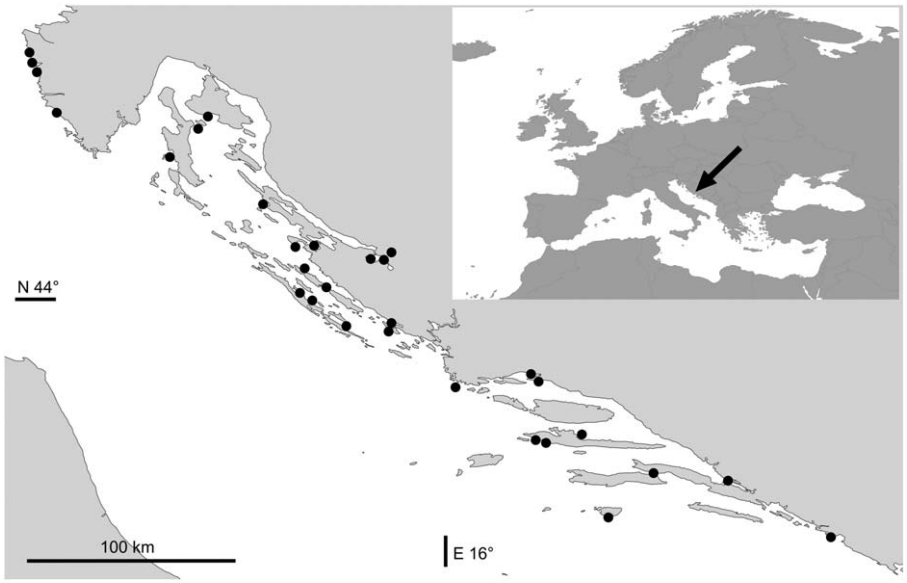

Fig. 1. Locations of findings of 30 bottlenose dolphin carcasses in the Croatian coast of the Adriatic Sea.

rewards for dolphin killings being paid after 1960 (Bearzi et al. 2004; Bearzi and Fortuna 2006). Finally, in 1995, when all marine mammals became protected by law in Croatia, dolphin killings became illegal. Today, the bottlenose dolphin remains the only resident marine mammal species in the Croatian part of the Adriatic Sea (Bearzi et al. 2004; Đuras Gomerčić et al. 2009a). However, knowledge on the present status of bottlenose dolphins in the Adriatic is rather limited. Research has mainly been concentrated on the pathological examination of stranded animals (Gomerčić et al. 1998b; Gomerčić et al. 2000; Đuras Gomerčić et al. 2009a,b); while social ecology and behavioral studies were performed only in the relatively small area of Kvarnerić, north-eastern Adriatic (Bearzi et al. 1997, 1999). Limited efforts have been made in determining the distribution and abundance of the bottlenose dolphin in the Adriatic. Bearzi et al. (1997) found extremely low bottlenose dolphin density in the area of Kvarnerić, an order of magnitude lower than in most places where the coastal bottlenose dolphin communities have been studied. Furthermore, the total number of bottlenose dolphins in the Croatian part of the Adriatic Sea in 1998 was estimated at 218 individuals by aerial census surveys (Gomercic et al. 2002).

Whether or not the wide culling campaign of the last century was severe and caused contraction in the size of the bottlenose dolphin population, is one of the major issues in population evolutionary history and future conservation and management plans. When a population suddenly contracts to a small size, a bottleneck, the genetic drift can result in sudden and dramatic changes in allele frequencies independently of selection, which can have profound effects on the evolutionary history of the population. Loss of polymorphism can lead to increased homozygosity, expression of recessive deleterious alleles, inbreeding depression and decreased adaptive potential (Prochazka et al. 2008). The aims of this study were to investigate the current level of genetic diversity of the eastern Adriatic bottlenose dolphin population and to test the hypothesis that it has experienced recent effective population size reduction, i.e. a bottleneck, as it is believed that the bottlenose dolphin numbers had been greatly reduced when hundreds of dolphins were culled by the early 1960s off the eastern Adriatic coasts (Bearzi et al. 2004).

Material and methods

From 1994 until 2003 we collected tissue samples of 30 bottlenose dolphin carcasses on the Croatian coast of the Adriatic Sea (Fig. 1), as part of a long-term project to investigate marine mammal strandings. Total genomic DNA was extracted using Wizard Genomic DNA Purification Kit, Promega.

