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A molecular genetic analysis of kinship in free-living groups of sperm whales

by

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for the degree of Doctor of Philosophy

at

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ABSTRACT

Mature female sperm whales (*Physeter macrocephalus*) are found in socially cohesive groups of 10-30, including immature whales of both sexes, within which there is apparent communal care of calves. The goal of this thesis was to investigate patterns of kinship within such groups in order to better understand the genetic basis for sperm whale sociality. For the molecular genetic analysis, highly polymorphic DNA markers were developed by cloning and sequencing eleven microsatellite loci from sperm whales. Primers for polymerase chain reaction (PCR) were successfully developed for amplification of alleles at five of these loci. Additionally, primers for PCR-amplification of the male-specific *SRY* gene were developed for molecular sexing. The sexing data revealed that one microsatellite marker was X-linked. To investigate social structure, free-living groups of whales found off the mainland coast of Ecuador were studied with non-invasive techniques. Social groups were delineated from photographic identifications of individuals and direct observations of behavioural interactions. DNA was obtained from several samples of skin naturally sloughed by whales; a large number of samples were collected from three distinct groups. Molecular sexing showed that all groups contained mostly females. Kinship was demonstrated by the non-random distribution of microsatellite allele variation within social groups. Several indices of allele sharing were higher amongst individuals within the same group than amongst individuals in different groups. Simulation modelling suggested that the observed groups were matrilineal in structure with restricted dispersal of females. Based on the sex ratio data, males were estimated to disperse from their natal groups at about age six years. The evidence for genetic relatedness described here indicates that kin selection may have been important in the social evolution of sperm whales.

LIST OF ABBREVIATIONS AND SYMBOLS

χ^2	chi-square statistic
μCi	microCurie
μl	microlitre
μg	micrograms
A	adenine
ATP	adenosine 5'-triphosphate
bp	nucleotide base pairs
C	cytosine
°C	degrees Celsius
df	degrees of freedom
DNA	deoxyribonucleic acid
dNTPs	deoxynucleoside-5'-triphosphates
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
G	guanine
G	Goodness-of-fit statistic
HWE	Hardy-Weinberg Equilibrium
IPTG	isopropylthio-β-D-galactoside
kb	1000 base pairs
M	molar
mg	milligrams
ml	millilitre
mM	millimolar

mtDNA	mitochondrial DNA
n	sample size
ng	nanograms
p	probability
PCR	polymerase chain reaction
PEG	polyethylene glycol
r	coefficient of relatedness
SD	standard deviation
SDS	sodium dodecyl sulfate
SE	standard error
sec	seconds
T	thymine
t	t statistic
tRNA	transfer ribonucleic acid
U	units
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

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Field data were collected by the whole crew of *Balaena*. Identification photographs of individual whales were catalogued by Susan Dufault, and length measuring photographs were analysed by Mary Dillon. Samples from stranded cetaceans were donated to this work by Dr. Robin Baird (Simon Fraser University and Marine Mammal Research Group), Dr.

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CHAPTER 1

INTRODUCTION AND REVIEW OF SOCIAL ORGANISATION IN SPERM WHALES

Sperm whales (*Physeter macrocephalus*) have engendered study and speculation since the earliest days of whaling (*e.g.* Beale 1839; Melville 1851). They are remarkable among the mammals for their complex social organisation (Best 1979), and are among the most numerous and largest of all cetaceans (*i.e.* whales, dolphins and porpoises). The sexes display a striking degree of geographical segregation, with mature females and young whales being restricted to tropical and sub-tropical waters (to about 40° latitude), while mature males have a distribution covering almost all deep, ice-free ocean areas (Best 1979). Physical dimorphism between the sexes is also extreme with fully mature males (up to 18 m and 57 metric tons) attaining about one and a half times the body length and three times the mass of fully mature females (Best 1979; Rice 1989). Both sexes are famous for their deep feeding dives. As a species, sperm whales appear to be ecologically very successful, probably consuming more food than all of the world's fisheries combined (Kanwisher and Ridgway 1983).

Social groups

The primary social groups encountered in sperm whales consist of mature females and young whales of both sexes (reviewed in Best 1979). Such groups are often termed 'mixed' and typically number 10-40 individuals (mean of about 25). Much larger numbers of whales have been seen together, but these seem to represent temporary aggregations of groups. Indeed, when individual groups are followed for a few days, they will often merge with other groups for a brief period of time and then separate (Whitehead and Arnbom 1987).

Social cohesion within groups

Members of mixed groups demonstrate tight social cohesion. Social facilitation has often been observed, as have 'standing-by', assisting and supporting behaviours (Caldwell and Caldwell 1966; Caldwell *et al.* 1966; Best *et al.* 1984). Entire groups frequently spend hours at the water surface, in close physical contact and often with intense physical interaction (Whitehead and Weilgart 1991). Group members are usually spread out in small clusters while on the water surface, but companionships within these clusters are very temporary and fluid in nature (Gordon 1987).

Structure of groups

In a few instances, it has been possible to sample most members in a single group, either because of mass stranding events (Rice *et al.* 1986) or because of whaling operations (Ohsumi 1971; Best 1979). Analyses of such data indicate that female membership in a mixed group is on average 78% (range 36-100%), with about three-fourths of the females being mature (Best 1979; Rice 1989). Females attain sexual maturity at about ten years of age (IWC 1982). There has been some suggestion that females might segregate into separate groups or sub-groups according to reproductive status (Rice *et al.* 1986), but several investigators have found immature, pregnant, lactating and resting whales together in the same groups in proportions not significantly different from random (reviewed in Rice 1989).

Marking studies (using artificial tags or photographs of natural tail patterns) indicate that some individuals remain together for periods of years (Ohsumi 1971; Best 1979; Gordon 1987). A more extensive study, focusing on the Galapagos Islands sperm whale population, found that specific individuals could be statistically allocated into groups based on association patterns constructed over periods of weeks; likelihood ratio tests indicated that, within years, groups with a mean size of twenty were approximately closed in membership (Whitehead and Arnbom 1987). However, with more data, it was found that

some groups seemed to retain their membership over years while others seemed to split (Whitehead and Waters 1990). The story became more complex, when analysis by Whitehead *et al.* (1991), using a long time-series of individual identifications, showed that a typical group is in fact stable only over a short period of time and seems to represent a temporary merging of smaller, but very stable, sub-groups. These stable sub-groups were termed 'units'. Units contain about a dozen whales that stay together for periods of years. Off the Galapagos, there are about two units travelling together at any given time, and they stay together for an average of a week or so as a coordinated 'group'. Within these short-term groups, there is a large amount of interaction between members of the different units, but associations tend to be strongest amongst individuals that are also from the same long-term unit (K. Richard and H. Whitehead unpublished data). Groups often occur in very temporary (a few hours) aggregations with other groups, but the size of both groups and aggregations seems to vary considerably with environmental conditions and geographic location (Whitehead *et al.* 1991; Whitehead and Kahn 1992). Evidence for the merging of stable units into short-term groups is not yet available for areas outside of the Galapagos Islands.

Whitehead *et al.* (1991) suggest that in their model a 'unit' may represent a family of whales. Short-term 'groups' may form in order to enhance cooperation or avoid mutual interference during foraging activities. It is not known whether there are preferential associations between particular units. If so, this might reflect some degree of genetic relatedness between them.

Dispersal from groups

Males disperse from their natal groups while still immature, probably at about 4-5 years on average, but at least by 15-20 years (Best 1979; Rice 1989). It is clear that all males must disperse since large males are never seen to be long-term members of a mixed group. Following dispersal, similar-sized males aggregate into loose groups, but

associations between individuals seem much less stable than in mixed groups (Caldwell *et al.* 1966; Best 1979). These groups can contain a few dozen individuals, but as the males mature, they form increasingly smaller groups and move to increasingly higher latitudes (Best 1979; Gaskin 1985). Males achieve physiological sexual maturity at about 18-21 years (Rice 1989), but do not appear to begin breeding until about 25-27 years (Best 1979). At this stage, they tend to be solitary and found in polar waters. These males are generally thought to be the principal breeders in the population, returning to tropical waters to visit mixed groups during the breeding season, probably as single individuals but perhaps sometimes in small groups. There is some suggestion that the number of males visiting mixed groups is limited by competitive fighting between males for dominance prior to the breeding season (Best 1979). The frequency and regularity of a male's visits to warm-water breeding grounds remain unknown, as do the geographic relationships between a male's natal group range, his feeding range, and his breeding range. Once on the breeding grounds, males rove between groups of females, remaining with individual groups for only hours at a time (Whitehead 1993, 1994).

The extent of female dispersal from natal groups has been unclear. Complete lack of dispersal would lead to stable and perfect matrilines. However, if all or some females disperse, then mixed groups should include some genetically unrelated females. Best (1979) summarizes the evidence supporting female dispersal. By comparing the observed proportion of mature females within mixed groups to the proportion within the whole population based on whaling data, Best concluded that nearly half of all juvenile females appear to be absent from mixed groups. Moreover, in instances when most individuals within a mixed group were killed, workers found an equal sex ratio amongst whales less than eleven years of age; this would be unexpected if juvenile dispersal is sex-biased. Finally, there are also several reports of groups that appear to contain only juveniles of both sexes, although the sighting frequency of such groups is much lower than the sighting frequency of mixed groups. If females do disperse, they probably join juvenile

groups which contain both sexes, and eventually join a mixed group again before puberty (Best 1979).

Communal care within social groups

Best (1979) suggested that social groups in sperm whales have evolved by selection for cooperative foraging and/or communal care of calves. Whitehead (1[illegible] examined the spatial organisation of groups of foraging sperm whales and concluded that it was unlikely that formation foraging was the major evolutionary force leading to gregariousness of female sperm whales. On the other hand, there are several observations indicating that communal care of calves does occur.

Calves are vulnerable to killer whales (*Orcinus orca*) and sharks (Best *et al.*1984; Arnbom *et al.* 1987; Rice 1989). They are also unable to dive as deep, nor as long, as adult whales must regularly dive in order to feed (Best 1979; Gordon 1987). Thus, in the normal course of her daily foraging, a female has to leave her calf at the surface for extended periods of time. However, calves, particularly small ones, are almost always found close beside an adult. Detailed studies of association patterns show that an individual calf spends most of its time with one mature female (presumed to be its mother), but is frequently seen with other older whales (usually female, but also male) when the putative mother is absent (Gordon 1987). Individual adults regularly associate with different calves (Gordon 1987), and dive patterns of non-parents within groups are staggered to the apparent benefit of calves (Whitehead submitted). In field observations, calves are often seen to move from one adult to another, or more commonly for one adult to move beside one calf as another adult leaves it, usually by diving deeply (pers. obs.). Thus, young whales seem to have social bonds to several adults in addition to their mothers. These observations suggest a babysitting function for mixed groups (or units), with different adults accompanying a calf at different times, thereby enabling mothers to feed without leaving their calves unduly vulnerable to predation or to getting lost.

Arnbom *et al.* (1987) describe an attack by *Orcas* against a group of sperm whales that included a calf. The adult sperm whales appeared to protect the calf by keeping it surrounded in the middle of the group. A similar case is recounted in Best *et al.* (1984) for an attack during an apparent birth of a sperm whale calf. However, communal defense may still be important even when there are no young calves to protect. During two other encounters between *Orcas* and sperm whales, no sperm whale calves were present, and in both cases the sperm whales formed a coordinated rank and within minutes chased the *Orcas* away (pers. obs.). Gregariousness might thus serve to reduce predation risk through mutual defense and vigilance.

Other data provide further evidence for communal care. There are several records of sperm whales directing epimeletic, or care-giving, behaviour towards injured group members in the course of whaling operations (Caldwell and Caldwell 1966). As well, during birthing events, several adults within the group support the mother and newborn calf (reviewed in Rice 1989).

The occurrence of allosuckling is suggested by observations of individual calves apparently suckling from different mature females, and in one instance, by two similar-sized calves apparently suckling from one mature female (Gordon 1987); twins are not thought to survive in cetaceans (Rice 1989). In an examination of whaling data, Best *et al.* (1984) found that the number of lactating females in a group exceeds the number of whales presumed on the basis of size or age to be suckling, and they suggest that this is most likely because calves actually do suckle from more than one female, including from some females who have no dependant calves of their own.

Weaning is thought to occur at an average age of about two years, but older adolescents still suckle to some degree (Best *et al.* 1984). Calving intervals are also very long (~5 years; IWC 1982). Such prolonged care within groups indicates the importance of sociality to individual calves. Moreover, the period of lactation is likely an important

time for social learning and bonding, enabling young animals to learn essential cultural experience and knowledge from their older companions (Brodie 1969; Best *et al.* 1984).

Post-reproductive females appear to exist in some odontocete species (Bigg *et al.* 1990; Marsh and Kasuya 1986), although there is no direct evidence for this in sperm whales. However, maximum longevity is long (60-70 years; Rice 1989) and both calving intervals and the duration of lactation are longer in older individuals (Best *et al.* 1984). Even if not strictly post-reproductive, females are likely to change roles with age, investing more in calf rearing than in calf bearing (*cf.* Marsh and Kasuya 1986). Such females may contribute to the communal care of calves in a variety of ways, perhaps by sharing critical survival experience or helping to suckle calves that are not their own. It may be that whole social units, not just mothers, are required for efficient calf rearing.

