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Mitochondrial DNA analysis of harbour porpoises (*Phocoena phocoena*) in the Baltic Sea, the Kattegat–Skagerrak Seas and off the west coast of Norway

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Abstract Incidental mortality of harbour porpoises, *Phocoena phocoena*, from captures in fishing gear has been documented consistently as severe in some European waters. Since fishing effort varies greatly among regions, management units must be defined for more effective conservation of this species. In this study, analysis of mitochondrial DNA (mtDNA) restriction fragments was performed to investigate the population structure of harbour porpoises in the Baltic Sea, the joint Kattegat–Skagerrak Seas and off the west coast of Norway. Mitochondrial DNA of 65 harbour porpoises collected from three regions was analysed with nine restriction enzymes. Analysis of the heterogeneity in the frequency distribution of haplotypes among provisional subpopulations revealed significant differences, which supports the recognition of three different subpopulations and thus three separate management units. Furthermore, indices of haplotypic diversity (range: 0.211 to 0.628) and nucleotide diversity (range: 0.070 to 0.168) of these provisional subpopulations were much lower than for western North Atlantic subpopulations, which is consistent with the view that harbour porpoises in European waters, particularly in the Baltic Sea, are depleted.

Introduction

The incidental mortality of harbour porpoises due to commercial fisheries has been identified as the major

threat to eastern North Atlantic harbour porpoises (Perrin et al. 1994). In some areas, incidental deaths have reached an annual level of 2.9 to 5% of the estimated population size which may be unsustainable (Smith et al. 1993; Carlström and Berggren 1996). In most areas, the lack of information on the population structure, distribution, abundance and mortality hinders a thorough assessment of the status of this species. Both the International Whaling Commission (IWC) and the Agreement on the Conservation of Small Cetaceans of the Baltic and North Seas (ASCOBANS) have recommended that research on these topics be conducted on the harbour porpoises in the North Atlantic. The need to investigate the population structure to facilitate an assessment of the status of harbour porpoises in the North Atlantic Ocean has been stressed by a recent comprehensive survey in the North Sea and adjacent waters (Hammond et al. 1995) and the launching of independent observer schemes to monitor bycatches in some areas.

Three global populations of harbour porpoises were proposed by Gaskin (1984) and supported by genetic analysis (Rosel et al. 1995): North Pacific, North Atlantic and Black Sea/Sea of Azov. Morphometric and meristic comparisons of skulls by Yurick and Gaskin (1987) showed that western and eastern North Atlantic porpoises were different and warranted classification as separate populations. Several studies have attempted to show genetic structuring within each of the two North Atlantic populations (Kinze 1985; Yurick and Gaskin 1987; Amano and Miyazaki 1992; Anderson 1993; Walton 1995) but conclusions have been contradictory. Thus, the population structure of harbour porpoises in the Baltic Sea, the Kattegat–Skagerrak Seas and off the west coast of Norway remains poorly understood. Preliminary results from morphometric studies (Börjesson and Berggren 1993) have indicated that harbour porpoises collected in the Swedish Baltic and Kattegat–Skagerrak Seas belong to different populations even though the geographic distance between these seas is not great. Bycatch of harbour porpoises occurs year-round

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in these areas (Berggren 1994) at levels believed to be unsustainable (Berggren 1995). Furthermore, a decline in the number of sightings of harbour porpoises in the Baltic Sea and Kattegat–Skagerrak Seas during the last decades has been reported by Berggren and Arrhenius (1995a). The present situation in the Baltic Sea appears to be the most serious with very few sightings reported during the last decade (Skora et al. 1988; Berggren and Arrhenius 1995b).

Mitochondrial DNA analyses have been used successfully for defining smaller management units within exploited populations of harbour porpoises, *Phocoena phocoena* (Rosel et al. 1995; Wang et al. 1996). In the present study, we analyzed restriction fragments of mtDNA from harbour porpoises captured incidentally in the Swedish Baltic Sea, the Kattegat–Skagerrak Seas and off the west coast of Norway to investigate whether porpoises from these areas belong to the same or separate genetic entities.

