Preliminary Analysis of Mitochondrial DNA Variation Within and Between the Right Whale Species *Eubalaena glacialis* and *Eubalaena australis*

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ABSTRACT

Skin biopsy samples have been obtained from 126 North Atlantic (*Eubalaena glacialis*) and 17 South Atlantic (*E. australis*) right whales. Samples varied in size from less than 0.05g to 1.31g with an average of 0.46g. The amount of DNA isolated from 20 samples averaged 600μg/g of tissue. Many of the DNA samples contained low molecular weight DNA, however, all but one sample had mtDNA of sufficient quality for restriction fragment length polymorphism (RFLP) analysis.

DNA samples from 10 North Atlantic and 10 South Atlantic whales were digested with 10 restriction enzymes and surveyed for RFLPs using the whole cloned mouse mitochondrial DNA as a probe. A total of seven composite restriction fragment length morphs were found which differed from one another by one or more restriction sites. Three composite morphs were present among the North Atlantic samples and four among the South Atlantic samples; all composite morphs were species specific. Within population nucleotide divergence (π) was estimated as 0.08% for E. glacialis and 0.24% for *E. australis*. The genetic distance between the North and South Atlantic right whales was 1.82% suggesting that the two diverged between approximately 0.9 to 1.8 million years ago.

Keywords: right whales; North Atlantic; South Atlantic; genetics; taxonomy; evolution.

INTRODUCTION

Prior to the 15th century, right whales (genus *Eubalaena*) are believed to have had a world wide abundance of 100,000 to 300,000 (Braham and Rice, 1984). Extensive whaling operations over the subsequent four and a half centuries severely depleted the right whale populations and current estimates suggest that there are less than 700 northern right whales (*E. glacialis*) (IWC, 1986), of which approximately 300 are located in the northwest

Atlantic population (Kraus, 1985; Gaskin, 1987), and an estimated 3,000 southern right whales (*E. australis*) (Braham and Rice, 1984; IWC, 1986) of which approximately 1,200 reside in the South Atlantic (Payne *et al.*, 1990).

The South Atlantic right whale is similar in appearance and breeding biology to the North Atlantic right whale, but, because of its geographical distribution, its reproductive cycle is six months out of phase (Braham and Rice, 1984). As a result, northern and southern right whales are assumed to be reproductively isolated and are considered to be separate species (Muller, 1954; Schevill *et al.*, 1986).

Northern and southern right whales have been protected internationally from commercial whaling since the 1930s. Despite this protection the North Atlantic population is not showing significant signs of recovery (Kraus, 1985; Gaskin, 1987). In contrast, the South Atlantic right whale population is estimated to be growing at a rate of 7.6% per year (Payne *et al.*, 1990).

In the current study, the amount of genetic variation within and between the North and South Atlantic right whales was assessed in order to examine their time of genetic divergence. The level of genetic variation was assessed using mitochondrial (mt) DNA restriction fragment length polymorphisms (RFLPs), an approach that requires very little tissue and can detect low levels of genetic variation (see Discussion below). Skin samples were obtained by remote biopsy darting of free-ranging right whales (Brown *et al.*, this volume; Lambertsen, 1987; Lambertsen *et al.*, 1988; Matthews *et al.*, 1988).

MtDNA has a number of characteristics that make it an attractive tool for genetic studies that look at closely-related individuals and species. Animal mtDNA is a double-stranded, circular molecule, approximately 16,500 base pairs (bp) in length, which evolves five to 10 times as quickly as nuclear DNA (Brown *et al.*, 1979; 1982). This high rate of evolution, which may be due to an ineffective repair system, results in a high level of intraspecific variation (Brown *et al.*, 1979; 1982). Further, mtDNA is inherited maternally by transmission through the egg cytoplasm without paternal leakage (Avise *et al.*, 1983; Lansman *et al.*, 1983; Kessler and Avise, 1985; Cann *et al.*, 1987), which means that mutational changes in the molecule are simpler and more easily modelled than are those within nuclear DNA, since the latter is also influenced by the effects of recombination (Monnat and Loeb, 1985). These characteristics make mtDNA RFLP analysis useful for the assessment of genetic variation both within and between closely related species.

PROCEDURES

Tissue samples

In 1988 and 1989, skin samples were collected from the North Atlantic right whales in the Bay of Fundy and the continental shelf of the Atlantic coast of Nova Scotia between Browns and Baccaro Banks, and from individuals from the South Atlantic population found off the shores of Argentina around Peninsula Valdes. The whales were darted at a range of 5–20m, using crossbows and arrows fitted with biopsy tips. [For details, see Brown *et al.*, this volume.]