Maintenance of social organisation

The epimeletic and communal care behaviours described above may be considered altruistic since they appear to benefit one whale (*e.g.* the calf or mother) at cost to another (*e.g.* from increased risk of injury during a predator's attack, lost foraging and resting times, or increased energetic expenditures from lactation). Socially complex behaviours of this sort are likely to have evolved and been maintained through kin selection or reciprocal altruism (*cf.* Hamilton 1964; Trivers 1971, 1985; Dawkins 1979; Kurland 1980; Axelrod and Hamilton 1981; Connor and Norris 1982; Grafen 1984). Kin selection and reciprocal altruism are not necessarily mutually exclusive, however, as there are likely benefits from reciprocating altruism among relatives (Trivers 1971; Krebs and Davies 1987).

Communal care would evolve by kin selection if individuals in a group profited through an increase in inclusive fitness from the beneficial effects of their actions on relatives. Thus, group members would necessarily have to be genetically related. In order for an altruistic behaviour to be maintained, an altruistic whale would have to direct its act towards genetic relatives, such that the gain in fitness (b) to the recipient (*e.g.* the calf)

exceeds the loss in fitness (c) to the altruistic whale by more than the degree of genetic relatedness (r) between them; *i.e.* $b/c > 1/r$ (*cf.* Hamilton 1964).

Some mammals displaying socially complex behaviours have been shown to form groups based on non-relatives (*e.g.* McCracken and Bradbury 1977). If sperm whale social groups also consist mostly of unrelated "friends", then communal care may have arisen by reciprocal altruism. With reciprocity, an altruistic act results in a deferred, but valuable repayment in one form or another from the recipient of the original altruistic act (Trivers 1971, 1985). For example, a babysitter might be repaid for its altruism by receiving help in the care of its own present or future offspring or by being allowed to remain with the group (to its own advantage). Such reciprocity can only be maintained if individuals interact frequently (Trivers 1971, 1985; Axelrod and Hamilton 1981), as sperm whales appear to do. Without regular interaction, an altruistic individual will lack sufficient opportunity to determine which individuals tend to reciprocate its own acts of altruism, and as a result may actually lose more from non-reciprocators than it gains from reciprocators. The reciprocity model thus requires a stable, long-term behavioural relationship between at least some members of a group who are able to identify each other and who are able to withhold their altruism from, or in other ways punish, any individual who does not reciprocate such actions. Under this model, one would expect communal care to be limited to members of the same long-term social group (or unit).

Connor and Norris (1982) describe a variation of reciprocity in which altruistic tendencies extend to other members of society in general. Their model allows for significant social fluidity and changing membership within groups. They envisage selection favouring one whale helping another whale even if that other whale cannot recompense it fully, if at all. The altruistic whale's fitness would increase as a result of a greater tendency for individuals who know of its altruistic tendencies to act altruistically towards it. This could generate selection pressures for generalized altruistic tendencies,

with individual whales being regarded as cheaters (and thus punished) if they fail to offer similar amounts of altruism as other whales.

Contribution of this thesis

The genetic structure underlying the social organisation of sperm whales has long remained conjectural (Best 1979). Elucidation of this structure is important for further progress in understanding the ecology and evolution of sperm whale sociality. Thus, the goal of this thesis was to examine kinship within social groups of sperm whales. My approach employed a molecular genetic analysis of relatedness within several free-living groups, using technology that has only recently become available. I developed a suite of highly polymorphic genetic markers for detection of kinship (described in Chapter 2), as well as male-specific markers for molecular sexing (Chapter 3). These molecular tools were employed to examine genetic variation in three separate groups of whales, studied with non-invasive techniques off the mainland coast of Ecuador (Chapter 4). The results of this work provide formal genetic evidence for a matrilineally-based social organisation in sperm whales.

CHAPTER 2
DEVELOPMENT OF HIGHLY POLYMORPHIC GENETIC MARKERS FOR SPERM WHALES

Introduction

The use of different genetic markers for studying relatedness has been extensively reviewed in the literature (*e.g.* Burke 1989; Harrison 1989; Hoelzel and Dover 1989; King 1989; Avise 1993; Queller *et al.* 1993). Variable Number of Tandem Repeat (VNTR) markers consist of several copies of a short DNA sequence motif arranged in a tandem array. These markers can be categorized into two forms, *i.e.* microsatellites (in which the motif is usually 2-5 bp in length) and minisatellites (in which the motif is usually 10-100 bp in length). VNTR markers are much more variable than all other known genetic markers. Moreover, they have the potential to positively identify close relatives (as opposed to simply excluding individuals from being close relatives). Most of the genetic variation revealed by VNTR markers results from differences between individuals in the number of copies of the repetitive motif.

In the original approach used to type individuals for minisatellites, a size-fractionated and Southern blotted sample of restriction-digested genomic DNA is screened by a probe that detects several similar minisatellite loci simultaneously, thereby generating a complex multilocus pattern of bands (termed a 'DNA fingerprint') that is unique to an individual (Jeffreys *et al.* 1985a, b). The bands represent length variant alleles from each of the loci, but one is not able to link a specific band to a specific locus. Although DNA fingerprints can be highly informative, one generally requires samples of known kinship to calibrate allele sharing for studies of wild populations (*e.g.* Packer *et al.* 1991). Unfortunately, samples of known kinship do not exist for sperm whales. Moreover, in test trials using DNA fingerprinting of sperm whales, DNA hypervariability appeared to be fairly low (K. Richard unpublished data). (This conclusion is tentative, however, as

the low grade quality of the available samples (Appendix A) appeared to significantly affect the clarity of the DNA fingerprints.) In published studies of other cetacean species (including humpback whales, killer whales and pilot whales), bandsharing amongst non-relatives usually appears to be quite high (Amos *et al.* 1991; Hoelzel and Dover 1991; Baker *et al.* 1993).

Variation at microsatellites is typically detected by polymerase chain reaction amplification (PCR; Saiki *et al.* 1988) using primers that target non-repetitive, unique sequence flanking the repetitive array, followed by size-fractionation of the PCR products in polyacrylamide (Fig. 2.1). Similar analysis of minisatellite markers is also possible, but cloning, PCR amplification, and scoring of allele sizes often prove to be much more difficult than with microsatellites (*e.g.* Burke *et al.* 1991). For this thesis, several microsatellite markers were developed from sperm whales. Microsatellites were chosen for development primarily because they are short in sequence and amenable to amplification by PCR. These qualities are particularly useful because of the small size and degradation of many of the samples obtained from sperm whales in this study (Appendix A).

Materials and methods

i. Construction of a mini-library of size-selected DNA

A 50 µg sample of genomic DNA (see Appendix A for a description of samples and procedures for organic extraction of DNA) from sloughed skin sample #GAL145 (mature male seen near the Galapagos Islands in 1989, photographically identified as whale #531) was digested with four restriction endonucleases, *Alu*I, *Hae*III, *Hinc*II, and *Rsa*I, and size-fractionated in 1.2% agarose. DNA fragments in the range of 200-500 bp were eluted (BioRad electroeluter) from the gel, phenol-extracted, and resuspended in 25 µl TE (10 mM Tris, 1 mM EDTA), to an estimated concentration of 112.5 ng/µl.

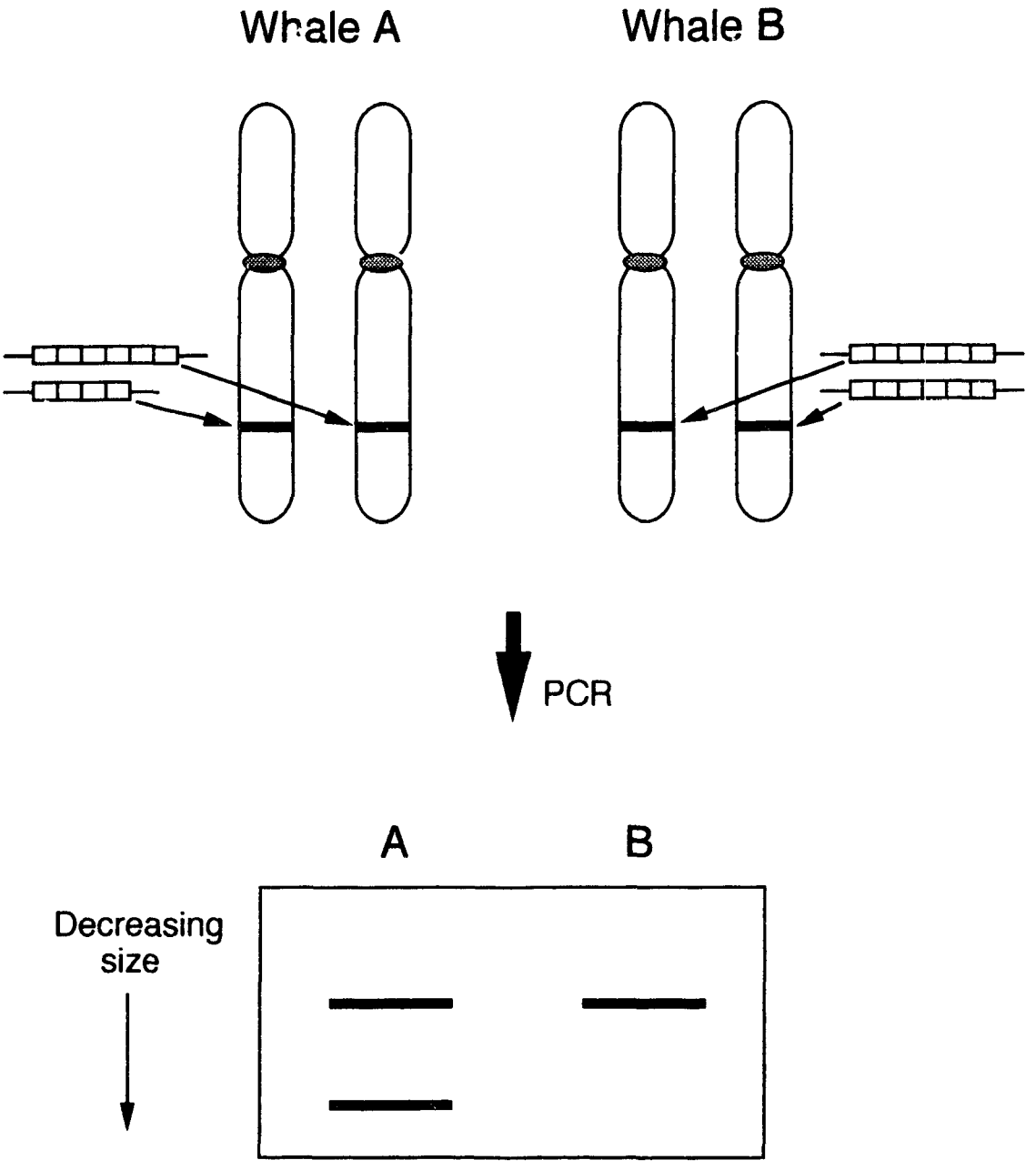

Fig. 2.1: Individual genotypes at a microsatellite locus determined by size fractionation of PCR amplification products. The sizes of PCR products reflect the number of repeat units borne by each allele. Amplification products are sufficiently small (< 300 bp) that allelic size variants can be scored precisely by comparison to DNA markers.

ii. Ligation and transformation of DNA

A 250-500 ng aliquot of the size-selected DNA was blunt-end cloned into 40-200 ng of M13mp8, *Sma*I cut, dephosphorylated vector (Amersham), using 1-4 units of T4 DNA ligase in a reaction volume of 11 µl containing 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM rATP, 5 mM DTT, 5% PEG, and incubated overnight at 14 °C. Ligation products were ethanol-precipitated and resuspended in 10 µl of TE, and then 5 µl used to transform MAX Efficiency DH5αF'IQ^{TM} Competent Cells (BRL), following the manufacturer's protocol. The cells were plated on 2X YT medium (1% (w/v) Bactotryptone, 1% (w/v) Yeast Extract, 0.5% (w/v) NaCl) with X-gal and IPTG to generate 100-600 recombinant plaques per plate.

iii. Screening of mini-library for microsatellites

Single stranded oligonucleotide probes, $(GA)_{15}$, $(GT)_{15}$, and $(GATA)_5$, were end-labelled by 5 units of T4 polynucleotide kinase in a 5 µl reaction containing 80-100 ng of oligonucleotide, 50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, and 5 µCi of ^{32}P-γ-ATP (3000 Ci/mmol; Amersham), incubated at 37 °C for 30 minutes. The entire end-labelling reaction mix was used as a probe in a single hybridization container without further purification or quantification of labelled product.

The plated transformations were blotted onto Hybond-N circular nylon membranes (Amersham) following the manufacturer's protocol. Typically, two filters were pre-hybridized together for at least one hour at 65 °C in a 10 ml solution of 5 x SSPE (750 mM NaCl, 50 mM Sodium phosphate, 5 mM EDTA), 5 x Denhardt's solution (0.1% (w/v) bovine serum albumin, 0.1% (w/v) Ficoll, 0.1% (w/v) polyvinylpyrollidone), 0.5% SDS, and 100 µg/ml tRNA. Probe was added directly to the hybridization solution and allowed to hybridize at 65 °C for approximately 20 hours. Membranes were washed under different stringencies, typically with 2 washes in 2 x SSPE, 0.1% SDS for 10-15 minutes at room temperature, but to a maximum stringency of 0.1 x SSPE, 0.1% SDS at 65 °C.