The samples were genotyped at 12 dinucleotide microsatellite loci: EV1Pm, EV14Pm derived from *Physeter macrocephalus*, EV37Mn, EV94Mn from *Megaptera novaeangliae* (Valsecchi and Amos 1996) and D08, D14, D18, D22, D28, TexVet3, TexVet5, TexVet7 from *Tursiops truncatus* (Shinohara et al. 1997; Rooney et al. 1999). For amplification three primer pairs were multiplexed in one polymerase chain reaction (PCR) using the QIAGEN Multiplex PCR Kit (QIAGEN GmbH, Hilden, Germany). PCRs were carried out in a 8-μl volume containing 80–120 ng of genomic DNA, 1× QIAGEN Multiplex PCR Master Mix (consisting of QIAGEN Multiplex PCR buffer with a final concentration of 3 mM $MgCl_2$, dNTP mix, and HotStarTaq DNA polymerase), 0.2 μM of locus-specific fluorescent-labeled forward primer (fluorescent dyes were FAM, JOE and TAMRA) and nonlabeled reverse primer. PCR cycling profile was 15 min at 95 °C; then 30 cycles of 30 s at 94 °C, 90 s at 55 °C, 60 s at 72 °C; then 30 min at 60 °C. The PCR products were run on an ABI PRISM, 310 Genetic Analyzer (Applied Biosystems) according to manufacturer's instructions. GeneScan Analysis Software 3.1 and Genotyper 2.5.2 (both Applied Biosystems) software were used to determine the allele sizes with GeneScan ROX 350 as the internal standard. In order to detect genotyping errors due to null alleles, short allele dominance and scoring errors due to stuttering, we used MICRO-CHECKER 2.2.3 (Van Oosterhout et al. 2004). We used the software GIMLET 1.3.3 (Valière 2002) to check for the presence of parent–offspring pairs, since related genotypes could bias estimations of genetic diversity, specially if the sample size is quite low. The same program was also used to calculate the probability of identity (P_{ID}), i.e. the probability that two individuals drawn at random from the population share identical multi-locus genotypes (Paetkau and Strobeck 1994); as well as the probability of identity among siblings (Evett and Weir 1998). Allelic diversity (number of alleles per locus), Nei's unbiased expected (He) heterozygosity (Nei 1978) and observed (Ho) heterozygosity were

Table 1
Number of individuals genotyped at each locus (*N*), allelic diversity (*A*), allelic richness (AR), Nei's unbiased expected heterozygosity (He), observed heterozygosity (Ho) and the probability of the data under the assumption of the null hypothesis of Hardy–Weinberg equilibrium (P_{HWE}).

Locus	*N*	*A*	AR	He	Ho	P_{HWE}
EV1Pm	27	7	6.405	0.414	0.333	0.363
D18	30	9	8.640	0.771	0.800	0.803
TexVet3	23	7	7.000	0.823	0.783	0.154
D14	26	6	5.884	0.762	0.654	0.408
D08*	30	5	5.000	0.786	0.633	0.249
TexVet5*	26	6	5.873	0.666	0.500	0.163
EV94Mn	30	8	7.469	0.782	0.667	0.178
TexVet7*	30	3	2.998	0.344	0.333	0.525
EV14Pm	26	6	5.884	0.757	0.692	0.109
EV37Mn*	30	15	13.951	0.906	0.867	0.686
D28	30	7	6.531	0.766	0.467	0.002***
D22	30	7	6.379	0.611	0.667	0.915
Mean$^{12\ loci}$ ± SE		7.167 ± 0.833	6.835 ± 0.705	0.692 ± 0.05	0.616 ± 0.05	
Mean$^{4\ loci}$ ** ± SE		7.25 ± 2.66	6.956 ± 2.4	0.676 ± 0.12	0.583 ± 0.11	

* Microsatellite loci used for comparison with other published data.
** Mean of the selected four loci (marked with *).
*** Significant P_{HWE} values.

