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RESEARCH ARTICLE

Mitochondrial Control Region and microsatellite analyses on harbour porpoise (*Phocoena phocoena*) unravel population differentiation in the Baltic Sea and adjacent waters

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Abstract The population status of the harbour porpoise (*Phocoena phocoena*) in the Baltic area has been a continuous matter of debate. Here we present the by far most comprehensive genetic population structure assessment to date for this region, both with regard to geographic coverage and sample size: 497 porpoise samples from North Sea, Skagerrak, Kattegat, Belt Sea, and Inner Baltic Sea were sequenced at the mitochondrial Control Region and 305 of these specimens were typed at 15 polymorphic microsatellite loci. Samples were stratified according to sample type (stranding vs. by-caught), sex, and season (breeding vs. non-breeding season). Our data provide ample evidence for a population split between the Skagerrak and the Belt Sea, with a transition zone in the Kattegat area. Among other measures, this was particularly visible in significant frequency shifts of the most abundant mitochondrial haplotypes. A particular haplotype almost absent in the North Sea was the most abundant in Belt Sea and Inner Baltic Sea. Microsatellites yielded a similar pattern (i.e., turnover in occurrence of clusters identified by STRUCTURE). Moreover, a highly significant association between microsatellite assignment and unlinked mitochondrial haplotypes further indicates a split between North Sea and Baltic porpoises. For the Inner Baltic Sea, we consistently recovered a small, but significant separation from the Belt Sea population. Despite recent arguments that separation should exceed a predefined threshold before populations shall be managed separately, we argue in favour of precautionary acknowledging the Inner Baltic porpoises as a separate management unit, which should receive particular attention, as it is threatened by various factors, in particular local fishery measures.

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Introduction

In order to assess the conservation status of any organism, a proper understanding of its population structure is an essential prerequisite. While the concept of Evolutionary Significant Units (ESU) aims at denominating units with a common evolutionary history over longer (evolutionary) timescales, the scope of conservation and management efforts is typically more limited, both in space and time (Moritz 1994, 1999). In this paper, units are to be identified, which (1) comprise individuals among which random mating can occur and (2) among which gene flow is restricted such that these "management units" can be distinguished from one another by statistically significant genetic differentiation. Such units have been also termed "demographically significant" (Dizon 2002).

While in disjunctively distributed organisms, the definition of such "units to conserve" is sometimes straightforward, it is less evident in continuously distributed species with potentially high dispersal abilities, such as marine mammals. As a null hypothesis here, one might envision a single random mating population inhabiting the entire distribution range. However, if genetic population structure exists such that the null hypothesis is rejected, there are two alternative hypotheses, i.e., (1) a continuous correlation between geographic and genetic distance among pairs of individuals due to isolation-by-distance or (2) the existence of distinct populations within the species, among which dispersal (and—as a consequence—genetic exchange) is limited. In the latter case, the exact location of the population boundaries, i.e., the barriers restricting dispersal, has to be identified.

On the scale of the entire North-East Atlantic Ocean, it has been recently demonstrated by microsatellite analysis that population connectivity exists among harbour porpoises over thousands of kilometres with significant isolation-by-distance (IBD, Fontaine et al. 2007). According to this study, however, gene flow can be significantly decreased due to oceanographic barriers, such as underwater ridges, eventually causing profound environmental differences among separate basins on a relatively small scale. We focus here on the population structure of the harbour porpoise, *Phocoena phocoena*, in the Baltic area, which comprises a series of basins separated by shallow underwater ridges, i.e., the Kattegat (KAT), the Belt Sea (BES), and the Inner Baltic Sea (IBS) (Fig. 1). Porpoise population structure within this region, relative to the adjacent Skagerrak (SKA) and North Sea (NOS), is not fully understood and has been a continuous matter of debate (see, e.g., Koschinski 2002): Studies of morphology, genetics, and contaminant loads have found significant differences between sample groups of harbour porpoises from the Inner Baltic Sea and the Skagerrak–Kattegat (Börjesson and Berggren 1997; Wang and Berggren 1997; Berggren et al. 1999). Other studies have found significant differentiation between porpoises from the Belt Sea and the North Sea (Andersen 1993; Kinze 1985), between the Kattegat/Belt Sea and the Skagerrak (Andersen et al. 2001; Kinze 1985; Teilmann et al. 2008), between the Belt Sea/Inner Baltic Sea and the North Sea (Tiedemann et al. 1996; Huggenberger et al. 2002), and between the Skagerrak–Kattegat Seas and the west coast of Norway (Wang and Berggren 1997). Further, some previous studies have indicated differences between porpoises in the Belt Sea and the Inner Baltic Sea on morphological and genetic grounds (Huggenberger et al. 2002; Tiedemann et al. 1996). In a recent review, Palmé et al. (2008) have challenged this view: These authors re-analysed existing mtDNA data from Wang and Berggren (1997) from the Inner Baltic Sea, Swedish waters ($n = 27$ from IBS in Fig. 1) and compared them to a combined sample of Kattegat and Skagerrak ($n = 25$ from KAT/SKA); they concluded that identification of Baltic porpoises as a separate conservation unit is premature and urged towards a resolution of the status of these porpoises. The debate is

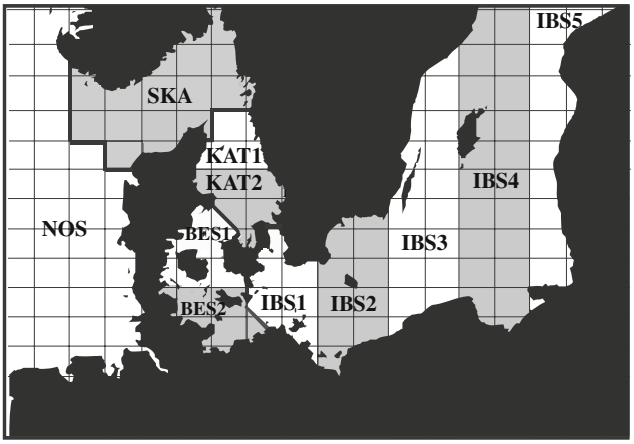

Fig. 1 Sampling locations (50 km × 50 km grids defined by the International Council for the Exploration of the Sea, ICES) and assignment to regions (*solid lines*). Regions are North Sea (NOS), Skagerrak (SKA), Kattegat (KAT), Belt Sea (BES), and Inner Baltic Sea (IBS). Except for the distinction between NOS and SKA, all boundaries (= *solid lines*) are defined by submarine ridges, i.e., a shallow area (50 m depth) between SKA and KAT, the Samsø sill (26 m depth) between KAT and BES, and the Darss sill (18 m depth) between BES and IBS. KAT, BES, and IBS are referred to as the "Baltic area" throughout the manuscript. Within this area, regions are further divided into subregions of 100 km width (subsequently numbered and indicated by alternating white/gray colouring)

however continuing, as a further independent analysis of the data re-confirmed the initial notion of a separate unit of porpoises in IBS (Berggren and Wang 2008).

A proper understanding of harbour porpoise population structure is crucial, as assessments of by-catch in commercial fisheries have led to concern over the status of this species in recent years. This is particularly true in the Baltic area (KAT, BES, and IBS in Fig. 1), where the harbour porpoise is found throughout the year. In KAT and BES it is the most abundant and in the Inner Baltic Sea even the only cetacean regularly encountered (Berggren 1994; Berggren and Arrhenius 1995a, b; Kinze 1995). Studies have shown that by-catch levels in gillnet fisheries may not be sustainable in the Skagerrak, Kattegat and in the Inner Baltic Sea (Berggren et al. 2002; Carlström 2003; Skóra and Kuklik 2003). Further, the occurrence of harbour porpoises in these areas declined drastically between the 1950s and the 1980s (Skóra et al. 1988; Määttänen 1990; Berggren and Arrhenius 1995a) with no indication of recovery in the Inner Baltic Sea (Gillespie et al. 2005). Porpoises have also become less common during this period in Danish waters (Andersen 1982; Clausen and Andersen 1988). Although the main threat to porpoises in the Baltic area has been identified as by-catch in commercial fisheries, other threats related to pollutants and boat traffic may also have a negative effect on the populations (Koschinski 2002). For example, studies of organochlorines indicate that PCBs levels detected in animals from the Baltic area may cause a health risk at the individual and/or population level based on similar findings in other species and geographical areas (Berggren et al. 1999).

The abundance of harbour porpoises in the Baltic area (excluding the Inner Baltic Sea) was estimated in 1994 during vessel based surveys as part of the Small Cetacean Abundance in the North Sea survey (SCANS). SCANS estimated that there were 36,046 (CV = 0.34) harbour porpoises in the Skagerrak, Kattegat, and Danish Great Belt Seas (SKA, KAT, and BES1 in Fig. 1) in July 1994 and 588 (CV = 0.48) in the Danish Little Belt and German Kiel Bight (BES 2) (Hammond et al. 2002). The abundance of harbour porpoises in the Inner Baltic Sea (IBS) was estimated during two aerial surveys conducted in 1995 and 2002. The survey in 1995 generated an abundance estimate of 599 porpoises (95% C.I. 200–3300) (Hiby and Lovell 1996) and in 2002 the estimated abundance was 93 (95% C.I. 10–460) (Berggren et al. 2004).