Materials and methods

Sampling areas and tissue collection

Skeletal muscle tissue was collected from 65 harbour porpoises killed incidentally by gillnet fisheries of the Swedish Baltic Sea (27), the Kattegat–Skagerrak Seas (25), and off the west coast of Norway (13) between 1985 and 1993 (Fig. 1). The samples were frozen (-20°C) initially for up to 8 years before being preserved in a solution of 20% dimethyl sulphoxide (DMSO), 0.25 M ethylenediaminetetraacetic acid disodium salt (sodium EDTA) and saturated with NaCl (Seutin et al. 1991). DNA extraction, agarose gel electrophoresis and Southern transfer followed standard methods with minor modifications (Southern 1975; Maniatis et al. 1982); mtDNA was hybridized to radioactively labelled pAM1 probe which contained the complete mouse mtDNA (Martens and Clayton 1979). For more details on methods and modifications, see Wang (1993) and Wang et al. (1996). The restriction enzymes selected were the same as in Wang et al. (1996) and had recognition sequences of six base pairs (bp) (*Bam*HI, *Bgl*II, *Eco*RI and *Hind*III) or four bp (*Alu*I, *Hae*III, *Hin*fI, *Mbo*I and *Taq*I).

Data organization

A letter designation was given to each restriction pattern following the patterns described in Wang et al. (1996). Each porpoise was described by a composite of nine letters representing the nine enzymes. The order of the letters in a composite corresponds to the alphabetical order of the enzyme names: *Alu*I, *Bam*HI, *Bgl*II, *Eco*RI, *Hae*III, *Hind*III, *Hin*fI, *Mbo*I and *Taq*I. Each unique combination of nine letters characterized a different mtDNA haplotype.

The molecular sizes of the restriction fragments of harbour porpoise mtDNA were estimated using standard curves of mobility versus molecular size generated by polynomial regression analysis from the mobilities of fragments of known size (computer program provided by R.G. Danzmann, University of Guelph, Guelph, Ontario, Canada). The fragments of known size were of bacteriophage lambda (λ) DNA digested with *Eco*RI and *Hind*III. As in Wang et al. (1996), fragments smaller than 360, 390 and 280 bp were not scored for *Alu*I, *Hin*fI and *Mbo*I digests, respectively, or for *Hae*III and *Taq*I, for which the lower limit was 290 bp.

The presence (or absence) of restriction sites was inferred from the fragment patterns on the assumption that changes in restriction sites were caused by single base substitutions within the recognition

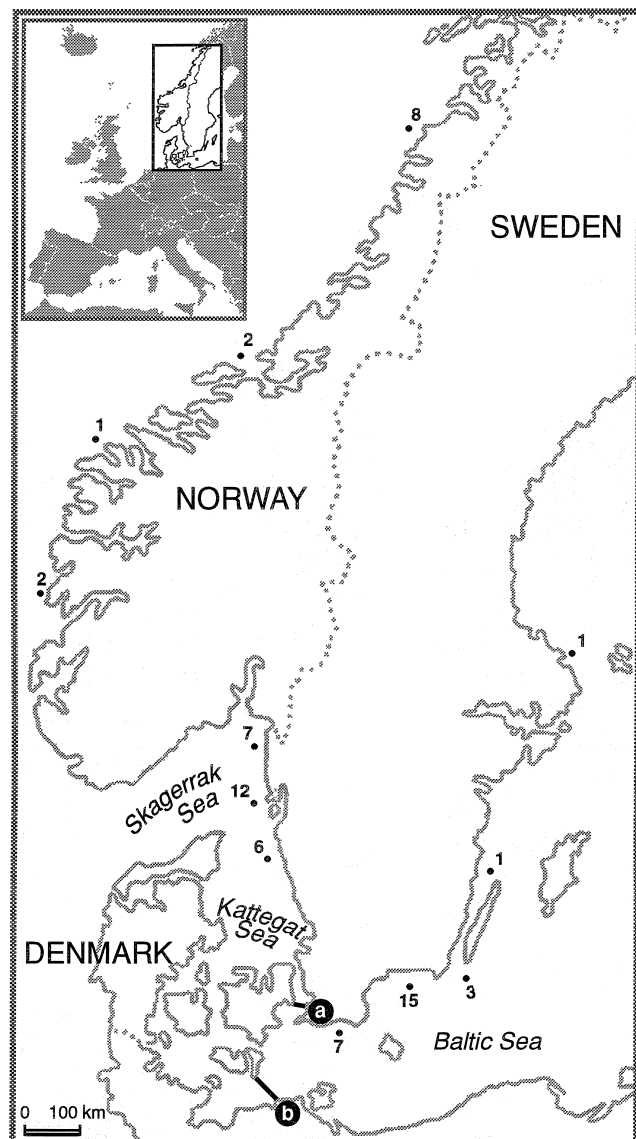


Fig. 1 *Phocoena phocoena*. Map showing the three sampling areas, the Baltic Sea, the Kattegat–Skagerrak Seas and the west coast of Norway. Numerals indicate the number of specimens and the location where they were collected. The Limhamn and Darss underwater ridges (a, and b, respectively) mark the borders of the Kattegat and Baltic Seas