obtained using Genetix 4.05 (Belkhir et al. 1996–2004). Allelic richness was calculated using the program FSTAT 2.9.3 (Goudet 2001). Deviations from Hardy–Weinberg equilibrium (HWE) were evaluated for all loci by calculating probabilities with the program GENEPOP 1.2 (Raymond and Rousset 1995) using complete enumeration for loci with up to four alleles and a Markov chain method for loci with more than four alleles. The same program was used in linkage disequilibrium testing for all pairs of loci; *P*-values were corrected for multiple statistical tests by the Bonferroni method. We further compared allelic diversity and expected heterozygosity of Adriatic bottlenose dolphins with the published data on 14 other bottlenose dolphin populations or stocks (Natoli et al. 2004, 2005, 2008). The comparison was based on the mean values obtained over four microsatellite loci (EV37Mn, TexVet5, TexVet7 and D08) selected because they were mutually used in all compared investigations. To investigate possible population structure, a Bayesian clustering analysis was performed using STRUCTURE 2.3.3 software (Pritchard et al. 2000), which probabilistically assigns individuals to populations based on their multi-locus genotypes. A total of 10,000 Markov Chain Monte Carlo iterations, after a burn-in period of 10,000 iterations, were run for each number of genetic clusters (K, ranging from 1 to 4) from the admixture model. To test for evidence of recent bottleneck events, we used three different approaches. The first approach assumes that in a recently reduced population the gene diversity will be higher than that expected at equilibrium. Gene diversity was estimated under three models of molecular evolution: the stepwise mutation model (SMM), the infinite allele model (IAM), and the two-phase model (TPM). The TPM has been shown to be the most appropriate for microsatellite DNA data (Di Rienzo et al. 1994). We used TPM with 95% single-step mutations and 5% multiple-step mutations, and a variance among multiple steps of 12, as recommended by Piry et al. (1999). Ten thousand iterations were used for each mutation model. To determine if the number of loci exhibiting heterozygosity excess was significant, the one-tailed Wilcoxon signed rank test for heterozygote excess was applied. Secondly, we tested distribution of allele frequencies to determine whether a bottleneck-induced mode shift has recently occurred. Mode shift is a transient distortion in the distribution of allele frequencies such that the frequency of alleles at low frequency becomes lower than the frequency of alleles in an intermediate allele frequency class (Luikart et al. 1998a). For these analyses we used the program Bottleneck, v. 1.2.02 (Piry et al. 1999). The third test that we used in detecting bottleneck is the *M* ratio test which is based on the ratio of the observed number of microsatellite alleles to the range of allele sizes. Because alleles are randomly lost as a result of genetic drift, the *M* ratio is expected to decrease in bottlenecked populations (Garza and Williamson 2001). This analysis was carried out with Critical M and M-P-Val programs from Garza and Williamson (2001). The programs simulate an equilibrium distribution of M in a constant size population assuming values for three parameters: θ, the parameter based on effective population size prior to the bottleneck and mutation rate; Δg, the average size of non one-step mutations; and p_s, the proportion of one-step mutations. We simulated two sets of parameter values: a reasonable parameterization of the two-phase mutation model is $p_s = 0.9$ and $\Delta g = 3.5$, as noted by Garza and Williamson (2001); and alternative parameter values, based on 29 fully resolved mutations found by Garza and Williamson (2001) in the literature, with $p_s = 0.88$ and $\Delta g = 2.8$. To account for the differences in effective population size and mutation rates, we tested four values of θ parameter (0.01, 0.1, 1 and 2). Although smaller values of θ increase the value of Mc, we set the value of θ at a maximum of 2, because θ is population specific. With the θ set at a value greater than 2, the effective population size prior to the bottleneck would be greater than the range from 1000 to 10,000 (assuming mutation rates of 5×10^{-4} to 5×10^{-5}), which is not probable for the bottlenose dolphin population in the Adriatic, whose current size is estimated to no more than several hundred individuals.

Results

All microsatellite loci are highly polymorphic (Table 1). Three to 15 alleles per locus were found and allelic diversity across loci was 7.167 ± 0.833. Allelic richness ranged between 2.998 and 13.951 and mean allelic richness was 6.835 ± 0.705. The mean expected heterozygosity was 0.692 ± 0.05 and ranged from 0.344 to 0.906, while mean observed heterozygosity was 0.616 ± 0.05 and ranged from 0.333 to 0.867. Significant deviation from HWE was observed at locus D28 and was combined with substantial heterozygote deficit. Furthermore, MICRO-CHECKER detected evidence for null alleles at that locus, while tests for short allele dominance and scoring errors due to stuttering were negative for all loci. Locus D28 was omitted from further analyses due to the evidence of null alleles being present.

Statistical tests for linkage disequilibrium for all combinations of loci were non-significant after Bonferroni correction, indicating independent segregation of loci. GIMLET did not identify any parent–offspring pairs in the sample. The probability of identity for unrelated individuals among 11 microsatellite loci was 6.771e−11 and 1.131e−04 for related individuals. In order to obtain reasonably low P_{ID} for related individuals (e.g. <0.01) at least five of the most informative microsatellite loci should be used, while adding

Table 2
Multi-locus probability of identity for unrelated (P_{ID}) and related (P_{IDsib}) dolphins. The values are calculated sequentially in increasing order of single-locus values starting with the most informative locus.

Locus	P_{ID}	P_{IDsib}
EV37	2.144e−02	3.098e−01
TV3	1.377e−03	1.126e−01
D08	1.217e−04	4.342e−02
EV94	1.098e−05	1.685e−02
D18	1.006e−06	6.636e−03
D14	1.027e−07	2.668e−03
EV14	1.111e−08	1.082e−03
TV5	1.728e−09	5.004e−04
D22	3.902e−10	2.533e−04
EV1	1.450e−10	1.620e−04
TV7	6.771e−11	1.131e−04

three more loci lowers the value of P_{ID} by an order of a magnitude (Table 2).

