Use of Corneal Cell Culture for R-Band Chromosome Studies on Stranded Cetaceans

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ABSTRACT

This paper presents a cell culture technique using corneas of postmortem animals and explores the application of fluorescent reverse banding (R-banding) chromosome analysis in stranded cetaceans. These techniques were used to look at heteromorphic (variable) regions in the karyotypes of five representative cetacean species which strand on U.S. coastlines: pygmy killer whale (*Feresa attenuata*); false killer whale (*Pseudorca crassidens*); short-finned pilot whale (*Globicephala macrorhynchus*); pygmy sperm whale (*Kogia breviceps*); and humpback whale (*Megaptera novaeangliae*). Numerous heteromorphic regions found in karyotypes of these species were compared with similar regions in karyotypes of bottlenose dolphins (*Tursiops truncatus*), a species for which extensive cytogenetic work has been done. An extra, unique chromosome was found in the karyotype of an individual *M. novaeangliae* which stranded in Hawaii. The results suggest that there are cytogenetic markers in these species that can be used, as they are in other cetaceans, to confirm relationships and examine regional population differences.

Introduction

Cytogenetic studies of stranded cetaceans have made an important contribution to the karyotypic data base for the Cetacea by providing an opportunity for chromosome analysis of species largely unavailable by other sampling methods (Arnason et al. 1977; Duffield 1977; Benirschke and Kumamoto 1978; Arnason 1980, 1981). To establish cell cultures for chromosome analysis, living tissue is essential. While sampling blood is a direct way of obtaining cells for standard leukocyte culture from live whales and dolphins (Duffield 1986; Duffield and Chamberlin-Lea 1990), obtaining viable, uncontaminated tissue samples from cetaceans dead for several days is a major problem. We have developed a technique for establishing cell cultures from the corneas of postmortem cetaceans. Corneal tissue

has excellent regenerative properties associated with high mitotic indices (Van Horn et al. 1977; Leeson and Leeson 1981) and postmortem cornea can remain viable up to several weeks (Doughman et al. 1976).

Corneal cell cultures have been used for a variety of in vitro studies, including chromosome analysis (Conrad 1970; Dahl et al. 1974; Manski and Whiteside 1974; Pimenova and Simonenko 1974; Kenney et al. 1986). Cell cultures initiated from the corneas of postmortem cetaceans were used in this study to provide a supply of mitotic cells for chromosome preparations. These preparations were stained using a fluorescent reverse banding (R-banding) technique, which, in cetaceans, is particularly useful for chromosome studies because it bands euchromatic regions of the chromosomes for homologue identification and simultaneously visualizes highly variable heterochromatic regions (chromosome heteromorphisms) present in the chromosomes (Duffield 1986; Lambertsen and Duffield 1987; Duffield and Chamberlin-Lea 1990).

In this paper, we present R-band karyotypes derived from corneal cell cultures for five cetacean species which are periodically handled by stranding networks along the coast of the United States. The heteromorphic regions of the chromosomes from these species were compared with those of *Tursiops truncatus*.

Methods

Animals

The R-band karyotypes were prepared for four species that frequently strand on the east coast of Florida: pygmy killer whale ([*Feresa attenuata*], N = 1; field No. C-83-20, male, 215 cm, 10 July 1983); false killer whale ([*Pseudorca crassidens*], N = 2; field No. SWF-8631B, female, 259 cm, 5 June 1986; Id. No. SWC-PC- 8326, female, 312 cm, 25 February 1986); short-finned pilot whale ([*Globicephala macrorhynchus*], N = 2; field No. SWF-8651B, female, 356 cm, 24 July 1986; Id. No. SWC-GM-8003, female, 272 cm, 5 July 1983); and pygmy sperm whale ([*Kogia breviceps*], N = 1; field No. SWF-KB-8330-B, female, 272 cm, 18 September 1983). The R-band karyotypes were also prepared for a humpback whale ([*Megaptera novaengliae*], N = 1; male calf, SLP-Mn-81, 7 March 1981) stranded in Hawaii. Both blood and corneal cell cultures were available for one of the false killer whales and one of the pilot whales.

Collection of Corneal Samples

The eye was removed from the socket by severing the eye muscles, associated connective tissue, and the optic nerve, care being taken to ensure that the eye remained intact. Sterile precautions were not necessary during collection. The eye was stored and shipped dry in a plastic bag at 4°C until culturing; fluid in the bag encouraged tissue decomposition and increased the possibility of contamination. The inner corneal layers were kept moist by the aqueous humor, so desiccation of the cornea was not a problem.

