MARINE MAMMAL SCIENCE, 8(3):275–283 (July 1992)

RESTRICTABLE DNA FROM SLOUGHED CETACEAN SKIN; ITS POTENTIAL FOR USE IN POPULATION ANALYSIS

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Abstract

Several species of cetaceans naturally slough visible quantities of skin. We have investigated the prospect of using this material as a viable alternative to the use of biopsy darts for the collection of samples for molecular analysis. Pieces of skin were collected from free-ranging individuals of three different species—the humpback (*Megaptera novaeangliae*), sperm (*Physeter macrocephalus*) and right whales (*Eubalaena glacialis*). DNA was extracted from 11 pieces of sloughed skin and DNA "fingerprint" profiles prepared. All samples contained DNA of both sufficient quality and quantity for genetic analysis. The applicability of this approach is discussed generally in relation to answering problems about the population structure and breeding systems of cetaceans.

Key words: sloughed skin, cetacean, DNA fingerprint.

The study of genetic variability can tell much about the structure of natural populations. Recent developments in analysis at the level of the DNA itself have yielded several new and powerful approaches, the uses and limitations of which are discussed by Hoelzel and Dover (1989*a*). Of these, DNA finger-

printing has attracted most publicity. Developed on humans at Leicester, this technique has proved capable of unambiguous identification and confident paternity analysis in humans (Jeffreys *et al.* 1985) to the extent that it has been accepted as valid evidence in court (Gill *et al.* 1985). This potential has since been applied to natural populations of animals (Wetton *et al.* 1987, Burke *et al.* 1989, for a review see Burke 1989). Preliminary studies have shown that a number of cetacean species are amenable to study using these techniques (Hoelzel and Amos 1988; Spilliaert *et al.* 1989; Amos and Hoelzel 1990).

Although, through its unprecedented level of genetic resolution, DNA fingerprinting has attracted most publicity, techniques focusing on other regions of the genome have similar potency in the study of natural populations. Two classes of DNA sequence allow a broader scale analysis and are, therefore, of particular importance for the study of cetacean populations, where gene flow between different geographic areas and the delimitation of 'stocks' are usually unknown. Firstly, the maternally inherited mitochondrial DNA (mtDNA) reveals patterns of matrilineal structure and provides a good indicator of bottlenecks (Wilson *et al.* 1985). Secondly, repeated DNA sequences, or gene families, are capable of identifying sub-populations or "stocks" (Dover 1982). Both mtDNA (e.g., Baker *et al.* 1990, Numachi *et al.* 1989, Hoelzel and Dover 1989*b*, Duffield *et al.* 1989) and repeated sequences (Amos and Dover 1991) are now being applied to the study of cetacean populations.

Most cetacean populations are logistically difficult to study, being both inaccessible and wide-ranging. Genetic analysis, applying techniques like DNA fingerprinting to such samples that may be obtained, offers potential insight into previously intractable questions about population structure. It has been reported recently that it is no longer necessary to have access to dead whales in order to get samples for DNA analysis. In addition to incidental catches and stranded material, small skin plugs can be taken from free-swimming whales using a biopsy dart apparatus (for example see Mathews *et al.*, 1988, Lambertson 1987, Hoelzel and Amos 1988). We report here a further source of material, sloughed skin.

Sloughed skin is routinely observed in the wake of sperm, *Physeter macrocephalus,* and humpback, *Megaptera novaeangliae,* whales in tropical waters (personal observations by H.W., J.G. and M.F.). Large quantities are released during certain behaviors and in certain situations. For instance, pieces of sloughed skin may be seen floating in the water by stationary whales being groomed by the fish "lae" or leatherback runners (*Scombroides lysan*) (Glockner and Venus 1983, Glockner-Ferrari and Ferrari 1985). Most skin is shed when whales come into physical contact. This might be a calf rubbing up against its mother or males physically competing for access to a female (Glockner-Ferrari and Ferrari 1985, Tyack and Whitehead 1983). The same is observed for social interaction in groups of sperm whales (Whitehead 1984). A further good source is following breaches (leaps from the water) and lobtails (the thrashing of flukes on the water surface) (Whitehead 1985). However, skin has also been collected from single sperm whales which were not engaged in energetic displays (Whitehead *et al.* 1990). Most good photographs of sperm whales, blue whales, *Balaenoptera*

musculus, and right whales, *Eubalaena* sp., show regions where skin has recently been sloughed (Whitehead 1984, Sears *et al.* 1987).