Comparisons of allelic diversity and expected heterozygosity with published data of 14 other populations and based on mean values of four microsatellite loci are presented in Table 3. Both allelic diversity (7.25) and expected heterozygosity (0.676) of the Adriatic population are above medians of all compared populations (6.75 and 0.668, respectively), confirming high level of genetic diversity in the investigated Adriatic population.

The STRUCTURE analysis of the data revealed no population structuring. The most likely number of subpopulations identified was $K = 1$, and higher values of K revealed lower likelihoods (results not shown). Re-analyses of the data using LocPrior model (Hubisz et al. 2009) with incorporated information on sampling areas (northern, middle and southern Adriatic coast) did not alter these results.

For the Bottleneck analysis, Wilcoxon signed rank tests were not significant under any of the three mutational models: TPM (with 95% single-step mutations), SMM and IAM ($P = 0.897$, 0.949 and 0.0737, respectively). The P-values were still >0.05 when alternative proportions (range 70–95%) were attributed to the single-step mutations in TPM. In addition, there is no evidence for a significant deviation from the normal L-shaped distribution of allele frequencies as expected for a stable population under mutation-drift equilibrium (Fig. 2).

The sample M ratio was calculated to be 0.736. Estimated critical M ratios varied between 0.748 and 0.857 for parameter values $p_s = 0.9$ and $\Delta g = 3.5$; and between 0.785 and 0.864 for parameter values $p_s = 0.88$ and $\Delta g = 2.8$, depending on the value of parameter θ. Therefore, it follows that the sample M ratio value was smaller than the simulated critical and average M ratio values in each parameter set and for each value of θ (Fig. 3). This result suggests a recent bottleneck in the investigated bottlenose dolphin population, contrary to the results obtained using the test for heterozygosity excess.

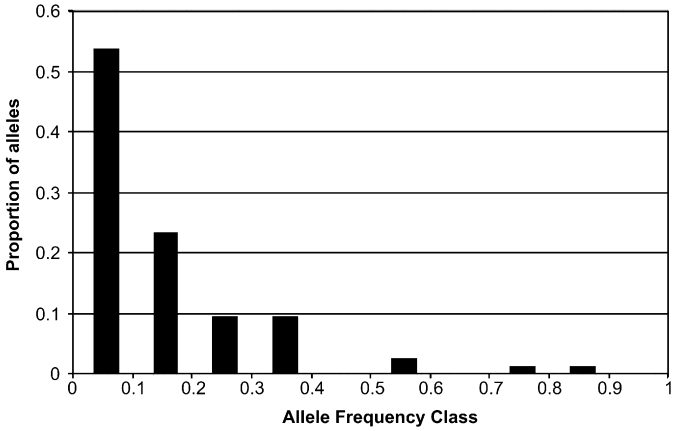

Fig. 2. Allele frequency distribution for 12 microsatellite loci in the Adriatic bottlenose dolphin population ($n = 30$ individuals). Bars represent the proportion of alleles found in each allele frequency class. The distribution is L-shaped, as expected for a stable population under mutation-drift equilibrium.

Discussion

This study investigates the level of genetic diversity in the bottlenose dolphin population from the Croatian part of the Adriatic Sea and a possibility of recent population size contraction, since there are indications that there has been intensive eradication operations in the mid 20th century (Bearzi et al. 2004) that might have caused reductions in the effective population size. Thus, we hypothesized that if such reductions in population size happened, they might have led to a decrease in the level of genetic diversity and/or might be detected with the tests for reduction in population size using data from microsatellite loci.

The results obtained in this study do not indicate reduction of genetic diversity in the population of bottlenose dolphins in the Croatian part of the Adriatic Sea, as measured by 12 microsatellite loci, since all the loci are highly polymorphic with a large number of alleles (7.167 ± 0.833), high allelic richness (6.835 ± 0.705) and heterozygosities (Ho, 0.616 ± 0.05; He, 0.692 ± 0.05) (Table 1). When comparing these results with the results of investigations of genetic diversity of other bottlenose dolphin populations or stocks, a high level of genetic diversity in the investigated Adriatic population is confirmed. In order to have comparable data, we used mean values of expected heterozygosity and allelic diversity over four microsatellite loci (EV37Mn, TexVet5, TexVet7 and D08) that were used in all compared populations. Given the fact that Natoli et al. (2005) identified only one, the eastern Mediterranean population, among the samples from the Adriatic Sea, the Ionian Sea and Israel, it is not surprising to find the Adriatic population's values of allelic diversity (7.25) and expected heterozygosity (0.676) to be the most similar to those of the Eastern Mediterranean population (7.50 and 0.668, respectively). Most of the populations used for comparison that have greater values of allelic diversity than the Adriatic population were represented by larger sample sizes. This comes as no surprise since allelic diversity is dependent on sample size. The exceptions are bottlenose dolphin populations from Western North Atlantic, having lower sample sizes but higher allelic diversities (both pelagic and coastal populations sample sizes were 27 individuals, and their respective allelic diversities were 11.25 and 8.00) (Table 3). Furthermore, the populations in South Africa that are indicative of founder event showed low levels of both allelic diversity (5.75 for the North Coast, 6.00 for the South Coast and 5.50 for Biopsies) and expected heterozygosity (0.553, 0.528 and 0.52, respectively), regardless of the large number of individuals analysed (39, 47 and 56, respectively) (Natoli et al. 2008) (Table 3). This suggests that among indications of a bottlenecked population are low levels of allelic diversity and expected heterozygosity. Nevertheless, the levels we found in the Adriatic bottlenose dolphin population were relatively high.