Corneal Cell Culture

The eye was well rinsed in running tap water and placed cornea down in a 1:750 dilution of 17% aqueous Zephiran chloride (Winthrop Laboratories, New York, NY 10016) for 2 to 3 minutes. This procedure removed gross bacterial contamination from the corneal surface and did not appear to damage the inner cell layers. The cornea was carefully rinsed with distilled water to remove any traces of disinfectant.

A piece of cornea (ca. 3-4 mm diameter) was excised aseptically. To ensure maximum viability, two to three pieces were taken from different portions of the cornea. The inner portion of the cornea (including Descemet's membrane) and the associated endothelial cell layer occasionally separated from the stroma. This membrane and cell layer must be included for cell growth. The corneal sample was soaked for 1 hour in culture medium (Ham's F-10 with L-glutamine [Gibco, Grand Island, NY 14072] supplemented with 10-15% fetal calf serum; penicillin-streptomycin (Gibco), final concentration 100 units/mL; and Fungizone, amphotericin B (Gibco), final concentration 2.5 mcg/ml), which was further supplemented with 10 × concentrations of antibiotic and fungicide. The explants were minced, transferred to culture flasks, fed with culture medium and the cell cultures were placed in a 36°C, 5% CO_2-air incubator.

Cytogenetic Analysis

Metaphase cells were collected by the addition of colcemid (Gibco; final concentration 0.1 μg/mL) overnight. Cells were harvested by standard techniques (Hack and Lawce 1980) using a one part 0.075M KCl and one part 20% fetal calf serum hypotonic solution for 12 minutes at 37°C. Cells were fixed in three parts absolute ethanol and one part glacial acetic acid. Chromosomes were banded with a fluorescent R-banding technique using chromomycin A-3 and distamycin A (Sahar and Latt 1978; Schweizer 1980). Photographs, using Kodak Technical Pan 2415 film, were taken on a Zeiss microscope equipped with an ultraviolet light source and epifluorescence. Two to five karyotypes were prepared for each animal. In these karyotypes, the chromosome pairs were arranged into four groups (A-D) based on centromere position and numbered consecutively. The resultant karyotypes were examined for R-band heteromorphisms, discrete chromosomal regions which showed size and/or intensity differences between homologues. Chromosome composites were constructed based on the similarity in banding pattern of these species to *Tursiops truncatus*; the latter species was chosen as the stan-

