A note on genetic isolation of Mediterranean sperm whales (*Physeter macrocephalus*) suggested by mitochondrial DNA

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ABSTRACT

Thirteen sperm whales were sampled, using sloughed skin, in the Mediterranean Sea during six distinct encounters. Individuals were discriminated using the results of molecular sexing, mitochondrial control region sequencing and microsatellite genotyping (3 loci). Samples from 57 specimens were available from sperm whale strandings on northern European coasts. The first ~200bp of the mitochondrial DNA (mtDNA) control region of each sample were sequenced and three different haplotypes were identified. The frequency of each haplotype was significantly different between the Mediterranean Sea and the eastern North Atlantic, suggesting that sperm whales in the two areas comprise different maternal entities.

KEYWORDS: SPERM WHALE; GENETICS; EUROPE; MEDITERRANEAN SEA; NORTH ATLANTIC; STOCK IDENTITY

INTRODUCTION

The sperm whale (*Physeter macrocephalus*) has a cosmopolitan distribution. Genetic analyses to date have detected comparatively low levels of variation in mitochondrial (mt) DNA on a global scale. While the levels of variation at nuclear loci are similar to those reported in other large whales, the degree of divergence within ocean basins among putative sperm whale populations is low in both genomes. For example, genetic inter-oceanic differentiation was detected in mtDNA between North Atlantic and Pacific sperm whales by Lyrholm and Gyllensten (1998) but no genetic heterogeneity was detected within North Pacific sampling areas. In contrast, Richard *et al.* (1996) detected significant levels of genetic heterogeneity among sperm whale pods (mature females accompanied by immature male and female individuals) at the Galapagos Islands, possibly due to matrilineal pod structure of sperm whales at low latitudes.

Sperm whales are the second most common large whales observed in the Mediterranean Sea after fin whales (*Balaenoptera physalus*). A central question to the management and conservation of the species in the Mediterranean Sea, where abundance and movements through the Strait of Gibraltar are poorly known, is whether sperm whales in the Mediterranean are isolated from the eastern North Atlantic populations. This study represents a first attempt to test the hypothesis of a homogeneous distribution of genetic variation among sperm whales in the Mediterranean and eastern North Atlantic. To test for any deviation from the null-hypothesis, nucleotide sequences from the first part of the maternally inherited mtDNA control region were collected and analysed.

MATERIAL AND METHODS

Sample collection

Skin samples ($n = 36$) were collected in the Mediterranean Sea during 1998, 1999 and 2001 summer surveys. All samples were collected as sloughed skin from free-ranging sperm whales observed in six distinct groups encountered in four different areas of the Mediterranean Sea: the Tyrrhenian Sea; the Ionian Sea; the North western Basin; and the Balearic Sea (Table 1 and Fig. 1). Group composition was extrapolated from the estimated size of the animals (Rice, 1989). All sightings were assumed to be of different groups unless at least one individual of the group was re-sighted (based on photo-identification).

Samples from the eastern North Atlantic (Table 2) were available from animals stranded in Scotland ($n = 26$), Ireland ($n = 4$), Belgium ($n = 5$) (Holsbeek *et al.*, 1999; Joiris *et al.*, 1991), Netherlands ($n = 3$) (Holsbeek *et al.*, 1999), Norway ($n = 2$) and Denmark ($n = 17$) (Kinze *et al.*, 1998). As was the case in the Mediterranean Sea, samples from the North Atlantic were from a wide-ranging area (Fig. 1), thereby ensuring that sampling was not biased to only a single group of sperm whales in either area (see Richard *et al.*, 1996). The Atlantic samples were mostly from male animals, some of them mass stranded (Table 2). After collection, samples were preserved in a saturated sodium chloride solution with 20% dimethyl sulfoxide.

Laboratory analysis

Two different methods of DNA extraction were employed: a standard phenol/chloroform extraction protocol (Sambrook and Russell, 2001) and an extraction kit (DNeasy Tissue Kit™, Qiagen Inc.). The first ~200bp of the 5' end of the mtDNA control region was amplified using a forward primer (MT4F) designed by Arnason *et al.* (1993) and Bp16071R (5'-CCTCAGTTATGTTATGATCATGGGC-3'). This approach was necessitated by the degraded nature of DNA extracted from the sloughed skin samples. The initial symmetric PCR amplifications were carried out in a total volume of 20 μL consisting of: 0.2 μM of each dNTP; 67mM Tris-Cl (pH 8.8); 2mM $MgCl_2$; 17mM NH_3SO_4; 10mM β-mercaptoethanol; 0.1μM of each primer; 0.4 units of *Taq* DNA polymerase. Negative and positive controls were included to detect possible contamination as well as loading

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Table 1

Details of the sperm whale tissue (sloughed skin) samples obtained from the Mediterranean Sea (Group composition: Ad=Adult; Juv=juveniles; N-b=new-born; N_{SS}=Number of sloughed skin samples collected; N_I=Number of individuals genetically identified) and results of molecular sexing (F=female; M=male; U=undetermined) and allele size (bp) for the 3 microsatellite loci analysed (*where electrophoresis was performed but no sufficient result was obtained).