Methods

Samples of sloughed skin were collected from three species of cetaceans: the sperm whale (2 independent samples), the humpback whale (11 samples, 8 analysed) and the right whale, *E. glacialis* (1 sample).

A piece of skin (*ca.* 500 cm^2) sloughed from a sperm whale, was collected by hand from the dinghy of a 12-m sailing vessel on 20 April 1988 off the Galapagos Islands, Ecuador. The skin was finely chopped and preserved in saturated salt solution. The sample was kept at ambient temperature (5–30°C), except for 17 d during which it was frozen at below −10°C, until processing began on 7 June 1988.

Further samples of sperm whale skin were collected in waters around the central groups of islands of the Azores Archipelago. Snorkelers were either towed on the bow of the research vessel or swam freely following in the wake of the whales. Pieces of skin approximately 20–40 cm^2 were collected by hand or by using a small aquarium net and placed in saturated salt solution, predominantly at *ca.* 0°C. Sloughed skin was always seen in the vicinity of groups of whales, but was more difficult to collect from single animals. Single whales are less tolerant of swimmers, swim faster and spend shorter periods at the surface. In addition, there appeared to be less sloughed skin in the water, on average, than when several whales were present. Since body contact is likely to release pieces of skin, this might be expected.

Eleven samples of sloughed skin were collected from humpback whales inhabiting the waters off the west coast of Maui, Hawaii, between January and May 1988 by M.F. and D.A.G.-F. The samples were collected benignly by hand using snorkeling gear while swimming from a 4.7 m inflatable Zodiac. Sample size varied from 1 to 4 cm^2. Upon collection, each sample was placed in a Kodak film canister filled with sea water. Two samples were later placed in a saturated solution of table salt (NaCl). No refrigeration was available until port was reached, a period of 1 to 8 h. Following this the samples were frozen. Prior to shipping, the samples were thawed, at least twice, and placed in 20% dimethylsulphoxide (DMSO) saturated with table salt. This solution has recently been shown to preserve cetacean skin samples for at least one year in the absence of refrigeration (Amos and Hoelzel 1991) and is regarded as the best alternative to freezing.

A single right whale skin sample was removed from the animal by hand by a researcher operating from an inflatable Zodiac in waters off Argentina. The sample was placed in sea water without refrigeration. Two control samples were also obtained from stranded animals, one of which was preserved by freezing, the other by sun-drying.

High molecular weight DNA was extracted as follows: pieces of skin were powdered in liquid nitrogen using a pestle and mortar, the cells lysed using proteinase K [in: 1% SDS (sodium dodecyl sulphate), 100 mM NaCl, 50 mM

Tris pH 8.0, 50 mM EDTA (ethylenediamine tetracetic acid)] and the DNA purified through organic solvent extraction (once each with phenol and then chloroform, Maniatis *et al.* 1982). The aqueous phase was then brought to 2.5 M LiCl, incubated at −20°C for 30 min and impurities removed by centrifugation (13 K × B for 5 min). DNA was precipitated from the supernatant with 2 volumes of ethanol. For the poorest sample (sun-dried right whale skin), a larger piece of material was used (0.3 cm^3) and the DNA concentrated by glass milk precipitation (Vogelstein and Gillespie 1979).