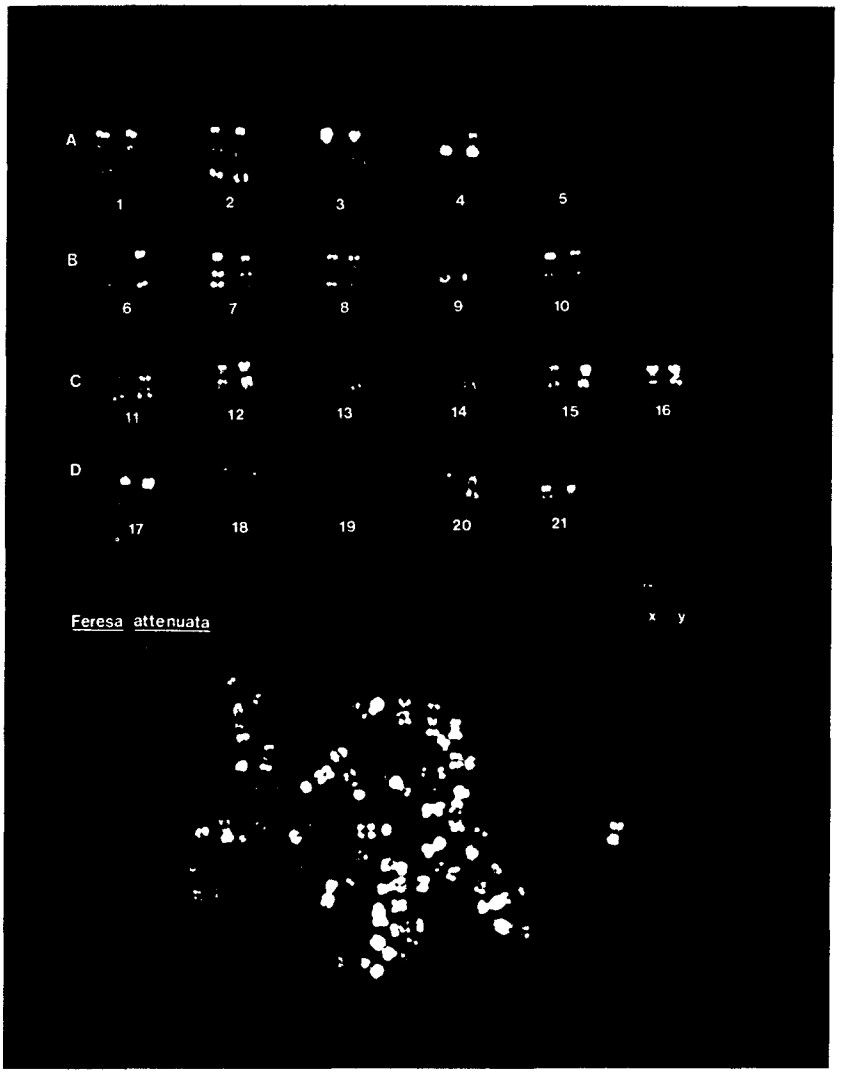

Figure 1
R-banded karyotype of a male *Feresa attenuata* (2N = 44).

dard because of its established R-band karyotype and range of heteromorphisms (Duffield and Chamberlin-Lea 1990). Differences among species in R-band heteromorphisms were noted.

Results

Cell cultures were established from eyes which varied in postmortem age from three days to two weeks. The corneal cells grew vigorously in culture. Outgrowth of cells from the original explant most commonly occurred within one day of the initiation of culture, no matter what the postmortem age of the eye. Significant amounts of cell migration from the explant were observed by the third or fourth days. For one eye, cell outgrowth was delayed for two or three weeks, but once established, this culture grew as well as those which had initially grown out more quickly.

The R-banded karyotypes of *F. attenuata*, *P. crassidens*, *G. macrorhynchus*, *K. breviceps*, and *M. novaeangliae* are shown in Figures 1–5. The *F. attenuata*, *P. crassidens*, and *G. macrorhynchus* karyotypes had a diploid chromosome number of 2N = 44. The *M. novaeangliae* calf had a 2N = 45 (+ mar), exhibiting an extra, small chromosome in its karyotype (Fig. 5). This chromosome stained faintly with R-banding and its origin was unknown, but it was consistently pres-

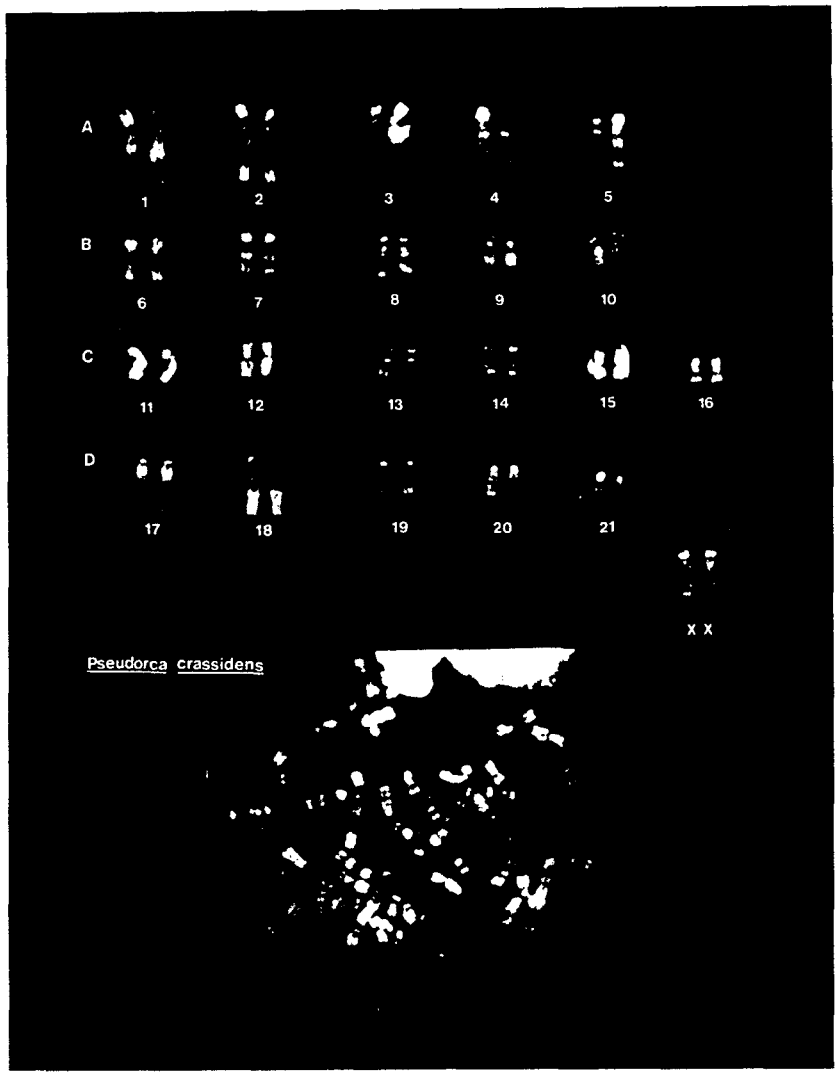

Figure 2
R-banded karyotype of a female *Pseudorca crassidens* (2N = 44).

ent in all cells examined. The karyotype of *Kogia breviceps* had a 2N = 42. For *P. crassidens* and *G. macrorhynchus*, the karyotypes obtained by corneal culture were consistent with those obtained from blood.