DNA extraction

Whole DNA (nuclear and mitochondrial) was extracted by first grinding the frozen tissue and 3.5 ml of 4M urea, 0.2M NaCl, 100mM Tris-HCl (pH 8.0), 0.5% n-lauroylsarcosine, and 10mM EDTA to a fine powder with a mortar and pestle cooled with liquid nitrogen. Samples were then incubated for two days at 37°C. Proteinase K (65 units) was added and the samples returned to 37°C for one day. To finish the extraction, each sample was extracted once with one volume of phenol/chloroform isoamyl alcohol and once with one

volume of chloroform isoamyl alcohol. Finally the DNA was precipitated by the addition of 0.3M sodium acetate and two volumes of 95% isopropanol at room temperature and centrifuged for 15 minutes at 20,000 x g. The resultant pellet was dissolved in 1.0ml 10mM Tris-HCl (pH8), 10mM NaCl, and 2mM EDTA (pH 8.0). The quality and quantity of the isolated DNA was determined by agarose gel electrophoresis (Maniatis *et al.*, 1982).

RFLP analysis

Total DNA (nuclear and mitochondrial) from 10 northern and 10 southern right whale samples was digested with 10 restriction enzymes recognizing sequences of four or six base pairs (*Apa* I, *Ava* I, *Bal* I, *BamH* I, *Bgl* I, *BstE* II, *Cla* I, *Hha* I, *Sin* I and *Stu* I) according to the manufacturer's (Bethesda Research Laboratories) directions. These enzymes were chosen from a large number of enzymes initially surveyed because they produced several easily scorable fragments. After digestion, the resultant DNA fragments were electrophoresed through 20cm 0.8% agarose gels at 30 volts for approximately 16 hours, transferred to nylon membranes by Southern blotting (Southern, 1975) and probed with a radioactively labelled cloned pAM 1 mtDNA probe which contains the entire mouse mtDNA (Martin and Clayton, 1979). The probe was labelled via primer extension (Maniatis *et al.*, 1982).

RESULTS

In the past two years a total of 143 samples have been collected, representing 126 North Atlantic and 17 South Atlantic individuals. The amount of tissue obtained from the first 20 samples processed varied from less than 0.05g to 1.31g, averaging approximately 0.46g per biopsy plug (Table 1). The amount of DNA isolated from these skin samples averaged 600μg/g of tissue. Most of the DNA contained some molecules less than 20kb in length, however, all but one sample had mtDNA of sufficient quality for the mtDNA analysis.

Table 1

Summary of DNA yields from North (Egl) and South (Eau) Atlantic right whale skin biopsy samples

Queen's I.D.No.	Amount of tissue (g)	DNA yield (μg)	Queen's I.D. No.	Amount of tissue (g)	DNA yield (μg)
Egl 01	0.60	133	Eau 06	0.25	75
Egl 02	1.10	1,194	Eau 07	0.21	250
Egl 03	0.80	358	Eau 09	0.11	75
Egl 04	0.40	198	Eau 10	0.09	433
Egl 05	0.25	133	Eau 11	0.18	833
Egl 06.	0.18	134	Eau 12	0.08	25
Egl 07	0.43	198	Eau 13	0.24	100
Egl 08	<0.05	8	Eau 14	0.29	149
Egl 10	1.12	198	Eau 15	0.36	166
Egl 11	2.29	135	Eau 16	0.14	167
Egl 12	0.35	298			
Egl 13	0.33	298			
Egl 14	0.12	49			
Egl 15	0.30	150			
Egl 16	<0.05	13			

The mtDNA was surveyed for RFLPs in 10 North Atlantic and 10 South Atlantic right whales using nine 6bp and one 4bp recognition site restriction enzymes and probed with pAM1. Forty-three to 45 restriction sites were scored for each individual representing 1.52% of the approximately 16,500bp mtDNA genome. The majority of the polymorphisms observed could be explained by the addition or deletion of a single restriction site. For example, *Cla* I generated three patterns (Fig. 1). Pattern B consisted of one 16.5 kb fragment, while patterns A and C both had two smaller fragments which summed to 16.5 kb (A:12.2 kb and 4.3kb; C:10.5 kb and 6 kb). The patterns generated by *Ava* I and *Hha* I did not fit this model. *Ava* I produced three patterns. Patterns A (7.5, 6.6, and 2.3 kb) and B (7.5, 4.1, 2.5, 2.3 kb) could be explained by a single restriction site change which produced a single 6.6 kb fragment in A and two fragments (4.1 and 2.5 kb) in B. Pattern C shared all the fragments in B except the 2.5 kb band but did not have any

