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Genetic isolation of a now extinct population of bottlenose dolphins (*Tursiops truncatus*)

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A number of dolphin species, though highly mobile, show genetic structure among parapatric and sometimes sympatric populations. However, little is known about the temporal patterns of population structure for these species. Here, we apply Bayesian inference and data from ancient DNA to assess the structure and dynamics of bottlenose dolphin (*Tursiops truncatus*) populations in the coastal waters of the UK. We show that regional population structure in UK waters is consistent with earlier studies suggesting local habitat dependence for this species in the Mediterranean Sea and North Atlantic. One genetically differentiated UK population went extinct at least 100 years ago and has not been replaced. The data indicate that this was a local extinction, and not a case of historical range shift or contraction. One possible interpretation is a declining metapopulation and conservation need for this species in the UK.

Keywords: ancient DNA; marine mammal; population genetics; metapopulation

1. INTRODUCTION

Data on population dynamics are critical for the effective conservation of large mammal species, whose populations are frequently fragmented, and for which critical habitat may be lost to anthropogenic disturbance. One key question is about the pattern of individual or group movement over time. If local habitat is critical, and local extinctions are possible, how long does it take before that habitat patch is reoccupied? From a conservation point of view, when is it necessary to conserve unoccupied but suitable habitat for a protected species? This information is important, but can be difficult to acquire for long-lived species (Elmhagen & Angerbjorn 2001). One way to address this is by comparison with historical populations, but for this to be effective, it is necessary to understand the nature of a given historical population and its relationship to extant populations. Here, we use ancient DNA (aDNA) to assess the status of a historical bottlenose dolphin (*Tursiops truncatus*) population, from a location where this species is no longer found in an established local population.

The bottlenose dolphin is a cosmopolitan species found throughout the world's temperate and tropical oceans. Resident populations have been reported throughout the species range (Leatherwood & Reeves 1990). Natoli *et al.* (2005) showed that at least five regional genetically differentiated populations exist between Scotland and the Black Sea, and that boundaries to gene flow correspond to habitat boundaries. Other studies have shown similar structure along the Gulf Coast of Florida (Sellas *et al.* 2005) and in the Gulf of California (Segura *et al.* 2006). This concentration into small, primarily resident populations has raised conservation concerns given potential local anthropogenic impact (Thompson *et al.* 2000). It also suggests local habitat dependence, and the possibility that a metapopulation model will be useful towards understanding the population biology of this species, though clear data on the dynamics of these populations in relation to metapopulation models are not yet available (and therefore a conclusion about the utility of this model cannot as yet be reached for this species).

We analysed samples from modern populations distributed around the coastal waters of the UK (and more distant populations for reference), and compared these with ancient samples from the Humber River estuary where no current population exists (figure 1). The modern distribution of bottlenose dolphins in UK waters is discontinuous (Evans 1993), and we obtained samples from all known regional concentrations. Sightings in the intervening regions are relatively rare, and often represented by individual animals (Evans 1993). Artefacts, including many butchered animal bones, excavated from the high status, middle-late Saxon settlement at Flixborough (Loveluck & Dobney 2001) dated between the seventh and tenth centuries AD, were well preserved due to the high alkaline wood ash content of the middens (Canti 1992). The sheer quantity of cetacean remains (primarily *T. truncatus*) found at the site is exceptional (157 specimens), and their presence across all strata indicates their usage by the inhabitants over the entire 400 years of occupation at the site. We consider and exclude the possibility that the Flixborough dolphins may have been caught and killed elsewhere in the UK. We then test the

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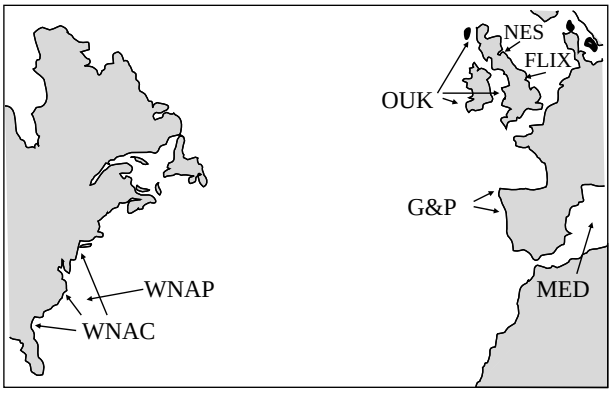

Figure 1. Sample locations. FLIX, Flixborough (on Humber River); NES, Northeast Scotland; OUK, United Kingdom (except Northeast Scotland) and Ireland; G&P, Galicia and Portugal; MED, Mediterranean Sea; WNAC, Western North Atlantic Coastal; WNAP, Western North Atlantic Pelagic.

hypothesis that the dolphin bones recovered at Flixborough represented a local insular population, and not a modern range contraction or distributional change of one of the extant populations. If correct, this would imply the abandonment of a local habitat patch, and the lack of its recolonization for at least 100 years.