To compare the distribution of observed heteromorphic regions, a composite karyotypic comparison was constructed using one chromosome of each chromosome pair from the five species examined (Fig. 6). This composite included *Tursiops truncatus* as a standard because many individuals of this species have been examined by R-banding and both the distribution of heteromorphic regions and the type of variation found is well documented (Duffield and Chamberlin-Lea 1990).

Discussion

From the results reported here, as well as from several years of trials using corneal tissue to establish cell cultures for chromosome analysis in Duffield's laboratory, we have observed that prolonged postmortem viability, freedom from contamination and ease of handling make cornea an extremely useful tissue for establishing cell cultures from stranded cetaceans. We have found that the chances for viability are greatest when eyes are taken either from animals that washed ashore dead or from animals that were returned to the water prior to death. Animals dying out of the water more often exhibited decreased corneal cell

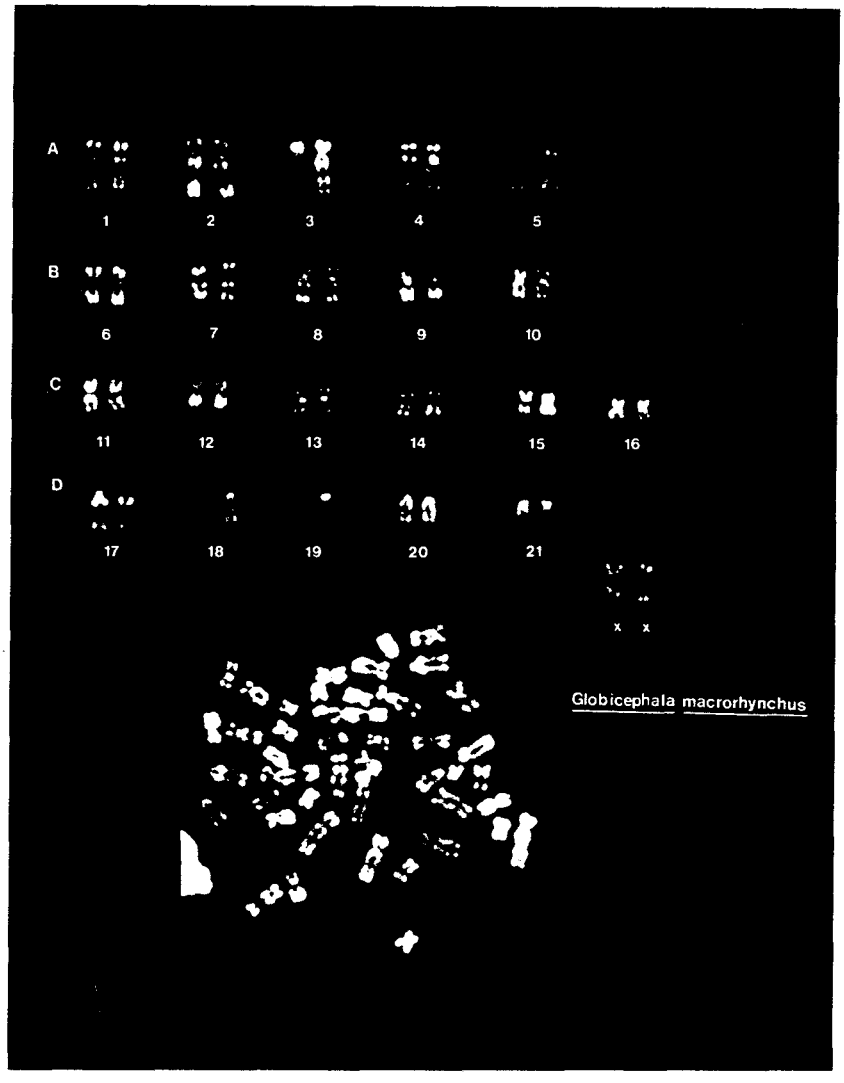

Figure 3
R-banded karyotype of a female *Globicephala macrorhynchus* (2N = 44).

viability, possible owing to extreme overheating (D. Duffield, pers. obs.). In contrast to other postmortem tissues, the inner cell layers of the cornea appear to remain uncontaminated until the eye has become severely decomposed. Because the eye can be removed intact from the animal and does not require sterile handling or preservation in culture media, samples are easy to obtain in the field.