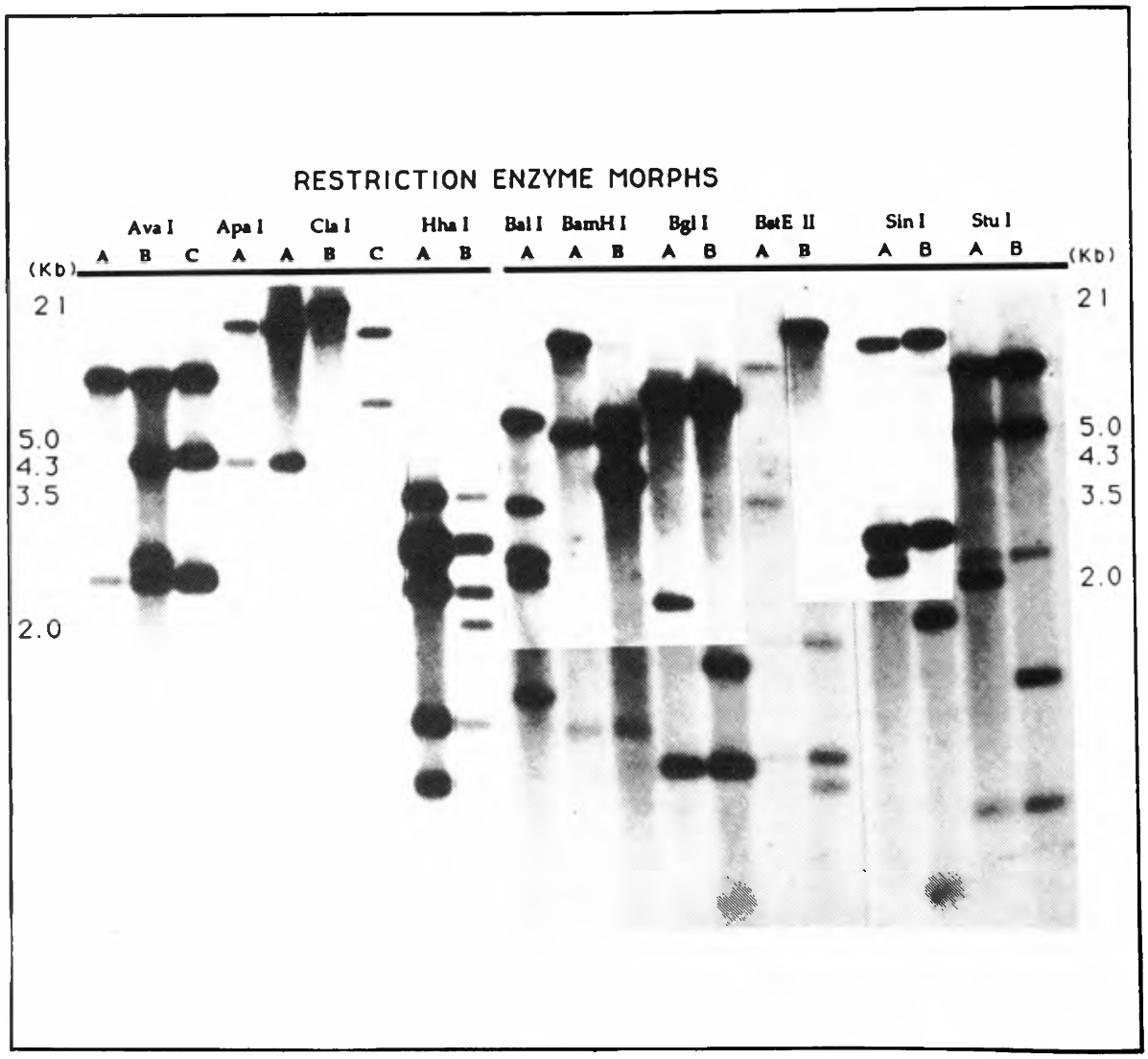

Fig. 1. Audioradiograph showing representative restriction morphs for the 10 enzymes used to survey *Eubalaena* mtDNA.

Table 2

Eubalaena composite restriction fragment length morphs. All enzymes except *Hha* I are six base pair recognition site restriction endonucleases. *Hha* I is a four base pair restriction enzyme.