2. MATERIAL AND METHODS

(a) *Samples*

Sample locations are shown in figure 1. A total of 157 cetacean specimens (vertebrae, ribs, cranial fragments and some teeth) were analysed from Flixborough, and grouped into a minimum number of potential animals (58 for *Tursiops*) based on non-repeatable elements, and stratigraphic position in the site. Modern samples from strandings and biopsies included some mitochondrial DNA (mtDNA) and locus D08 data from previous studies (Hoelzel *et al.* 1998; Parsons *et al.* 2002; Natoli *et al.* 2005). Samples from Saxon Flixborough were compared with recent populations from the Moray Firth, western UK and Ireland, northwest Spain and Portugal, the Mediterranean, and coastal and offshore populations from the western North Atlantic (figure 1).

(b) *DNA extraction and amplification*

All aDNA extractions and polymerase chain reaction (PCR) set-ups were carried out in a dedicated aDNA laboratory where no work on modern or post-PCR DNA was carried out. Furthermore, this was the first time this species had been worked on in that laboratory. All materials and work surfaces used for aDNA were cleaned before and after each use with a 10% dilution of bleach. All solutions used were autoclaved and filtered through a 0.2 µm syringe filter. All samples were wiped down with a 10% dilution of bleach, polished with sand paper and exposed to UV irradiation at a distance of 3–10 cm for a minimum of 15 min before drilling. The densest area of each bone was chosen for drilling and tooth samples were taken from the pulp cavity. Displaced bone powder was collected and volumes ranging from 0.1 to 0.5 ml of powder were digested in 0.425 M EDTA, 0.5% SDS, 0.05 M Tris and 0.333 mg ml^{-1} proteinase K. A range of 3–8 ml of digestion buffer was used, depending on sample size, and samples were incubated on a rotating wheel at 55°C overnight and then at 37°C for 24 h. A 1.4 ml aliquot of each digest was used in a slightly modified version of the QIAquick PCR Purification Kit method recommended in Yang *et al.* (1998). For this study, samples were washed twice with QIAquick PE buffer and additionally centrifuged for 1 min to remove all traces of the buffer before elution in 50 µl of 1X TE buffer. DNA was not quantified and was stored at −20°C. Extraction controls that underwent each step, but which began with no bone powder, were included in addition to standard negative controls. All ancient amplifications were replicated from independent extractions (all work done in Durham).

DNA preservation in the zooarchaeological assemblage at Flixborough was confirmed by the independent amplification of aDNA from bones of domestic and wild geese (Barnes *et al.* 2000). The mtDNA amplicons were not cloned since 92.3% of directly sequenced individuals (all with replication) had the same single haplotype, and that sequence was consistent with expectations given the geographical position of the site. Modern samples were extracted by standard methods.

(c) *Mitochondrial DNA*

A 171 bp segment of the 5′ control region was amplified (excluding primers). The primers were designed to maximize specificity to *T. truncatus* DNA: 5′-TTAGTCTCTCCTTG-TAAAT and 5′-GGTGATTAAGCTCGTGAT. For each aDNA reaction, 5 µl of the DNA extract was used in 25 µl PCR mixture containing 15 mM Tris–HCl (pH 8.0), 50 mM KCl, 0.2 mM of each dNTP, 2.5 mM $MgCl_2$, 0.08 µg $µl^{-1}$ bovine serum albumin (BSA), 26 ng $µl^{-1}$ of each primer and 0.5 units PE Applied Biosystems AmpliTaq Gold hot start Taq polymerase. Samples underwent 46 reaction cycles with an annealing temperature of 57°C. PCR products were purified using a Qiagen QIAquick PCR Purification Kit and sequenced using the ABI BigDye Terminator sequencing kit. Nucleotide diversity (π) was calculated using Arlequin v. 2.0 (http://lgb.unige.ch/arlequin/), as was ϕ_{ST} with 1000 permutations. Mantel (1967) tests correlating geographical and genetic distance (for both mtDNA and microsatellite data) were tested for significance by 1000 permutations.