Significant deviations from HWE at locus D28 and evidence for null alleles, as detected by MICRO-CHECKER, should call for caution if using the locus in future genetic analyses.

In addition to reflecting the extent of genetic variability in the population, probabilities of identity provide an estimate of the minimum number of loci needed to discriminate among individuals. For genetic investigations including individual identification of the bottlenose dolphins from the Croatian part of the Adriatic Sea (e.g. biopsies), using five to eight highly informative loci (Table 2) should be statistically powerful yet economically viable.

Table 3
Comparison of allelic diversity (*A*) and expected heterozygosity (He) of Adriatic bottlenose dolphins with published data of other bottlenose dolphin populations, based on the mean values obtained over four microsatellite loci (EV37Mn, TexVet5, TexVet7, D08). Populations are arranged in ascending order from the lowest value (bottom). The Adriatic bottlenose dolphin population is highlighted.

A	Population	*n*	Reference	He	Population	*n*	Reference
12.25	Mediterranean Sea	45	Natoli et al. (2004)	0.870	Western North Atlantic pelagic	27	Natoli et al. (2004)
11.25	Western North Atlantic pelagic	27	Natoli et al. (2004)	0.769	Eastern North Atlantic	35	Natoli et al. (2005)
11.00	Western Mediterranean	42	Natoli et al. (2005)	0.746	Mediterranean Sea	45	Natoli et al. (2004)
10.50	Eastern North Atlantic	35	Natoli et al. (2005)	0.740	Western Mediterranean	42	Natoli et al. (2005)
8.00	Western North Atlantic coastal	27	Natoli et al. (2004)	0.728	Gulf of Mexico	22	Natoli et al. (2004)
7.50	Eastern Mediterranean	32	Natoli et al. (2005)	0.694	Western North Atlantic coastal	27	Natoli et al. (2004)
7.25	Adriatic	30	This investigation	0.676	Adriatic	30	This investigation
6.75	Gulf of Mexico	22	Natoli et al. (2004)	0.668	Eastern Mediterranean	32	Natoli et al. (2005)
6.25	Eastern North Atlantic	27	Natoli et al. (2004)	0.666	Eastern North Pacific	14	Natoli et al. (2004)
6.00	South Coast, South Africa	47	Natoli et al. (2008)	0.614	Eastern North Atlantic	27	Natoli et al. (2004)
5.75	North Coast, South Africa	39	Natoli et al. (2008)	0.604	Scotland	20	Natoli et al. (2005)
5.50	Biopsies, South Africa	56	Natoli et al. (2008)	0.553	North Coast, South Africa	39	Natoli et al. (2008)
5.50	Scotland	20	Natoli et al. (2005)	0.528	South Coast, South Africa	47	Natoli et al. (2008)
4.75	Black Sea	16	Natoli et al. (2005)	0.520	Biopsies, South Africa	56	Natoli et al. (2008)
4.75	Eastern North Pacific	14	Natoli et al. (2004)	0.497	Black Sea	16	Natoli et al. (2005)
6.75	Median			0.668	Median		

n, number of individuals analysed for each population.

We did not detect evidence of population structuring. However, this result may be limited by the relatively small sample size of the current study. For a more powerful analysis additional samples are required, including those of the neighbouring regions.