Membranes were exposed overnight to Kodak XAR5 X-ray film with intensifying screens at -70 °C. Recombinant (clear) plaques that matched positive signals on the film were removed as a plug from the agar plate and suspended in 1 ml of 2X YT; each plaque suspension was replated and rescreened until all plaques matched a positive signal on film (Sambrook *et al.* 1989).

iv. DNA sequencing

Single stranded and double-stranded (RF) templates were prepared from the plaque suspensions, according to Sambrook *et al.* (1989). All clones were sequenced by the Sanger dideoxy chain-termination method (Sanger *et al.* 1977). Most clones were partially sequenced with the Klenow fragment of DNA polymerase I (Stratagene DSK ^{35}S Sequencing Kit), but complete sequences were obtained only by using T7 DNA polymerase (Pharmacia ^{T7}Sequencing Kit). Most sequences were obtained entirely from single-stranded template. Sequences of some clones were completed by sequencing an overlapping segment of the opposite strand (*i.e.* by using reverse sequencing primers on double stranded RF template) because the polymerase appeared unable to extend beyond the repeat region in one direction for these particular clones.

v. PCR primers

PCR primers were designed by eye, considerations being that the primers be ~17-20 base pairs in length, the GC content be ~40-50%, the relative proportion of each base be approximately equal between primers, and that the PCR products be ~120-160 bp in size. Primers were also designed to avoid both high GC content on the 3' end of the primers and high complementarity within and between primers. Primer sites were not placed immediately adjacent to the repetitive array in order to avoid possible cryptic simplicity (Tautz *et al.* 1986) and consequent instability in the target template. It was often not possible to select primers that met all these conditions. For some sequences, a second

set of primers were designed if the first set failed to reliably amplify the expected product. Primers originally isolated from pilot whale DNA and published by Tautz (1989) were also tested for amplification of sperm whale DNA.

vi. Radiolabelling of PCR primers

PCR oligonucleotide primers were end-labelled in a reaction containing 0.5 U/µl T4 polynucleotide kinase (Pharmacia), 50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, 1 µM primer, and 0.5 µCi/µl ^{35}S-γ-ATP (high specific activity, *i.e.* ≥1000 Ci/mmol; ICN or NEN), incubated at 37 °C for ~4 hours.

vii. Preparation of template DNA for PCR reactions

DNA was PCR-amplified directly from tissue samples, adapting a protocol developed for human forensic work (Walsh *et al.* 1991). Thus, DNA templates were prepared by boiling the skin sample in the presence of a chelating resin, either 5% Chelex 100 or 6% Instagene Purification Mix (BioRad). A small (~1-5 mg) and thin piece of tissue was rinsed in water, placed in 200 µl of resin mix contained in a 1.5 ml Sarstedt screw-cap tube, incubated at 56 °C for several minutes to overnight, vortexed for ten seconds, and then boiled for eight minutes. After boiling, the samples were again vortexed for 10 seconds and centrifuged at 16 000 x g for 3 minutes. An aliquot of the supernatant was added directly to the PCR reaction.

viii. PCR amplifications

All amplifications were performed in a Perkin Cetus Elmer thermal cycler, typically with 7 cycles of denaturation at 96 °C for 1 minute, annealing at 48-58 °C for 1 minute and extension at 72 °C for 5 seconds, followed by 21-23 cycles in which the denaturation temperature was reduced to 90 °C. Reactions contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.001% (w/v) gelatin, 1-4 mM $MgCl_2$, 200 µM each dNTP, 0.1-0.4 µM primers,

0.025 U/µl *Taq* Polymerase, and were brought to a total volume of 10 µl with Chelex-extracted template (40-79% of the total volume), overlaid with 2-3 drops of mineral oil. Only one primer of the pair was end-labelled with ^{35}S-γ-ATP (and it was not diluted with unlabelled primer). PCR products were size-fractionated on 8% denaturing polyacrylamide in a S2 sequencing gel apparatus (BRL), using sequencing products for the M13mp18 + strand vector (Pharmacia) as size markers. Standardization between gels was ensured by including a known sample as an additional size reference. Gels were fixed, dried and exposed to Kodak XAR5 X-ray film, typically for 1-3 days.

Results and discussion

i. Screening of mini-library

In total, approximately 1750 recombinant plaques were surveyed for GT, GA, and GATA repeats. Rescreening indicated that only 11 of the original 22 plaques picked after the initial hybridizations were true positives; 3 clones were identified by the GA probe, 8 by the GT probe, and 0 by the GATA probe. Thus, 0.63% of the inserts in the mini-library contained microsatellites. This compares to a figure of ~4.5% for a mini-library from rainbow trout (*Oncorhynchus mykiss*) prepared and probed concomitantly with the sperm whale library (K. Richard unpublished data).

ii. Microsatellite sequences

Sequences were obtained for eleven microsatellite loci (Fig. 2.2; Appendix B). Sequencing of the clones appeared to be hampered by some aspect of the repetitive array. This problem was overcome by using T7 polymerase instead of Klenow, and, for clones SW7, SW9, SW14 and SW17, by sequencing overlapping segments of both strands.

SW2

5'-AGCTGGGTAA TTTGTAAATG TCAATCAGGC AACGGGGCAC AGAGAGAGAG
AGAGAGAGAG AGAGAGAGAG AAAGGGGCC-3'

SW10

5'-CTTGCTAACC TAAGGATGGA GATGATACAC ATGCCCTCAT TAGAAGACCA
GATGTGCGTG CGTGCGTGCG TGCGTGCGTG CGTGTGTGTG TGTGTGTGTG
TGTGTGTGTG TGTTTCCTTC TCCCTCATCC TTTGCAGTTG CAGACCTGGG
AAATCCTGTG GTATGCATAA AACTTGATCA TTTCGACAGG CTCTCCTTAC
AAAATCACAG GCTTGGATGC ATA-3'

SW13

5'-AGCTGTCTTA ATGAAATTCC CGTGTGTGTG TGTGTGTGTG TGTGTGTGTG
TGTGTGTGTG TTCTCAAGGA AGTAACATTC AGGGAAACAA TCAGGGTTTT
TAATTTTCTG GGTCATTTTT ATAATTTATT TGTTTTAAGA AAACAGCATC
ATTTACGTGG GAGTCATGAT GGGACTTTTG AAGGAAATTT TAACTGTTTC
CTTGATGGCT CGTTTATG-3'

SW15

5'-CCATGCTATG GGAGCCCCTA GACAGAACTT GTCTTGGACA ATCATCTATG
GATGAGAGAG CCATTGTGGA AGTCCACGTT TCCAGCAGAA AATTTCCAGC
ACATTGTTGG AATAACAACA ATGACAACAA CAGGATGTAT GTGTGTGTGT
GTATATATAT ATATATATAT ATATATCTAT AGATAGATAG ATAGATAGAC
AGATAGATAT GTAATATATA CACATACATA TACATATATC ATATATATAC
TAGCATATGT GAGTGTGTGT GTGCGTGTAT GTGCATGTGT GTGTGCATGT
GTTTGGATGC ATTGCAGAGG GCAAGAGAAG CAGTTGACTT TACATGTGCT
GAGCTAGAGC CTGCGTCTGA GACGTAGAGA TCTGAGGCT-3'

SW19

5'-CTCTCATGAC TAAAATAGTT ATGTAGTTTT CTTTAACAGT AATGTAGAGA
GAGTGTGTGT GTGTGTGTGT GTGTGTGTGT GTGTGTGTGT GTGTGTGTGT
GTGTGATGGA GGGGGCGGGG AAGGGATAAT AATAGGTGAA AAGCCCAGAA
CTGAGAGTCA GTTCCACCAT CCCTATACCC TAACCAGTCC CACTTATGCA
TTGATCTATT TCTATTCTTA CTCATATGCC CCAAGACAGT CAGG-3'

Fig. 2.2: Sequences cloned from the 5 microsatellites used in the construction of multilocus profiles. Underlined sequences indicate target sites for PCR primers.

iii. PCR of the microsatellite arrays

Extractions using the chelating resin provided sufficient template for several PCR amplifications, proved less time-consuming than organic extractions, and required only minuscule amounts of tissue. Ten of the microsatellite clones contained enough sequence flanking the repetitive array to design PCR primers (Fig. 2.2; appendices B, C). PCR conditions were varied for several parameters including annealing temperature, magnesium concentration, primer concentration, template concentration, and number of cycles (Table 2.1).

Five of the primer sets produced polymorphic amplification products of the expected size range (Figs. 2.3 - 2.7). PCR products usually included artifactual shadow bands as seen in other studies (Schlötterer and Tautz 1992), but these did not affect scoring of the gels (Appendix D). For each primer set, the yield of replicate reactions was inconsistent, but samples that failed to amplify in one trial could usually be amplified in a second or third trial. Inconsistent yields were possibly due to degradation of the DNA samples (Appendix A), *e.g.* if the quality of DNA negatively affected copying of the target DNA during the early cycles of the PCR, the total yield of the reaction, because of exponential amplification, would be greatly diminished. Experimental error was controlled for by preparing all reactions for each PCR trial from one master mix that contained all reagents except the template.

PCR amplification of the most polymorphic locus, SW19, was uniformly inefficient (Fig. 2.7), and one-quarter of the samples could not be scored at this particular locus with the time and resources available to me (Appendix E). It appears unlikely that the gaps in the data set could be explained by null, or non-amplifying, alleles (Callen *et al.* 1993) since all samples proved very difficult to amplify. Four different combinations of primer pairs were tested for this locus, but options for primer design were restricted by the limited sequence data available (Fig. 2.2; Appendix C).

Table 2.1: PCR conditions for each microsatellite marker.

Locus	Primer sequences (* = labelled with ^{35}S)	Annealing temp (°C)	$[Mg^{2+}]$ (mM)	Concentration of each primer (μM)	No. of cycles
SW2	5'-AGCTGGGTAATTTGTAA*	55	1	0.1	< 25
	5'-GGCCCCTTTCTCTCTCT				
SW10	5'-ACCTAAGGATGGAGATG	58	1.5	0.2	25-30
	5'-ATTTCCCAGGTCTGCAA*				
SW13	5'-AGCTGTCTTAATGAAATTCCC	55	1	0.1	28-30
	5'-ACGTAAATGATGCTGTT*				
SW15	5'-GGAAGTCCACGTTTCCA	58	2	0.1	28-30
	5'-TGCCCTCTGCAATGCAT*				
SW19	5'-GTAGTTTTCTTTAACAGTAATG	48-55	1.5	0.2	30
	5'-AGTTCTGGGCTTTTCACCTA*				

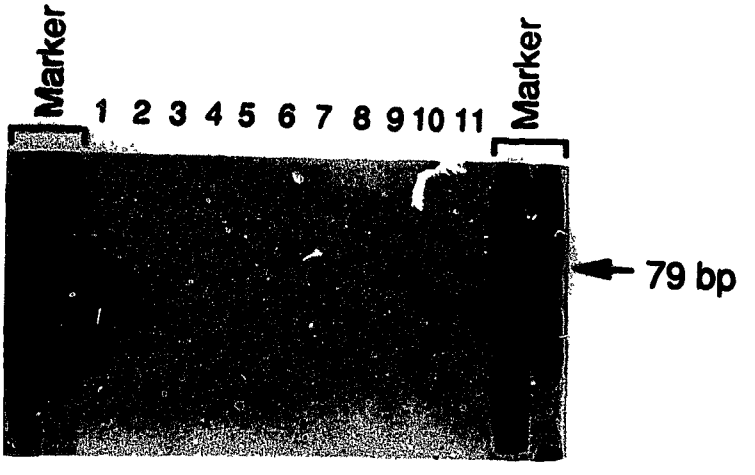

Fig. 2.3: PCR amplification products for microsatellite locus SW2. Primer sites are shown in Fig. 2.2. DNA was amplified directly from sloughed skin samples prepared with the Chelex resin. The size of each amplified allele was estimated by reference to co-migrating M13 sequence fragments (C, G and T combined in one lane; A in single lane) run on either side of the sperm whale samples. The genotypes scored for the 11 samples of sperm whale DNA are presented in appendix D.

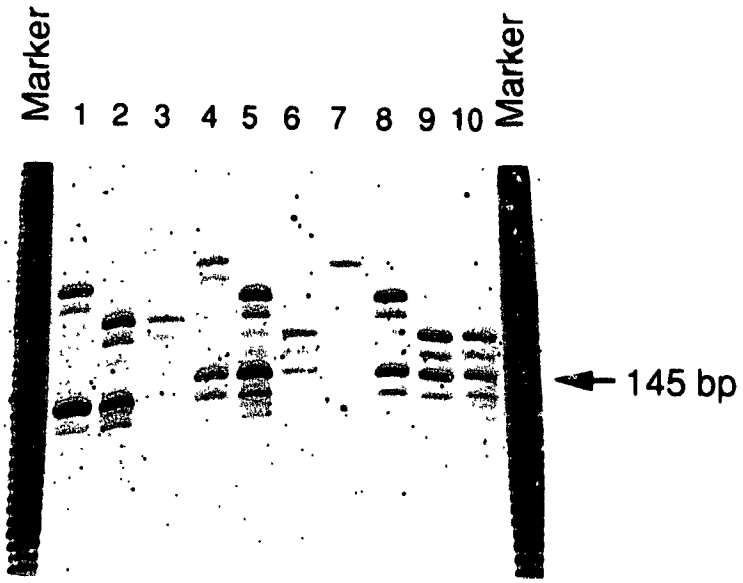

Fig. 2.4: PCR amplification products for microsatellite locus SW10. Primer sites are shown in Fig. 2.2. DNA was amplified directly from sloughed skin samples prepared with the Chelex resin. The size of each amplified allele was estimated by reference to co-migrating M13 sequence fragments (A, C, G and T combined in one lane) run on either side of the sperm whale samples. The genotypes scored for the 10 samples of sperm whale DNA are presented in appendix D.