(d) *Microsatellites*

Loci studied were Ttru AAT_{44} (Caldwell *et al.* 2002); D08, D18, D22 (Shinohara *et al.* 1997); and MK8 (Krutzen *et al.* 2001) chosen for their relatively short lengths. Reactions for aDNA samples were in 25 µl using a 5 µl aliquot of DNA extract, 15 mM Tris–HCl (pH 8.0), 50 mM KCl, 0.2 mM of each dNTP, 1.3–1.8 mM $MgCl_2$ (specific to each primer), 0.08 µg $µl^{-1}$ BSA, 26 ng $µl^{-1}$ of each primer (including 10% fluorescently labelled primer) and 0.5 units PE Applied Biosystems AmpliTaq Gold. Reactions were run for 46 cycles with annealing temperatures of 52°C for Ttru AAT_{44}, 56°C for D22, 59°C for D08, and 62°C for D18 and MK8. Products were visualized on an ABI Prism 373 or 377 slab gel automated sequencer.

Amplifications from ancient samples were replicated from independent extracts 2–13 times, depending on the size and quality of the sample. Genotypes were accepted only when one or two alleles were consistently amplified (samples with ambiguous amplifications of multiple alleles were discarded for that locus). Homozygotes at microsatellite DNA loci were accepted after two to eight replications (average = 3).

Allelic richness was calculated using Fstat v. 2.9.3.2 (http://www2.unil.ch/popgen/softwares/fstat.htm) with an adjusted sample size of $n = 19$. Observed heterozygosity was tested against Hardy–Weinberg expectations in Arlequin v. 2.0 using a Fisher exact test and the Markov chain method (100 000 iterations and 5000 dememorization steps). Population

Table 1. F_{ST} based on five microsatellite DNA loci in the upper diagonal (all significant after Bonferroni correction; $p<0.003$), and ϕ_{ST} based on mtDNA control region sequence in the lower diagonal ($^{*}p<0.05$, $^{**}p<0.001$).

	FLIX	NES	OUK	G&P	MED	WNAP	WNAC
FLIX		0.163	0.140	0.098	0.153	0.144	0.210
NES	0.037		0.049	0.127	0.196	0.163	0.222
OUK	0.130*	0.145**		0.057	0.130	0.093	0.224
G&P	0.332**	0.352**	0.013		0.027	0.014	0.142
MED	0.664**	0.654**	0.288**	0.155*		0.046	0.160
WNAP	0.549**	0.544**	0.242**	0.106*	0.056*		0.138
WNAC	0.868**	0.872**	0.622**	0.635**	0.565**	0.643**	
MPOP[a]	0.376 (0.252, 0.508)	0.316 (0.202, 0.437)	0.115 (0.073, 0.161)	0.060 (0.029, 0.095)	0.087 (0.049, 0.130)	0.053 (0.027, 0.083)	0.274 (0.180, 0.376)

[a] Population-specific F_{ST} values indicating the extent to which each local population differs genetically from the metapopulation as a whole (see text); values in parentheses are 95% high probability density intervals (HPDIs). Population abbreviations are the same as given in figure legend 1.

differentiation was measured using F_{ST} as calculated by FSTAT v. 2.9.3.2 using permutation to test for significance (1000 iterations). An assignment test was performed in GENECLASS2 (http://www.montpellier.inra.fr/URLB/index.html), with assignment probabilities calculated using Monte–Carlo resampling (1000 simulated individuals, $\alpha=0.01$).

The most probable number of putative populations (K) that best explains the pattern of genetic variability was estimated using the program STRUCTURE v. 2.1 (Pritchard *et al.* 2000). We assumed admixture and a correlated allele frequency model. Burn-in length was 500 000 replications; simulation length was 1 000 000 repetitions. K was set to $2\leq K\leq 10$, and each value was replicated thrice.

(e) *Individual population* F_{ST}

The hierarchical Bayesian method used (Foll & Gaggiotti 2006) estimates individual F_{ST} values for each local population and relates them to environmental factors using a generalized linear model. We first applied this method to estimate individual F_{ST} values for all the European and West Atlantic populations without the inclusion of environmental data. We then carried out a second analysis in order to investigate the potential effects of the age of the samples and their geographical origin. In this case, we considered two factors: (i) age of samples (modern or ancient) and (ii) the geographical area (West or East Atlantic). The consideration of two factors leads to nine alternative models and the method provides posterior probabilities for each one of them using a Reversible Jump MCMC approach. The model with the highest posterior probability is the one that best explains the data.