Fluorescent R-band karyotypes have been previously reported for *M. novaeangliae* (Lambertsen and Duffield 1987; Lambertsen et al. 1988), but not for the other stranded species. The diploid number for *M. novaeangliae* is 2N = 44. The stranded calf examined here had a extra, small chromosome (2N = 45, + mar). The origin and significance of the extra chromosome seen in the karyotype of the stranded humpback whale calf is not clear, but a similar extra chromosome has been noted in a live Atlantic humpback whale that was sampled by skin biopsy (Lambertsen and Duffield 1987). An extra chromosome has also been reported for *T. truncatus* (Duffield et al. 1985; Duffield and Wells 1988). In this species, a small, unique marker chromosome was found in certain individuals belonging to a resident female bottlenose dolphin social unit in Sarasota Bay, FL. In contrast to the extra chromosome in the humpback whale, the extra chromosome in the bottlenose dolphin was brightly staining with satellites

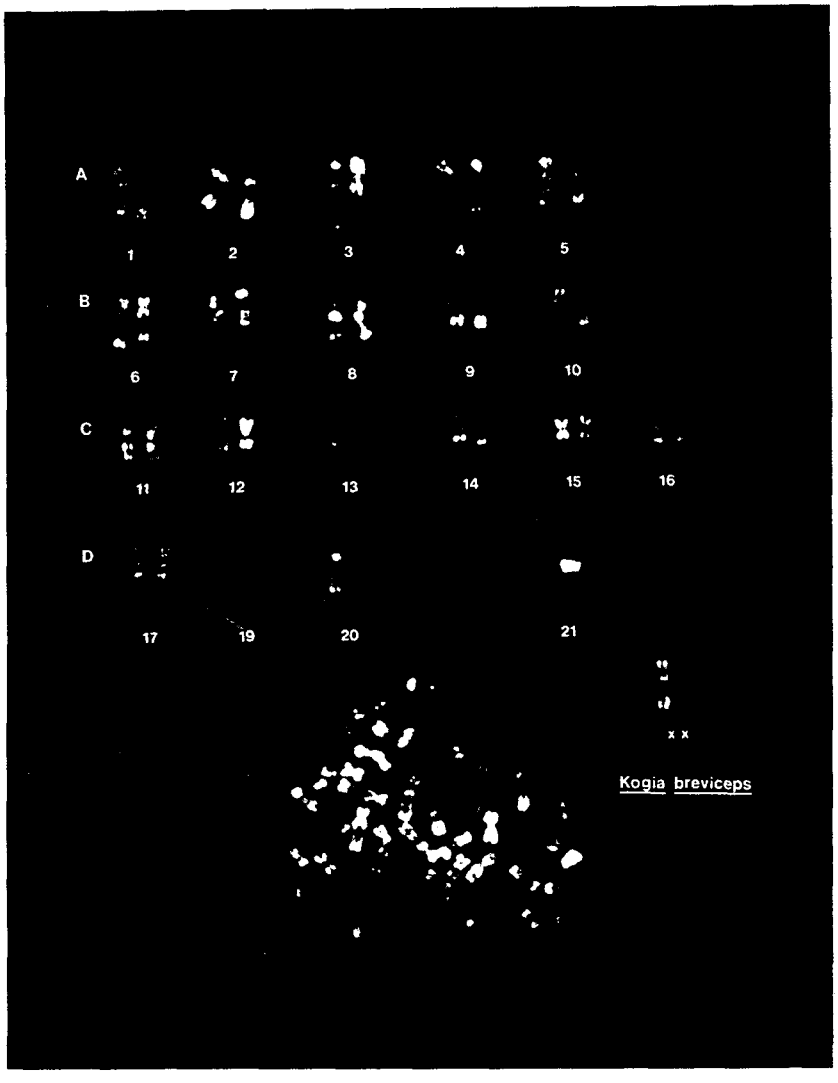

Figure 4
R-banded karyotype of a female *Kogia breviceps* (2N = 42).

and carried nucleolar organizer regions. This marker chromosome had been inherited for three generations and did not appear to have a phenotypic effect in any of the animals. The inheritance pattern of the marker confirmed long-term observational and behavioral data suggesting that resident female bands in the Sarasota study area were composed largely of related individuals (Wells et al. 1980; Irvine et al. 1981; Wells 1986). Because they are relatively rare and found in specific animals, these marker chromosomes are particularly useful for tracing the relationships and reproductive interactions of individuals and groups with neighboring or distant groups of the same species.