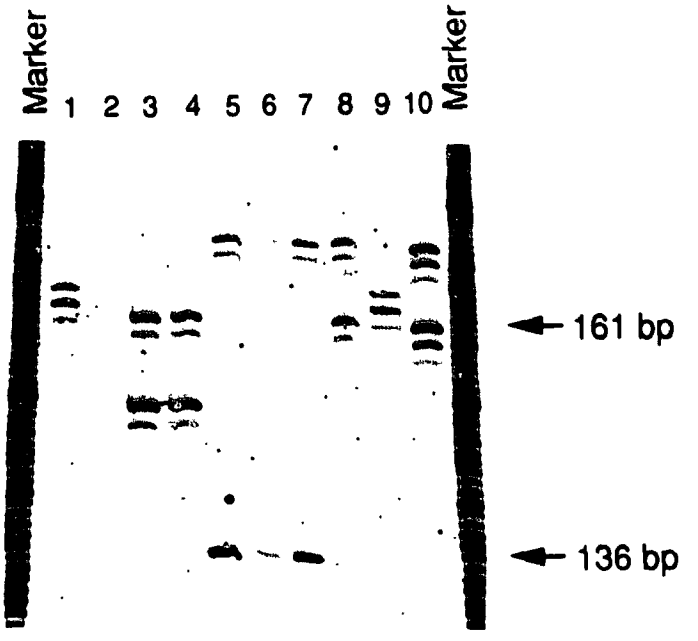

Fig. 2.5: PCR amplification products for microsatellite locus SW13. Primer sites are shown in Fig. 2.2. DNA was amplified directly from sloughed skin samples prepared with the Chelex resin. The size of each amplified allele was estimated by reference to co-migrating M13 sequence fragments (A, C, G and T combined in one lane) run on either side of the sperm whale samples. The genotypes scored for the 10 samples of sperm whale DNA are presented in appendix D.

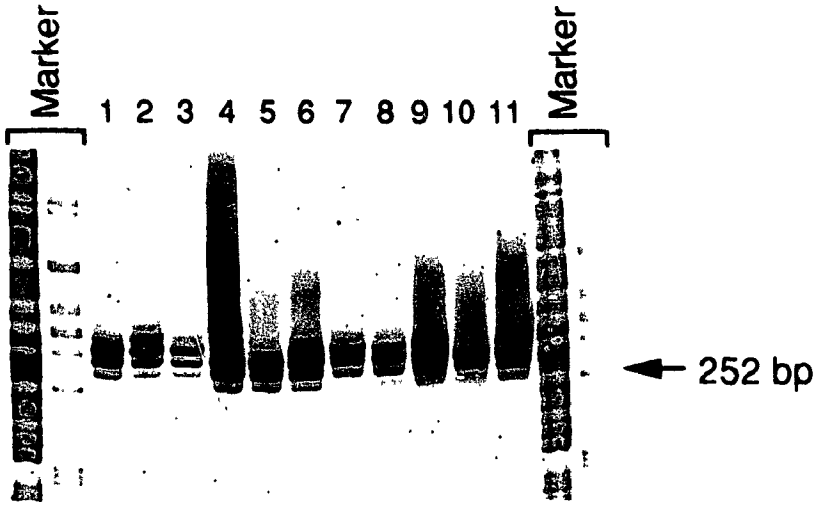

Fig. 2.6: PCR amplification products for microsatellite locus SW15. Primer sites are shown in Fig. 2.2. DNA was amplified directly from sloughed skin samples prepared with the Chelex resin. The size of each amplified allele was estimated by reference to co-migrating M13 sequence fragments (C, G and T combined in one lane; A in single lane) run on either side of the sperm whale samples. The genotypes scored for the 11 samples of sperm whale DNA are presented in appendix D.

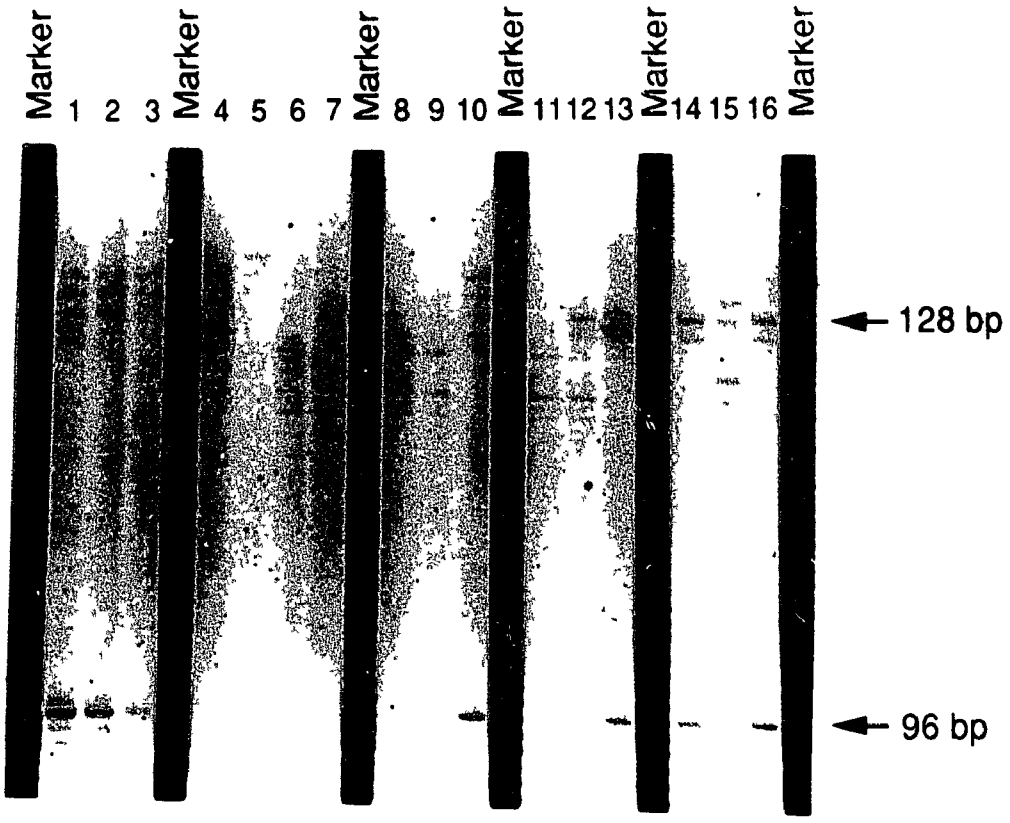

Fig. 2.7: PCR amplification products for microsatellite locus SW19. Primer sites are shown in Fig. 2.2. DNA was amplified directly from sloughed skin samples prepared with the Chelex resin. The size of each amplified allele was estimated by reference to co-migrating M13 sequence fragments (A, C, G and T combined in one lane) run on either side of the sperm whale samples. The genotypes scored for the 16 samples of sperm whale DNA are presented in appendix D.

Primer sets for the other microsatellites failed to amplify visible product in the PCR trials, or generated several fragments of DNA that could not be clearly scored. These microsatellites were also the ones that proved most difficult to sequence, which may indicate secondary structure at these loci. The primer set from pilot whale sequence also failed to amplify a visible product.

iv. Chromosomal location of microsatellites

Molecular sexing (see Chapter 3) indicated that microsatellite locus, SW15, was located on the X-chromosome. Most females (76%; $n = 63$) were heterozygous at this locus, but all males ($n = 28$, including samples from outside of Ecuador) each displayed only one amplification product (that varied between males). These results suggested that male genotypes were haploid and therefore X-linked.

The other four microsatellite markers did not display sex-specific patterns and were assumed to be autosomal and unlinked. Results of statistical tests for genotypic linkage disequilibria were consistent with these assumptions (as described in Chapter 4). Indeed, mammalian genomes typically contain tens of thousands of microsatellite sequences (Tautz and Renz 1984) spread throughout all chromosomes ($2n = 42$ in sperm whale (Rice 1989)), and the probability of cloning any two loci close together on a chromosome would clearly be remote.

As in other studies employing microsatellites, markers were assumed to be non-coding and selectively neutral (Queller *et al.* 1993).

v. Scoring of genotypes

A genetic profile was constructed for different samples by scoring allele patterns at the five useful microsatellite loci (Appendix E; see also Chapter 4 for a description of the samples). All allele patterns were scored independently at least twice, and many samples were PCR-amplified in more than one reaction. No inconsistencies were detected between

replicate analyses of a sample. Polymorphism at each locus ranged from 5-18 alleles, and observed heterozygosities were very high (Table 2.2).

There was no evidence in the data for null alleles, *i.e.* no locus displayed excess homozygosity (tests for Hardy-Weinberg Equilibrium are presented in Chapter 4; also *cf.* Pemberton *et al.* 1995). The microsatellite markers isolated from sperm whale were assumed to display a pattern of Mendelian co-dominant inheritance. Samples of known genealogy were not available from sperm whales in order to test this assumption directly. However, data from a variety of other mammals, including another cetacean species, *Globicephala melas* (Amos *et al.* 1993), are consistent with this pattern of inheritance. Calculated mutation rates for dinucleotide microsatellites are usually high relative to most other types of marker (*e.g.* 10^{-3} per locus per gamete (Weber and Wong 1993)), but still low enough that alleles remain stable for several successive generations. Jeffreys *et al.* (1988) describe the relationship between heterozygosity and mutation rate for five minisatellite loci; mutation rates remain below 10^{-2} for heterozygosities < 99%. In any event, although clearly important in paternity exclusion analyses, mutation rates at microsatellite markers should have only negligible effects on estimates of kinship (Queller *et al.* 1993).

Summary

Five highly polymorphic microsatellite markers were developed from sperm whales. One marker was X-linked. Allelic size variation was scored by polymerase chain reaction analysis. PCR amplifications were inefficient but genotypes could usually be scored with repeated trials.

Table 2.2: Characterization of genetic variation in sperm whales at 5 microsatellite markers. Samples include all 80 unique individuals listed in appendix E. Estimates of the number of repeat units assume that length variation is determined by the number of copies of the major dinucleotide motif in the repetitive array.

Locus	Repetitive sequence of the cloned allele	Observed polymorphism (no. of alleles)	Observed heterozygosity	Estimated size of PCR-amplified allele (bp)	Estimated no. of dinucleotide repeat units	No. of copies in sample set	Observed frequency
SW2	$(AG)_{15}$	5	0.55	73	12	1	0.006
				75	13	13	0.081
				77	14	91	0.569
				79	15	42	0.263
				81	16	13	0.081
						n = 160	

Table 2.2 continued.

Locus	Repetitive sequence of the cloned allele	Observed polymorphism (no. of alleles)	Observed heterozygosity	Estimated size of PCR-amplified allele (bp)	Estimated no. of dinucleotide repeat units	No. of copies in sample set	Observed frequency
SW10	$(GTGC)_7(GT)_{16}$	12	0.84	137	10	1	0.006
				139	11	1	0.006
				141	12	3	0.019
				143	13	8	0.050
				145	15	32	0.200
				147	16	18	0.113
				149	17	34	0.213
				151	18	35	0.219
				153	19	19	0.119
				155	20	3	0.019
				157	21	5	0.031
				159	22	1	0.006
						n = 160	

Table 2.2 continued.

Locus	Repetitive sequence of the cloned allele	Observed polymorphism (no. of alleles)	Observed heterozygosity	Estimated size of PCR-amplified allele (bp)	Estimated no. of dinucleotide repeat units	No. of copies in sample set	Observed frequency
SW13	$(GT)_{20}$	11	0.83	136	-9	10	0.063
				151	16	4	0.025
				159	20	9	0.056
				161	21	12	0.075
				163	22	56	0.350
				165	23	24	0.150
				167	24	18	0.113
				169	25	10	0.063
				171	26	11	0.069
				173	27	3	0.019
				175	28	5	0.019
						n = 160	

Table 2.2 continued.

Locus	Repetitive sequence of the cloned allele	Observed polymorphism (no. of alleles)	Observed heterozygosity	Estimated size of PCR-amplified allele (bp)	Estimated no. of dinucleotide repeat units	No. of copies in sample set	Observed frequency
SW15	Complex ~150 bp	5	0.76*	252	?	11	0.077
			*Because of X-linkage, heterozygosity calculated only for females (n = 63).	256	?	52	0.364
				258	?	60	0.420
				260	?	9	0.063
				262	?	11	0.077
						n = 143	
SW19	$(AG)_4(TG)_{26}$	18	0.95	90	6	2	0.017
				96	9	13	0.112
				103	~13	3	0.026
				110	16	2	0.017
				112	17	5	0.043
				114	18	1	0.009

Table 2.2 continued.