sequence (Nei and Tajima 1981). Changes in restriction sites were assessed based on minimizing the number of mutational steps and homoplasious events required to produce the different fragment patterns from the most common pattern, “A” (see Wang 1993). Because restriction fragment information from enzymes recognizing four bp was incomplete (many fragments were too small to detect), many site gains or losses could not be analyzed. However, there was no reason to believe this loss of information affected the inference of site changes or biased the estimations of pairwise divergences among the haplotypes since both site gains and losses would be equally undetectable.

Data analysis

Potential geographic heterogeneity in the frequency distribution of haplotypes among harbour porpoises was tested using the chi-

square contingency test. A Monte Carlo bootstrapping technique was used to overcome the problem of small sample sizes (Roff and Bentzen 1989). In the present study, 10 000 randomizations were executed for each pairwise comparison using the MONTE program of the restriction enzyme analysis package (REAP) Version 4.1 (McElroy et al. 1992). With three provisional subpopulations, three pairwise comparisons were performed. To ensure that the level of significance for the experiment remained at $\alpha = 0.05$ the sequential Bonferroni correction technique (Rice 1989) was used to determine the critical value for each pairwise comparison.

Haplotype diversity (Nei and Tajima 1981) was estimated for each provisional subpopulation. This index of genetic variability is a function of the number and frequency of haplotypes within a sample without accounting for the relationships among haplotypes. This measure is analogous to heterozygosity estimates for nuclear DNA polymorphisms, and values can range from zero (monomorphism) to one (all individuals are unique).

Divergence among mtDNA haplotypes was estimated separately for 4- and 6-bp-recognizing restriction enzymes with the program MAXLIKE (written by F. Tajima and revised in 1988 by L. Jin, Center for Demographic and Population Genetics, University of Texas, Houston, Texas, USA) using the maximum-likelihood restriction site approach of Nei and Tajima (1983). Estimates of nucleotide diversity (Nei 1987) and nucleotide divergence (Nei and Tajima 1983) were also obtained with MAXLIKE. A phenogram was produced with the distance matrix of pairwise divergences among haplotypes using the neighbor-joining approach of Saitou and Nei (1987) by the NEIGHBOR algorithm of the phylogenetic inference package (PHYLIP), Version 3.41 (distributed by J. Felsenstein); this phenogram was rooted with respect to Haplotype 54, which was selected because of its distance from other haplotypes and its relationship to a haplotype found in the western North Atlantic. The neighbor-joining approach makes no assumptions about equal or constant rates of evolution among lineages unlike the unweighted pair group method of averages (UPGMA) of Sneath and Sokal (1973).

Results

Mitochondrial DNA of all 65 samples was examined with each of the nine enzymes. Approximately 590 bp or 3.6% of the molecule was surveyed (see Wang 1993). As with North American porpoises (Wang et al. 1996), all restriction enzymes produced polymorphic restriction patterns, with the exception of *Bam*HI. Five restriction patterns not observed previously (Wang et al. 1996) were detected, one for each of five restriction enzymes: *Alu*I (pattern "H"); *Hind*III ("C"); *Hinf*I ("N"); *Mbo*I ("Q") and *Taq*I ("N") (see Table 1). Eleven haplotypes were found of which seven had not been observed previously among North American porpoises (Wang et al. 1996). Four of these haplotypes (Nos.: 52, 53 and 58) were the direct result of the five "new" restriction patterns; the other three haplotypes were the products of novel combinations of restriction patterns shared with western North Atlantic porpoises (Table 2). Samples from the Baltic Sea, the Kattegat-Skagerrak Seas and the west coast of Norway comprised mainly Haplotype 2 (80, 89 and 62%, respectively). In contrast, Haplotype 1 was the predominant haplotype (31%) in the western North Atlantic (Wang et al. 1996).