In all cases, we used 10 pilot runs of 2000 iterations to obtain the parameters of the proposal distributions used by the MCMC. We also used an additional burn-in of 1.5×10^5 iterations and a thinning interval of 50. All estimates were obtained from a sample size of 20 000.

3. RESULTS

Dolphin bones from Flixborough represented a minimum of 58 individuals (determined primarily through an assessment of skeletal element representation and stratigraphic integrity). Of these, DNA was successfully extracted from 39 samples (primarily from vertebrae and other bones; see §2). Unique genotypes confirmed the identification of individuals. The authenticity of genotypes was supported by replication from independent extracts, novel combined genotypes and the failure of aDNA extracts to amplify for larger fragments (over approx. 250 bp).

The mtDNA data (new sequence GenBank accession numbers: EF540867–EF540876) show the greatest similarity between Flixborough and other UK/Irish samples, and these results are not consistent with the Saxon era sample representing a population now distributed elsewhere. The dominant haplotype found in Flixborough (present in 36 out of 39 samples; 92.3%) is fixed in the Moray Firth and present in 62.2% of samples from the western UK. Further south and west, the haplotype becomes less common (42.9% of samples from northern Spain, 28.6% of samples from the Mediterranean and not found in the western North Atlantic). Mantel tests comparing $F_{ST}/(1-F_{ST})$ against ln geographical distance among all coastal populations were highly significant (mtDNA, $p=0.002$; microsatellite DNA, $p=0.01$). Population structure was evident from F_{ST} data for both microsatellite and mtDNA (table 1).

Multilocus individual genotypes at Flixborough assigned back to Flixborough 78.9% of the time (using GENECLASS; see §2). A likelihood assignment test clustering individuals by genotype (implemented in STRUCTURE; see §2) defined the Flixborough (FLIX), Mediterranean (MED) and Western North Atlantic Coastal (WNAC) putative populations most clearly (figure 2). The most probable number of populations identified by STRUCTURE was five (the estimated ln probability for $K=4$ was -3080.7, for $K=5$ was -2998.7 and for $K=6$ was -3022.7; posterior probability of $K=5$ was 1.00), but differences among all putative populations could be seen in the assignment proportions (figure 2).

Population-specific F_{ST} values (table 1) measuring the degree of differentiation between each local population and the putative metapopulation as a whole (Foll & Gaggiotti 2006), indicate that the genetic composition of FLIX is quite distinct, and two other local populations (NES and WNAC) have similar values. The analysis that considers the effect of age of samples and geographical area assigns the highest posterior probability (0.57) to the null model (excluding age and geographical area). Thus, neither of the two factors considered explains well the observed genetic structure. The model that includes only the age of the sample has a posterior probability of 0.26 indicating that some degree of temporal genetic differentiation between FLIX and the extant metapopulation may have accumulated between the time of extinction and the present time. The model that includes only the geographical origin of the sample has a low posterior probability (0.11), while all other models have negligible posterior probabilities. The

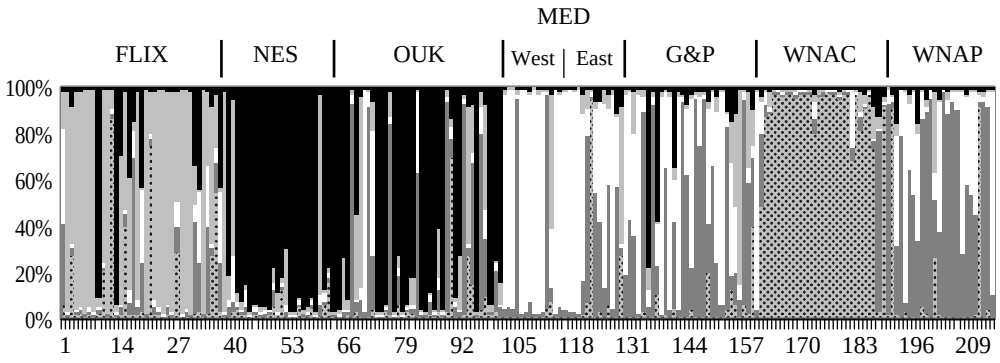

Figure 2. Likelihood assignments based on individual genotypes at five microsatellite DNA loci. Abbreviations are the same as given in figure legend 1. (See electronic supplementary material for colour version of figure 2.)