Locus	Repetitive sequence of the cloned allele	Observed polymorphism (no. of alleles)	Observed heterozygosity	Estimated size of PCR-amplified allele (bp)	Estimated no. of dinucleotide repeat units	No. of copies in sample set	Observed frequency
SW19 continued				116	19	3	0.026
				118	20	8	0.069
				120	21	4	0.034
				122	22	8	0.069
				124	23	9	0.078
				126	24	14	0.121
				128	25	14	0.121
				130	26	9	0.076
				132	27	11	0.095
				134	28	4	0.034
				158	40	1	0.009
				160	41	5	0.043
						$n = 116$	

CHAPTER 3
DEVELOPMENT OF MARKERS FOR MOLECULAR SEXING OF SPERM WHALES

Introduction

There are few direct methods for reliably determining the sex of individual sperm whales observed at sea. While fully mature males are easily identified by their massive size, age-related variation makes it difficult to discern the sex of most other individuals, since males do not become markedly bigger than the largest females until they are about twenty years of age (Lockyer 1981). The presence of a callus (a whitish patch of hard, thickened tissue) on the dorsal fin often indicates a mature female (Kasuya and Ohsumi 1966). However, calluses can be difficult to discern in the field (Arnbom and Whitehead 1989). In addition, about one-fourth of mature females do not have a callus, while about one-third of immature males do (Kasuya and Ohsumi 1966). Immature sperm whales display no secondary sexual characters that are readily apparent at sea. Observation of the genital region of individual whales requires suitable field conditions, intense effort and animals of approachable temperament. Finally, because sperm whale calves show regular associations with several different adults, both male and female (see Chapter 1), it is difficult to ascribe sex-related behaviours to specific individuals.

With the development of methods for collecting skin tissue samples from living whales (Chapter 4), it has become practical to determine sex by genetic analysis. Methods for molecular sexing of mammals exploit DNA sequences that are located on the sex chromosomes. These sequences include two genes on the Y chromosome that have been found to be highly conserved in many different mammals, namely *ZFY* (the zinc finger protein gene; Page *et al.* 1987) and *SRY* (the sex-determining region Y gene; Sinclair *et al.* 1990).

ZFY was initially considered a candidate for the gene that determines sex (Page *et al.* 1987), but later work has suggested otherwise (Sinclair *et al.* 1988; Koopman *et al.* 1989; Palmer *et al.* 1989). Nevertheless, the human *ZFY* clone has been successfully used as a probe to identify male-specific restriction fragments on Southern blots of genomic DNA from a variety of mammals, including belugas, *Delphinapterus leucas* (Brown *et al.* 1991), and humpback whales, *Megaptera novaeangliae* (Baker *et al.* 1991). *ZFY* has also been found to have a homologous gene on the X chromosome, termed *ZFX* (Schneider-Gädicke *et al.* 1989). Several workers have used PCR to investigate sex-specific differences resulting from sequence polymorphisms between *ZFY* and *ZFX* (Ebensperger *et al.* 1989; Aasen and Medrano 1990; Palsbøll *et al.* 1992; Pollevick *et al.* 1992). Palsbøll *et al.* (1992) designed PCR primers from the published human sequence to amplify a 1060 bp fragment from both *ZFY* and *ZFX* in four species of mysticetes (minke whale, *Balaenoptera acutorostrata*, blue whale, *B. musculus*, fin whale, *B. physalus*, and humpback whale, *M. novaeangliae*) and two species of dolphins (beluga, *D. leucas*, and harbour porpoise, *Phocoena phocoena*). The PCR products were digested with the restriction endonuclease *Taq*I, and all species, except the fin whale, displayed sex-specific restriction fragment length polymorphisms (RFLPs), because of an additional restriction site in *ZFX* not found in *ZFY* (Fig. 3.1). This sex-specific RFLP pattern appears identical in all cetaceans (except the fin whale), and is somewhat different than the pattern observed in humans. Results from an earlier study with ungulates show sex-specific RFLPs for three out of five species tested (Aasen and Medrano 1990).

The currently favoured candidate for the primary sex determining gene is *SRY*. It encodes a testis-specific transcript and does not have a homologous gene on the X chromosome (Berta *et al.* 1990; Gubbay *et al.* 1990; Sinclair *et al.* 1990; Koopman *et al.* 1991). Using the published human sequence, Palsbøll *et al.* (1992) designed PCR primers complementary to regions within a highly conserved domain of the *SRY* gene. A single, male-specific product was generated in PCR amplifications with these primers (Fig. 3.2),

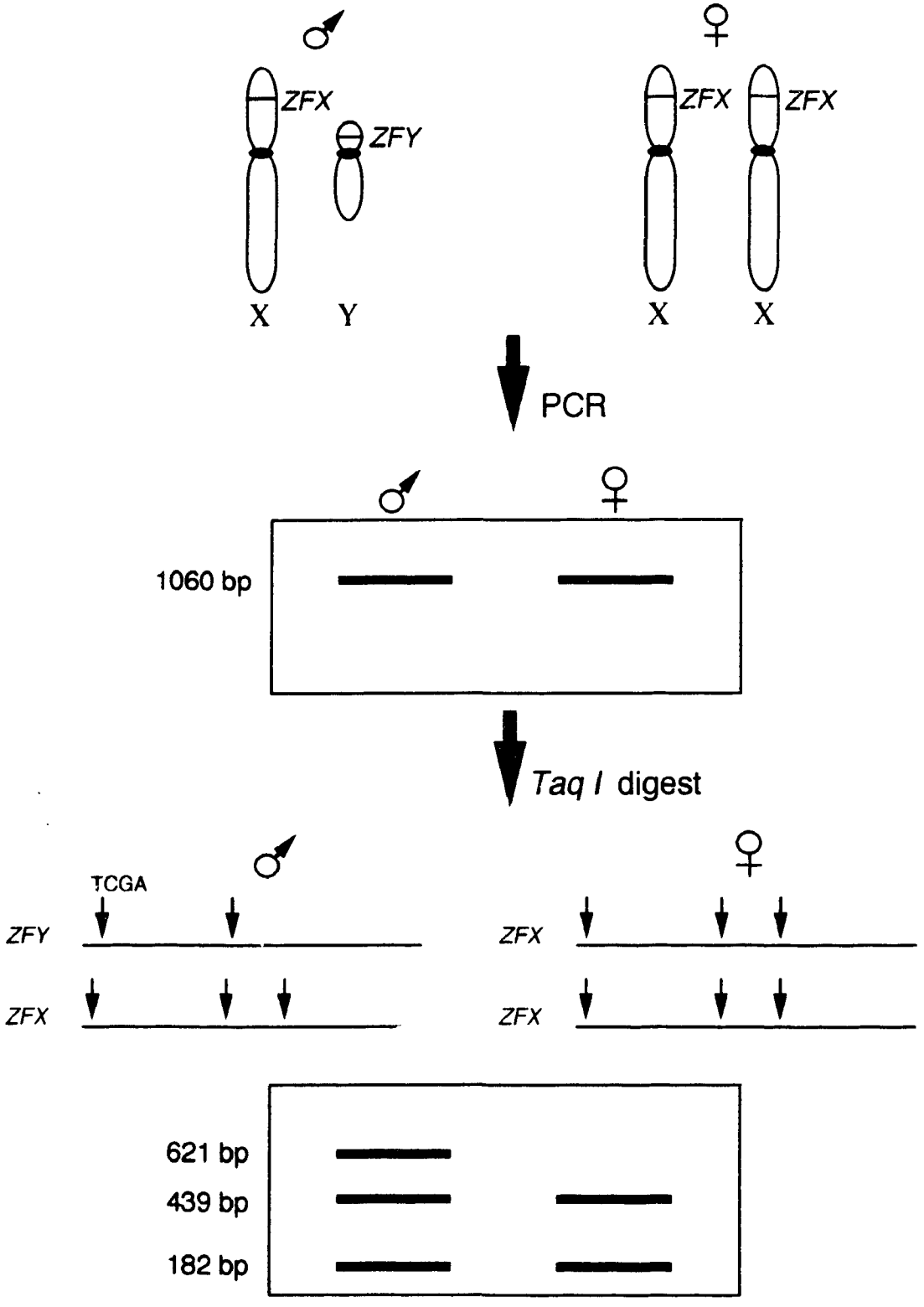

Fig. 3.1: Molecular sexing by PCR amplification of part of the *ZFY* and *ZFX* genes. Sex is determined from restriction polymorphisms between the sexes (based on Palsbøll *et al.* 1992).

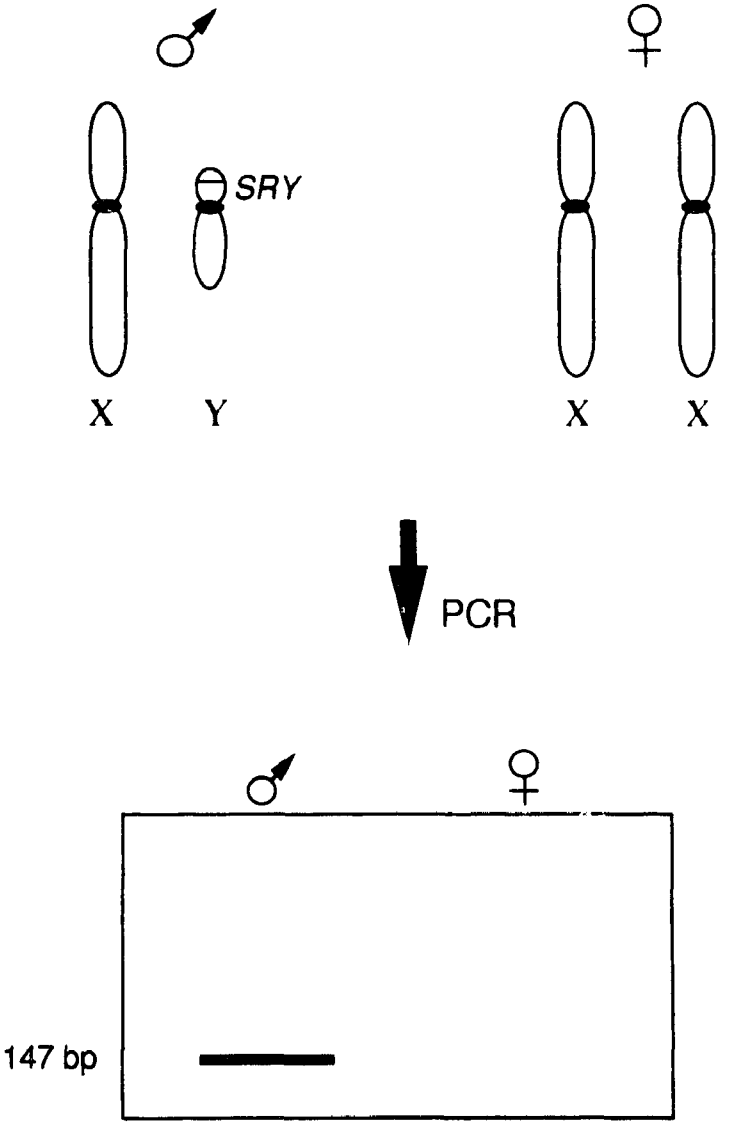

Fig. 3.2: Molecular sexing by PCR amplification of part of the *SRY* gene.

using DNA templates from human and all six cetacean species tested (the same species as listed above). Other workers have designed primers that have been shown to amplify *SRY* in an even wider range of mammalian species (Griffiths and Tiwari 1993). However, a general problem associated with the use of such 'universal' primers is the susceptibility of the assay to amplification of contaminating human DNA (Kwok and Higuchi 1989).

Prior to this work, McCarrey (1993) attempted to PCR-amplify male sperm whale DNA using the *SRY* primers from Palsbøll *et al.* (1992). He obtained very inconsistent results, but was able to clone and sequence a fragment of the *SRY* gene that had been amplified in a successful PCR reaction (Fig. 3.3).

In this Chapter, I describe a method for reliably sexing sperm whales by PCR amplification of part of the *SRY* gene. The problems of inconsistent PCR results and potential human contamination when using universal primers were obviated by using new primers based on the sequence obtained specifically from the sperm whale *SRY* gene. As an independent assay for determining sex, I also show that sex-specific RFLP patterns are obtained from fragments of *ZFY/ZFX* amplified from sperm whale DNA using universal primers designed by Palsbøll *et al.* (1992).

Materials and methods

i. Samples of known sex

Two samples of sloughed skin were collected from known mature male sperm whales in the Ecuador 1991 study (described in Chapter 4) and three more in similar studies near the Galapagos Islands. Donated samples were from four stranded male sperm whales and one stranded female sperm whale, as well as samples from 1-7 male specimens of pygmy sperm whale (*Kogia breviceps*), northern bottlenose whale (*Hyperoodon ampullatus*), long-finned pilot whale (*Globicephala melaena*), Atlantic white sided dolphin (*Lagenorhynchus acutus*), and fin whale (*Balaenoptera physalus*).