Although there were subtle differences in R-banding pattern among the five species examined here, their chromosomes were similar enough that the numbers on the chromosome pairs refer to the same homologous chromosome pair in each species. Pygmy sperm whales, as well as sperm and beaked whales, have a chromosome number of 2N = 42 (Arnason and Benirschke 1973; Arnason et al. 1977; Duffield 1977). A fusion between two of the acrocentric chromosome pairs may account for the 2N = 42 karyotype of *K. breviceps* (observation from the visual comparison of the R-band karyotypes, Fig. 6). However, detailed banding analyses have not been completed to determine the extent of similarities and differences in R-banding

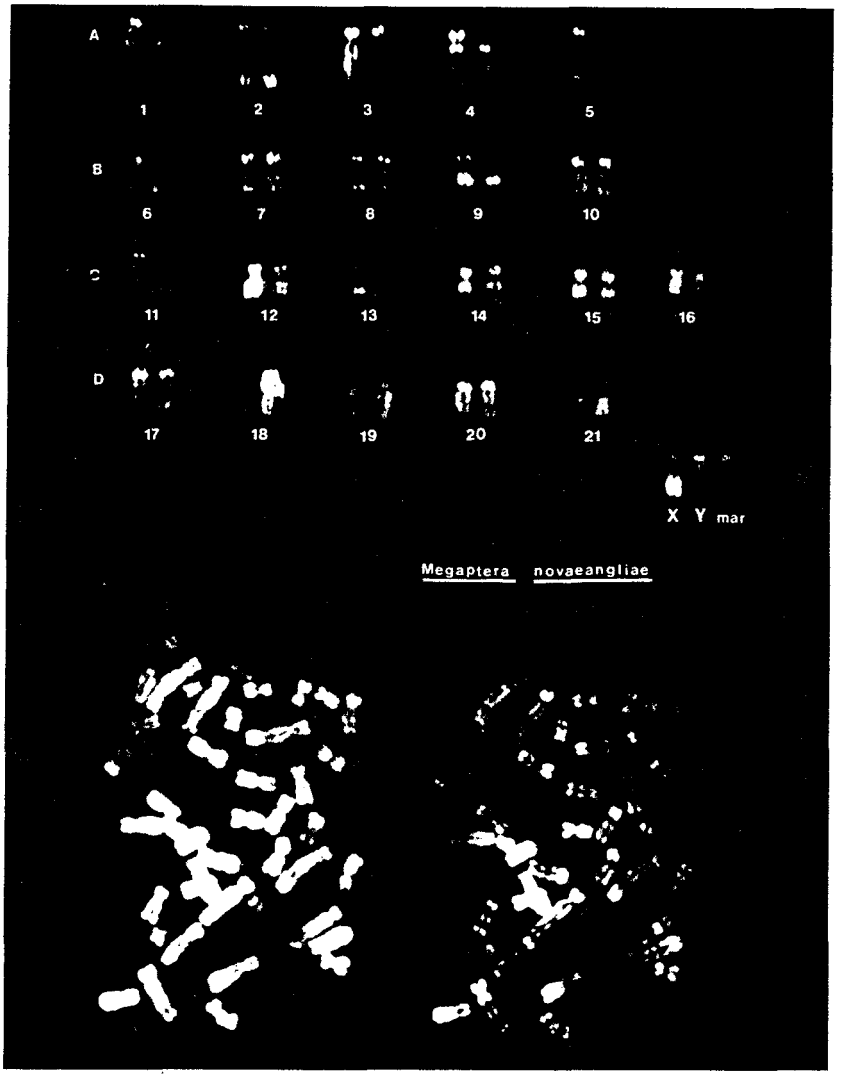

Figure 5
R-banded karyotypes of a male *Megaptera novaeangliae* (2N = 45, + mar). Two intensities of the metaphase spread for *M. novaeangliae* are included to show the faint staining extra chromosome. The presence of this extra chromosome was confirmed with standard Giemsa staining (not shown).

patterns and the basis for the change in number in *K. breviceps*. Instead, this paper concentrates on the presence in these species' karyotypes of chromosomal heteromorphisms which are visualized by R-banding.