Table 2. Comparative measures of diversity. (AR, allelic richness; CR, mtDNA control region. Population abbreviations are the same as given in figure legend 1. Asterisk indicates significance at $p=0.05$ after Bonferroni correction.)

	FLIX	NES	OUK	G&P	MED	WNAP	WNAC
AAT_{44} N	33	27	31	28	29	27	27
N alleles	5	6	7	8	8	7	4
AR	4.500	5.111	5.837	7.471	7.295	6.826	3.407
H_o	0.485	0.593	0.645	0.857	0.690	0.778	0.630
H_e	0.560	0.606	0.720	0.795	0.816	0.781	0.535
D22 N	22	26	31	29	28	26	27
N alleles	4	5	11 (1)	8	8	8	7 (1)
AR	3.998	4.461	9.420	7.422	7.685	7.594	6.618
H_o	0.455	0.500	0.742	0.759	0.571	0.808	0.852
H_e	0.511	0.560	0.775	0.817	0.779	0.852	0.782
D08 N	20	27	29	29	29	26	27
N alleles	4	3	8 (1)	8 (1)	9 (1)	11 (1)	7
AR	4.000	2.994	6.620	7.388	8.157	9.786	5.813
H_o	0.650	0.407	0.379	0.724	0.793	0.808	0.667
H_e	0.671	0.440	0.469	0.771	0.831	0.870	0.600
MK8 N	23	20	29	24	28	22	19
N alleles	4	5	7	8	8	8	6 (1)
AR	3.973	4.950	6.299	7.576	7.479	7.847	6.000
H_o	0.609	0.800	0.552	0.917	0.786	0.773	0.789
H_e	0.706	0.685	0.787	0.824	0.832	0.832	0.713
D18 N	29	27	35	29	30	26	27
N alleles	5	4	12 (2)	10	9	12 (1)	4
AR	4.847	3.997	9.424	9.557	8.438	11.302	3.704
H_o	0.517	0.556	0.514*	0.862	0.767	0.846	0.630
H_e	0.564	0.664	0.728	0.887	0.853	0.901	0.650
CR N	39	28	37	14	34	25	29
N Hap.	2	1	11	9	14	11	6
π	0.002 ± 0.002	0	0.021 ± 0.012	0.024 ± 0.014	0.029 ± 0.016	0.029 ± 0.016	0.010 ± 0.006

model-averaged F_{ST} values obtained from this analysis are almost identical to those obtained from the analysis that did not consider environmental data. Moreover, estimates of population-specific F_{ST} values differ little among the alternative models. There is a small difference observed for FLIX, whose F_{ST} is largest under the model that includes age (mean = 0.389, HPDI = (0.271, 0.527)) and smallest under the null model (mean = 0.366, HPDI = (0.248, 0.494)). This suggests that the effect of the age of the sample is only minor.

All the genetic analyses carried out strongly suggest that FLIX is a distinct local population, rather than representing a range shift or a subsample from a historically more extensive neighbouring population. It is also unlikely that the Flixborough dolphins were caught further a field and transported to the site unless they were all caught in the same (unknown) local population (otherwise, its local population F_{ST} would have been low).

There were no significant heterozygote deficiencies for the FLIX sample after Bonferroni correction. Allelic dropout was detected from multiple amplifications of heterozygotes, but replication was apparently sufficient to address this for the acceptance of homozygotes (replicated up to eight times, see §2). Diversity levels were very similar at FLIX, NES and WNAC (table 2; cf. Hoelzel *et al.* 1998), all showing relatively low levels of diversity, especially for mtDNA. The other more diverse populations may be open to greater migration from pelagic habitats. The one pelagic sample, Western North Atlantic pelagic (WNAP), shows the lowest individual population F_{ST} (table 1) and the greatest diversity (table 2). Relatively high diversity at OUK could reflect the sampling of

multiple populations, though there is no apparent Wahlund effect (no evidence for significant heterozygote deficiency in this sample).

4. DISCUSSION

This study sheds light on the relationship between extant and a now extinct population of bottlenose dolphins. The now extinct population is among several populations located at the northeastern margin of the species' range, and all statistical genetic analyses indicate that it represents a well-defined discrete local population that went extinct at least 100 years ago.

