

Genetic variation and population structure of western Mediterranean and northern Atlantic *Stenella caeruleoalba* populations inferred from microsatellite data

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The patterns of genetic differentiation and levels of genetic diversity among striped dolphin (*Stenella caeruleoalba*) populations from the North Atlantic Ocean (N=45 individuals) and the central and western Mediterranean Sea (N=78) were investigated using five polymorphic microsatellite loci. A North Pacific sample (N=14) was added as an out-group. Two of the markers were tetranucleotide repeats tested for the first time in this species. The Mediterranean, Atlantic and Pacific samples displayed a mean number of alleles per locus of 11.2, 13.4, and 9.6 respectively, suggesting a high but variable polymorphism across loci. The Mediterranean sample displayed particular characteristics: (i) the lowest allelic richness and expected heterozygosity ($H_{e_{\text{Mediterranean}}}=0.76$, while $H_{e_{\text{Atlantic}}}=0.83$ and $H_{e_{\text{Pacific}}}=0.85$); (ii) a significant departure from Hardy–Weinberg equilibrium ($P<0.001$; $F_{IS}=0.050$); and (iii) a significant linkage disequilibrium between two pairs of loci. These last two features, present neither in the Atlantic sample nor in the Pacific one, suggest that the western Mediterranean population might possibly be further subdivided. Significant genetic differentiation was detected between the Mediterranean and Pacific populations, and between the Mediterranean and Atlantic populations. However, pairwise Wright's F_{ST} was not significantly different from zero between the two geographically isolated Atlantic and Pacific populations.

INTRODUCTION

The striped dolphin *Stenella caeruleoalba* (Meyen, 1833) is cosmopolitan in most of the world's warm and temperate seas, and is generally considered as a relatively abundant species (Archer II & Perrin, 1999; Reeves et al., 2003). According to the International Union for the Conservation of Nature and Natural Resources (IUCN), the species is at 'lower risk' but 'conservation dependent' (Cetacean Specialist Group, 1996). In the western Mediterranean, the striped dolphin is the most abundant cetacean species according to transect survey estimations (95% confidence interval: 68,379 to 214,800 individuals after Forcada et al. (1994), although this estimate is not based on the most robust current prospecting methods). However, the Mediterranean population has recently been facing various threats such as morbillivirus epizootics, pollution, or fishing interactions, the effects of which are difficult to quantify precisely (Aguilar, 2000).

In the humanized areas of south-western Europe, assessing the striped dolphin's population genetic structure and genetic diversity within populations could be an early step towards the design of proper conservation policies (Beebee & Rowe, 2004). In a study of mitochondrial DNA (mtDNA) restriction polymorphism, García Martínez et al. (1999) found that none of the 27 restriction haplotypes was shared between a Mediterranean sample (N=76) and an Atlantic sample (N=22). However, due to the maternal mode of inheritance of the mtDNA, studying nuclear autosomal markers is also useful, especially in species displaying a complex social

behaviour. Previous studies on cetaceans (e.g. Bérubé et al., 1998) have suggested that a nuclear gene flow may occur, whereas mtDNA reveals a strong geographical structure among populations. Using polymorphic nuclear markers, Valsecchi et al. (2004) found a significant differentiation between striped dolphin samples from the North Sea (N=6) and the Mediterranean Sea (N=98). The present study compares the patterns of genetic differentiation and levels of genetic diversity, using selectively neutral markers, between striped dolphin samples from the Mediterranean Sea (N=78), the Atlantic Ocean (N=45) and the North Pacific Ocean as an outgroup (N=14).

MATERIALS AND METHODS

Sample collection

Samples, consisting of muscle, skin and blubber, liver, or kidney from 123 dead stranded individuals, were collected in the western Mediterranean and the North Atlantic between 1989 and 2004. Samples from 14 dead by-caught individuals from the North Pacific were added as an out-group (Figure 1). Samples were stored at -20°C .

Laboratory protocols

Deoxyribonucleic acid (DNA) extraction was performed using standard phenol:chloroform methods (Sambrook & Russell, 2000). Individuals were genotyped at five microsatellite loci. Three loci were dinucleotide repeats:

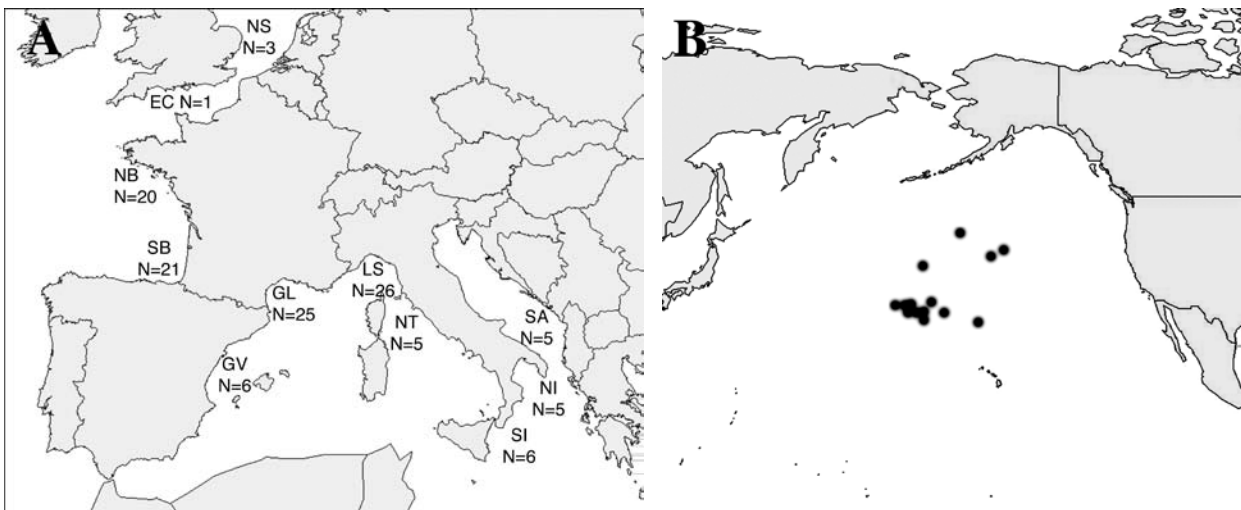


Figure 1. (A) Sampling locations of the 78 Mediterranean and 45 Atlantic stranded individuals. The Mediterranean samples came from the Gulf of Valencia (GV), the Gulf of Lions (GL), the Ligurian Sea (LS), the northern Tyrrhenian Sea (NT), southern Ionian Sea (SI), the northern Ionian Sea (NI) and the southern Adriatic Sea (SA). The 'Atlantic' samples came from the southern Bay of Biscay (SB), the northern Bay of Biscay (NB), the English Channel (EC) and the North Sea (NS); N, number of individuals sampled at each location; (B) sampling locations of the 14 dead by-caught individuals from the North Pacific; each black spot indicates a sampling location.

MK6 and MK9 (isolated from *Tursiops aduncus*, Krützen et al., 2001), EV92Mn (isolated from *Megaptera novaeangliae*, Valsecchi & Amos, 1996). Two loci were tetranucleotide repeats: GATA053 and GATA098 (isolated from *Megaptera novaeangliae*, Palsbøll et al., 1997). Polymerase chain reactions (PCR; Mullis & Faloona, 1987) were conducted in 20 μ l volumes containing 10 μ l of Qiagen Multiplex Mix (Qiagen GmbH, Hilden, Germany), primers to a final concentration of 0.2 to 0.5 μ M, and purified water to a final volume of 20 μ l. One primer of each pair (provided by Prologo, France) was fluorescently labelled. Reactions were cycled using the Eppendorf Gradient Mastercycler 5331 (Eppendorf AG, Hamburg, Germany) as follows: 15 min at 95 °C, then 34 cycles of 30 s at 94 °C, 90 s at 51 °C and 90 s at 72 °C. A final extension step of 10 min at 72 °C was added. Migrations were performed on a ABIprism automated sequencer (Applied Biosystems, Foster City, CA, USA) with the Genescan 500 TAMRA size marker, and gels were analysed using ABI GENESCAN and GENOTYPER softwares (Applied Biosystems, Foster City, CA, USA).

Statistical analyses

The allelic richness (R) in each sample was computed as described in the FSTAT v. 2.9.3.2 package (Goudet, 1995). The unbiased expected heterozygosities (H_e) and genetic distances were estimated after Nei (1978) and computed using the GENETIX (Belkhir et al., 1996–2004) package. Significance of the difference between the mean variability indices among the basins was tested using Student's paired *t*-test. The Hardy–Weinberg (HW) exact test was performed after Guo & Thompson (1992) using the GENEPOP (Raymond & Rousset, 1995) package. Frequencies of null alleles were estimated using the MICROCHECKER (Van Oosterhout et al., 2004) package. Population structure was analysed computing Wright's (1969) F-statistics according to Weir & Cockerham (1984), using the GENETIX package. Population differentiation was estimated using F_{ST} , whereas

departure from HW equilibrium (HWE) within the samples was measured using F_{IS} . Values of R_{ST} , accounting for allele sizes, were computed after Michalakis & Excoffier (1996) using the GENEPOP package. Significance of the deviation from zero of Nei's (1978) genetic distances (D) and Wright's (1969) F-statistics were assessed by permutation tests.