Table 1 *Phocoena phocaena*. Fragment size estimates, in base pairs, for all endonucleases that produced polymorphic restriction patterns in harbour porpoise mitochondrial DNA. Letter designations for each pattern follow Wang et al. (1996). Sums of the fragments are presented in the bottom row (- not present)

<i>Alu</i> I	<i>Bgl</i> II			<i>Eco</i> RI			<i>Hae</i> III			<i>Hind</i> III			<i>Hinf</i> I			<i>Mbo</i> I			<i>Taq</i> I							
	A	C	E	H ^a	A	B	A	B	C	A	B	A	B	C ^a	A	D	N ^a	A	B	E	Q ^a	A	D	H	N ^a	
990	990	990	-	990	4720	4720	1990	-	-	8570	-	-	-	-	2460	1430	-	2460	2180	2460	2460	-	2230	2230	-	2490
-	-	870	-	17870	4520	-	1930	-	-	-	1410	-	-	-	-	-	1410	-	2180	-	2460	2230	-	2230	-	-
830	830	830	830	4240	4240	1160	1160	-	6670	-	1340	-	-	-	1700	-	1340	1700	1700	1700	1700	2060	2060	2060	2060	2060
620	620	620	620	3950	-	1000	1000	-	3680	3680	1270	1270	1270	1270	1160	1160	1160	1160	1160	1160	1160	1840	1840	1840	1840	1840
560	560	560	560	2640	2640	920	920	2640	3130	3130	1060	1060	1060	1060	1110	1110	1110	1110	1110	1110	1110	-	-	-	1710	-
-	-	-	550	560	-	810	810	-	2360	-	920	920	920	920	780	780	780	780	780	780	780	1550	1550	1550	1550	1550
450	450	450	450	560	-	760	760	-	520	520	790	790	790	790	-	-	-	-	-	-	690	-	870	870	1070	-
-	410	-	-	720	720	720	720	-	-	-	740	-	-	-	620	620	740	620	620	620	620	800	800	800	800	870
400	360	360	360	420	420	620	620	-	650	650	650	650	650	430	430	430	660	430	430	430	430	750	750	750	750	750
360	-	-	-	420	420	420	420	-	-	-	520	-	-	420	420	420	420	420	420	420	420	710	710	710	710	710
-	-	-	-	410	410	410	410	-	480	480	480	480	480	360	360	360	480	360	360	360	360	530	530	530	530	530
-	-	-	-	290	290	290	290	-	460	460	460	460	460	280	280	280	460	280	280	280	280	450	450	450	450	450
-	-	-	-	290	290	290	290	-	430	430	430	430	430	280	280	280	430	280	280	280	280	410	410	410	410	410
-	-	-	-	390	390	390	390	-	390	390	390	390	390	420	420	420	390	420	420	420	420	290	290	290	290	290
Sum	4210	4220	4090	4760	16110	16120	10870	10810	15900	16360	14070	9480	9150	9950	9650	9370	9870	10340	9870	9870	10340	11690	11780	11080	11080	11950

^aPatterns not observed by Wang et al. (1996)

Table 2 *Phocoena phocoena*. Distribution of mtDNA haplotypes for harbour porpoises sampled from the Baltic Sea, Kattegat–Skagerrak Seas and the west coast of Norway

Haplotype	Haplotype No. ^a	Baltic Sea	Kattegat–Skagerrak Seas	Norway
AAAAAAAAA	1	0	0	1
AAAABAAAA	2	24	20	8
AAAAAAAEA	5	0	2	0
AAAAABAAA	14	0	0	1
AAAABAAAN ^b	52	0	0	2
HAAAACAAA ^b	53	0	1	0
AABCAADBH ^b	54	0	1	0
AAAABAAQA ^b	55	0	1	0
AAAABAAAD ^b	56	1	0	0
CAAAAABA ^b	57	0	0	1
AAAAANAA ^b	58	2	0	0

^aBased on Wang et al. (1996)

^bHaplotypes that were not observed by Wang et al. (1996)

Tests of heterogeneity in the distribution of mtDNA haplotypes

Analysis of the heterogeneity in the frequency distribution of mtDNA haplotypes indicated significant differences among the samples collected from the Swedish Baltic, the Kattegat–Skagerrak Seas and the west coast of Norway at the 0.05 level of significance (Table 3).