Chromosome heteromorphisms have been noted in cetacean karyotypes both by C-banding (Arnason 1974, 1980; Arnason et al. 1977, 1980, 1985; Duffield 1977; Stock 1981; Worthen 1981) and fluorescent R-banding (Duffield 1982, 1986; Lambertsen and Duffield 1987; Duffield and Chamberlin-Lea 1990). C-banding is a chromosome banding technique that leaves tightly coiled C-band positive areas of the chromosome darkly stained (Bradbury et al. 1981) but denatures euchromatic regions. It, therefore, requires the sequential application of an additional banding technique (such as G-banding or R-banding) to identify homologous chromosome pairs. Fluorescent R-banding, in contrast, uses a staining agent which binds to guanine-cytosine (GC) rich DNA areas of the chromosomes. It bands euchromatin areas for homologue identification, while simultaneously binding strongly to GC-rich heteromorphic (variable) regions (Schweizer 1980). A significant number of GC-rich heteromorphic regions have been reported in the chromosomes of cetacean species (Duffield 1986; Lambertsen and Duffield 1987; Duffield and Chamberlin-Lea 1990). In *Tursiops truncatus*, *Orcinus orca*, and *Megaptera novaeangliae* at least half of the chromosome

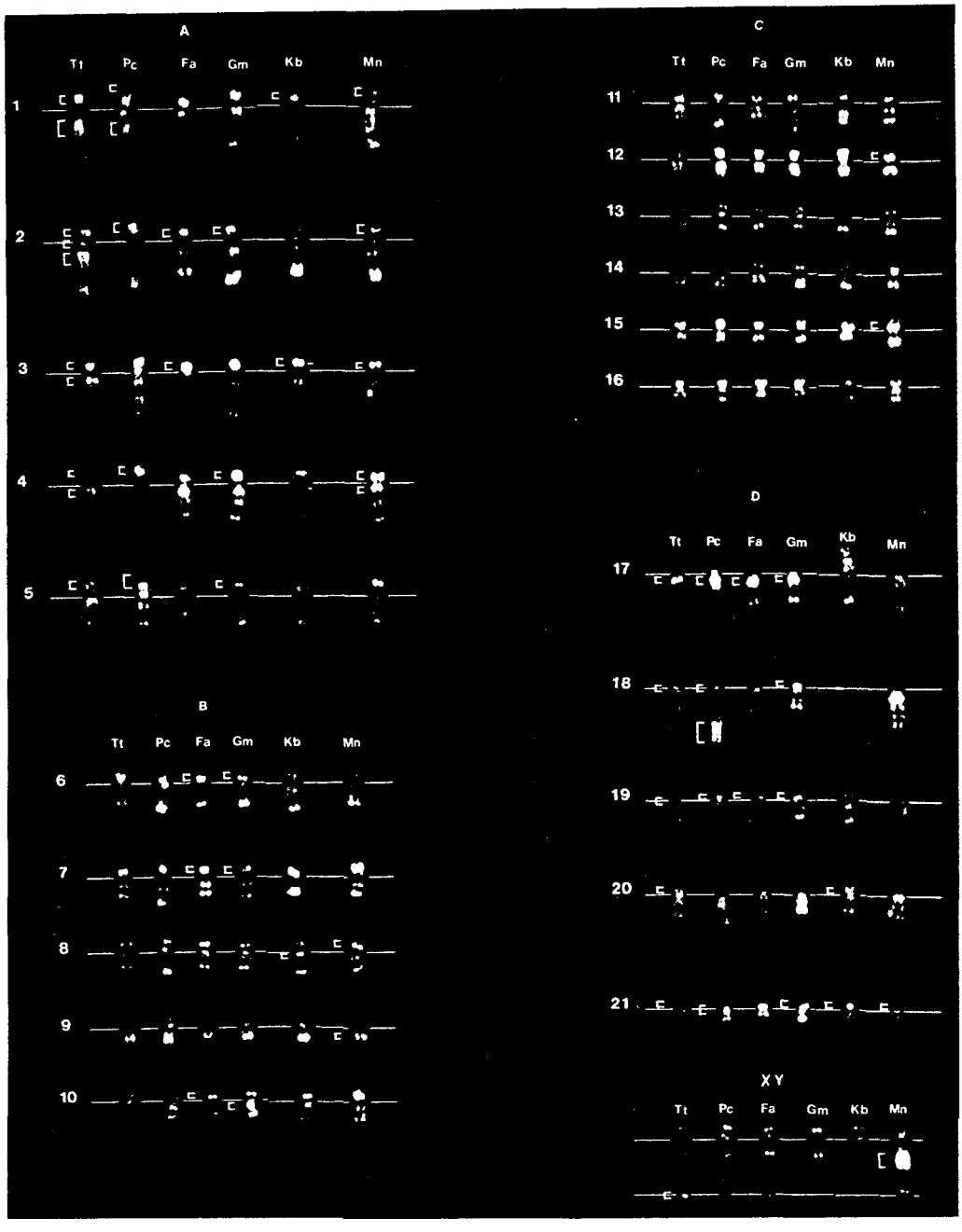

Figure 6

One chromosome of each chromosome pair from the karyotypes of *Tursiops truncatus* (Tt) compared to those of *Pseudorca crassidens* (Pc), *Feresa attenuata* (Fa), *Globicephala macrorhynchus* (Gm), *Kogia breviceps* (Kb), and *Megaptera novaeangliae* (Mn), to demonstrate the positions of known R-band heteromorphic regions in the chromosomes of these species (brackets). Note the unusual blocks of R-band heteromorphic material in *P. crassidens* (D-18) and in *M. novaeangliae* (X chromosome).

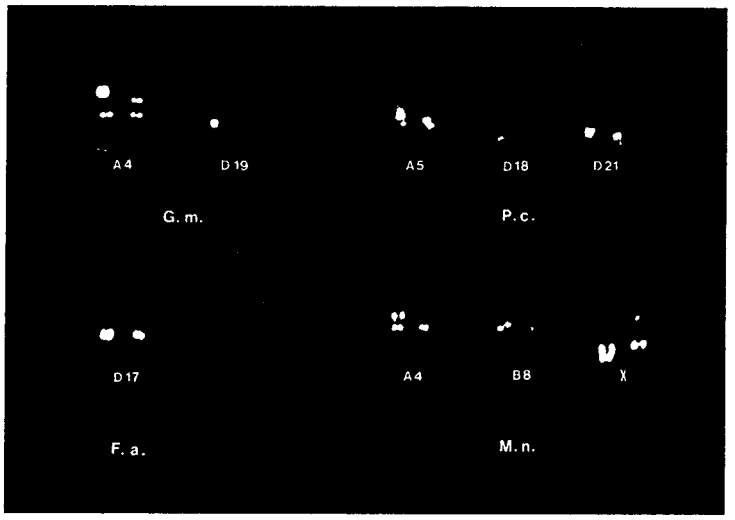

Figure 7
Examples of the types of R-band variants seen in various heteromorphic regions in the karyotypes of *Globicephala macrorhynchus* (Gm), *Pseudorca crassidens* (Pc), *Feresa attenuata* (Fa), and *Megaptera novaeangliae* (Mn).

pairs in their karyotypes exhibit these heteromorphic regions which may vary in size and in intensity of staining.