Here we present the by far largest and geographically most complete genetic assessment of harbour porpoises in the Baltic area. We chose the mitochondrial DNA Control Region as one molecular marker, because (1) it has been proven to be informative for delineating porpoise populations both on global (Rosel et al. 1999b) and regional scales (Tiedemann et al. 1996; Walton 1997) and (2) mtDNA puts particular emphasis on female dispersal patterns (Tiedemann et al. 2000). We complemented this analysis with an assessment of 15 nuclear microsatellites, a marker system affected by dispersal of both sexes, in order to arrive at a comprehensive picture of porpoise population structure in the Baltic area.

From a logistical point of view, sampling of porpoises is necessarily opportunistic, as direct sampling is not feasible and samples arise from stranded and by-caught specimens. As our samples originate both from strandings and by-catches and cover all seasons, we were also able to evaluate the impact of sampling status (stranding vs. by-caught; winter vs. breeding season) on the reliability of the estimates of population genetic variability and divergence.

Materials and methods

Sample collection and DNA extraction

We collected 497 skin and liver samples from harbour porpoises from fishery by-catches ($n = 231$) and strandings ($n = 266$), including 39 samples from an earlier study (Tiedemann et al. 1996). Exact sampling location was known for all specimens and was assigned to a 50 km × 50 km grid system defined by the International Council for the Exploration of the Sea (ICES; Fig. 1, Online supplementary Fig. 1). Each such grid was assigned to the respective region, i.e., North Sea (NOS, $n = 94$), Skagerrak (SKA, $n = 42$), Kattegat (KAT, $n = 85$), Belt Sea (BES, $n = 187$), and Inner Baltic Sea (IBS, $n = 89$). Except for the distinction between NOS and SKA, all boundaries among regions (= solid lines in Fig. 1) are defined by submarine ridges, i.e., a shallow area (50 m depth) between SKA and KAT, the Samsø sill (26 m depth) between KAT and BES, and the Darss sill (18 m depth) between BES and IBS (Köster and Schwarzer 1996). KAT, BES, and IBS are referred to as the "Baltic area" throughout the manuscript.

DNA was extracted from skin or liver samples after an initial Proteinase K treatment, using either the standard phenol/chloroform method, the Super Quik Gene DNA extraction kit (Analytical Genetic Testing Center, Denver, USA), the DNeasy Tissue Kit™ (QIAGEN, Hilden, Germany), or the G NOME® Kit (Qbiogene, California, USA), according to manufacturer's instructions.

Sexing

Samples of unknown gender were Polymerase Chain Reaction (PCR) sexed using ZFX and SRY specific primers; PCR conditions were as in Rosel (2003).

mtDNA analysis

The 5′ end of the mitochondrial Control Region was amplified according to Tiedemann et al. (1996), sequenced either with the Thermosequenase Dye Terminator Cyclesequencing Kit (Amersham) and analyzed on an ABI 373 automatic sequencer (Applied Biosystems) or sequenced with the BigDye Terminator Kit (Applied Biosystems) and analyzed on an AB 3100 capillary sequencer (Applied Biosystems). Control Region sequences were aligned in BioEdit v. 7.0.0 (Hall 1999). Mitochondrial haplotypes were defined based on 414 bp sequence in comparison to haplotype PHO1 (GenBank No. Y13872, Tiedemann et al. 1996). A haplotype network was constructed using TCS 1.13 with default parameter settings (95% connection limit, Clement et al. 2000).

Microsatellite analysis

305 samples were genotyped at 15 previously published polymorphic microsatellite loci: PPHO104, PPHO130, PPHO131, PPHO137, PPHO142 (Rosel et al. 1999a), lgf-1 (Kirkpatrick 1992), EV94, GATA053, Gt011 and Gt015 (Valsecchi and Amos 1996; Palsbøll et al. 1997; Bérubé et al. 1998; Andersen et al. 2001), KWM12a (Hoelzel et al. 1998), and Tex Vet3, Mk6, Mk8 and Mk9 (Rooney et al. 1999; Krützen et al. 2001). About 100 ng of genomic DNA were used as template. PCRs were carried out in a standard volume of 37.5 µl, containing 1 mM Tris–HCl, pH 9.0, 5 mM KCl, 0.15 mM $MgCl_2$, 0.2 mM of each dNTP, 0.13 µM of both forward (fluorescence-labelled at 5′-end) and reverse primers, and 0.75 U *Taq* polymerase (Qbiogene). For all loci except EV94 the thermal cycling profile consisted of an initial hot start for 30 s at 95°C, followed by 40 cycles of denaturation for 30 s at 94°C, 30 s at the locus-specific annealing temperature, 30 s at 72°C, and a final extension for 10 min at 72°C, and was performed in a Biometra thermocycler. Annealing temperatures were: 41°C for KWM12a and PPHO130, 42°C for PPHO142, 43°C for lgf-1, 50°C for Tex Vet3, Mk6 and GT015, 51°C for GT011, 52°C for Mk8 and Mk9, and 55°C for PPHO104, PPHO131, PPHO137 and GATA053. In the case of EV94, a 2-Step PCR was run with 3 cycles for 3 min at 94°C, 1 min at 48°C and 1 min at 72°C, followed by 27 cycles of 15 s at 94°C, 30 s at 50°C, 1 min at 72°C, and a final extension for 10 min at 72°C. All reactions included both positive and negative controls. Fragment size of amplified DNA was determined on an AB 3100 automatic sequencer, using the GENEMAPPER version 3.5 software and an internal size standard (LIZ500, Applied Biosystems). We tested for linkage disequilibrium across all pairs of loci using GENEPOP on the web (http://genepop.curtin.edu.au/). Furthermore, the same software was used to test for deviation from Hardy–Weinberg equilibrium (HWE) for each locus in each population using Fisher's exact test and the Markov chain method (1,000 demorization steps, 100 batches, with 10,000 iterations per batch set). Levels of significance of the HWE and linkage disequilibrium tests were Bonferroni corrected for multiple comparisons (Rice 1989).

Population analysis

Based on geographic origin, samples were assigned to 50 km × 50 km grids (Fig. 1, Online supplementary Fig. 1). Each grid was unambiguously assigned to a region, i.e., Danish and German North Sea (NOS), Skagerrak (SKA), Kattegat (KAT), Belt Sea (BES), Inner Baltic Sea (IBS, cf. Fig. 1). For additional geographic stratification, the regions KAT, BES, and IBS were further divided into 100 km wide stretches (called "subregions" hereafter; Fig. 1). Note that subregion IBS5 contained only 2 samples; therefore IBS5 was not included in any analysis regarding subregions. Given the opportunistic sampling strategy, relying on stranding and by-caught casualties (see above), we checked for consistency of measures calculated for (a) all samples, relative to subsets of samples, i.e., (b) by-caught samples only (excluding strandings, which might have drifted prior to detection); (c) females only (excluding males as the potentially more dispersing sex); (d) summer only (April–September, including the known breeding season of harbour porpoises; excluding winter samples when potential migration occurs; Koschinski 2002); (e) summer by-caught only, and (f) summer females only.

For the mtDNA data, we calculated standard measures of genetic diversity (haplotype diversity δ, Nei 1987; nucleotide diversity π, Tajima 1983) as well as of genetic divergence among adjacent regions/subregions (fixation index F_{ST}, Weir and Cockerham 1984), as implemented in the software package ARLEQUIN 3.11 (Excoffier et al. 2005). Divergence among regions was further evaluated by an exact test of sample differentiation based on haplotype frequencies, using a Markov Chain Monte Carlo (MCMC) approach (10,000 steps in Markov Chain, 1,000 dememorization steps; Raymond and Rousset 1995). Since F_{ST} values and other tests of population divergence were calculated among adjacent areas *only* (i.e., a priori planned orthogonal testing scheme, cf. Sokal and Rohlf 1995), a correction for multiple tests was not strictly required. However, as the most conservative interpretation, we also provide threshold values after Bonferroni correction for multiple tests for the full table of all possible pairwise comparisons (Rice 1989).

For the microsatellite data, the program FSTAT 2.9.3.2 (Goudet 2001) was used to estimate allelic richness within

populations (Petit et al. 1998). Allele frequency and private alleles were assessed with the Excel Add-In software GENALEX 6 (Peakall and Smouse 2006). Observed (H_O) and expected (H_E) heterozygosities were calculated using ARLEQUIN 3.11 (Excoffier et al. 2005). A Bayesian clustering method was performed using the software STRUCTURE (Pritchard et al. 2000), which applies MCMC methods to evaluate the likelihood of different subgroups. This approach uses individual multilocus genotype data to cluster individuals into K groups, while minimizing Hardy–Weinberg disequilibrium and gametic phase disequilibrium among loci within groups. Genetic subdivision was evaluated estimating the likelihood and sample composition of independent runs of subgroups ($K = 1$–5), assuming correlated allele frequencies and an admixture model, with a burn-in of 100,000 iterations and a data collection period of 900,000 iterations. To check for convergence of the Markov Chain parameters, three replicate runs for each value of K were performed. As for the mtDNA, the level of genetic differentiation was estimated by both calculating pairwise F_{ST} values and performing exact MCMC tests among adjacent regions/subregions, using ARLEQUIN and assuming an infinite allele model.