Nucleotide divergence and diversity indices

The nucleotide divergence among the provisional subpopulations (range: 0.000 to 0.003%) were all one to two orders-of-magnitude lower than the divergence within subpopulations or nucleotide diversity (range: 0.070 to 0.168%)(Table 4). Haplotypic diversity values varied from 0.211 to 0.628. Both estimates of genetic diversity indicate that the Baltic sample had the least amount of genetic variation (Table 5).

Table 3 *Phocoena phocoena*. Pairwise comparisons of the frequency distribution of mtDNA haplotypes of harbour porpoises sampled from the Baltic Sea, Kattegat–Skagerrak Seas and the west coast of Norway. Adjusted probabilities and their standard deviations (in *parentheses*) were obtained by the Monte Carlo bootstrapping method of Roff and Bentzen (1989) (*above diagonal*). The critical probabilities for each pairwise comparison corrected with the sequential Bonferroni technique are shown *below the diagonal* ($\alpha = 0.05$)

	Baltic Sea	Kattegat–Skagerrak Seas	Norway
Baltic Sea		0.035* (0.002)	0.007* (0.001)
Kattegat–Skagerrak Seas	0.050		0.021* (0.001)
Norway	0.017	0.25	

* $p < 0.05$

Table 4 *Phocoena phocoena*. Nucleotide divergence ($\times 100$) estimates between provisional subpopulations of harbour porpoises in Swedish and Norwegian waters. These values have been corrected for nucleotide diversity within subpopulations (values along the diagonal). Standard deviations are shown in *parentheses*

	Baltic Sea	Kattegat–Skagerrak Seas	Norway
Baltic Sea	0.070 (0.038)	0.001 (0.053)	0.003 (0.034)
Kattegat–Skagerrak Seas		0.168 (0.094)	0.000 (0.055)
Norway			0.137 (0.047)

Table 5 *Phocoena phocoena*. Haplotypic and nucleotide diversity indices for three provisional subpopulations of harbour porpoises in Swedish and Norwegian waters. Haplotypic diversity was estimated using Eq. 7 of Nei and Tajima (1981), and nucleotide diversity within subpopulations was estimated using the maximum-likelihood method (Eq. 28) of Nei and Tajima (1983)

Provisional subpopulations	Haplotypic diversity	Nucleotide diversity
Baltic Sea	0.211	0.070
Kattegat–Skagerrak Seas	0.363	0.168
Norway	0.628	0.137
Western North Atlantic Subpopulations	range: 0.819–0.858 ^a	0.341–0.434 ^a
Humpback whale Subpopulations	range: 0.000–0.751 ^a	0.000–0.196 ^b

^aFrom Wang et al. (1996); humpback whale haplotypic diversity values were calculated from data presented in Baker et al. (1990)

^bFrom Baker et al. (1990)

The neighbor-joining phenogram did not reveal obvious clusters of haplotypes corresponding to sampling locations. However, Haplotype 54 (AABCAADBH), represented by a single individual collected from the Skagerrak Sea, was separated from all other haplotypes (Fig. 2). The mean divergence between Haplotype 54 and the others was approximately 1.4% as compared to the mean divergence between all haplotypes (excluding Haplotype 54) of 0.3%. The rare haplotype appears to be related closely to Haplotype 23 (AABCIADBH) of Wang et al. (1996), which was represented by a porpoise from the Bay of Fundy. The difference between these two haplotypes is the loss of two *HaeIII* restriction sites in Haplotype 54 with respect to 23. The significance of the presence of these two rare but closely related haplotypes in different populations is unknown.

Discussion

Population structuring

The present study provides molecular evidence for the existence of separate harbour porpoise subpopulations in the Baltic Sea, the Kattegat–Skagerrak Seas and the