Resultant DNA was tested for quality on a 0.6% agarose gel. In all samples except those from the right whale it was found to be either predominantly above 14 Kb in length (all humpback samples and the sperm whale samples) or somewhat degraded (below 3 Kb). This might be interpreted in terms of a mixture of living cells and dead cells. The DNA yield was found to be rather variable, as might be expected. Samples from the humpback whales were the best and yielded up to about 20 $\mu g/cm^2$). The sperm whale samples gave yields of about 2–5 $\mu g/cm^2$. Both yield and quality of right whale DNA was poor (*ca.* 2 $\mu g/cm^2$), with considerable degradation (average molecular weight <10 Kb).

DNA fingerprints were obtained as described elsewhere (Amos and Hoelzel 1990) by digesting each sample with the restriction enzyme *Dde 1,* fragment separation by electrophoresing on a 0.7% agarose gel (2 V/cm for 2–3 days) in TBE (Tris/borate/EDTA) buffer, Southern blotting onto nylon "Hybond" (Amersham) membranes and probing with the human polycore probe 33.15 (donated by Alec Jeffreys). The probe was radioactively labelled using primer extension to incorporate α32P dATP. Hybridisations were carried out overnight at 1× SSC in the presence of 6% polyethylene glycol (PEG 6000) and 0.5% SDS. Heparin (50 $\mu g/ml$) and transfer RNA (5 $\mu g/ml$) were used as blocking agents. Resulting autoradiograms were exposed for 1 to 10 days with intensifying screens. For each species, a control sample (2 for the right whale) from stranded material was run alongside the sloughed skin samples in order to control for contamination.

Results

DNA fingerprints from three cetacean species are shown in Figure 1a, b, and c. All samples analysed yielded DNA fingerprints, although only the 3 most intense humpback fingerprints are shown. This was in spite of the fact that none of these samples were collected and preserved under conditions now known to be fully adequate (*i.e.,* in 20% DMSO saturated with NaCl). Nevertheless, even the right whale stranding sample, which was subjected to considerable decay and only sun-dried for preservation, gave fingerprints of sufficient clarity for useful information to be obtained.

Since the samples collected were all extremely thin (except for the right whale) and with very low DNA content, there is a possibility of contamination from clinging planktonic life. This was partially controlled for by the inclusion of samples from known individuals of the same species collected by alternative