	Enzyme phenotypes									
Morph	ApaI	AvaI	BalI	BamHI	BglI	BstEII	ClaI	HhaI	SinI	StuI
1	A	A	A	A	A	A	A	A	A	A
2	A	A	A	B	A	A	A	A	A	A
3	A	A	A	A	A	B	A	A	A	A
4	A	B	A	A	A	A	B	B	B	A
5	A	B	A	A	A	A	C	B	B	A
6	A	C	A	A	B	A	B	B	B	A
7	A	C	A	A	A	A	B	B	B	A

additional bands to account for the missing 2.5 kb piece. Similarly, the bands from the second pattern (B) generated by *Hha* I accounted for only 14.4 kb, leaving approximately 2.1 kb unaccounted for. The samples which generated patterns *Ava* I C and *Hha* I B were tested a number of times to establish that they were not the result of incomplete digestion. There could be additional recognition sites present which reduce the DNA to small fragments which were not seen on the audioradiographs.

Each distinctive pattern (morph) obtained for a given enzyme was assigned a letter (Fig. 1). A composite restriction morph was then established for each individual consisting of a series of letters, each of which represented the pattern expressed for a specific enzyme (Lansman *et al.*, 1983).

Seven out of the ten enzymes tested generated more than one banding pattern producing a total of seven composite restriction morphs among the 20 samples surveyed (Table 2); three composite restriction morphs were present in the northern samples and four different ones were present in the southern (Table 3).

The average nucleotide divergence between the composite restriction morphs was estimated by a pairwise comparison of shared and unshared restriction sites, where the fewer shared sites there are between two samples, the less closely related they are (Nei and Li, 1979). Because mtDNA is a circular molecule, the number of restriction sites equals the number of restriction fragments (bands) observed. (For these calculations *Ava* I pattern C was assumed to have at least five bands, *Hha* I pattern B was assumed to have at least nine.) Among the North Atlantic samples the average nucleotide divergence was 0.08% and among the South Atlantic samples it was 0.24%.

Table 3

Distribution and frequency of *Eubalaena* composite restriction fragment length morphs. For the North Atlantic samples N = 10, for the South Atlantic samples N = 10. All composite morphs were species specific.

Morph	Location	Frequency	Morph	Location	Frequency
1	North Atlantic	0.50	4	South Atlantic	0.50
2	North Atlantic	0.40	5	South Atlantic	0.30
3	North Atlantic	0.10	6	South Atlantic	0.10
			7	South Atlantic	0.10

Interspecific variation was present for four of the ten enzymes tested. All of the seven composite morphs produced were species specific. The genetic distance (d, the divergence between the two species after adjustment for within population diversity – Nei and Li, 1979) between the North Atlantic and South Atlantic samples was 1.82%.

DISCUSSION

Remote biopsy darting of the free ranging right whales has proven to be a successful method for obtaining the DNA necessary for genetic analyses. Sufficient DNA for many of the molecular techniques currently being used for population studies (e.g. Hoelzel and Amos, 1988; Stevens *et al.*, 1989; Baker *et al.*, 1990; Helbig *et al.*, 1990) can be isolated from a single skin sample. Hence, this relatively nonintrusive procedure (Brown *et al.*, this volume) should permit the sampling and subsequent genetic analysis of species which previously have been inaccessible due to the lack of nondestructive methods of acquiring the necessary DNA.

Due to their geographic locations, the North and South Atlantic right whales are assumed to be reproductively isolated (Braham and Rice, 1984). Skeletal data also provide evidence that the right whales are separate species (Muller, 1954). Preliminary mtDNA RFLP data support this classification since none of the seven composite restriction morphs identified were common to both the North and South Atlantic whales. Furthermore, the RFLP analysis indicated that the nucleotide diversity (d) between the North and South Atlantic right whales was 1.82%. Assuming a 1–2% per million years rate of mtDNA divergence (Brown, 1983; Wilson *et al.*, 1985), this suggests that the two are genetically isolated, having diverged approximately 0.91 to 1.82 million years ago. The time of divergence will be re-assessed by amplifying and directly sequencing portions of the mtDNA. However, because right whales were depleted severely by commercial whaling, the current within population variance (d=0.08% in the northern samples and 0.24% in the southern samples) is probably much lower than it was when the two species diverged. As a result, the apparent time of divergence between the current composite morphs may be greater than the time of genetic isolation of the two populations (Nei, 1987).

Although this preliminary analysis allows only a relatively low level of resolution, the low within species genetic distances obtained for the North and South Atlantic whales (nucleotide distance π= 0.08% and 0.24%, respectively) are consistent with the hypothesis that genetic variation has been diminished in these species by over-hunting.

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