For both mtDNA and microsatellites, analyses of molecular variance (AMOVA) were performed with ARLEQUIN 3.11 (Excoffier et al. 2005), assigning genetic variation to three levels, i.e., (1) among regions, (2) among subregions within regions, and (3) within subregions. Isolation-by-distance (IBD) was evaluated by correlating pairwise F_{ST} values among all 50 km × 50 km grids containing at least 5 samples and closest swimming distance (in km) among the midpoints of the grids. For the same data, a spatial autocorrelation analysis among individuals was performed using GENALEX 6 (distance classes of 100 km width; 999 permutations; confidence intervals of the correlation coefficients (r) estimated in 1,000 bootstrap replicates; Peakall and Smouse 2006).

Results

Based on a 414 bp mitochondrial Control Region sequence in 497 harbour porpoise specimens, we found 36 polymorphic sites (one transversion at position 23, one indel at position 126; 34 transitions), defining 42 distinct haplotypes (PHO1–PHO49 in Fig. 2; see Online supplementary Table 1 for sequence information). The two haplotypes PHO1 and PHO7, separated by a single point mutation at position 355, were by far the most abundant and occurred in 164 (33.0%) and 207 (41.6%) of the samples, respectively (Online supplementary Table 2). These haplotypes were found in all regions, but occurred regionally in strikingly different frequencies: PHO1 was the most abundant type in the North Sea (NOS) as well as in Skagerrak (SKA), whereas PHO7 was the predominant type in south Kattegat (KAT2), the Belt Sea (BES) and—although less common—in the Inner Baltic Sea (IBS; Online supplementary Table 2). The northern part of the Kattegat (KAT1) comprised a transition zone between the two types. This pattern was consistently found, regardless of whether the entire sample set was considered or whether analyses were restricted to samples from by-catches, summer season or one sex, i.e., females. This is particularly evident for the most abundant Baltic haplotype PHO7 (Online supplementary Table 2). Estimates of mtDNA genetic diversity (Table 1) were the lowest in the Belt Sea (BES summer by-caught samples; haplotype diversity $\delta = 0.479$, nucleotide diversity $\pi = 0.144\%$) and the highest in the North Sea (NOS, summer by-caught samples; $\delta = 1.000$, nucleotide diversity $\pi = 0.646\%$). The North Sea (NOS, all samples) showed also the highest number of private haplotypes, i.e., 13 haplotypes not found elsewhere in this study.

All 15 microsatellite loci were polymorphic with the number of alleles per locus ranging from 4 to 26. Linkage disequilibrium was neither evident in populations across all loci nor in pairwise locus comparisons (data not shown). Significant departures from HWE were only detected at locus GT015; they were generally associated with positive F_{IS} values (0.314–0.359, with $P < 0.005$) indicating heterozygote deficiency (Online supplementary Table 3). Omission of this locus did not significantly change the level of differentiation among regions; therefore it was retained for all further analyses. Differences in microsatellite diversity among regions were less pronounced than in mtDNA (Table 2): Allelic Richness was the lowest in the Belt Sea (BES, summer by-caught samples; AR = 7.595) and the highest in Skagerrak (SKA, all samples; AR = 9.490). Observed heterozygosity was the lowest in the Belt Sea and the Kattegat (BES, summer females, KAT, summer by-caught samples; $H_O = 0.724$) and the highest in Skagerrak (SKA, by-caught samples; $H_O = 0.799$). Expected heterozygosity was the lowest in the Belt Sea (BES all samples; $H_E = 0.770$) and the highest in Skagerrak (SKA summer samples and SKA summer females, $H_O = 0.824$). The highest number of private alleles when assessing all samples was found in the North Sea (11), while the overall highest number of private alleles was found in the Inner Baltic Sea (13 in IBS, by-caught samples).

The Bayesian clustering approach implemented in STRUCTURE detected a slight trend of population structuring. Results over three replicated runs tested for each K (mean likelihood: −18877 for $k = 1$; −18664 for $k = 2$; −18787 for $k = 3$; −18806 for $k = 4$; −19216 for $k = 5$)

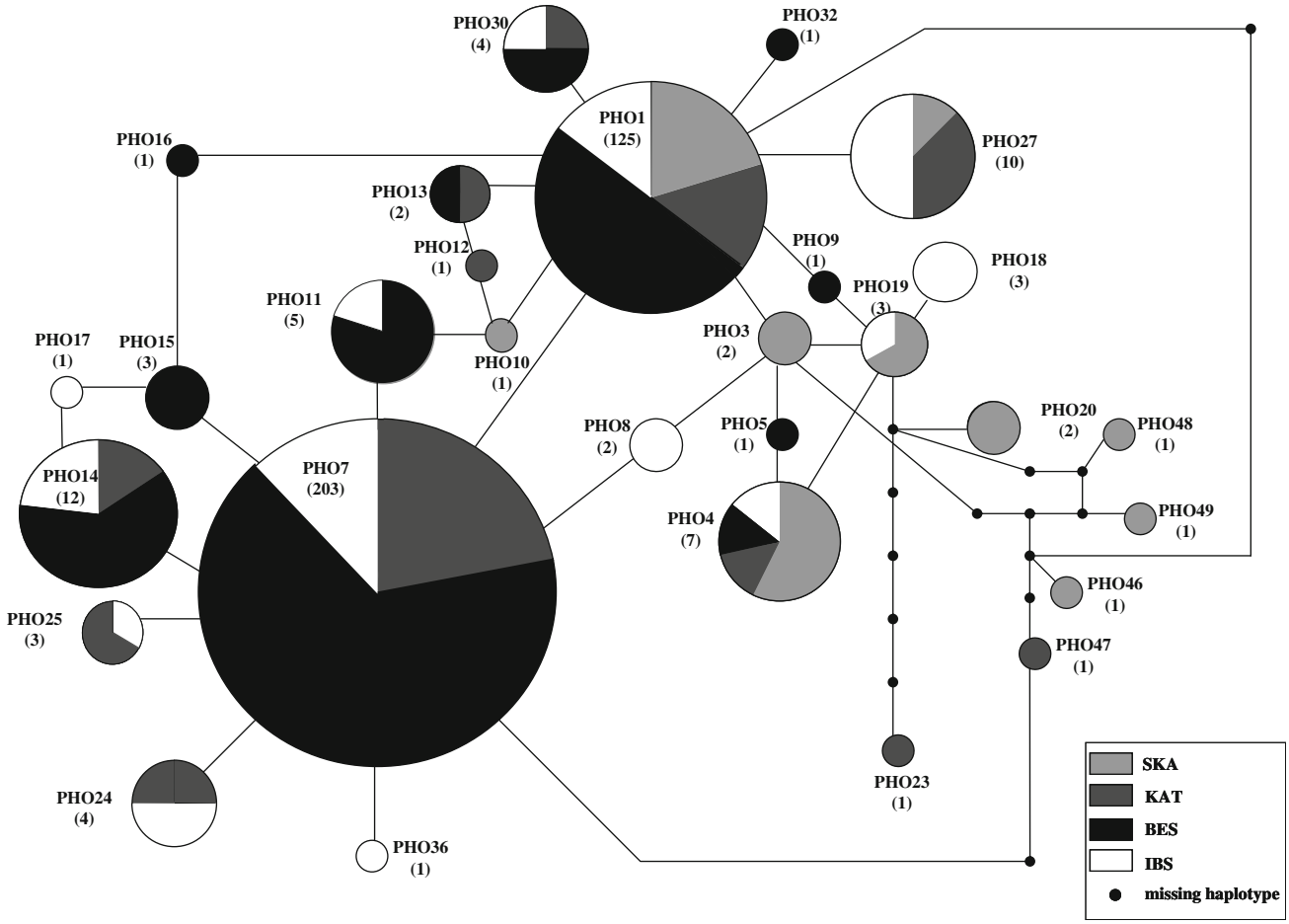

Fig. 2 Haplotypic network of haplotypes occurring in Skagerrak (SKA), Kattegat (KAT), Belt Sea (BES), and Inner Baltic Sea (IBS). Circle diameter is proportional to relative frequency of haplotypes. Numbers in parentheses give absolute frequency of occurrence

showed the highest likelihood for $K = 2$ clusters (Fig. 3). Although many specimens were assigned to one or the other cluster with quite high probabilities, these clusters did not show a clear geographic division. Nonetheless, there was a tendency of more frequent assignment of individuals from North Sea (NOS), Skagerrak (SKA), and Kattegat (KAT) to cluster 2 and of individuals from Belt Sea (BES) to cluster 1 (Fig. 4). Among the individuals from the Inner Baltic Sea (IBS), there was no such tendency to be preferentially assigned to one or the other cluster (Figs. 3 and 4).