RESULTS

Amplifications were successful for the five loci, among which were GATA098 and GATA053, two tetranucleotide markers which had not been previously amplified in this species.

Linkage disequilibrium

No significant linkage disequilibrium was detected when all samples were pooled. However, when pooling the samples by basin, significant linkage disequilibrium was observed only within the Mediterranean, between two pairs of loci: GATA098/GATA053 ($P=0.038$) and MK9/GATA053 ($P=0.028$).

Genetic diversity

The number of alleles per locus and population ranged from six (GATA053 in the Mediterranean and the Pacific) to 21 (MK6 in the Atlantic) (Table 1). The allelic richness (R) per locus and population ranged from 3.8 (GATA053 in the Mediterranean) to 13.0 (MK6 in the Pacific). The unbiased expected heterozygosity (H_e) per locus and per population ranged from 0.497 (GATA053 in the Mediterranean) to 0.931 (MK6 in the Pacific), suggesting a high but variable level of polymorphism across loci. The observed heterozygosity (H_o) per locus and per population ranged from 0.323 (GATA053 in the Mediterranean) to 1.000 (MK6 in the Pacific).

The mean number of alleles per locus in each population ranged from 9.6 in the Pacific to 13.4 in the Atlantic (Table 1). The mean allelic richness (R) was significantly higher

Table 1. Genetic variation at five microsatellite loci in *Stenella coeruleoalba*.

Locus/ parameters	Mediterranean	Atlantic	Pacific	Total
<i>GATA098</i>				
n	73	42	14	129
N _A	11	11	10	11
R	6.980	8.014	9.152	7.923
He	0.839	0.871	0.897	0.865
Ho	0.808	0.833	0.857	0.821
<i>MK9</i>				
n	65	42	14	121
N _A	11	13	9	16
R	6.395	8.865	8.132	7.865
He	0.754	0.882	0.868	0.827
Ho	0.754	0.786	0.857	0.777
<i>GATA053</i>				
n	62	41	14	117
N _A	6	9	6	10
R	3.760	5.861	5.142	4.822
He	0.497	0.621	0.669	0.564
Ho	0.323	0.610	0.714	0.470
<i>MK6</i>				
n	65	40	12	117
N _A	19	21	15	25
R	9.141	11.086	12.998	10.536
He	0.850	0.914	0.931	0.895
Ho	0.908	0.925	1.000	0.923
<i>EV92Mn</i>				
n	56	38	10	104
N _A	9	13	8	15
R	6.853	8.060	8.000	8.093
He	0.840	0.848	0.863	0.865
Ho	0.625	0.526	0.500	0.577
<i>Mean ±SD</i>				
n	64.2 ±6.14	40.6 ±1.67	12.8 ±1.79	
N _A	11.2 ±4.82	13.4 ±4.56	9.6 ±3.36	
R	6.63 ±1.92	8.38 ±1.88	8.68 ±2.83	
He	0.756 ±0.150	0.827 ±0.118	0.846 ±0.102	
Ho	0.684 ±0.226	0.736 ±0.164	0.786 ±0.189	

n, number of genotyped individuals; N_A, number of different alleles detected; R, allelic richness based on a minimum sample size of ten individuals; He, Nei's (1978) unbiased expected heterozygosity; Ho, observed heterozygosity; SD, standard deviation.

Table 3. Multilocus indices of genetic differentiation between three basins. Values below diagonals were computed using data from the five microsatellite loci, while values above diagonals were computed excluding the data from locus *EV92Mn*. For *D* and *F_{ST}*, asterisks indicate significant deviations from zero (*, P<0.05; **, P<0.01; ***, P<0.001).

D	Med	Atl	Pac	F _{ST}	Med	Atl	Pac	R _{ST}	Med	Atl	Pac
Med		0.066***	0.106***	Med		0.023***	0.036***	Med		0.003	0.032
Atl	0.085***		0.045***	Atl	0.024***		0.009	Atl	0.013		-0.009
Pac	0.166***	0.092***		Pac	0.042***	0.015*		Pac	0.026	-0.010	

(A)

(B)

(C)

(A), pairwise values of Nei's (1978) genetic distance; (B) pairwise values of Wright's F_{ST}; (C) pairwise values of R_{ST}; Med, Mediterranean; Atl, Atlantic; Pac, Pacific.