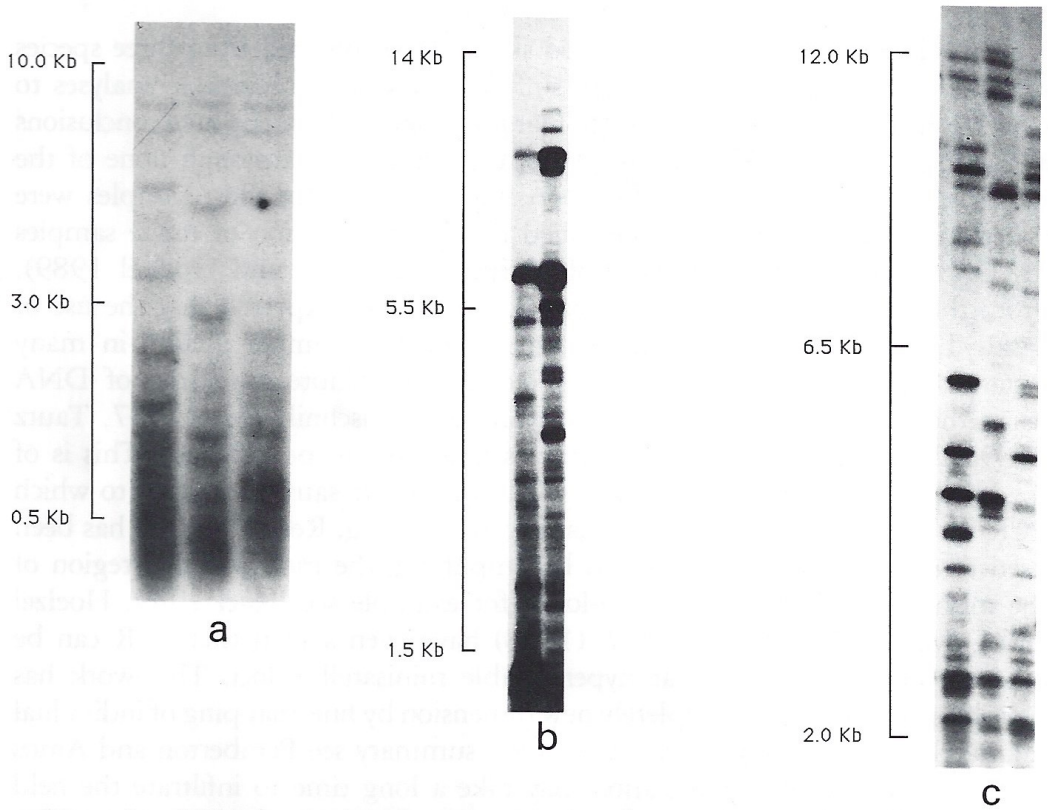

Figure 1. DNA fingerprints from three species of cetaceans. The size of the fragments is given in kilobases (Kb). a: Fingerprints prepared from three right whales. The lefthand sample is derived from sloughed skin. The other two lanes are both prepared from stranded individuals. b: Fingerprints prepared from two sperm whales. The lefthand sample derives from sloughed skin. The righthand sample is a control prepared from a stranded animal. c: Fingerprints prepared from sloughed skin from 3 humpback whales.

methods. Although it is very difficult to eliminate completely the possibility of contamination without testing a wide spectrum of possible contaminating species, the overall banding profiles of a DNA fingerprint are often characteristic of a species (Van Pijlen *et al.* 1991; Amos, personal observations). Thus the gross similarity observed between the control and the sloughed samples, both in intensity of hybridisation and distribution of band molecular weights, is an indication that the majority of DNA in the sloughed skin is of the species from which it was shed. Conversely, the lack of any gross dissimilarities between samples suggests that large amounts of contaminating DNAs from a single planktonic species are not present. It would be useful to take this further and to test a variety of possible contaminating planktonic species and so ascertain the amount required to cause significant alteration of fingerprint patterns. Within the limits of sample quality, all three species show considerable variability (comparison includes both control and sloughed samples). In particular, the humpback whale has very high levels and should prove amenable to genetic dissection of family relationships.

Discussion

We demonstrate here that sloughed skin can be collected from three species of cetaceans and that it can contain sufficient DNA for molecular analyses to be performed. Our sample sizes are not large enough to draw any firm conclusions about the proportion of such samples which are usable, although none of the samples we tested failed to yield DNA fingerprints. Since these samples were collected, a method has been published for the preservation of tissue samples for DNA analysis in the absence of refrigeration (Amos and Hoelzel 1989). From the results presented in that paper, it might be expected that the use of a salt/DMSO preservative solution would improve sample quality in many instances. Furthermore, enzymic amplification of minute quantities of DNA using the polymerase chain reaction (PCR, see Wrischnik *et al.* 1987, Tautz 1989) allows very much smaller samples than ours to be analysed. This is of special relevance to the study of sloughed skin, where sample quality (to which PCR is relatively insensitive) and quantity are limiting. Recently, PCR has been used to study cetacean populations by amplifying the most variable region of the mitochondrial genome, the D-loop (for example see Rosel 1989, Hoelzel and Dover 1989*b*). Jeffreys *et al.* (1988) have even shown that PCR can be used to uncover variability at hypervariable minisatellite loci. This work has since been extended to a completely new dimension by fine-mapping of individual minisatellite loci (Jeffreys *et al.* 1990, for a summary see Pemberton and Amos 1990). This level of sophistication may take a long time to infiltrate the field of population genetics. However, it is clear that, even at its simplest level, PCR can extend the degree to which sloughed skin samples may be used in the study of natural populations.

To date, except for a preliminary study by Whitehead *et al.* (1990) little attention has been given to the occurrence of sloughed skin. We do not know for any species what proportion of animals are sloughing skin at any time or how regularly it can be collected. Nor do we know whether the greater frequency of observations of sloughed skin in warmer waters is due to greater water clarity or a faster turnover of epidermis in warm and/or sunny conditions. These parameters clearly need to be determined.