Analyses of molecular variance (AMOVAs) revealed significant divergence among subregions in both marker systems, regardless of the type of samples considered (mtDNA: F_{ST} between 0.097 and 0.285; all $p < 0.001$; microsatellites F_{ST} between 0.008 and 0.017; all but one $p < 0.001$; one $p = 0.012$; Table 3). Most genetic variation occurred within subregions (mtDNA: between 71.5 and 90.3%; microsatellites: between 98.3 and 99.2%). The second largest variance component was attributed to divergence among regions (mtDNA: between 8.9 and 26.4%; microsatellites: between 0.5 and 0.9%), while the smallest fraction of the variance was due to divergence among subregions within regions (mtDNA: between 0.8 and 4.4%; microsatellites: between 0.2 and 0.8%).

For mtDNA, the pairwise comparison of genetic divergence among Skagerrak and Kattegat (SKA–KAT F_{ST} between 0.167 and 0.225; Table 4) always showed consistent highly significant divergence (regarding F_{ST}, MCMC-exact test, and the occurrence of the most abundant haplotype PHO7), regardless of whether all samples were simultaneously analyzed or divided into subsets (by-caught, females, summer only; Table 4). A second comparison, i.e., Belt Sea and Inner Baltic Sea (BES–IBS) was always significant in the MCMC-exact test, while the F_{ST} (0.049) and the frequency difference regarding haplotype PHO7 was only significant when the analysis was restricted to by-caught specimens. In order to determine more precisely the geographic location of potential population splits, these pairwise comparisons were repeated among subregions (Online supplementary Table 4). In this analysis, the SKA–KAT split was positioned within the Kattegat

Table 1 mtDNA diversity measures for (a) all samples, (b) by-caught only, (c) females only, (d) summer only, (e) summer by-caught only, and (f) summer females only

Region	*n*	δ	π (%)	Most common haplotypes	Number and frequency of private haplotypes	Number and frequency of private haplotypes occurring more than once
(a) All samples						
NOS	94	0.729 ± 0.034	0.493 ± 0.309	PHO1: 41.5%	13 (14.9%)	1 (2.1%)
SKA	42	0.635 ± 0.082	0.415 ± 0.274	PHO1: 59.5%	5 (14.3%)	1 (4.8%)
KAT	85	0.593 ± 0.051	0.284 ± 0.204	PHO7: 60.0%	3 (3.5%)	0 (0.0%)
BES	187	0.586 ± 0.028	0.181 ± 0.149	PHO7: 56.7%	3 (2.7%)	1 (1.6%)
IBS	89	0.704 ± 0.039	0.290 ± 0.207	PHO7: 47.2%	4 (7.9%)	2 (5.6%)
(a) By-caught only						
NOS	5	0.900 ± 0.161	0.532 ± 0.411	PHO1: 40.0%	1 (20.0%)	0 (0.0%)
SKA	17	0.728 ± 0.114	0.402 ± 0.278	PHO1: 52.9%	3 (17.6%)	0 (0.0%)
KAT	57	0.639 ± 0.060	0.308 ± 0.218	PHO7: 56.1%	4 (7.0%)	0 (0.0%)
BES	83	0.505 ± 0.061	0.166 ± 0.141	PHO7: 68.7%	3 (9.6%)	2 (8.4%)
IBS	69	0.698 ± 0.044	0.283 ± 0.205	PHO7: 46.4%	3 (8.7%)	2 (7.2%)
(c) Females only						
NOS	49	0.749 ± 0.042	0.472 ± 0.302	PHO1: 38.8%	5 (10.2%)	0 (0.0%)
SKA	23	0.676 ± 0.103	0.369 ± 0.257	PHO1: 56.5%	4 (17.4%)	0 (0.0%)
KAT	53	0.626 ± 0.061	0.303 ± 0.216	PHO7: 56.6%	4 (9.4%)	1 (3.8%)
BES	97	0.597 ± 0.041	0.183 ± 0.151	PHO7: 56.7%	2 (3.1%)	1 (2.1%)
IBS	47	0.747 ± 0.054	0.365 ± 0.248	PHO7: 46.8%	4 (12.8%)	1 (6.4%)
(d) Summer only						
NOS	71	0.710 ± 0.038	0.512 ± 0.320	PHO4: 42.3%	7 (12.7%)	2 (5.6%)
SKA	32	0.569 ± 0.102	0.338 ± 0.237	PHO1: 65.6%	4 (12.5%)	0 (0.0%)
KAT	65	0.630 ± 0.053	0.278 ± 0.202	PHO7: 55.4%	2 (3.1%)	0 (0.0%)
BES	136	0.588 ± 0.030	0.170 ± 0.143	PHO7: 55.1%	3 (3.7%)	1 (2.2%)
IBS	54	0.658 ± 0.047	0.219 ± 0.171	PHO7: 51.8%	0 (0.0%)	0 (0.0%)
(e) Summer by-caught only						
NOS	4	1.000 ± 0.177	0.646 ± 0.514	–	2 (50.0%)	0 (0.0%)
SKA	15	0.733 ± 0.124	0.424 ± 0.292	PHO1: 53.3%	3 (20.0%)	0 (0.0%)
KAT	48	0.664 ± 0.058	0.319 ± 0.225	PHO7: 52.1%	3 (6.3%)	0 (0.0%)
BES	52	0.479 ± 0.080	0.144 ± 0.130	PHO7: 71.2%	3 (9.6%)	1 (1.9%)
IBS	41	0.663 ± 0.057	0.232 ± 0.180	PHO7: 51.2%	0 (0.0%)	0 (0.0%)
(f) Summer females only						
NOS	40	0.664 ± 0.053	0.441 ± 0.288	PHO4: 47.5%	5 (12.5%)	0 (0.0%)
SKA	21	0.676 ± 0.111	0.383 ± 0.265	PHO1: 57.1%	4 (19.0%)	0 (0.0%)
KAT	43	0.679 ± 0.055	0.333 ± 0.232	PHO7: 48.8%	3 (9.3%)	1 (4.7%)
BES	66	0.617 ± 0.045	0.186 ± 0.153	PHO7: 54.5%	3 (10.6%)	2 (9.1%)
IBS	30	0.609 ± 0.071	0.194 ± 0.161	PHO7: 56.7%	0 (0.0%)	0 (0.0%)

n number of samples analyzed; δ haplotype diversity; π nucleotide diversity; ±standard deviations

(i.e., between KAT1 and KAT2; F_{ST} between 0.049 and 0.100). This fits well with the transition in haplotype frequency within the Kattegat (Fig. 4). The second split (BES–IBS) occurred within the Belt Sea (between BES1 and BES2). As in the comparison among regions (Table 4), this second split was only supported in some of the statistical analyses (see Online supplementary Table 4 for details).

Regarding the microsatellite data, none of the MCMC exact tests yielded significant results (Table 4). Apart from that, all other tests (F_{ST}, frequency of STRUCTURE Cluster 1) in all sample partitions indicate a split between Kattegat and Belt Sea (KAT–BES; F_{ST} between 0.006 and 0.010; Table 4). A second split between Belt Sea and Inner Baltic Sea (BES–IBS; F_{ST} between 0.004 and 0.009) was supported in all sample sets, except for the female only

Table 2 Microsatellite diversity measures for (a) all samples, (b) by-caught only, (c) females only, (d) summer only, (e) summer by-caught only, and (f) summer females only

Region	*n*	PA	NA	AR	H_O	H_E
(a) All samples				$n_{AR} = 15$		
NOS	68	11	12.533	9.010	0.765 ± 0.193	0.815 ± 0.169
SKA	17	5	9.800	9.490	0.797 ± 0.209	0.822 ± 0.200
KAT	43	6	11.400	8.867	0.742 ± 0.191	0.801 ± 0.196
BES	112	7	11.600	8.023	0.727 ± 0.203	0.770 ± 0.203
IBS	65	3	11.333	8.399	0.744 ± 0.191	0.793 ± 0.201
(b) By-caught only				$n_{AR} = 13$		
NOS	1	–	–	–	–	–
SKA	14	5	8.933	8.767	0.799 ± 0.227	0.817 ± 0.210
KAT	23	8	10.067	8.475	0.730 ± 0.192	0.810 ± 0.193
BES	50	6	10.200	7.665	0.740 ± 0.200	0.779 ± 0.195
IBS	46	13	10.800	7.988	0.741 ± 0.190	0.790 ± 0.202
(c) Females only				$n_{AR} = 14$		
NOS	33	9	11.267	8.992	0.789 ± 0.190	0.821 ± 0.156
SKA	15	4	9.267	9.107	0.794 ± 0.213	0.821 ± 0.196
KAT	37	8	11.333	8.788	0.743 ± 0.186	0.803 ± 0.191
BES	62	8	10.867	7.915	0.731 ± 0.208	0.778 ± 0.208
IBS	36	1	10.267	8.293	0.739 ± 0.202	0.792 ± 0.210
(d) Summer only				$n_{AR} = 14$		
NOS	49	11	11.867	8.656	0.769 ± 0.202	0.810 ± 0.164
SKA	14	3	9.267	9.267	0.786 ± 0.211	0.824 ± 0.194
KAT	31	7	11.200	8.853	0.737 ± 0.187	0.809 ± 0.185
BES	87	5	11.000	7.790	0.728 ± 0.198	0.774 ± 0.197
IBS	40	3	10.267	8.055	0.735 ± 0.199	0.789 ± 0.205
(f) Summer by-caught only				$n_{AR} = 13$		
NOS	1	–	–	–	–	–
SKA	13	8	8.867	8.867	0.790 ± 0.226	0.820 ± 0.210
KAT	21	11	9.867	8.523	0.724 ± 0.198	0.812 ± 0.190
BES	33	7	9.467	7.595	0.749 ± 0.186	0.782 ± 0.183
IBS	28	9	9.467	7.730	0.735 ± 0.190	0.784 ± 0.194
(f) Summer females only				$n_{AR} = 14$		
NOS	27	8	10.600	8.853	0.775 ± 0.200	0.817 ± 0.155
SKA	14	4	9.267	9.267	0.786 ± 0.211	0.824 ± 0.194
KAT	30	9	11.200	8.938	0.742 ± 0.184	0.811 ± 0.184
BES	48	7	10.467	7.901	0.724 ± 0.203	0.778 ± 0.202
IBS	23	2	9.467	8.372	0.749 ± 0.204	0.800 ± 0.203

n number of samples analyzed; *PA* private alleles; *NA* average number of alleles across all loci; *AR* average allelic richness (based on sample size n_{AR}); H_O average observed heterozyogyosity; H_E average expected heterozyogsity; ±standard deviations

subsample. Here, the comparison among subregions (Online supplementary Table 4) confirmed the BES–IBS split in two out of four sample partitions, while no other split received consistent statistical support.