The evidence for a genetic bottleneck in the Adriatic bottlenose dolphin population was ambiguous. The Bottleneck analysis did not detect sufficient evidence for a recent bottleneck. We used the Wilcoxon signed rank test because it is the most appropriate and powerful if fewer than 20 loci are analysed (Piry et al. 1999). Although the IAM is recommended for allozyme data and the SMM is generally more appropriate when testing microsatellite data (i.e. dinucleotide repeat loci) (Luikart et al. 1998a), we used both SMM and IAM because they represent two extreme models of mutation along a continuum of possible models. However, all loci will follow a mutation model somewhere in-between the two extreme models (Piry et al. 1999). When testing both the extreme models and in-between models (TPM with proportions of single-step mutations in range between 70 and 95%), the null hypothesis of the Wilcoxon's test (no significant heterozygosity excess on average across loci) cannot be rejected and thus it suggests that there is no sufficient evidence for a recent bottleneck in the Adriatic bottlenose dolphin population. Moreover, analysis of allele frequency distribution failed to detect a mode-shifted distribution of allele frequencies (Fig. 2), also suggesting that a bottleneck is not likely to have occurred in the recent past. On the other hand, the *M* test suggested a recent bottleneck in the investigated bottlenose dolphin population. The discrepancy between the results of the bottleneck analyses first led us to question our sample size. We genotyped 30 individuals, which should ensure that the majority of the alleles in the population were sampled. According to Garza and Williamson (2001), the number of individuals genotyped has to be equal to at least twice the number of alleles at the most variable locus, which in our study was locus EV37Mn with 15 alleles found. Piry et al. (1999) suggest typing at least 10 polymorphic loci and sampling at least 30 individuals in order to achieve a reasonably high statistical power. We therefore conclude that our sample size, although at the lower end, meets both requirements. Luikart and Cornuet (1998b) noted that bottlenecks can go undetected if they were either not very severe or were very recent. The lack of evidence for genetic bottleneck obtained by using the test for heterozygosity excess and the high genetic diversity found, might suggest that the fishermen eradication actions against bottlenose dolphins in the eastern Adriatic Sea were overestimated and the population has not experienced severe reduction of its population size. Further, it is possible that the sampled individuals were not representative of the bottlenecked population or that the bottlenecked population was not completely isolated and contained genes from immigrants that have obscured the genetic effects of the bottleneck, both of which could explain the lack of evidence of bottleneck in the investigated population when using the test for

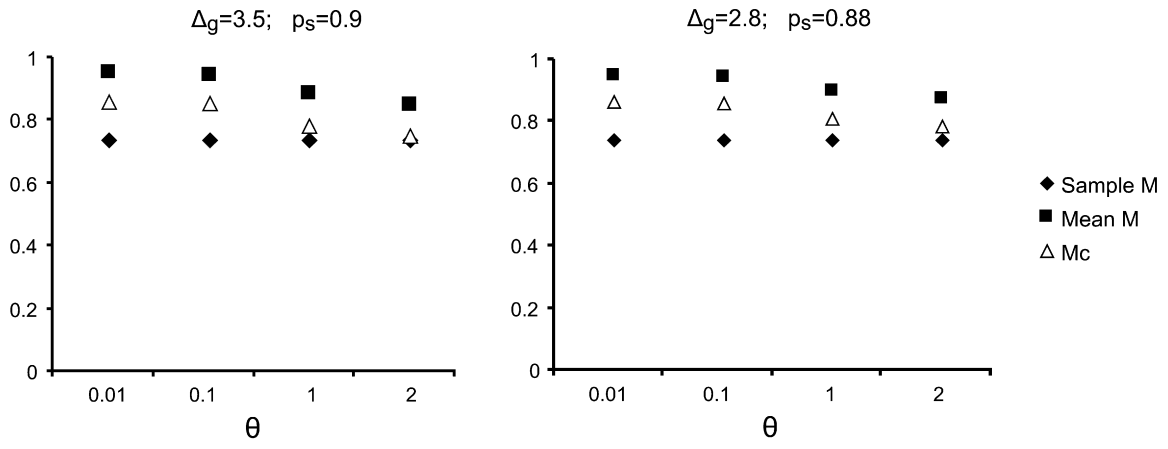

Fig. 3. *M* ratio graphics for two simulated sets of parameter values (p_s and Δg), each with four values of θ. Calculated sample M (♦), estimated critical M (Mc, △) and mean M (■) ratios are presented for each simulated scenario.

heterozygosity excess (Piry et al. 1999). However, as the *M* ratio test suggested that a bottleneck did happen, we speculate that its effect on heterozygosity might have been obscured by the genes from immigrants from the neighbouring populations (i.e. Ionian Sea). The idea that the Adriatic bottlenose dolphin population is not isolated from the rest of the eastern Mediterranean populations was already implied by Natoli et al. (2005). If correct, our speculation of gene flow between Adriatic and neighbouring dolphin populations would further reinforce this idea. The lack of genetic structuring in the data set analysed would therefore be expected. Regional fine-scale population structuring and gene flow among bottlenose dolphins in the eastern Mediterranean Sea should be investigated more thoroughly. Further research including more extensive sampling is needed to identify population boundaries and the extent of isolation of the individuals from the Croatian part of the Adriatic Sea from those in the surrounding areas. Future investigations should include biopsy sampling, as the certainty of origin of a biopsied individual is higher and there can be biases associated with stranded samples (Valsecchi et al. 2004). The understanding of the population boundaries is of vital importance for the biology and conservation plans of the Adriatic bottlenose dolphins living in the semi-enclosed basin of the Adriatic Sea, and whose demographic history and population status are poorly known. With putative evidence of past dolphin culling campaigns in mind, we are more inclined to accept the evidence for a recent bottleneck found by the *M* ratio test, taking a more conservative approach to the interpretation of ambiguous bottleneck results. Therefore, we suggest maintaining the current level of bottlenose dolphin protection in the area and careful monitoring of the population in the future both with field observations and genetic analyses.