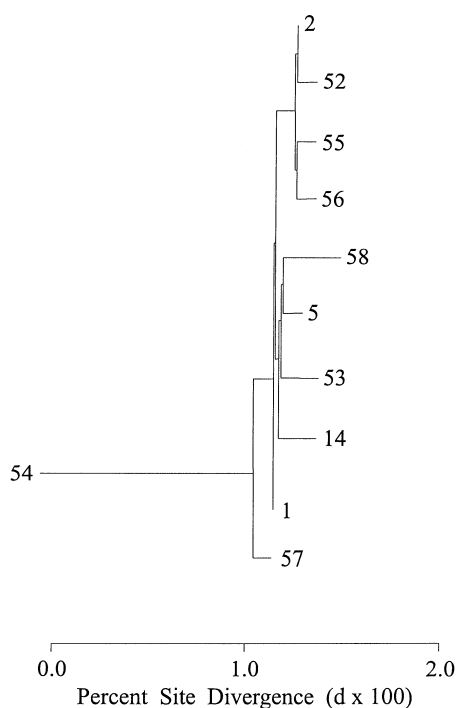


Fig. 2 *Phocoena phocoena*. Neighbor-joining (Saitou and Nei 1987) phenogram of pairwise percent divergences among mitochondrial DNA haplotypes of harbour porpoises produced by the NEIGHBOR algorithm of PHYLIP Version 3.41 (distributed by J. Felsenstein). Numbers at the branch termini represent the haplotypes. The tree is rooted to Haplotype 54

west coast of Norway. These findings support preliminary results of cranial morphometric analyses which showed differences between porpoises of the Baltic Sea and Kattegat–Skagerrak Seas (Börjesson and Berggren 1993). Several distinct groupings of harbour porpoises within populations were also found in the western North Atlantic by Wang et al. (1996) and in the eastern North Pacific by Rosel et al. (1995). However, due to limited sampling from the eastern North Atlantic finer structuring within this population was not detected (Rosel et al. 1995). Our ability to detect structuring within the eastern North Atlantic population complements the previous studies. The identification of at least three genetically different subpopulations of harbour porpoises in this region helps greatly in the understanding of the biology and conservation of this species. However, because there are shared haplotypes among the subpopulations, the level of genetic exchange that exists still needs to be studied to determine the distinctiveness of each subpopulation.

Even though there is evidence for separating these subpopulations, their boundaries remain unknown and must also be identified for effective conservation actions. The boundary between subpopulations may be fluid depending on many factors (e.g. differences in seasons, annual weather conditions, prey species distribution) and thus presents a difficult subject to understand. The migration of harbour porpoises into the Baltic Sea in early spring and out of the area during late autumn was de-

scribed by Andersen (1982) based on anecdotes and catch statistics. However, incidental catches by Swedish fisheries showed that at least some individuals remain in the Baltic Sea during the winter (Berggren 1994). Furthermore, incidental catches also occur throughout the year in the Kattegat–Skagerrak Seas (Berggren 1994) and off the west coast of Norway (Aarefjord et al. 1995). Future investigations into the movement patterns of individual porpoises may help to clarify these contradicting reports and define the boundary of each subpopulation.

Genetic diversity

Although five “new” restriction patterns and seven “new” haplotypes were observed, the estimates of genetic diversity for eastern North Atlantic subpopulations were all noticeably lower than the levels found for subpopulations within the western North Atlantic (Wang et al. 1996)(see Table 5). The harbour porpoises of the eastern North Atlantic (especially in the Baltic Sea) have been exploited for a long time at levels suspected to be high and possibly unsustainable (Andersen 1982; Gaskin 1984; Berggren 1994, 1995). The vaquita (*Phocoena sinus*), restricted to the upper Gulf of California, Mexico (Brownell 1986), also suffer from bycatches not likely sustainable (Vidal 1995). The life history of the vaquita is very similar to the harbour porpoise (Hohn et al. 1994) which makes this species suitable for comparison. The small vaquita population (327 individuals: Gerrodette 1994) has no detectable genetic variation in the mitochondrial genome indicating a population that had passed through a severe “bottleneck” and consequent inbreeding (Rosel and Rojas-Bracho 1993). Furthermore, the levels of genetic diversity in the eastern North Atlantic subpopulations of harbour porpoises are comparable to those found for subpopulations of humpback whales, *Megaptera novaeangliae*, some of which have been harvested commercially (Baker et al. 1990)(Table 5). The paucity of genetic diversity in the eastern North Atlantic subpopulations of harbour porpoises, particularly in the Baltic Sea, is consistent with other lines of evidence that suggest a depleted population (Skora et al. 1988; Berggren and Arrhenius 1995a, b).

Our study has provided some important information relevant to the conservation of harbour porpoises in the eastern North Atlantic. Based on differences in the frequency distribution of haplotypes, we recommend applying the precautionary approach in recognizing at least three distinct management entities (Baltic Sea, Kattegat–Skagerrak Seas and the west coast of Norway) until more information is available regarding genetic exchange among subpopulations. Furthermore, the low genetic diversity in the eastern North Atlantic subpopulations increases the urgency for expeditious and effective management actions to ameliorate the high level of incidental mortality caused by commercial fisheries in these waters.

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