Chromosome pairs exhibiting R-band heteromorphisms in this study are indicated in each of the karyotypes (Figs. 1-5). A comparison of the distribution of heteromorphic regions in the chromosomes of the five stranded cetacean species and *T. truncatus* (Fig. 6) shows that many of the R-band heteromorphic regions are in the same chromosomal locations in all six species; however, there are also species differences (*see, for example*, the X chromosome in *M. novaeangliae* and D-18 in *P. crassidens*). Examples of the types of heteromorphisms seen in this study are illustrated in Figure 7. In *T. truncatus*, 52 heteromorphisms for 11 chromosome pairs have been found among 104 animals studied (Duffield and Chamberlin-Lea 1990). This number of variants makes R-band heteromorphism analysis extremely useful in population studies, especially when hypotheses of specific relationships need to be tested. As more individuals of each of the stranded species are karyotyped, the number of recognizable heteromorphic regions and the range of heteromorphisms within these regions will be established.

In cetaceans, R-band chromosome heteromorphisms have been used both as genetic markers for determining parentage in captive breeding programs (Duffield et al. 1986; Duffield and Chamberlin-Lea 1990; Hewlett et al. 1989) and for investigating population structure in the field (Lambertsen and Duffield 1987; Duffield and Wells 1988; Duffield et al. 1989). An exciting application of R-band heteromorphisms analysis for stranded cetaceans lies in its potential for confirming suspected relationships among animals stranding together, as in Florida, for example, where *K. breviceps* often strand in adult female-calf pairs or in adult female-calf pairs accompanied by a juvenile or adult male (D. Odell, pers. obs.). Similarly, chromosome heteromorphism analysis could be useful in a mass stranding situation to investigate relationships among specific individuals.

The use of cornea to establish cell cultures from postmortem cetaceans opens up the possibility for cytogenetic studies on stranded cetaceans which have been dead for several days. Fluorescent R-band chromosome heteromorphisms in the karyotypes of these species, as well as the existence of unique marker chromosomes, provide cytogenetic markers for assessing relationships within groups of stranded animals and for looking at regional population differences in these species.

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