Acknowledgements

The work was supported by grants from the Ministry of Science, Education and Sports of the Republic of Croatia (Project No. 053-0533406-3640) and Gesellschaft zur Rettung der Delphine e.V., Munich, Germany, and was carried out according to the annual permission of the State Institute for Nature Protection, Ministry of Culture, Republic of Croatia. We are grateful to Snježana Džijan and Ivan Biruš of the Josip Juraj Strossmayer University of Osijek.

References

Baird, R.W., Walters, E.L., Stacey, P.J., 1993. Status of the Bottlenose Dolphin, *Tursiops truncatus*, with special reference to Canada. Can. Field Nat. 107, 466–480.

Bearzi, G., Notarbartolo di Sciara, G., Politi, E., 1997. Social ecology of bottlenose dolphins in the Kvarnerić (northern Adriatic Sea). Mar. Mammal Sci. 13, 650–668.

Bearzi, G., Politi, E., Notarbartolo di Sciara, G., 1999. Diurnal behavior of free-ranging bottlenose dolphins in the Kvarnerić (northern Adriatic Sea). Mar. Mammal Sci. 15, 1065–1097.

Bearzi, G., Holcer, D., Notarbartolo di Sciara, G., 2004. The role of historical dolphin takes and habitat degradation in shaping the present status of northern Adriatic cetaceans. Aquat. Conserv.: Mar. Freshw. Ecosyst. 14, 363–379.

Bearzi, G., Fortuna, C.M., 2006. Common bottlenose dolphin *Tursiops truncatus* (Mediterranean subpopulation). In: Reeves, R.R., Notarbartolo di Sciara, G. (Eds.), Status and Distribution of Cetaceans in the Black Sea and Mediterranean Sea. IUCN Centre for mediterranean Cooperation, Málaga, Spain, pp. 64–73.

Bearzi, G., Fortuna, C.M., Reeves, R.R., 2009. Ecology and conservation of common bottlenose dolphins *Tursiops truncatus* in the Mediterranean Sea. Mammal Rev. 39, 92–123.

Belkhir, K., Borsa, P., Chikhi, L., Raufaste, N., Bonhomme, F., 1996–2004. GENETIX 4.05, logiciel sous Windows TM pour la génétique des populations. Laboratoire Génome, Populations, Interactions, CNRS UMR 5171. Université de Montpellier II, Montpellier, France.

Di Rienzo, A., Peterson, A.C., Garza, J.C., Valdes, A.M., Slatkin, M., Freimer, N.B., 1994. Mutation processes of simple-sequence repeat loci in human populations. Proc. Natl. Acad. Sci. U.S.A. 91, 3166–3170.

Đuras Gomerčić, M., Galov, A., Gomerčić, T., Lucić, H., Škrtić, D., Ćurković, S., Vuković, S., Gomerčić, H., 2009a. Human-induced cetacean mortality in the Adriatic Sea. In: Abstract Book: 23rd Annual Conference of the European Cetacean Society, Istanbul, Turkey.

Đuras Gomerčić, M., Galov, A., Gomerčić, T., Škrtić, D., Ćurković, S., Lucić, H., Vuković, S., Arbanasić, H., Gomerčić, H., 2009b. Bottlenose dolphin (*Tursiops truncatus*) depredation resulting in larynx strangulation with gill-net parts. Mar. Mammal Sci. 25, 392–401.

Evett, I., Weir, B., 1998. Interpreting DNA Evidence: Statistical Genetics for Forensic Scientists. Sinauer Associates, Sunderland.

Garza, J.C., Williamson, E.G., 2001. Detection of reduction in population size using data from microsatellite loci. Mol. Ecol. 10, 305–318.

Gomerčić, H., Huber, D., 1989. Istraživanja i zaštita morskih sisavaca Jadrana. IV konferencija o zaštiti Jadrana. Neum, 19 and 20 oktobar 1989. godine. Program rada, p. 19 (in Croatian).

Gomercic, H., Huber, D., Gomercic, A., Gomercic, T., 1998a. Geographical and historical distribution of the cetaceans in Croatian part of the Adriatic Sea. Rapp. Commun. Int. Mer Médit. 35, 440–441.

Gomerčić, H., Huber, Đ., Gomerčić, V., Škrtić, D., Gomerčić, A., Vuković, S., 1998b. Dolphin morbilliviral infection from the Mediterranean Sea did not spread into the Adriatic Sea. Acta Veterinaria Hungarica 46, 127–134.