The most complete census data for UK cetacean sightings have been recorded over the last 30 years; however, there are records for the Humber region dating back at least until the late nineteenth century (see review in Evans 1993). The last record indicating regular sightings of bottlenose dolphins in the Humber estuary was by Caton-Haigh (1892). Since then, records (including census and stranding records published since 1927) indicate that sightings have been rare, as they are today (Evans 1993). However, zooarchaeological data from Flixborough suggest the existence of an active dolphin fishery in Saxon times, possibly exploiting a resident population (Dobney *et al.* in press). The implication is that this population went to extinction at least 100 years ago, and the region remains abandoned today. If the sightings in Caton-Haigh (1892) reflected transitory individuals rather than an established population, then extinction could have been anytime back to approximately 1000 years ago. However, solitary dolphins (as are often seen along the UK coast) were typically identified as such, and the wording in Caton-Haigh (1892; though brief) suggests that sightings were common at that time.

Although much information is necessarily missing from an analysis of a now extinct population, our results raise important questions about population dynamics. Studies suggesting dependence on local habitat patches, and reduced gene flow among patches for this species (Natoli *et al.* 2005; Sellas *et al.* 2005) meet part of the criteria for metapopulation structure. Under that model, the dynamics of local patch extinction and recolonization would determine the temporal fate of an abandoned patch. In this case, FLIX has apparently remained empty for at least 100 years, or five or more dolphin generations, and potentially much longer. There are three possible explanations, considered within the framework of a metapopulation model. First, recolonization is slow enough for five generations to be within the normal range. Second, the habitat patch at the Humber estuary has been effectively removed as suitable habitat by pollution, prey species depletion or similar disturbance. Third, the broader metapopulation in UK waters is in decline. If locally defined habitat patches were unimportant to this species, then none of this would apply, but there are considerable data to suggest that local habitat dependence is important for bottlenose dolphins (see review in Natoli *et al.* 2005).

The first hypothesis would imply long-term recolonization dynamics and has important implications for habitat conservation for this species. The power for comparisons of migration rates is limited by the number of loci that could be amplified from the ancient material. However, estimated gene flow rates between extant populations based on nine polymorphic loci and the coalescent model implemented in MIGRATE (Beerli & Felsenstein 2001) ranged from 3.07 to 16.63 per generation (Natoli *et al.* 2005), suggesting opportunity for recolonization. These are highly mobile animals, known to be capable of excursions much further than the distance between the NES and FLIX sites (Reynolds *et al.* 2000). Further, a recent simulation study (Vuilleumier & Perrin 2005) indicates that landscape connectivity in species with high cognitive abilities (such as dolphins) is expected to be high enough to allow for long-distance dispersal, and at the same time maintain genetic structuring.

The second two possibilities may be more probable. The Humber estuary is a major shipping area with 84 million tones of cargo passing through its ports last year (Fletcher & Burman 2005), and long-term concerns over pollution both in the water column and in the sediment (Edwards *et al.* 1997). However, it is not known whether these factors would exclude bottlenose dolphins, and this species is found in other regions with high levels of boat traffic and pollution (e.g. near New Orleans at the mouth of the Mississippi (Fertl *et al.* 1999), Santa Monica Bay in Los Angeles (Bearzi 2005), etc.). Further, the site was apparently abandoned by bottlenose dolphins at least 100 years ago, and both boat traffic and pollution would have been much less pronounced at that time (though some other environmental factors could in theory be responsible). The area was primarily agricultural until the Victorian era, and it remains an important site for water birds (ranked among the top five important sites in the UK by *English Nature*). There are, on the other hand, corroborating data suggesting the decline of bottlenose dolphins in North Sea waters (Harmer 1927; Fraser 1953; Kayes 1985; Evans 1993; Parsons *et al.* 2002), implying that the lack of recolonization could reflect a declining metapopulation. The lack of a positive equilibrium point is a potential cause for metapopulation extinction (Hanski 1991). Kayes (1985) reviews the evidence for a decline in bottlenose dolphin numbers along the Dutch North Sea coast since the late 1960s. However, Wilson *et al.* (1999) suggest that there is no clear trend for the population at the Moray Firth.

The data from aDNA permitted the exclusion of possible alternative explanations (range contraction or redistribution), and illustrated that a genetically unique population has been lost at a site that has not been repopulated. Given the propensity for this species to develop local resource specializations (see review in Hoelzel in press), and apparent habitat dependence (Natoli *et al.* 2005; Sellas *et al.* 2005), the abandonment of a site raises questions about population dynamics. Although metapopulation dynamics have not been fully demonstrated for this species, our data could imply a decline for a broader UK metapopulation, and a conservation need for this species in UK waters.

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