For both marker systems, we tested for an isolation-by-distance (IBD) pattern by comparing pairwise F_{ST} values among single 50 km × 50 km grids to the geographical distance (i.e., shortest swimming distance) among the grids. Both marker systems exhibited a slight, but significant positive correlation between genetic and geographic distance, if the entire study area was considered. This IBD-pattern accounted for 6% (mtDNA) and 34% (microsatellite) of the variation, respectively (solid lines in Fig. 5). If the most distant grids sampled in the German/Danish North Sea are excluded from this analysis (NOS, cf. Fig. 1, Online supplementary Fig. 1), the analysis for the "Baltic area" (KAT, BES, IBS) and the adjacent Skagerrak (SKA) did not reveal any IBD regarding the mtDNA, while

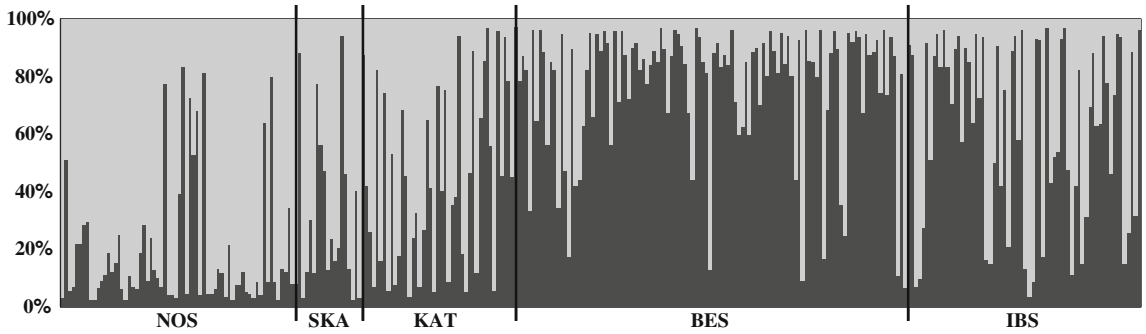

Fig. 3 Bayesian STRUCTURE analysis of proportional population structure of the five regions over 15 microsatellite loci. Each individual is represented by one vertical column divided into $k = 2$ coloured clusters. The length of each colour line is proportional to the individual's estimated membership coefficient in the two segments. Cluster 1 is coloured in dark gray, Cluster 2 in light gray

a minor IBD effect remained for the microsatellites, accounting for 4% of the variation (dashed lines in Fig. 5). Spatial autocorrelation analysis showed a significantly positive correlation (i.e., specimens are genetically more alike than expected in random permutations) within a distance of 100 km for mtDNA and 200 km for microsatellites (Fig. 6).

As our STRUCTURE analysis on the microsatellite data did not reveal any clear-cut geographical pattern, it was not straightforward to look for a potential correlation between mitochondrial haplotypes and multilocus microsatellite genotypes. However, as STRUCTURE repeatedly supported subdivision of the entire data set into two clusters, we looked for a potential association between the most common mitochondrial haplotypes (PHO1, PHO4, and PHO7) and the highest probability of assignment (to cluster 1 or 2) of the respective specimen. The rationale behind this analysis is that—under the hypothesis of panmixia—any clustering suggested by nuclear microsatellites should be decoupled from the haplotype occurrence at the genetically unlinked mtDNA. Under the alternative hypothesis of a (subtle) population structure potentially obscured by migrants, we expect a correlation between mtDNA haplotypes and assignment to one of the two STRUCTURE clusters based on microsatellites. In this analysis, specimens were counted (1) for the cluster they were assigned to with a probability >50% and (2)—in a more stringent iteration—for the cluster they were assigned to with a probability >60%. Note that in (2) sample size is reduced, as 33 (out of 299) specimens with assignment probabilities between 40% and 60% to both clusters were not included. Association between mitochondrial haplotype and the cluster assignment of the respective individual was statistically highly significant, as detected by an χ^2 replicated goodness-of-fit test (Table 5). Evaluating the occurrence of single haplotypes in the respective microsatellite clusters showed a highly significant association of PHO7 to cluster 1 and of PHO4 to cluster 2 (in fact, all specimens bearing PHO4 were assigned to cluster 2), while no significant association with any cluster was observed for PHO1. This pattern was robust, with similar *p*-values, regardless of the assignment criterion adopted (i.e., >50% vs. >60% probability).

Discussion

Population genetic signal in samples stratified by season, sex, and strandings versus by-caughts

It is often argued that in cetaceans by-caught specimens from the breeding season allow the most accurate assessment of genetic population structure, as they are most likely to represent the breeding population. However, in highly endangered and not easily accessible species such as porpoises, ambitious sampling schemes might be logistically constrained, motivating the opportunistic inclusion of samples from other sources (e.g., strandings and samples outside the breeding season). Because of the considerably large data set presented here, we were able to evaluate the validity of the population structure conclusions drawn from such "suboptimal" samples. As outlined above, we conclude that inclusion of stranding and non-breeding season specimens generally resembles the pattern of genetic diversity and divergence found in our analysis of the summer by-caught samples. Including strandings and non-breeding season might put a bias on all estimates towards overestimation in genetic diversity and towards underestimation in genetic divergence, as admixture might occur due to either *post mortem* drifting or seasonal migration (in winter). Across our entire study, there is however no indication for any systematic bias due to sample type, as both diversity measures and differentiation patterns are largely consistent across sample types. We argue that this consistency makes a point for the possibility to draw meaningful conclusions about genetic diversity and