Gp	Date of encounter	Location	Gp size	Group composition	N_{SS}	N_I	Sex	EV001	GATA 053	GT011
A	21/06/98	Tyrrhenian Sea	5	3 Ad, 1 Juv, 1 N-b	4	3	F F U	121/128 120/124 121/134	200/216 200/216 202/223	* * *
B	14/07/98	Ionian Sea	7	4 Ad, 2 Juv, 1 N-b	3	1	F	121/139	*	*
C	05/08/99	Northwestern basin	1	1 Ad	1	1	M	128/141	207/223	*
D	29/06/00	Ionian Sea	5	3 Ad, 1 Juv, 1 N-b	5	4	F F F M	128/130 120/130 120/124 124	208/223 199/212 212/224 212/223	108/114 101/114 * 100/115
E	08/07/01 12/07/01 13/07/01	Baleares	6	2 Ad, 2 Juv, 1 N-b	22	3	F F M	124 124 124	203/211 203/211 203/207	116/120 114/118 119/121
F	19/08/01	Northwestern basin	1	1 Ad	1	1	1 M	124	*	116/120
Total			**24**		**36**	**13**				

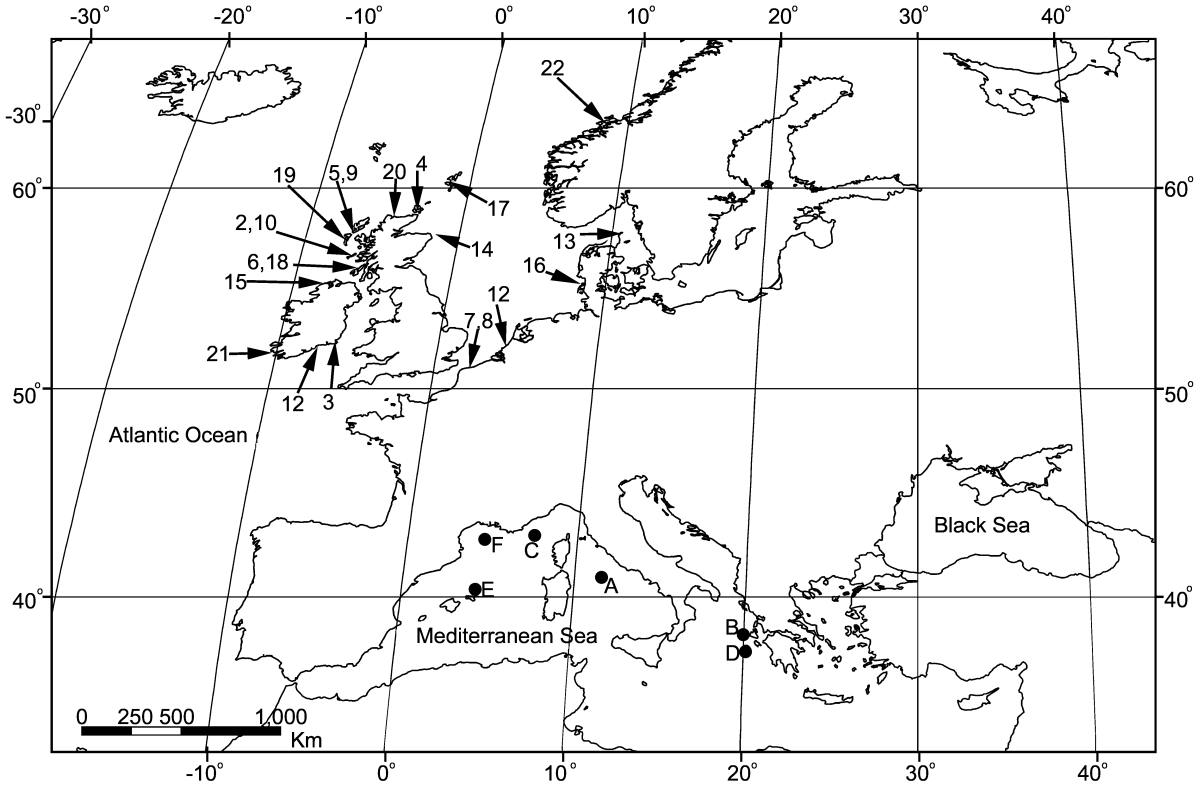

Fig. 1. Map showing the sampling sites in the Mediterranean Sea (letters refer to sightings described in Table 1) and along the North eastern Atlantic coast (numbers refer to stranding described in Table 2).