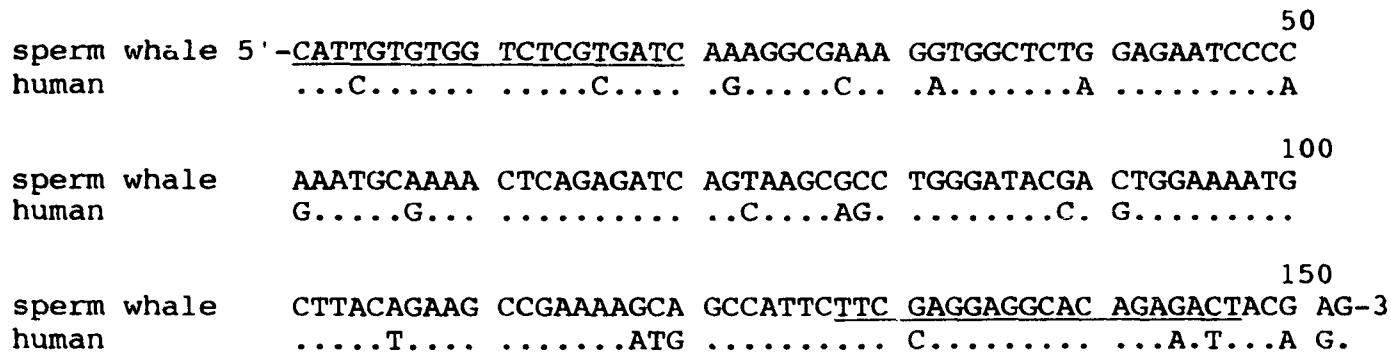

Fig. 3.3: Sequence of part of the *SRY* gene in sperm whales (based on McCarrey 1993) aligned against the homologous sequence in humans (based on Sinclair *et al.* 1990). Dots denote sequence identity. Underlined sequences indicate target sites for PCR primers.

ii. Species-specific primers for PCR amplification of SRY

I designed a new set of primers for PCR amplification of part of the *SRY* gene based on the sequence obtained directly from sperm whale, *i.e.* 5'-CATTGTGTGGTCTCGTGATC-3' and 5'-AGTCTCTGTGCCTCCTCGAA-3' (Fig. 3.3). Using these primers, PCR amplifications were performed with the samples from the sperm whale and the five other cetacean species, in a reaction volume of 25 µl, containing 19 µl of the Chelex/Instagene supernatant (refer to Chapter 2), 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 0.001% gelatin, 200 µM each dNTP, 0.2 µM each primer and 0.625 units of *Taq* polymerase, overlaid with 20 µl mineral oil. PCR reactions with human template used 10-75 ng of purified DNA. Amplification was carried out with 30 cycles denaturation at 96 °C for 1 minute, annealing at 58 °C for 1 minute, and extension at 72 °C for 5 seconds in a Perkin Cetus Elmer DNA Thermal Cycler. PCR products were size-fractionated in 13.5% polyacrylamide or 1.5% Synergel (Diversified Biotech) and visualized by ethidium bromide staining.

In some PCR reactions, the *SRY* fragment was simultaneously amplified with the X-linked microsatellite, SW15 (*i.e.* 'multiplex PCR'). Reactions included 0.1 µM of all primers, with one primer from each pair end-labelled with ^{35}S. Other reaction components and PCR conditions were as described for SW15 in Chapter 2. PCR products were resolved and scored on sequencing gels as described in Chapter 2.

iii. PCR amplification of ZFY/ZFX

DNA was amplified at the *ZFY/ZFX* loci in sperm whale samples with primers designed by Palsbøll *et al.* (1992), *i.e.* 5'-CATCCTTTGACTGTCTATCCTTG-3' and 5'-CATTATGTGCTGGTTCTTTTCTG-3', using PCR reagents identical to those described for the *SRY* primers. Amplification was carried out with 30 cycles at 96 °C for 1 minute, 60 °C for 1 minute and 72 °C for 1 minute. After amplification, PCR products

were digested with the restriction endonuclease *Taq*I (Pharmacia), then size-fractionated by gel electrophoresis in 1.5% Synergel and visualized by ethidium bromide staining.

Results and discussion

i. SRY assay

Using the primers designed from the *SRY* sequence of sperm whale (Fig. 3.3), the PCR amplification of sperm whale DNA from nine known males consistently produced good yields of the expected size fragment (147 bp), with no visible artifacts; the one known female sperm whale sample produced no PCR products (Fig. 3.4a). The new primers also performed well in multiplex PCR reactions (Fig. 3.5).

Using the same PCR conditions as for sperm whales, the same-sized fragment was amplified from samples of known male DNA from all other cetacean species tested (two toothed whales, two dolphins, and one baleen whale), although product yields were sometimes lower than in sperm whales (data not shown). DNA variation at the primer sites of *SRY* appears to be less between cetacean species than between the sperm whale and human, as no product was amplified from human male DNA in seven separate trials using the same primers and identical PCR conditions as for successful amplification of *SRY* from cetacean DNA (Fig. 3.4a). The sperm whale oligonucleotide primers differ from the human template sequence at a total of five nucleotide positions (Fig. 3.3). This seems sufficient to prevent amplification of the human *SRY* gene by these primers, at least under conditions that will amplify *SRY* from cetacean DNA. The use of these cetacean-specific primers thus eliminated the possibility of incorrect sexing owing to human contamination during the collection and preparation of samples.

ii. ZFY/ZFX assay

Amplification of the *ZFY/ZFX* locus from sperm whale DNA, using the universal primers, generated a product of approximately the expected size, 1060 bp (Fig. 3.4b).

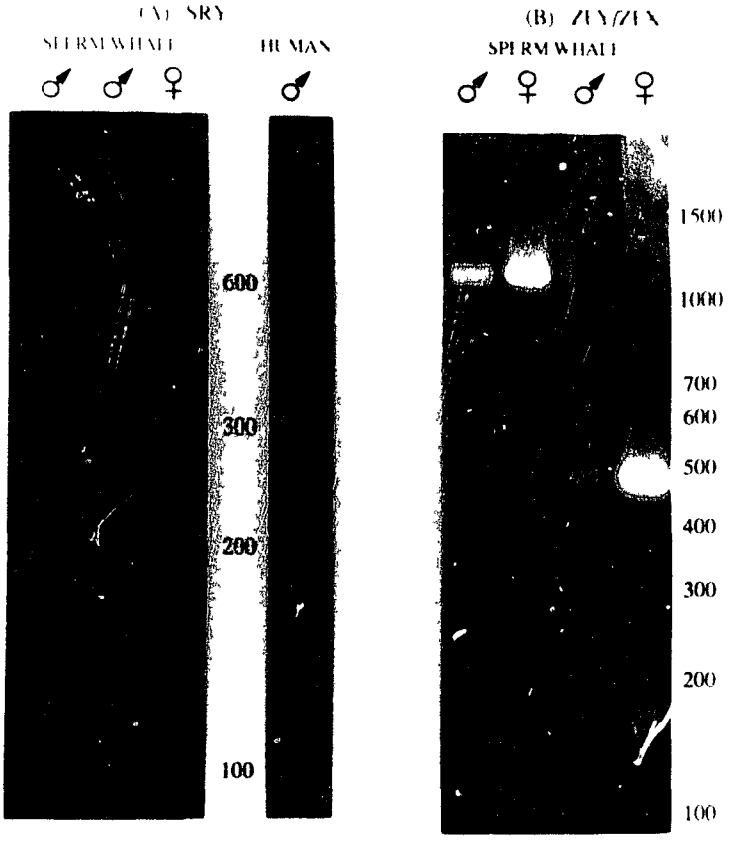

Fig. 3.4: Male-specific fragments amplified by PCR. (A) PCR amplification of the *SRY* fragment using primers based on sperm whale *SRY* sequence (Fig. 3.3). The 147 bp fragment was amplified from male sperm whale DNA, but not from female. No products were amplified from human male DNA under the same conditions employed with sperm whale samples. PCR products were size-fractionated by gel electrophoresis in 13.5% polyacrylamide. (B) In the two leftmost lanes, PCR amplification of the *ZFY/ZFX* fragments, using primers based on the human sequence, generated similar-sized products in male and female sperm whales. Digestion of these fragments with the restriction endonuclease *Taq*I produced a sex-specific pattern, *i.e.* male DNA displayed a unique fragment of approximately 650 bp. The small fragment (<200 bp) was much fainter in males, and in this figure is no longer visible after reproduction. PCR and digestion products were size-fractionated by gel electrophoresis in 1.5% Synergel. Numbers indicate size of DNA in base pairs, estimated from the 100 bp DNA ladder (Gibco BRL).

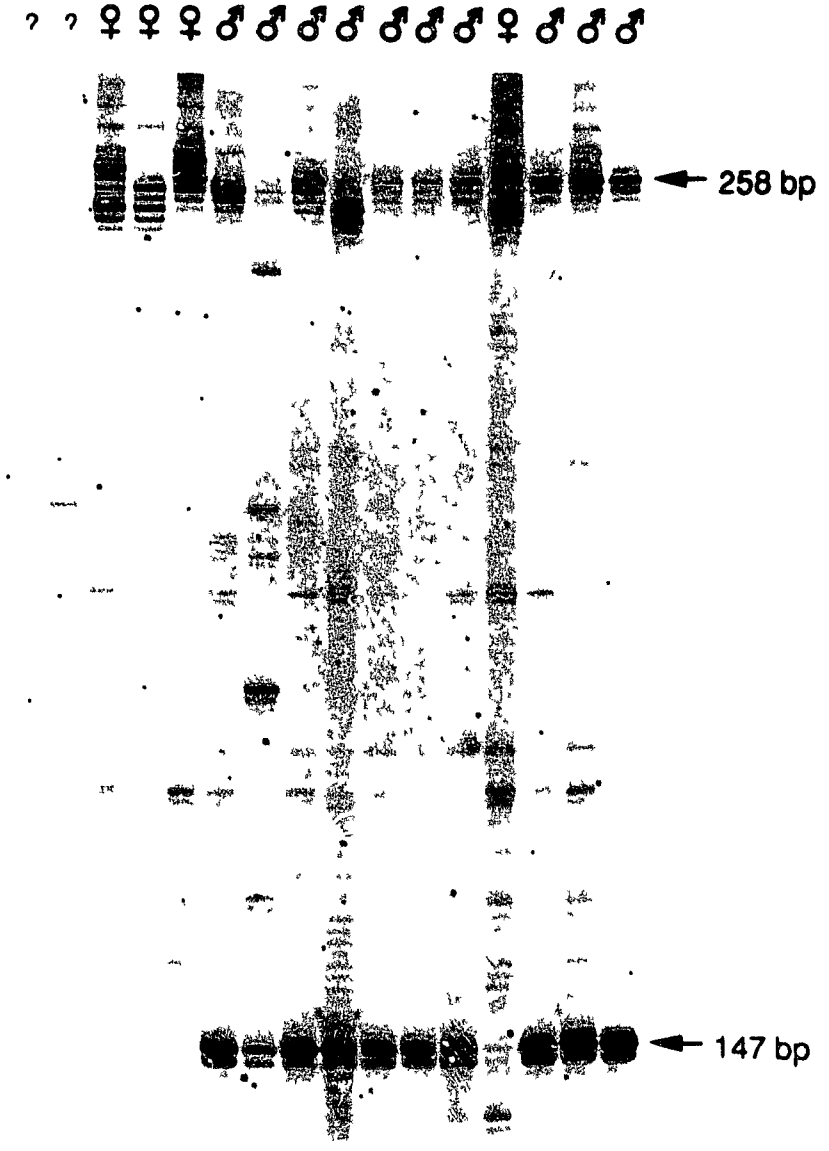

Fig. 3.5: Multiplex PCR-amplification products for the *SRY* gene and the X-linked, microsatellite locus SW15. DNA was amplified directly from sloughed skin samples prepared with Chelex resin. Primer sites are shown in Figs. 3.3 and 2.2, respectively. The 147 bp band is the male specific *SRY* fragment. PCR-amplified alleles at SW15 ranged in size from 252 - 262 bp; females were frequently heterozygous, but males always displayed only 1 allele. "?" indicates a failed PCR reaction.

Restriction endonuclease digestion of the amplified *ZFY/ZFX* products with *Taq*I produced a sex-specific pattern similar to that reported by Palsbøll *et al.* (1992) for other cetaceans. The diagnostic difference between the sexes was a fragment of approximately 650 bp that occurred only in the restriction pattern of males (Fig. 3.4b). Palsbøll *et al.* (1992) have demonstrated in other cetaceans that this male-specific fragment results from a missing restriction site in *ZFY* compared with *ZFX*. The smaller fragment (< 200 bp) was often not evident in males (Fig. 3.4b), but it is not actually female-specific. Rather, it results from an additional restriction site in *ZFX* not found in *ZFY*, and thus appears fainter in males, since males have only one copy for every two in females.

iii. Reliability of assay results

The use of species-specific primers for the *SRY* analysis increased the reliability of the PCR assay, presumably because of perfect complementarity between the primers and template. The PCR-amplified fragment was expected to be male-specific in sperm whales because of the conservation of *SRY* on the Y chromosome across a very broad range of mammals (both placental and marsupial; Sinclair *et al.* 1990; Foster *et al.* 1992) and because of the primary role of this gene in mammalian sex determination (Koopman *et al.* 1991). As well, the *SRY* fragment appears to be male-specific in all other tested cetaceans, namely belugas, harbour porpoise, and fin, blue, minke, and humpback whales (Palsbøll *et al.* 1992). The reliability of the sex-specific *ZFY/ZFX* digestion patterns was supported by the consistency of similar patterns in other cetacean species (Palsbøll *et al.* 1992). Results with sperm whale samples of known sex were consistent with sex-specific patterns for both markers, although it was only possible to test one known female. However, the results of the *SRY* and *ZFY/ZFX* assays were in complete agreement when several samples of unknown sex were scored at both markers (data not shown).

In general, it is valuable to score samples using both sex markers. Mutual verification by the two markers can detect occurrences of technical failure in the analyses.

For instance, a null result obtained from PCR amplification with *SRY* primers may suggest either that the sample is female DNA or that the sample is male DNA that failed to be amplified by the PCR reaction (for several possible artifactual reasons). For my analyses, I found that a more efficient approach was to simultaneously amplify the *SRY* fragment and the X-linked, microsatellite marker, SW15 (Fig. 3.5). A heterozygous genotype at SW15 would confirm that the individual was female. A null result at *SRY* in combination with a homozygous genotype at SW15 would suggest that the individual was female, rather than that PCR amplification failed to work, particularly if this result was obtained in repeated trials. However, a null result for the SW15 amplification would suggest that a null result at *SRY* may likely have resulted from a failed reaction.