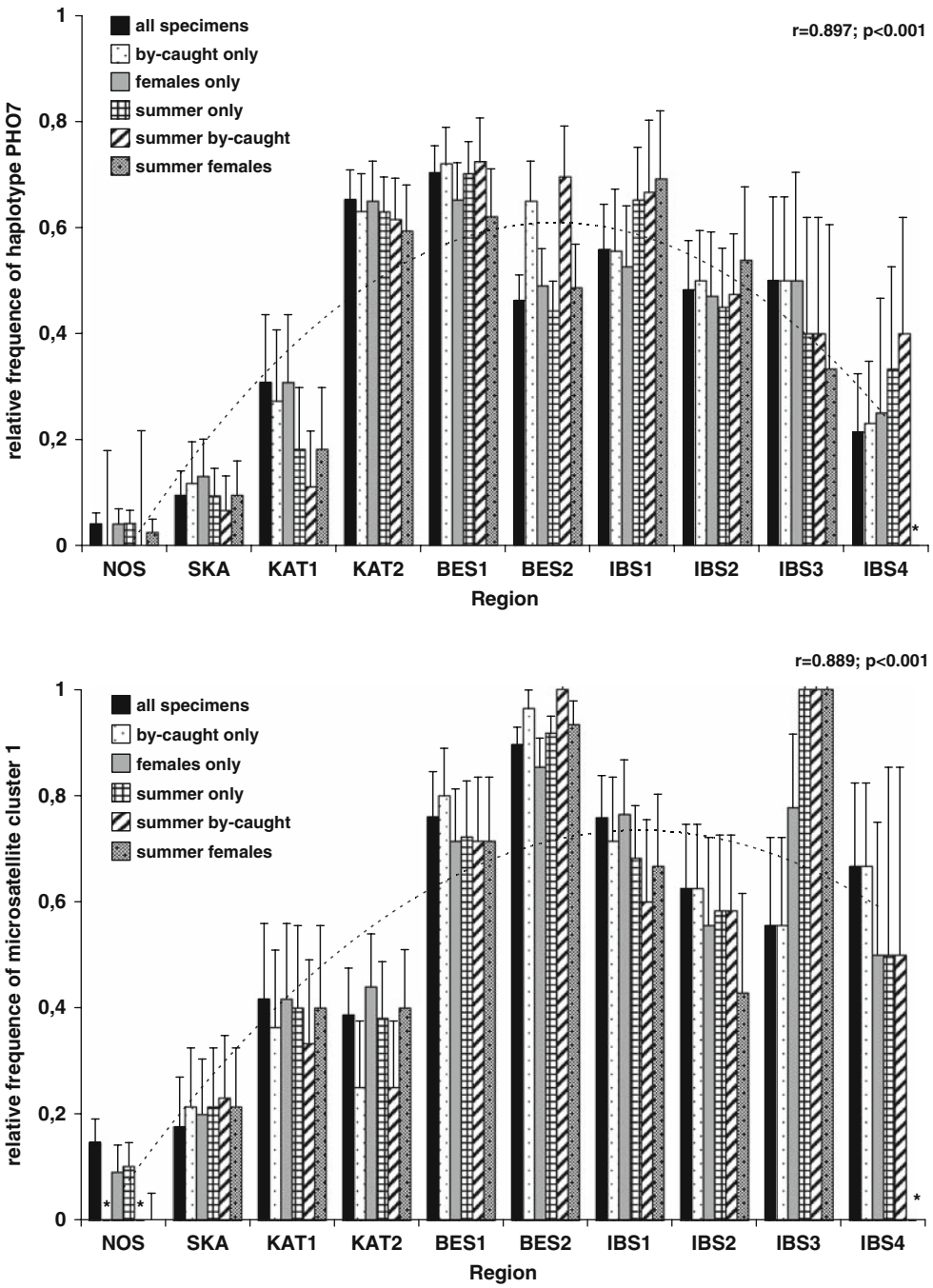

Fig. 4 Relative frequencies (mean and standard error) of the most abundant mitochondrial haplotype (PHO7; *upper graph*) and the microsatellite STRUCTURE cluster 1 (*lower graph*) in the analyzed regions (cf. Fig. 1), separately given for all samples, by-caught samples only, females only, summer (April–September) samples only, by-caught summer samples only, summer female samples only. * Lack of samples. The dotted lines and associated *r*-, *p*-values indicate a quadratic regression (based on data for all samples)

differentiation also from stranded and/or off-season samples, at least in the area studied here. There are, however, at least two cases where sample stratification makes a difference: (1) Regarding our North Sea samples (NOS), the haplotype PHO4 is most abundant in summer, while it is outnumbered by PHO1 in the combined sample set. This is an indication of seasonal immigration (in winter) into that area, as PHO1 is an ubiquitous haplotype in the eastern North Atlantic (type A in Walton 1997; type N1 in Tolley and Rosel 2006), abundant from Norway through the whole North Sea and around UK down to France. Conversely PHO4 has been found to be particularly frequent only in the North Sea (type L in Walton 1997; type S2 in Tolley and Rosel 2006). As a consequence, significant divergence between North Sea and Skagerrak (NOS–SKA) is most pronounced in summer (Table 4, Online supplementary Table 4). (2) The abundance of PHO7 in northern Kattegat (KAT1) is consistently lower in summer than when samples are combined (Fig. 4, Online supplementary Table 2), rendering KAT1 more alike to the adjacent Skagerrak (SKA). This pattern might indicate off-season migration out of KAT2/BES1, where PHO7 is particularly abundant.

Table 3 Analyses of molecular variance (AMOVA) for (a) all samples, (b) by-caught only, (c) females only, and (d) summer only

Regions	Degrees of freedom	Percentage of variation	F-statistic	*p* value of F-statistic
mtDNA				
All samples				
Among regions	4	19.5	$F_{CT} = 0.195$	0.082
Within regions among subregions	6	2.3	$F_{SC} = 0.028$	0.006
Within subregions	468	78.2	$F_{ST} = 0.218$	<0.001
By-caught				
Among regions	4	8.9	$F_{CT} = 0.090$	0.035
Within regions among subregions	5	0.8	$F_{SC} = 0.009$	0.282
Within subregions	223	90.3	$F_{ST} = 0.097$	<0.001
Females				
Among regions	4	19.9	$F_{CT} = 0.199$	0.102
Within regions among subregions	6	4.4	$F_{SC} = 0.054$	0.011
Within subregions	262	75.7	$F_{ST} = 0.243$	<0.001
Summer				
Among regions	4	26.4	$F_{CT} = 0.264$	0.067
Within regions among subregions	5	2.1	$F_{SC} = 0.029$	0.008
Within subregions	348	71.5	$F_{ST} = 0.285$	<0.001
Microsatellites				
All samples				
Among regions	4	0.9	$F_{CT} = 0.009$	0.038
Within regions among subregions	6	0.2	$F_{SC} = 0.003$	0.322
Within subregions	599	98.9	$F_{ST} = 0.011$	<0.001
By-caught				
Among regions	4	0.5	$F_{CT} = 0.005$	0.044
Within regions among subregions	5	0.3	$F_{SC} = 0.003$	0.403
Within subregions	258	99.2	$F_{ST} = 0.008$	0.012
Females				
Among regions	4	0.7	$F_{CT} = 0.007$	0.076
Within regions among subregions	6	0.5	$F_{SC} = 0.005$	0.200
Within subregions	355	98.8	$F_{ST} = 0.011$	<0.001
Summer				
Among regions	4	0.9	$F_{CT} = 0.009$	0.065
Within regions among subregions	5	0.8	$F_{SC} = 0.008$	0.032
Within subregions	432	98.3	$F_{ST} = 0.017$	<0.001

Genetic variability

The genetic variability pattern found in this study for the mtDNA (based on all 497 specimens) confirms the estimates of an earlier study on a much smaller data set ($n = 39$; Tiedemann et al. 1996) remarkably well: Diversity was $\delta = 0.73$ and $\pi = 0.49\%$ for the North Sea (NOS) (compared to $\delta = 0.88$ and $\pi = 0.42\%$ in Tiedemann et al. 1996), $\delta = 0.59$ and $\pi = 0.18\%$ for the Belt Sea (BES) (compared to $\delta = 0.59$ and $\pi = 0.15\%$ in Tiedemann et al. 1996), and $\delta = 0.70$ and $\pi = 0.29\%$ for the Inner Baltic Sea (IBS) (compared to $\delta = 0.60$ and $\pi = 0.23\%$ in Tiedemann et al. 1996). We found private haplotypes in all five regions (Table 1), in particular in the North Sea which might imply that our North Sea sample might be part of a North Sea population geographically not fully covered by our sampling scheme. If we focus on those private haplotypes occurring more than once, such haplotypes were found in the North Sea (1, accounting for 2% of the samples), the Skagerrak (1; 5%), the Belt Sea (1; 2%), and the Inner Baltic Sea (2; 6%).

Genetic population structure and population splits

Our combined mtDNA and microsatellite study on almost 500 porpoises from the Baltic area and adjacent waters

Table 4 Pairwise comparisons among adjacent regions (cf. Fig. 1) for (a) all samples, (b) by-caught only, (c) females only, and (d) summer only

Regions	NOS–SKA	SKA–KAT	KAT–BES	BES–IBS
mtDNA				
All samples	94/42	42/85	85/187	187/89
F_{ST}	0.060	0.225	0.003	0.005
p F_{ST}	**0.008**	**<0.001**	0.205	0.144
p MCMC	**0.004**	**<0.001**	**<0.001**	**<0.001**
p freq PHO7	0.252	**<0.001**	0.510	0.156
By-caught	5/17	17/57	57/83	83/69
F_{ST}	0.000	0.167	0.025	0.049
p F_{ST}	1.000	**<0.001**	**0.027**	**0.004**
p MCMC	0.460	**0.003**	**0.030**	**<0.001**
p freq PHO7	1.000	**0.002**	0.154	**0.008**
Females	49/23	23/53	53/97	97/47
F_{ST}	0.122	0.170	0.000	0.022
p F_{ST}	**0.008**	**<0.001**	1.000	0.051
p MCMC	**0.030**	**<0.001**	**0.034**	**0.001**
p freq PHO7	0.319	**<0.001**	1.000	0.289
Summer	71/32	32/65	65/136	136/54
F_{ST}	0.166	0.192	0.000	0.008
p F_{ST}	**<0.001**	**<0.001**	0.461	0.158
p MCMC	**<0.001**	**<0.001**	**0.031**	**0.001**
p freq PHO7	0.372	**<0.001**	1.000	0.748
Microsatellites				
All samples	68/17	17/43	43/112	112/65
F_{ST}	0.004	0.000	0.006	0.003
p F_{ST}	0.119	0.695	**<0.001**	**0.015**
p MCMC	1.000	1.000	1.000	0.690
p freq Clu 1	0.718	0.136	**<0.001**	**0.004**
By-caught	1/14	14/23	23/48	48/48
F_{ST}	0.017	0.000	0.010	0.005
p F_{ST}	0.531	0.951	**0.002**	**0.014**
p MCMC	1.000	1.000	1.000	0.528
p freq Clu 1	1.000	0.710	**<0.001**	**0.007**
Females	33/15	15/37	37/62	62/40
F_{ST}	0.006	0.000	0.006	0.003
p F_{ST}	0.103	0.870	**0.005**	0.106
p MCMC	1.000	1.000	1.000	0.622
p freq Clu 1	0.360	0.203	**<0.001**	0.160
Summer	49/14	14/31	31/91	91/40
F_{ST}	0.012	0.000	0.007	0.009
p F_{ST}	**0.005**	0.941	**0.001**	**<0.001**
p MCMC	1.000	1.000	1.000	0.460
p freq Clu 1	0.361	0.321	**<0.001**	**0.013**

p values <0.05 are in bold

n_1/n_2 sample sizes for any pairwise comparison. *p MCMC p* value for Markov Chain Monte Carlo test; *p freq PHO7 p* value of Fisher's Exact test on frequency of mtDNA haplotype PHO7; *p freq Clu 1 p* value of Fisher's Exact text on frequency of microsatellite Cluster 1. Bonferroni correction for all $k = 6$ possible pairwise comparisons would yield a threshold for significance of $p = 0.008$

clearly rejects the null hypothesis of a single panmictic population. It has been previously shown at the larger scale of the entire Eastern Atlantic ocean that porpoise populations might be structured due to isolation-by-distance over thousands of kilometres in the absence of oceanographic barriers, while barriers separating oceanic basins can cause profound population structure even on a much smaller geographic scale (Fontaine et al. 2007). These patterns of population structure appear applicable in our study area as well:

Kattegat, Belt Sea, and Inner Baltic Sea are basins of considerable depth (down to 124, 80, and 459 m), separated by shallow underwater ridges from 18 to 50 m deep (Köster and Schwarzer 1996). As a consequence, there is a latitudinal salinity gradient from marine conditions in the Skagerrak through brackish areas to almost fresh-water conditions in the Northern part of the Inner Baltic Sea (IBS). Because of the profound underwater structure, the margins of basins may constitute abrupt—rather than continuous—changes in environmental conditions as a consequence of limited water exchange (Zettler et al. 2007). Our finding that only a small amount of genetic variation can be attributed to isolation-by-distance might be explained by this apparent lack of a continuous environmental gradient in the Baltic area.

It is evident from all our analysis that porpoises in the Belt Sea (BES) belong to a population separate from the one in North Sea (NOS) and Skagerrak (SKA). The single most striking characteristic of this "Belt Sea" population is the high abundance of the peculiar PHO7 haplotype, a single mutational step away from the ubiquitous type PHO1. PHO7 can be truly considered a Baltic haplotype, as it has only very rarely appeared in our NOS and SKA samples, but is apparently virtually absent elsewhere: Previous studies on altogether 538 porpoises from the Eastern North Atlantic, from Norway, the North Sea, France, and Portugal down to Western Africa (Walton 1997, Tolley and Rosel 2006) revealed only 2 specimens (0.4%) bearing haplotype PHO7 (from France; S11 in Tolley and Rosel 2006), compared to a frequency of around 60% in Kattegat and Belt Sea revealed in our study. Such a population split in the Kattegat (KAT) corroborates previous genetic (Tiedemann et al. 1996; Andersen et al. 2001) and morphometric studies, which suggested a division between Kattegat/Skagerrak and Belt Sea (Huggenberger et al. 2002). At first glance, mtDNA and microsatellite data seem not fully consistent regarding the exact location of this split, as mtDNA points to a split between KAT1 and KAT2, while microsatellites support divergence between KAT2 and BES1 (Fig. 4, Online supplementary Table 4). Taking into account possible sex differences in dispersal, such a pattern could indicate a

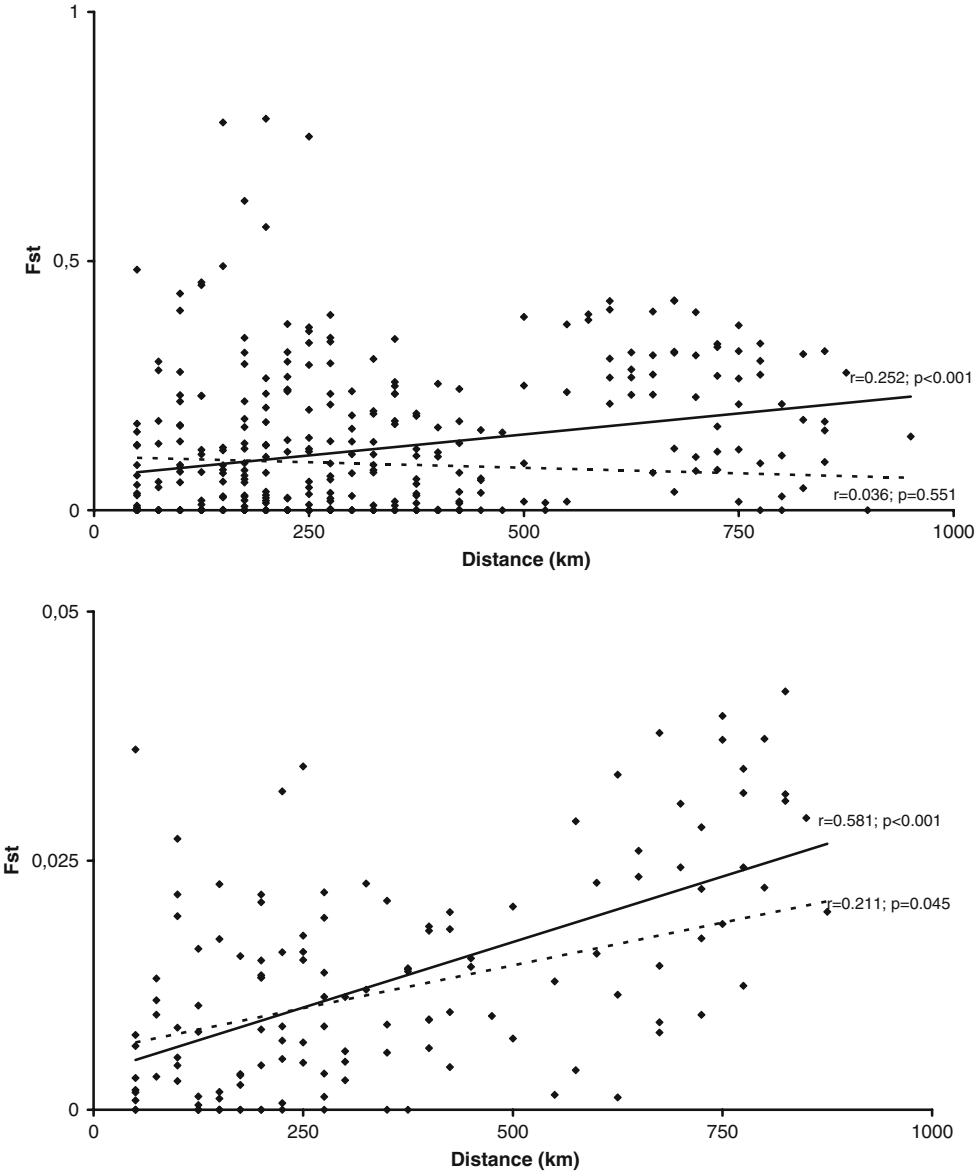

Fig. 5 Pairwise F_{ST} values among single 50 km × 50 km grids (only those with $n \geq 5$ considered), relative to the shortest swimming distance among grids for mtDNA (*upper graph*) and microsatellites (*lower graph*). Linear regression is given for the entire area (*solid line*) and without NOS (*dashed line*)

more resident female population (mainly determining the mtDNA pattern) with genetic exchanges through immigrating males causing a southward shift with regard to a split in microsatellite patterns.

Much debate has been going on whether harbour porpoises inhabiting the Baltic area could be further divided into distinct western and eastern Baltic populations, and if that was the case, where to draw the geographic line to separate them. Recent morphometric comparisons (Huggenberger et al. 2002) propose oceanographic borders at the Darss and Limhamn underwater ridges as the western limit of an Inner Baltic porpoise population (corresponding to Inner Baltic Sea IBS in our study) and a Western subpopulation including the areas of the Belt Sea (BES) and potentially part of Kattegat (KAT2, Fig. 1). We could not detect a clear trend for the Control Region data between BES and the three westernmost 100 km stretches of Inner Baltic Sea (IBS1-3) where high to medium frequencies of haplotype PHO7 were observed (Fig. 4). The frequency, however, declined in samples from central Poland (IBS4 in Fig. 4). Statistical analysis on mtDNA provides some support for a split between BES1 and BES2. Geographically, these two parts are separated by the Danish isle of Funen. Regarding microsatellites, a second split was consistently recovered between BES2 and IBS1, exactly the same location also suggested by morphometric analysis (Huggenberger et al. 2002).