errors. Reactions were performed on thermal cyclers (MJ Research Inc.) and consisted of 2 minutes of denaturing at 94° Celsius, followed by 28-33 cycles of denaturing at 94° Celsius, for 1 minute; annealing at 54° Celsius for 1 minute and extension at 72° Celsius for 4 minutes. Cycling sequencing was conducted according to the manufacturer's instructions (Dye Terminator Cycle Sequencing Ready Reaction Kit™, Applied Biosystems, Inc.). The order of sequencing products was resolved using an Applied Biosystems ABI Prism™ 377 automated sequencer. Sex was determined using the multiplex approached presented by Bérubé and Palsbøll (1996). To ensure only sloughed skin samples collected from different individuals were included in the test we determined the genotype at three microsatellite loci; EV001 (Valsecchi and Amos, 1996), GATA 053 (Palsboll *et al.*, 1997) and GT011 (Bérubé *et al.*, 1998). Amplifications were conducted as described in the original primer notes with fluorescent end labelling. The

Table 2

Details of tissue samples obtained from sperm whales stranded on the Northeastern Atlantic coast. N=Number of animals sampled (MS=Mass stranding). Type of tissue used (necropsy): S=Skin; M=Muscles; Sex (from field observation) M=male; F=Female; H=haplotype (as defined in Table 3), *n*=number of samples, empty cells correspond to failed DNA extraction or sequencing.

ID No.	Date of stranding	Country	Location	N	Tissue	Sex	Size range (m)	H (*n*)
1	12/02/89	Belgium	Unknown	1	M	M	Adult	2 (1)
2	21/11/93	Scotland	Lochailort, Highland	1	S	M	15	2 (1)
3	26/09/93	Ireland	Kilmore Quay, Wexford	1	S	F	11.07	-
4	07/12/94	Scotland	Backaskail Bay, Sanday Orkney	11 (MS)	S	All M	12-13.4	1(8), 2(3)
5	13/03/94	Scotland	Hougharry, Western Isles	1	M	M	13	2 (1)
6	27/03/94	Scotland	Campa Islay, Argyll	1	M	M	16	1 (1)
7	18/11/94	Belgium	Koksijde	3	M	M	14.4-15.4	1(2), 2(2)
8	18/11/94	Belgium	Nieuwpoort	1	M	M	18.2	-
9	19/01/95	Scotland	Benbecula Western Isles	1	M	M	14	-
10	23/03/95	Scotland	Carse of Ardersier, Highland	1	S	M	13.7	1 (1)
11	15/06/95	Ireland	Youghal, Co. Donegal	1	S	F	7.22	1 (1)
12	12/01/95	Netherlands	Scheveningen	3	M	M	15.2-15.4	1 (3)
13	25/01/96	Denmark	Hulsig	1		M	13.1	2 (1)
14	28/01/96	Scotland	Cruden Bay, Grampian	6 (MS)	S	All M	12.1-12.8	1(1), 2(5)
15	20/03/96	Ireland	Tory Island, Co. Donegal	1	S	M	14.8	-
16	27/03/96	Denmark	Rømø	16 (MS)	S	All M	11.9-13.2	1(9), 2(7)
17	08/09/96	Scotland	Mousa, Shetland	1	M		'Large'	3 (1)
18	27/12/96	Scotland	Traigh Angus, Islay Strathclyde	1	S	M	14.4	2 (1)
19	20/03/98	Scotland	West Gerinish, Western Isles	1	S	M	~10	-
20	06/08/98	Scotland	Bettyhill, Highland	1	S	M	12.2	1 (1)
21	20/05/99	Ireland	Brandon, Co. Kerry	1	S	M	~15	-
22	28/04/99	Norway	Storjford	1	S	M	15.4	1 (1)
23	2001	Norway	Unknown	1	S			2 (1)

amplification products were separated and sized using an Applied Biosystems ABI Prism™ 377 automated sequencer.

Data analysis

The assessment of the degree of genetic differentiation between Mediterranean and Atlantic samples was based on the comparison between the observed and expected mtDNA haplotype frequencies and was tested using a G-test (or likelihood ratio test) for goodness of fit (Sokal and Rohlf, 1995).