Table 2. Values of F_{IS} and results of the Hardy-Weinberg equilibrium exact test. The 'HWE' rows indicate the P-value of HWE exact test.

Locus/parameters	Mediterranean	Atlantic	Pacific
<i>GATA098</i>			
F _{IS}	0.0363	0.0437	0.0459
HWE	0.2927	0.4984	0.8816
<i>MK9</i>			
F _{IS}	-0.0002	0.1105*	0.0127
HWE	0.4243	0.0230	0.9177
<i>GATA053</i>			
F _{IS}	0.3531*	0.0182	-0.0700
HWE	0.0000	0.6532	0.1915
<i>MK6</i>			
F _{IS}	-0.0691	-0.0119	-0.0776
HWE	0.4546	0.8143	0.8428
<i>EV92Mn</i>			
F _{IS}	0.2579*	0.3823*	0.4340*
HWE	0.0000	0.0000	0.0213
<i>All loci</i>			
F _{IS}	0.0965*	0.1115*	0.0746
HWE	< 0.001	< 0.001	0.3007

*, indicates that the F_{IS} value is significantly different from zero (P<0.05).

both in the Atlantic (R=8.38) and the Pacific (R=8.68) than in the Mediterranean (R=6.63) (P<0.05). The mean multilocus He was also significantly higher in the Atlantic (He=0.827) and the Pacific (He=0.846) than in the Mediterranean (He=0.756) (P<0.05) with no significant difference between the Atlantic and the Pacific. The mean multilocus Ho was lower in the Mediterranean (Ho=0.684) than in the Atlantic (Ho=0.736) and the Pacific (Ho=0.786), but the pairwise differences in Ho were not significant (P>0.05).

Hardy-Weinberg equilibrium (HWE)

Most of the loci were in HWE. However, the heterozygote deficiency was significant for five locus/basin combinations among 15 (indicated by asterisks in Table 2). The multilocus computation of the F_{IS} and HWE exact test showed a significant heterozygote deficiency in the Mediterranean and the Atlantic. One of the markers (*EV92Mn*) was

suspected of bearing null allele(s) in the three basins, with an estimated frequency of 0.12 in the Mediterranean, 0.19 in the Atlantic and 0.19 in the Pacific. However, results for genetic variability and HWE remained similar when excluding EV92*Mn* from the analyses (data not shown, available upon request), except that the departure from HWE in the Atlantic was no longer significant ($P=0.142$, $F_{IS}=0.0413$).

Genetic differentiation between the basins

The overall multilocus F_{ST} value (accounting for allele identity) was 0.025 considering all loci, and 0.023 excluding EV92*Mn*. The overall multilocus R_{ST} value (accounting for allele size) was lower with values of 0.011 (all loci) and 0.005 (without EV92*Mn*).

Pairwise Nei's (1978) genetic distance (D), F_{ST} and R_{ST} values indicated that the highest nuclear genetic differentiation occurred between the Mediterranean and Pacific populations (Table 3). Computation of multilocus pairwise Nei's (1978) genetic distance (D) and Wright's (1969) F_{ST} indicated a slight but highly significant differentiation between the Mediterranean and Atlantic populations ($P<0.001$; Table 3A,B). Most analyses indicated a higher or more significant differentiation between the Mediterranean and the Atlantic than between the Atlantic and the Pacific.

DISCUSSION

A higher genetic diversity within the Atlantic

In order to compare the patterns of genetic differentiation and the levels of genetic diversity among striped dolphin populations, 45 Atlantic samples mainly from the Bay of Biscay and 78 Mediterranean samples were analysed. The values computed for R and H_e within each basin showed a higher nuclear genetic diversity within the Atlantic than within the Mediterranean; this trend was also observed for the mean number of alleles (N_A) per locus largely dependent on the number of individuals sampled. This conclusion is consistent with Valsecchi et al.'s observations (2004) suggesting a higher level of allelic diversity in a North Sea sample ($N=6$) than in a Mediterranean sample ($N=98$), although this difference was not significant. Regarding mitochondrial DNA, García Martínez et al. (1999) found that mtDNA nucleotide diversity was higher in an Atlantic sample ($N=22$) than in a Mediterranean sample ($N=76$). Thus, the Atlantic population would be significantly more polymorphic than the Mediterranean population, regarding both nuclear and mitochondrial DNA.

Various hypotheses could explain the Atlantic population's higher genetic diversity. One explanation would be that the older a population is, the more polymorphic it is, as suggested by García Martínez et al. (1999). A second explanation would be a higher effective population size of the Atlantic population in relation to its large geographical range. The genetic diversity in the Pacific sample, despite its low number of individuals ($N=14$), is approximately similar to, or even slightly higher than, the diversity in the Atlantic sample.