It should be mentioned that this second split is less prominent than the first one: The transition in the Kattegat is associated with a pronounced shift in haplotype composition (increased frequency of haplotype PHO7) as well as microsatellite allele composition (increased frequency of

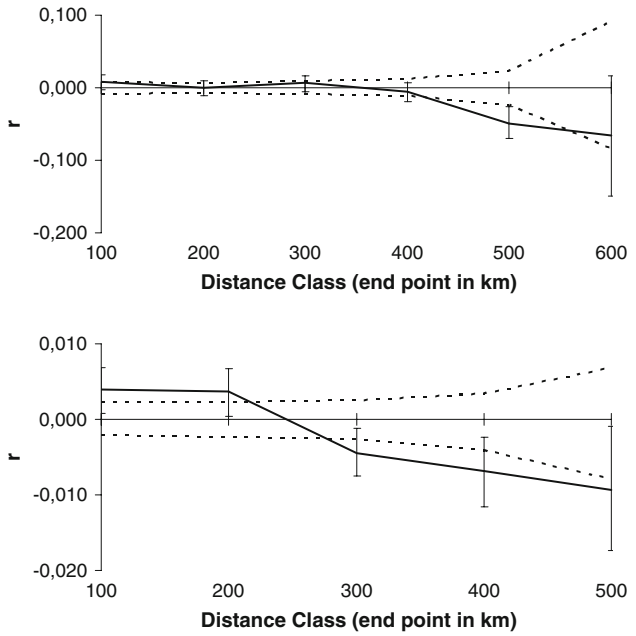

Fig. 6 Spatial autocorrelation coefficients (r, with 95% confidence intervals) of genotypic identity, conditional on shortest swimming distance among individuals (specimens from 50 km × 50 km grids with $n \geq 5$, NOS excluded), for mtDNA (*upper graph*; 350 specimens; $n = 61075$) and microsatellites (*lower graph*; 177 specimens; $n = 15576$). *Dashed lines* represent 95% confidence intervals for the null hypothesis of no autocorrelation ($r = 0$)

Cluster 1 specimens, reaching almost 100% in BES2, Fig. 4). This evidence clearly fulfils the criterion of demographic independence suggested by Dizon (2002) for the identification of a "Management Unit". In the Inner Baltic Sea (IBS), there is no such clear-cut transition relative to the adjacent Belt Sea (BES). Nonetheless, statistic analyses on both marker types consistently pick up a significant separation between IBS and BES. In light of the small absolute amount of this differentiation, it is difficult to argue in favour of a "demographic independency" of the Inner Baltic Sea population. Nonetheless, it is evident from our analysis that BES and IBS porpoises are not fully panmictic either.

Harbour porpoises are highly mobile animals (Teilmann et al. 2007). Thus one would expect moderate levels of gene flow between groups of porpoises from different areas. In fact, our data—while supporting genetic structure—point to an overarching of this structure by admixture, presumably due to sampling of migrants. It is evident that migration leads to genetic exchange only if it is followed by reproduction in areas other than the natal ones. Our significant association between microsatellite cluster assignment and the occurrence of two regional abundant mitochondrial haplotypes (PHO4, PHO7, cf. Table 5) can be taken as an indication that—although confirming migration—gene flow is apparently restricted enough not to distort this microsatellite/mtDNA "linkage". This pattern provides additional support for the interpretation of real population splits in our study area and is further corroborated by a significantly positive spatial autocorrelation for both marker systems (Fig. 6). Observing the haplotype frequencies for which the association with microsatellite clusters was significant (i.e., PHO4 and PHO7) more closely shows that PHO4 is largely restricted to harbour porpoises frequenting the German North Sea, UK North Sea waters and the Shetland Island only (Tiedemann et al. 1996; Walton 1997; Tolley et al. 1999; Tolley and Rosel 2006). This population apparently is resembled by cluster 2 in the microsatellite STRUCTURE analysis (note that all specimens bearing mtDNA haplotype PHO4 were assigned

Table 5 Association between the three most common haplotypes (PHO1, PHO4, PHO7) and the highest assignment probability to cluster 1 or 2 as inferred from the STRUCTURE analysis ($k = 2$)

	N	Mitochondrial haplotype			
		PHO1	PHO4	PHO7	Other
(a) Assignment probability >50%					
Replicated goodness of fit: $X^2 = 48.225$; $p < 0.001$					
Microsatellite Cluster 1	168	51	0	83	34
Microsatellite Cluster 2	131	52	24	28	27
Haplotype specific comparison among clusters		$p = 0.172$	$p < 0.001$	$p < 0.001$	$p = 0.943$
(b) Assignment probability >60%					
Replicated goodness of fit: $X^2 = 56.377$; $p < 0.001$					
Microsatellite Cluster 1	154	48	0	77	29
Microsatellite Cluster 2	112	48	24	18	22
Haplotype specific comparison among clusters		$p = 0.117$	$p < 0.001$	$p < 0.001$	$p = 0.881$

Assignment to either cluster was performed for (a) individuals with an assignment probability of >50% and (b) individuals with an assignment probability of >60% respectively. Statistic significance across clusters and haplotypes was analyzed using a χ^2 replicated goodness-of-fit test. Haplotype-specific occurrence was tested with a χ^2 test. *N* refers to number of samples for a given cluster assignment/haplotype combination

to cluster 2). Our haplotype network (Fig. 2) reflects a similar pattern with fewer relationships between PHO4 and other haplotypes and PHO4 being very rare among the Belt Sea individuals. We can see a fairly equal distribution of PHO1 (ubiquitous in the North Atlantic) within all sampled areas, while PHO7 clearly dominates within the Baltic area, especially in BES, and shows a highly significant association with specimens assigned to cluster 1 in the microsatellite STRUCTURE analysis. This strengthens the hypothesis of a separate population within the Belt Sea and southern Kattegat.

Conservation implications

Our geographically stratified data on mitochondrial DNA and microsatellites of 497 porpoises suggest the existence of three geographical units which differ from one another by significant differences in mtDNA haplotype and microsatellite allele composition (rejection of panmixia), i.e., (1) Skagerrak/Northern Kattegat (SKA/KAT1) (2) southern Kattegat/Belt Sea (sometimes called "Inner Danish Waters"; KAT2/BES), and (3) Inner Baltic Sea (IBS). From an evolutionary point of view, these differences are very subtle, as the two most abundant haplotypes PHO1 and PHO7 only differ by a single mutation and most haplotypes are closely related (Fig. 2). Clearly, the entire area is sufficiently connected through genetic exchange to be considered as belonging to the same "Evolutionary Significant Unit" (Moritz 1994, 1999). This is not surprising as (1) the Baltic Sea in its present form is less than 7000 years old (e.g., Köster and Schwarzer 1996), (2) no absolute barrier exists, and (3) porpoises are known to move considerably (Teilmann et al. 2008). In former times, seasonal movements through the Little Belt, supposedly coming from the Inner Baltic Sea, were so intense that porpoises were exploited by hunters in the Little Belt (Dudok van Heel 1962). The ability to seasonally migrate is essential for Baltic porpoises, as the Baltic Sea can almost completely freeze in harsh winters and can cause ice entrapments and mass death of porpoises (Teilmann and Lowry 1996). However, seasonal migration needs not necessarily to translate into dispersal and genetic exchange, as there is indication for philopatry and faithfulness to particular breeding areas, especially in females (Siebert et al. 2006; Verfuß et al. 2007). It has been recently argued that the sole rejection of panmixia should no longer be considered sufficient to the identification of a "management unit" (MU) (Palsbøll et al. 2006). Instead, it is suggested to define a threshold value of divergence below which significantly diverged geographical populations shall be lumped into the same MU. The authors correctly argue that—with high statistical power (e.g., with large sample sizes)—panmixia can be rejected, although populations might be demographically correlated through dispersal. In this context, a threshold of 10% dispersal has been suggested (Hastings 1993). In our study, if applying the F_{ST}-based Nm calculation implemented in ARLEQUIN as a rough estimate, mtDNA data yield an estimate of 6.5 and 7.5 migrants per generation across the inferred splits between KAT1/KAT2 and BES1/BES2, respectively. Although we are well aware of the limitations of this simple calculation (cf. Whitlock and McCauley 1999), these estimates would translate into a dispersal of around 1% per generation, if related to the BES2 population estimate (588; Hammond et al. 2002), clearly below the suggested 10% threshold. We hence argue that—although we have not exact gene flow estimates—genetic influx from the KAT2/BES1 into BES2/IBS should be limited enough not to cause full demographic connectivity: Given the very asymmetric size of these two populations, with tens of thousands porpoises on the one and only a few hundred at maximum on the other side (Hammond et al. 2002; Berggren et al. 2004; Hiby and Lovell 1996), even low dispersal rates would translate into a high number of migrants (relative to the size of the latter population), such that significant genetic differences (as observed here) could not be maintained. From a statistical point of view, splitting (into two MUs) or lumping (into a single MU) can either be the biologically correct decision or comprise a statistical error of type I (erroneous split of a single demographically coherent set of specimens) or type II (erroneous lumping of two demographically independent sets of specimens). However, the scientific habit of mainly controlling the type I statistical error should be re-considered in the conservation realm: In the case of the Baltic harbour porpoise, it can be considered "safer" to specifically conserve and protect the Inner Baltic porpoise population identified by both genetic and morphological means (Berggren and Wang 2008), although some demographic correlation might exist with the population of the Southern Kattegat/Northern Belt Sea, e.g., through male dispersal indicated by the less pronounced divergence at microsatellites, relative to the mtDNA. The alternative of managing KAT, BES, and IBS as a single population has the potential to become a "self-fulfilling prophecy": Adopting that management strategy would translate into a common tolerable by-catch estimate for the entire Baltic area. Because of the relatively large size of the population in the KAT/BES area, such management would likely condemn a separate Inner Baltic population to extinction in the foreseeable future.

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