RESULTS

DNA was extracted successfully from all sloughed skin samples from the Mediterranean Sea and for 52 of the necropsy samples from the eastern North Atlantic sperm whales. Approximately 25mg of tissue was sufficient to extract DNA from necropsy samples, however 40mg was needed from sloughed skin samples. Whale sex was successfully identified in 31 sloughed skin samples. Samples for which microsatellite loci analyses failed or where the genotype at a locus was ambiguous were discarded from the statistical analysis. From the combined results of the sexing, mtDNA control region sequences and microsatellite loci genotypes 13 samples were included from the Mediterranean Sea (Table 1), all of which differed at a minimum of one of the three loci.

Approximately 200 nucleotides of the 5' end of the mtDNA control region were successfully sequenced. Only two polymorphic sites were identified, defining three distinct different haplotypes (Table 3). Haplotype 1 was most frequent and observed in 64% of all samples, followed by haplotype 2, which was observed in 35% of all samples. Haplotype 3 was rare and observed only in a single individual (Table 3). The polymorphic nucleotide position defining the last haplotype has not previously been reported. The comparison between the Atlantic and Mediterranean populations was based on the proportion of each different haplotype among the samples collected in each area. All individuals sampled in the Mediterranean shared the same haplotype: haplotype 1 (Table 3). In contrast haplotype 1 was observed in 54% of the Atlantic individuals and haplotype 2 in 44%. Thus, while no nucleotide diversity was observed in the Mediterranean samples (one unique lineage), three haplotypes were observed among the eastern North Atlantic samples (nucleotide diversity of 1.5). The frequencies of haplotypes were significantly different between the Mediterranean Sea and eastern North Atlantic ($G_{[2df]} = 14.0$, $p < 0.01$).

Although females and immature whales in other areas have been seen to form long-term stable groups within which there is substantial genetic similarity (Dillon, 1996; Richard *et al.*, 1996; Dufault *et al.*, 1999), it is unlikely that the results have been affected by intra-group homogeneity in mtDNA control region as the Mediterranean sequences appeared to be monomorphic (a single haplotype). However, the difference in the haplotype frequency was also tested by including only one sample from each group. Although this reduced by half the sample size from the Mediterranean Sea, the difference was still significant ($G_{[2df]} = 12.3$, $p < 0.01$).

DISCUSSION

Although this analysis found a low level of intra-specific variation in the mtDNA control region as reported by Lyrholm *et al.* (1996), the spatial distribution of this variation was not homogenous. The significant level of divergence between the Mediterranean Sea and the eastern North Atlantic is consistent with the notion of restricted movement of groups between the two areas and suggests a resident sperm whale population in the Mediterranean Sea. A similar discreteness in the distribution of variation at the mtDNA control region has previously been observed between the Mediterranean and the eastern North Atlantic in striped dolphin, *Stenella coeruleoalba*, (Archer, 1996) and fin whale (Bérubé *et al.*, 1998).

Table 3

The mitochondrial control region haplotypes detected in the study and the respective frequencies in the two sampling areas.

Nucleotide position*	9	58	62	105	121	184	Sampling area
Published**	C	T/C	C/T	C/T	C/T	T/C	
Haplotype 1	C	T	T	C	C	T	Mediterranean Sea (n=13) Eastern North Atlantic (n=28)
Haplotype 2	C	T	C	C	C	T	Eastern North Atlantic (n=23)
Haplotype 3	A	T	T	C	C	T	Eastern North Atlantic (n=1)

*Position with reference to the sequences reported by Lyrholm and Gyllensten (1998); **Lyrholm and Gyllensten (1998).

The exclusive maternal inheritance of the mitochondrial genome means that the results reflect different maternal structures between the two areas, but nothing with respect to inter-breeding between the two areas. Differentiation in mtDNA haplotypes is consistent with the behaviour of female sperm whales, which have been observed to show fidelity to areas. The observation of newborn calves in different areas of the Mediterranean basin also suggests that females remain in the Mediterranean Sea to breed. However, these data cannot answer the question of whether Atlantic male sperm whales enter the Mediterranean to breed with females, or whether 'resident' males and females co-exist in the Mediterranean without interbreeding with Atlantic animals. Visual surveys in the area of the Strait of Gibraltar, the only possible passage between the two areas, suggested that sperm whales are present in the area for foraging rather than migratory purposes and do not support the hypothesis of a consistent migration pattern through the Strait (De Stephanis *et al.*, pers. comm.). Widespread inter-breeding with Atlantic males seems unlikely but additional analyses of nuclear mendelian inherited loci are needed to establish conclusively whether Mediterranean Sea sperm whales form a distinct population from the eastern North Atlantic or two different maternal entities as concluded from this preliminary study.

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