Despite these unknowns, sloughed skin clearly presents a possible source of DNA which could be exploited to study population structure in free-ranging cetaceans using a range of molecular techniques. Which approaches are best applicable is dependent on whether the samples are collected from individuals or from groups. Broadly speaking, three classes of samples may be distinguished: those linked to individuals, those linked to small groups (say <10 animals) and those collected from larger groups.

Where samples are attributable to individual whales it may be possible to link them to a photograph of natural markings (Katona and Whitehead 1981, Arnbom 1987, Sears *et al.* 1987), and hence to an identified individual. Such samples will then be useful for examining family relations using DNA fingerprinting, particularly for species such as the humpback whale where large photographic catalogues already exist and levels of variability are particularly high.

For samples from small groups of whales, familial analysis may still be feasible.

Individual pieces of skin taken from a "mixed bag" can be fingerprinted until all the observed individuals are accounted for. In conjunction with the use of Y chromosome specific probes (Baker *et al.* 1989) and morphological indications of an animal's age it may be possible to gain considerable insight into the structure of the group. For example, the presence of mother : calf, father : calf and sib : sib relationships may be determined. This analysis will be increasingly facilitated by the accumulation of fingerprint information for each species being studied (band frequencies, possible allelic systems and even population specific patterns). Recent studies using one highly polymorphic minisatellite locus, the equivalent of using a single-locus probe, in the long-finned pilot whale (*Globicephala melas*) illustrate the detail that may be revealed. For instance, paternal alleles in suckling offspring can show up polygony, and female genotypes within a group can demonstrate a matrilineal structure (Amos *et al.* 1991).

For larger groups, this detective work becomes increasingly impractical. Nonetheless, such samples can be useful for looking at relationships between social groups and larger population subdivisions using mtDNA, the ribosomal genes or other repetitive components of the genome. Of these, the mtDNA lends itself particularly to the use of PCR.

These considerations will determine how large and representative a set of samples of sloughed skin can be collected in any study, and consequently how useful the collection will be for different kinds of projects. In general, representative samples which can be considered random, are important in studies of population biology, and samples which can be linked to particular individuals or small groups, and thus behavior, are important for studies of social organization and behavioral ecology.

Many of the recent advances in our knowledge of living cetaceans have come from field studies carried out over long periods by very committed, but poorly funded, groups and individuals. In these studies data is often collected in conjunction with whale watching tours or other activities where biopsy darting can be inappropriate. Under these circumstances, whilst not replacing biopsy darts in all situations, the non-invasive collection of sloughed skin by hand, dip-net or snorkeller must now be viewed as a valuable alternative sampling strategy. Particularly in clearer waters, the lack of disturbance caused may make sloughed skin the preferred option. Together with simple techniques of sample preservation, use of sloughed skin will therefore allow integration of the important observational studies with emergent DNA technology, adding to our knowledge of living cetaceans.

Acknowledgments

We thank the Estacion Cientifica Charles Darwin, the crew of "Song of the Whale," Department of Oceanography and Fisheries of the Azores, Humphreyetta Back. Godfrey Merien, Jonathan Wright and, especially, Susan Waters helped in the collection, preservation and transmission of samples. Funding came from the Natural Sciences and Engineering Research Council of Canada, the International Fund for Animal Welfare and Eurogroup. Laboratory work was supported by the Natural Environment Research Council of Great Britain.

Literature Cited

Amos, W., and G. A. Dover. 1991. The use of satellite DNA sequences in determining population differentiation in the minke whale. Report of the International Whaling Commission (Special Issue 13):235–244.

Amos, W., and A. R. Hoelzel. 1990. DNA fingerprinting cetacean biopsy samples for individual identification. Report of the International Whaling Commission (Special Issue 12):79–85.