Summary

Two techniques for molecular sexing were refined for use in sperm whales. A fragment of the male *SRY* gene was PCR-amplified using primers designed from sperm whale DNA. This cetacean-specific assay was efficient and reliable, and eliminated the risk of human contamination in the PCR reaction. The results of the *SRY* assay were confirmed by multiplexing with an X-linked microsatellite locus or by analysis of the *ZFY/ZFX* genes using methods previously reported to work for other cetacean species.

CHAPTER 4
ANALYSIS OF KINSHIP IN SPERM WHALES

Introduction

The deep ocean habitat of sperm whales has not made them very amenable to detailed and long-term study. Indeed, much of our present understanding of social organisation is derived from anecdotal and qualitative clues suggested by observations and materials collected during whaling operations (Caldwell *et al.* 1966; Ohsumi 1971; Best 1979; Best *et al.* 1984). Over the last decade, however, methods that are nonlethal and relatively non-intrusive have been developed to study free-living groups of sperm whales for extended periods of time, allowing systematic observations to be made (Whitehead and Gordon 1986). Over 90% of individual sperm whales can be distinguished from good quality photographs of scar patterns on the tailflukes (Arnbom 1987), and this has enabled longitudinal studies of interacting individuals, as described in Chapter 1. Many of these methods were successfully employed in this thesis to study free-living groups of whales in the eastern tropical Pacific. In order to examine kinship within these groups, it was necessary to also develop new methods for the non-invasive collection of genetic samples.

The most common technique for collecting tissue samples from cetaceans involves using a darting device to obtain small biopsies from individual whales (IWC 1991a). However, current dart designs have proven ineffective for collecting good-sized samples of tissue from sperm whales (Whitehead *et al.* 1990). Biopsy darting is very successful for many species of baleen whale (IWC 1991b). These whales are generally much more solitary and less skittish than sperm whales and it is routinely possible to approach single, identifiable individuals. Directed sampling of individual sperm whales is hampered by the inability of observers to distinguish between and keep track of different individuals while at sea. In addition, sperm whales are easily perturbed and always display signs of disturbance during biopsy trials (Whitehead *et al.* 1990). When disturbed, a sperm whale

will usually submerge quickly beneath the water surface without raising its tail, and thus cannot be photographically-identified.

Although fresh biopsies are essential for certain genetic analyses (*e.g.* cell culturing, chromosome studies), other samples of lower quality can still prove highly informative, particularly when using PCR-based technology. In this regard, many cetacean species naturally slough visible pieces of skin (Amos *et al.* 1992). For the analysis presented in this chapter, field techniques were first developed to systematically collect large numbers of skin samples sloughed by individuals within social groups. Kinship within groups was then tested by characterising genetic variation amongst these samples using the molecular tools developed in the previous two chapters.

Materials and methods

i. Field methods

(a) Tracking and observing free-living groups of sperm whales

Research was conducted from the 13 m auxiliary cutter, *Balaena*, which remained at sea for about two weeks at a time. Data were collected between January 30 - March 12, 1991, in an area a few hundred kilometres off the coast of mainland Ecuador (approximately 1° N - 3° S, 81° W - 82° W). Groups of sperm whales were first located by passive acoustic tracking using a directional hydrophone (Whitehead and Gordon 1986). Whales were followed closely for up to several days at a time, until either acoustic contact was accidently broken during the night or until it was necessary to return to shore for supplies. In some instances, it was not possible to deliberately track a single group for extended periods because of the formation and subsequent breakup of temporary aggregations of different groups.

Sperm whales display two broadly defined modes of behaviour: foraging and socializing (Whitehead and Weilgart 1991). When foraging, group members can be spread out in a rank which may be over a kilometre wide. Individuals typically dive for a period

of ~40 minutes, followed by a ~10 minute period resting at the surface (Papastavrou *et al.* 1989). However, sperm whales are extremely gregarious, and at regular intervals (often in the afternoons) most members of a group will socialize together in clusters at the water surface. During several of these periods, I made written notes of behavioural interactions and cluster sizes that aided in delineation of groups for the genetic analysis.

(b) Photographic data

During daylight hours, individual whales or clusters of whales were discretely approached from behind and followed for several minutes, until they eventually submerged. By positioning the boat 50-100 m behind a whale swimming at the water surface, it was possible to photographically-identify the whale as it raised its tail to commence a feeding dive. During foraging periods, the number of identification photographs were maximized by trying to systematically move between whales at the surface. During socializing periods, individuals were generally not photographically-identified, since the whales seldom lifted their tails at these times.

The body lengths of different whales could be estimated from side photographs of the group taken from the crow's nest (Gordon 1990). Length measurements were usually only of unidentified individuals, again because most whales cannot be distinguished individually by an observer in real time.

(c) Collection of genetic samples

From the deck at the bow of the boat, members of the crew constantly searched for pieces of sloughed skin floating in the wake of a whale or group of whales swimming at the water surface. As well, several samples were invariably found near sites of active surface behaviours. These behaviours included physical interactions amongst individuals, breaches (leaping from the water) and lob-tailing (thrashing the tail-flukes onto the water surface). During surface activity, one can often directly see pieces of skin falling off the

body of a whale (pers. obs.). Samples of skin were scooped out of the water, either with a small net handled by a snorkler or with a net on a pole handled from the deck of the boat.

Tissue samples were manipulated with tweezers or gloved hands, rinsed with distilled water and then placed in a sample bottle containing a pickling solution of 20% dimethlysulfoxide (DMSO) saturated with NaCl (Amos and Hoelzel 1991). Each piece of skin was stored in a separate sample bottle unless there was no doubt that different pieces came from the same whale. Sample bottles were labelled on the inside and outside of the container, including skin sample number, photograph number (*i.e.* any identification photographs of whales from which the skin was obtained), date, time, and location. Upon return to the laboratory, samples were stored at -20 °C. Thus, some samples were left at ambient temperature (~15-32 °C) for several weeks.

ii. Delineation of social groups

Social groups for genetic analysis were delineated by comparison of skin sample collection records to written field observations and photographic identification records. This comparison identified days on which most, if not all, of the samples collected were from individuals belonging to a single group.

iii. Laboratory methods

DNA was obtained from sloughed skin either by organic extraction (Appendix A) or more often by boiling in a mix of Chelex resin (Chapter 2). Most samples were collected from unidentified individuals, and many were expected to be duplicates, collected from the same individual on different occasions. Thus, it was necessary to develop a suite of markers that could genetically-identify individuals. Kinship assessment also required highly polymorphic markers for comparison of genetic similarity between individuals. For these purposes, a multilocus profile was constructed for each sample by scoring allelic patterns at 4-5 different microsatellite markers (Chapter 2). Sex was determined for most

samples by multiplex PCR of the *SRY* fragment and the X-linked microsatellite, SW15, but sometimes also by the *ZFY/ZFX* assay (Chapter 3).

iv. Statistical computations

Computer programs for the various analyses of allele sharing described below were written in Microsoft QuickBASIC, version 4.0, using an IBM-compatible 386dx PC. *G*, *t*, and χ^2 statistics were calculated with the spreadsheet program, Quattro Pro, version 4.0. Three of the genetic tests (specifically indicated below) were performed with the computer program, GENEPOP, version 1.2 (Raymond and Rousset 1994).

v. Genetic identification of individuals

Samples with completely identical multilocus microsatellite profiles were assumed to be duplicates, collected from the same whale. To test this assumption, the similarity of microsatellite genotypes was determined for all possible pairwise comparisons of the unique multilocus profiles. The rationale for this comparison was that if it was true that two different whales never matched at five out of five markers, then it would also be expected that very few pairs of individuals would match at four out of five markers. More pairs (but still a small number) would be expected to be identical at three out of five markers, and so on. These comparisons were also made just among a subset of samples known *a priori* to be different (since they had been collected from photographically-identified whales).

The probability of randomly sampling two sperm whales that each display the most common multilocus genotype was estimated from the allele frequencies observed in the data set (Risch and Devlin 1992). This calculation assumed random associations of alleles within and among loci.

vi. Microsatellite allele frequencies

(a) Associations of alleles within loci

Genotype frequencies were tested for deviations from Hardy-Weinberg Equilibrium (HWE) using two different methods. The first method employed goodness-of-fit χ^2 tests of the total number of observed homozygotes (Lessios 1992). The second method calculated unbiased estimates of probability values for exact tests of Hardy-Weinberg proportions with multiple alleles (performed with GENEPOP, which is based on the Markov chain method algorithm of Guo and Thompson (1992)).

(b) Associations of alleles between loci

For each pair of loci, statistical independence between loci was tested using a Markov chain method to calculate unbiased estimates of the exact probability values for the observed combinations of alleles. All calculations were performed with GENEPOP, which employs the composite genotypic disequilibrium coefficients described in Weir (1990).

(c) Allele frequencies within groups

Allele frequency distributions at each locus within each group were inspected for evidence of enrichment, *i.e.* to determine if any alleles occurred in unusually high frequency within one group compared to the other groups (*cf.* Amos 1993).

Allelic composition was statistically compared between groups for each locus using GENEPOP. The program employs a Markov chain method to calculate unbiased estimates of the exact probabilities for Fisher's exact test on R x C contingency tables (Sokal and Rohlf 1981), where rows included the three groups, A, B and C, and columns the frequency of each allele. Probability values from the tests of each locus were combined (Sokal and Rohlf 1981) to obtain an overall significance value for the null hypothesis that there is no difference in allelic composition between groups. Estimates of Fisher's exact

probabilities were also calculated separately for each pair of groups for each locus (*i.e.* the rows in the contingency tables now included only two groups).

vii. Measures of microsatellite allele sharing

The microsatellite allele data were compared in a variety of different ways to measure genetic similarity of individuals within groups and between groups:

(a) Similarity of multilocus profiles

Unique multilocus profiles were compared for all possible pairwise comparisons of individuals within groups and for all possible pairwise comparisons of individuals between groups. Overall similarity (s) of the multilocus profiles was calculated as:

$$s = \frac{\text{total \# alleles shared by all individuals}}{\text{total \# alleles compared between all individuals}} \times 100$$

The statistical significance of s was tested using Monte Carlo simulations. In the Monte Carlo procedure, alleles were randomly shuffled and reassigned to different individuals, while still retaining the same structure in the data set (*i.e.* the same number of groups, the same number of individuals per group, and the same number of gaps in the multilocus profiles as in the observed data). The data were permutated in this way 100-200 times, and the value of s in the observed data compared to the value of s in each permutation of the data to determine if observed within and between group values deviated from random expectations. The ratio of within-group s to between-group s was similarly tested.

(b) Frequencies of allele sharing values

To obtain a finer resolution of allele sharing within groups, the number of shared alleles was compiled for every pairwise comparison amongst all individuals. The

frequency of each allele sharing value (*i.e.* 0/10, 1/10, 2/10...10/10 shared alleles) was determined for pairs within groups and for pairs between groups. Differences between frequency distributions were detected using the *G*-test statistic.

(c) Potential parent-offspring relationships

The parent-offspring relationship is the only genetic relationship for which specific pairs can be directly excluded (since, barring mutation, the two whales must share at least one allele at every single marker, unlike, for example, full sib relationships in which it is possible to share as many as all alleles or as few as none). The proportion of all possible pairwise comparisons that could be excluded from being potential parent-offspring pairs was determined within and between groups. Observed values were tested for deviations from random expectations using Monte Carlo simulations.

viii. Simulations of groups with defined kinship structure

Simulated groups of defined kinship structure were created for comparison to the observed groups. Simulated multilocus profiles were created with probabilities determined by the allele frequencies in the observed data set. To approximate sampling of the observed groups, the simulated structures were modelled to arrive at a total group size of 24, but then 5 whales were randomly removed (since some members of the observed groups were not sampled genetically, *cf.* Table 4.1). Fifty groups of each structure were constructed, as follows:

(a) Random groups: Multilocus profiles were generated from random expectations based on allele frequencies sampled off Ecuador. Females were generated with a probability of 0.78 to match the observed sex ratio (Appendix E).

(b) unrelated adults with offspring: As a simple model of nursery groups formed by genetically unrelated females, the simulated groups contained eight randomly constructed female profiles. Two offspring were created for each female, assuming an equal likelihood

that a calf would be male or female. The two offspring were half-sibs, *i.e.* each had a different father.

(c) single matrilines: Matrilines were modelled using population parameter estimates from the International Whaling Commission (1982), including pregnancy rate (0.2 yr^{-1}), age of female maturity (10 yrs), age of male dispersal (8 yrs), mortality of calves (0.093 yr^{-1}), and the mortality of males and females older than one year (0.066 yr^{-1} and 0.055 yr^{-1}, respectively). Profiles for founder females and all fathers were randomly generated. It was assumed that only male offspring dispersed from the groups and that there were no within-group paternal siblings (since most groups contain only 0-2 calves in any year class and breeding males spend only hours with any given group (Whitehead and Kahn 1992; Whitehead 1993)).

(d) two matrilines per group: Two separately created matrilines, each with 12 individuals, were combined to form a single group. This simulation approximated the structure modelled by Whitehead *et al.* (1991).