Genetic isolation between the basins

Overall, based on autosomal microsatellites allelic frequencies information (F_{ST}), the differentiation between

Stenella coruleoalba populations is low. The fact that the greatest genetic differentiation occurs between the Mediterranean and Pacific samples is consistent with the permanent isolation between those two basins since the worldwide radiation of *S. coruleoalba* (LeDuc et al., 1999).

The computation of Nei's (1978) genetic distance and Wright's (1969) F_{ST} indicates a slight but significant genetic differentiation between the Atlantic and the Mediterranean samples, suggesting a partial genetic isolation. When assuming an island model (Wright, 1969), the estimated number of migrants per generation (Nm) can be computed from F_{ST} , where N is the effective population size and m the fraction of the population replaced by migrants at each generation. The computation of Nm gives a rough estimate of 10.30 with all loci, and 10.60 without EV92*Mn*. Due to sampling characteristics and model assumptions, this estimation may not reflect an actual number of migrants. However, it seems to differ notably from the mtDNA Nm estimation of 0.98 by García Martínez et al. (1999), where N is the effective number of females (Slatkin, 1989). The magnitude difference between these two rough estimates arising from different markers might suggest that the male-mediated gene flow could be higher than the female-mediated gene flow, but a precise quantitative assessment remains to be undertaken.

Surprisingly, most indices of genetic diversity indicate that the nuclear genetic differentiation between the Mediterranean and the Atlantic populations is greater than the differentiation between the Atlantic and the Pacific populations. The striped dolphin is essentially a tropical and temperate water species, and it is usually found neither in the waters off southern Argentina or Chile, nor in the Canadian Archipelago (Archer II & Perrin, 1999). Moreover, the closure of the Panama seaway dates back to the late Pliocene (3–4 million years ago; Coates et al., 2004). Consequently, the low genetic differentiation between the Atlantic and Pacific samples is hardly accounted for by a significant current gene flow between these two oceans.

A significant heterozygote deficiency within the Mediterranean sample only

Though the Atlantic and Pacific samples are in Hardy–Weinberg equilibrium, the Mediterranean sample displays significant heterozygote deficiency, in particular regarding GATA053, one of the newly-amplified markers. Several hypotheses could explain this observation (Crouau-Roy, 1988), such as a bias due to the sampling of stranded animals, a significant inbreeding within the population, or a 'Wahlund effect' due to the sampling of two or more reproductively distinct populations. The hypothesis that stranded individuals might be, in some cases, more homozygous than the population they represent is discussed by Valsecchi et al. (2004). In the present study, though the Atlantic sample consists solely of stranded individuals, it does not display such a significant heterozygote deficiency. Furthermore, significant linkage disequilibrium (involving the two newly-tested tetranucleotides) is observed only within the Mediterranean sample, which can also be the consequence of the sampling of two or more reproductively distinct Mediterranean populations. So the Mediterranean

striped dolphin population would itself be subdivided (this would be consistent with the bimodal distribution of the allelic frequencies observed within this basin for the tetranucleotide locus GATA098). Performing assignment tests, Valsecchi et al. (2004) found some slight indices of genetic differentiation according to geographical distance between sampling regions within the Mediterranean (e.g. Gulf of Valencia, Ligurian Sea). Gaspari (2004) also found significant genetic differentiation within the Mediterranean, between 'inshore' and 'offshore' striped dolphin groups. Further studies, such as analyses based on haplotypic data, could be carried out to investigate the exact relationship between possible Mediterranean distinct lineages.

Conservation of the Mediterranean striped dolphins

Though it does not seem to be in imminent danger of extinction, the Mediterranean striped dolphin population lives in a highly humanized area, and faces a range of threats whose effects are difficult to quantify. This study shows that, regarding nuclear markers, the Mediterranean population as a whole is genetically less variable than the Atlantic or Pacific populations, and is partially reproductively isolated. Given its genetic characteristics, the Mediterranean striped dolphin population should therefore benefit from an adequate conservation effort.

We are grateful to W. Dabin, A. Dewez, F. Dhermain, J.-L. Fabre, B. Jakobsen, B. Lafitte, A.-S. Lemaire, and E. Valsecchi who provided samples, and to M. Bonhomme, F. Magné and A. Rozzi for technical laboratory assistance. We are also grateful to P. De la Rúa, J. Galián, A. Ortiz and J. Serrano for advice at various stages of the project and helpful comments on the manuscript, and to M. Oñate and B. Séverac for reviewing the English.

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Submitted 15 June 2006. Accepted 17 October 2006.