Gomerčić, H., Huber, Đ., Gomerčić, V., Vuković, S., Škrtić, D., Gomerčić, T., Dobranić, V., Lucić, H., Đuras, M., Ćurković, S., Gomerčić, A., Kardoš, Lj., 2000. Fatty liver and subcutaneous edema in a free-living bottlenose dolphin (*Tursiops truncatus*, Montagu 1821) from the Adriatic Sea; light- and electron-microscopical study. Vet. Arh. 70, 259–277.

Gomercic, H., Huber, C., Mihelic, D., Lucic, H., Gomercic, T., Curas, M., 2002. Estimation of the bottlenose dolphin (*Tursiops truncatus*) population in the Croatian part of the Adriatic Sea. In: 9th International Congress on the Zoogeography and Ecology of Greece and Adjacent Regions, Abstracts, The Hellenic Zoological Society. Thessaloniki, 2002, p. 43, http://www.vef.hr/dolphins/.

Goudet, J., 2001. FSTAT, A Program to Estimate and Test Gene Diversities and Fixation Indices (Version 2.9.3), Available at: http://www2.unil.ch/popgen/softwares/fstat.htm.

Hubisz, J.M., Falush, D., Stephens, M., Pritchard, J.K., 2009. Inferring weak population structure with the assistance of sample group information. Mol. Ecol. Res. 9, 1322–1332.

Jefferson, T.A., Leatherwood, S., Webber, M.A., 1993. Marine Mammals of the World, FAO Species Identification Guide, United Nations Environment Programme. Food and Agriculture Organization of the United Nations, Rome.

Luikart, G., Allendorf, F.W., Cornuet, J.-M., Sherwin, W.B., 1998a. Distortion of allele frequency distributions provides a test for recent population bottlenecks. J. Hered. 89, 238–247.

Luikart, G., Cornuet, J.-M., 1998b. Empirical evaluation of a test identifying recently bottlenecked populations from allele frequency data. Conserv. Biol. 12, 228–237.

Natoli, A., Peddemors, V.M., Hoelzel, A.R., 2004. Population structure and speciation in the genus *Tursiops* based on microsatellite and mitochondrial DNA analyses. J. Evol. Biol. 17, 363–375.

Natoli, A., Birkun, A., Aguilar, A., Lopez, A., Hoelzel, A.R., 2005. Habitat structure and dispersal of male and female bottlenose dolphins (*Tursiops truncatus*). Proc. R. Soc. B 272, 1217–1226.

Natoli, A., Peddemors, V.M., Hoelzel, A.R., 2008. Population structure of bottlenose dolphins (*Tursiops aduncus*) impacted by bycatch along the east coast of South Africa. Conserv. Genet. 9, 627–636.

Nei, M., 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89, 583–590.

Paetkau, D., Strobeck, C., 1994. Microsatellite analysis of genetic variation in black bear populations. Mol. Ecol. 3, 489–495.

Piry, S., Luikart, G., Cornuet, J.-M., 1999. Bottleneck: a computer program for detecting recent reductions in the effective population size using allele frequency data. J. Hered. 90, 502–503.

Pritchard, J.K., Stephens, M., Donnelly, P., 2000. Inference of population structure using multilocus genotype data. Genetics 155, 945–959.

Prochazka, P., Bellinvia, E., Fainova, D., Hajkova, P., Elhalah, A., Alomari, K., 2008. Immigration as a possible rescue of a reduced population of a long-distant migratory bird: reed warblers in the Azraq Oasis, Jordan. J. Arid Environ. 72, 1184–1192.

Raymond, M., Rousset, F., 1995. GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. J. Hered. 86, 248–249.

Rooney, A.P., Merritt, D.B., Derr, J.N., 1999. Microsatellite diversity in captive Bottlenose dolphins (*Tursiops truncatus*). J. Hered. 90, 228–231.

Shinohara, M., Domingo-Roura, X., Takenaka, O., 1997. Microsatellites in the bottlenose dolphin *Tursiops truncatus*. Mol. Ecol. 6, 695–696.

Valière, N., 2002. GIMLET: a computer program for analysing genetic individual identification data. Mol. Ecol. Notes 2, 377–379.

Van Oosterhout, C., Hutchinson, W.F., Wills, D.P.M., Shipley, P., 2004. MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. Mol. Ecol. Notes 4, 535–538.

Valsecchi, E., Amos, W., 1996. Microsatellite markers for the study of cetacean populations. Mol. Ecol. 5, 151–156.

Valsecchi, E., Amos, W., Raga, J.A., Podesta, M., Sherwin, W., 2004. The effects of inbreeding on mortality during a morbillivirus outbreak in the Mediterranean striped dolphin (*Stenella coeruleoalba*). Anim. Conserv. 7, 139–146.