Amos, W., and A. R. Hoelzel. 1991. Long-term preservation of whale skin for DNA analysis. Report of the International Whaling Commission (Special Issue 13):99–103.

Amos, W., T. M. O. Majerus and G. A. Dover. 1989. Conservation of DNA fingerprint profiles between whale stocks. Report of the International Whaling Commission. SC/S89/GEN4. 9 pp.

Amos, W., J. A. Barrett and G. A. Dover. 1991. Breeding behaviour of pilot whales revealed by DNA fingerprinting. Heredity 67:49–56.

Arnbom, T., 1987. Individual identification of sperm whales. Report of the International Whaling Commission 37:201–204.

Baker, C. S., D. A. Gilbert, R. H. Lambertsen, M. T. Weinrich and S. J. O'Brien. 1989. Using molecular techniques to determine the sex and individual identification of humpback whales. Report of the International Whaling Commission. SC/S89/GEN24. 7 pp.

Baker, C. S., S. R. Palumbi, R. H. Lambertsen, M. T. Weinrigh, J. Calambokidis and S. J. O'Brien. 1990. Influence of seasonal migration on geographic distribution of mitochondrial DNA halotypes in humpback whales. Nature 344:238–240.

Burke, T. 1989. DNA fingerprinting and other methods for the study of mating success. Trends in Ecology and Evolution 4:139–144.

Burke, T., N. B. Davies, M. W. Bruford and B. J. Hatchwell. 1989. Parental care and mating behaviour of polyandrous dunnocks *Prunella modularis* related to paternity by DNA fingerprinting. Nature 338:249–251.

Dover, G. A., 1982. Molecular drive: a cohesive mode of species evolution. Nature 299:111–117.

Duffield, D. A., T. A. Stevens, P. Goldie, R. Wells and E. D. Asper. 1989. Mitochondrial DNA analysis of population structure in *Orcinus orca* and *Tursiops truncatus*. Report of the International Whaling Commission. SC/S89/GEN14. 9 pp.

Gill, P., A. J. Jeffreys and D. J. Werrett. 1985. Forensic applications of DNA 'fingerprints'. Nature 318:577–579.

Glockner, D. A., and S. C. Venus. 1983. Identification, growth rate, and behavior of humpback whale, *Megaptera novaeangliae,* cows, and calves in the winter waters off Maui, Hawaii, 1977–79. Pages 223–258 *in* R. Payne, ed. Communication and behavior of whales. AAAS Symposia Series, Westview Press, Boulder, CO.

Glockner-Ferrari, D. A., and M. J. Ferrari. 1985. Individual identification, behavior, reproduction and distribution of humpback whales, *Megaptera novaeangliae,* in Hawaii. Report of the Marine Mammal Commission. NTIS PB85-200772. 36 pp.

Hoelzel, A. R., and W. Amos. 1988. DNA fingerprinting and 'scientific' whaling. Nature 333:305.

Hoelzel, A. R., and G. A. Dover. 1989*a*. Molecular techniques for examining genetic variation and stock identity in cetacean species. Pages 81–120 *in* G. P. Donovan, ed. The comprehensive assessment of whale stocks: the early years. Report of the International Whaling Commission (Special Issue 11).

Hoelzel, A. R., and G. A. Dover. 1989*b*. Analysis of mitochondrial D-loop variation within and between populations of the minke whale. Report of the International Whaling Commission. SC/S89/GEN15. 17 pp.

Jeffreys, A. J., V. Wilson and S. L. Thein. 1985. Hypervariable 'minisatellite' regions in human DNA. Nature 314:67–73.

Jeffreys, A. J., V. Wilson, R. Neumann and J. Keyte. 1988. Amplification of human

minisatellites by the polymerase chain reaction: towards DNA fingerprinting of single cells. Nucleic Acids Research 16:10953–10971.

JEFFREYS, A. J., R. NEUMANN AND V. WILSON. 1990. Repeat unit sequence variation in minisatellites: a novel source of DNA polymorphism for studying variation and mutation by single molecule analysis. Cell 60:473–495.

KATONA, S. K., AND H. P. WHITEHEAD. 1981. Identifying humpback whales using their natural markings. Polar Record 20:349–444.

LAMBERTSEN, R. H. 1987. A biopsy system for large whales and its use for cytogenetics. Journal of Mammalogy 68:443–445.

MANIATIS, T., E. F. FRITCH AND J. SAMBROOK. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbour Laboratory, Cold Spring Harbour, NY. 545 pp.

MATHEWS, E. M., S. KELLER AND D. B. WEINER. 1988. A method to collect and process skin biopsies for cell culture from free-ranging grey whales (*Eschrictius robustus*). Marine Mammal Science 4:1–12.

NUMACHI, K., T. KOBAYASHI, S. WADA AND E. SHIMURA. 1989. Mitochondrial DNA variation and its use for cetacean population study. Report of the International Whaling Commission. SC/S89/GEN20. 23 pp.

PEMBERTON, J., AND W. AMOS. 1990. DNA fingerprinting: a new dimension. Trends in Genetics 6:101–103.

ROSEL, P. E. 1989. Differentiation of harbour porpoise, *Phocoena phocoena,* populations using the mitochondrial D-loop. Report of the International Whaling Commission. SC/S89/GEN16. 9 pp.

SEARS, R., F. W. WENZEL AND J. M. WILLIAMSON. 1987. The blue whale. A catalogue of individuals from the western North Atlantic (Gulf of St. Lawrence). Mingen Island Cetacean Study (MICS Inc.), St. Lambert, Quebec, Canada.

SPILLIAERT, R., A. PALSDOTTIR AND A. ARNASON. 1989. DNA fingerprinting in two species of baleen whale: fin (*Balaenoptera physalus*) and sei (*B. borealis*) using a human hypervariable region, αglobin 5′ HVR. Report of the International Whaling Commission. SC/S89/GEN19. 13 pp.

TAUTZ, D. 1989. Hypervariability of simple sequences as a general source of polymorphic DNA markers. Nucleic Acids Research 17:6462–6471.

TYACK, P., AND H. WHITEHEAD. 1983. Male competition in large groups of wintering humpback whales. Behaviour 83:132–154.

VAN PIJLEN, W. AMOS AND G. A DOVER. 1991. Multilocus DNA fingerprinting applied to population studies of the minke whale *Balaenoptera acutorostrata.* Report of the International Whaling Commission (Special Issue 13):245–254.

VOGELSTEIN, B., AND D. GILLESPIE. 1979. Preparative and analytical purification of DNA from agarose. Proceedings of the National Academy of Sciences 76:615–619.

WETTON, J. H., R. E. CARTER, D. T. PARKIN AND D. WALTERS. 1987. Demographic study of a wild house sparrow population by DNA fingerprinting. Nature 327: 147–149.

WHITEHEAD, H. 1984. The unknown giants. National Geographic Number 166:774–784.

WHITEHEAD, H. 1985. Humpback whale breaching. Investigations on Cetacea 17:117–155.

WHITEHEAD, H., J. C. D. GORDON, E. A. MATHEWS AND K. RICHARD. In press. Obtaining skin samples from living sperm whales. Marine Mammal Science.

WILSON, A. C., R. L. CANN, S. M. CARR, M. GEORGE, U. B. GYLLENSTEN, K. M. HELM-BYCHOWSKI, R. G. HIGUCHI, S. R. PALUMBI, E. M. PRAGER, R. D. SAGE AND M. STONEKING. 1985. Mitochondrial DNA and two perspectives on evolutionary genetics. Biological Journal of the Linnean Society 26:375–400.

WRISCHNIK, L. A., R. G. HIGUCHI, M. STONEKING, H. A. ERLICH, N. ARNHEIM AND A. C. WILSON. 1987. Length mutations in human mitochondrial DNA: direct sequencing of enzymatically amplified DNA. Nucleic Acids Research 15:529–541.

Received: August 14, 1989
Accepted: July 11, 1990