(e) half-sibs only: Groups contained only half-sibs (coefficient of relatedness = 0.25). This structure is unrealistic for sperm whales, but was created as a reference for allele sharing amongst individuals with relatively high genetic relatedness.

ix. Coefficient of relatedness

Data from single locus markers can be used to obtain actual statistical estimates of relatedness, *r* (Pamilo 1989). I estimated *r* using the formula of Queller and Goodnight (1989):

$$r = \frac{\Sigma\Sigma\Sigma\Sigma\,(p_{i(-j)m} - p_{(-i)m})}{\Sigma\Sigma\Sigma\Sigma\,(p_{ijm} - p_{(-i)m})}$$

where $p_{(-i)m}$ is the frequency of an allele *m* in the population not including group *i*, p_{ijm} is the frequency of an allele *m* in individual *j* in group *i*, and $p_{i(-j)m}$ is the frequency of an

allele m in group i excluding individual j. Summations are across allelic positions, loci, individuals and groups. The estimates of population frequencies were based on all unique multilocus profiles ($n = 80$) in the Ecuador data.

Tukey's Jackknife method was used to calculate the jackknifed estimate of r with confidence intervals (Sokal and Rohlf 1981), by jackknifing over loci as recommended by Queller and Goodnight (1989) for a small number of groups.

I also simulated groups containing precisely known genetic relationships in order to compare estimated r to true r. These simulations consisted of 1565 groups, each containing a single matriline with 19 individuals. Other simulations included 1000 groups of randomly generated individuals (assumed $r = 0$). The Queller-Goodnight r value was estimated for each simulated group, using the Ecuador samples ($n = 80$) for population frequencies.

x. Age of male dispersal

Age of male dispersal was estimated from sex ratios using a model developed by Hal Whitehead (pers. comm.). The model is age structured and assumes an equilibrium population with equal numbers of each sex at age 1. Assuming a constant mortality rate and no dispersal of females (*i.e.* an infinite geometric series), the total number of females is estimated as $1/\beta$, where β is the annual mortality of females older than 1 year. Similarly, assuming a finite geometric series, the total number of males is estimated as $(1 - (1 - \alpha)^d) / \alpha$, where α is the annual mortality of males older than 1 year and d is the age of male dispersal. If the ratio of males to females is $1:f$, then:

$$\frac{1}{f} = \frac{(1 - (1 - \alpha)^d) / \alpha}{1/\beta}$$

Thus: $$d = \frac{\text{Log}(1 - \alpha / (\beta f))}{\text{Log}(1 - \alpha)}$$

Estimated mortality rates ($\alpha = 0.066$ and $\beta = 0.055$) were from the International Whaling Commission (1982). Confidence intervals were calculated from binomial theory. For these calculations:

$g = \frac{f}{1 + f}$, i.e. the proportion of females in the population

$\text{Var}(g) \approx \frac{g(1 - g)}{n}$, where n is the total sample size

$$95\%\ \text{c.i.}\ (g) \approx g \pm 2\sqrt{\frac{g(1 - g)}{n}}$$

$$= \frac{f \pm 2\sqrt{f / n}}{1 + f}$$

$$\text{So, 95\% c.i. } (d) = \frac{\text{Log}\left(1 + \frac{\alpha(-1 \pm 2\sqrt{f / n})}{\beta(f \pm 2\sqrt{f / n})}\right)}{\text{Log}(1 - \alpha)}$$

The age of male dispersal was also estimated by comparison of the relative proportion of males to females in the groups to the theoretical age composition of sperm whales published by Best (1979). This estimate assumes that most females remain in their natal groups and that male dispersal is sharp, rather than gradual, or at least occurs at a constant rate.

Results

i. Sample collection

During the 19 contact days with whales, a total of 331 sloughed skin samples were collected, without apparent disturbance to the whales, nor disruption of the daily socializations that go on within the groups (Fig. 4.1). Of these, 55 were collected from different photographically-identified individuals. Most samples, therefore, were linked to a particular group of whales rather than to single individuals. No samples were known to be collected from first-year calves.

Although it was often not possible to photograph a whale at the time a sample was collected, a sample could be attributed to an identified whale if that individual was the only whale near the collection site, since sloughed skin sinks quite quickly (sinking rate estimated in a bucket as ~0.5 - 1 cm/sec). There were 11 instances in which samples were thought to be collected from the same whale on more than one occasion. In all cases, the putative duplicate samples displayed identical microsatellite multilocus profiles. This confirmed that sloughed skin samples can be reliably linked to individual whales under suitable collection circumstances. Because the skin samples did not float for very long, potential collection sites had to be approached rapidly in order to detect and collect the pieces before they disappeared. Success at collecting sloughed skin samples also depended on external factors such as water clarity, sea state, time of day, and approachability of the whales. In general, more samples were collected by having two people each handle nets from the deck of the boat, rather than by having a snorkler jump into the water.

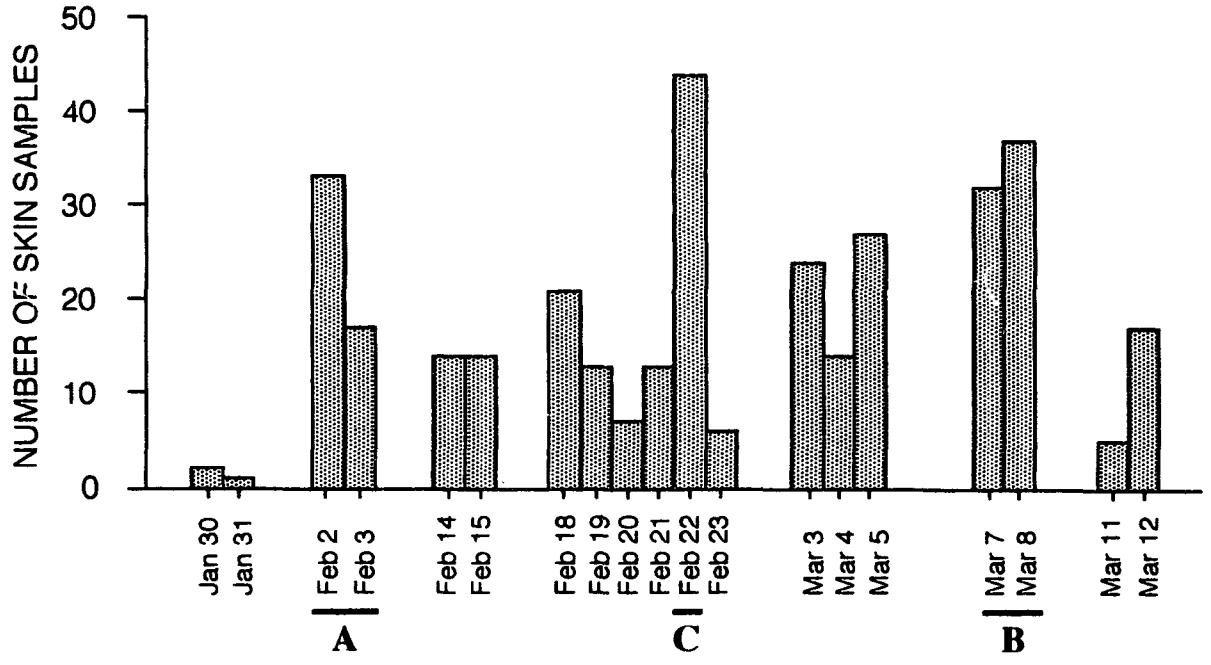

Fig. 4.1: Number of sloughed skin samples collected during each contact day with sperm whales over the course of the field study (January-March 1991). The days on which samples were collected from each group, A, B and C, are also indicated.

ii. Social groups

(a) Delineation of groups

Based on field observations and photographic records, I identified three distinct social groups from which several genetic samples had been collected (Table 4.1). Groups A and B were each followed for two days, during which time they did not aggregate with other groups. Group A had split off from a large aggregation (~50 whales) about two hours before the first samples were collected. No aggregations were observed immediately prior to or after the observations of group B. Photographic records indicated that the same individuals were repeatedly seen throughout the two day periods when the groups were followed; *i.e.* only 24 different individuals were photographed in group A from a total of 87 photographs, and only 24 different individuals in group B from a total of 113 photographs. During the afternoon socialization periods, the surface clusters contained 20-24 whales, consistent with photographic records. Group A contained a small calf and it was observed to move between different adults within the group. Group B did not contain a small calf but did display particularly tight social cohesion during an encounter with killer whales (*Orcinus orca*). (During this encounter, the sperm whales formed a close rank and actively chased the killer whales away.) Group C was defined differently than groups A and B; it included only samples that were collected very close together in time from a highly interactive group of whales. The group contained 2-3 dozen whales (including one small calf) that were all in intimate contact during an intense and exceptionally animated social interaction at the water surface. The interaction ended after 40 minutes with the whales submerging beneath the water surface. Twenty minutes later, a smaller number of whales (~12 whales including the calf) began a second interaction that lasted about 20 minutes. Samples were collected during both interactions. None of the group members were photographically identified, but photographic records indicated that several different groups had been followed before and after this interaction. Thus, social bonds amongst whales in group C were less clear than in the other groups.

Table 4.1: Characteristics of group samples.

Group	No. of samples assayed	No. of genetically-identified individuals*	Sex ratio (Female:Male)
A	47	18 (17)	2:1
B	57	20 (18)	4:1
C	33	18 (15)	8:1

*Based on multilocus profiles of 4-5 microsatellite loci (numbers in parentheses indicate profiles scored at all 5 markers).

An additional 24 samples were assayed from different photographically-identified individuals that did not belong to groups A, B, or C . The social relationships amongst these whales were unknown, but some individuals were probably members of the same groups. These whales were included as additional samples from the population for some of the analyses described below.

(b) Group structure

Measurement photographs taken of groups A and B showed a broad range of body sizes (7-12 m), and molecular sexing revealed that most individuals were female (Table 4.1). These results were consistent with a group structure composed of several adult females and immature whales of both sexes. All males in groups A, B and C must have been juvenile because none of the observed whales were of the distinctively large size (14-18 m) of mature males.

iii. Construction of genetic profiles

Allelic size variation at microsatellites was scored by PCR analysis. In total, multilocus profiles were constructed for 164 samples, mostly from the three groups A, B and C, but also from the 24 other whales linked to distinct identification photographs. Together, these samples displayed 80 unique multilocus profiles, representing 63 females and 17 males (Appendix E).

iv. Genetic identification of individuals

Because most samples were collected from unidentified individuals, it was likely that several were collected from the same individual on different occasions. To assess how well the suite of microsatellite markers could distinguish between individuals, I compared the unique multilocus profiles for all possible pairwise comparisons (Fig. 4.2). For each comparison, I determined how many markers had identical genotypes in both individuals.

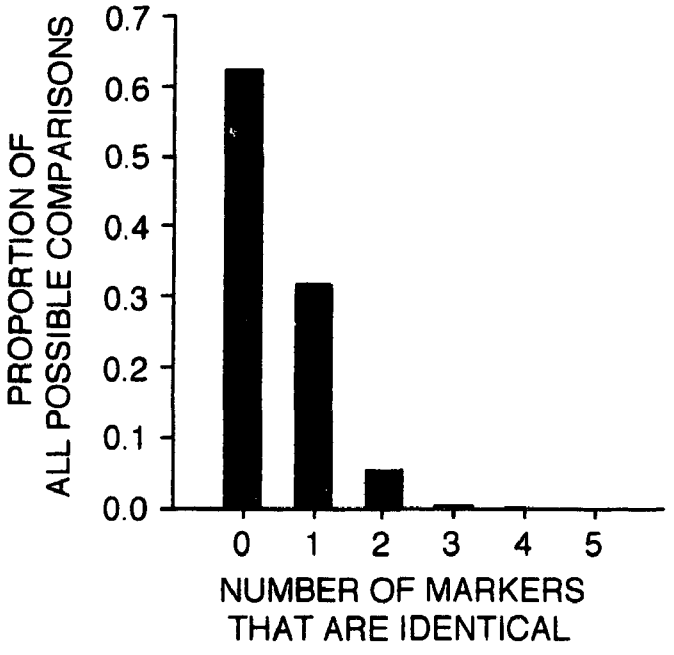

Fig. 4.2: Number of markers in multilocus profile that are identical between 2 whales for all possible pairwise comparisons of the 80 unique profiles.

Among 47 samples assayed from known different individuals (based on photographic identifications), no two multilocus profiles were identical at more than three markers. Of the 3160 possible pairwise comparisons among all 80 distinct profiles, 99.5% matched at only two or fewer loci, and 0.35% matched at three. Only one pair was identical at four out of five markers among the 58 unique profiles scored at all five microsatellites. Because so few pairs were identical at three and four markers, I concluded that samples with completely identical profiles were probably collected from the same individual. Thus, duplicates were removed from the analysis of kinship. False exclusion of samples would have made the analysis more conservative by reducing observed similarity within groups, since all duplicates were collected from within the same group.

Based on the allele frequencies amongst the 80 individuals from Ecuador, the probability of sampling two unrelated whales that both have the most common multilocus genotype for all five markers was estimated to be 1×10^{-9} (*i.e.* the most common genotype occurs with a frequency of about 1 in 31600). For samples scored at all microsatellites except SW19, the estimated probability of sampling two identical whales increased to about 1×10^{-6} (*i.e.* the most common multilocus genotype occurs with a frequency of about 1 in 920). These estimates should be accepted with reservation, as population frequencies were based on a small sample set that was not collected randomly (although there was little evidence for associations among alleles within or between loci, as described in the following section). Nonetheless, the estimates do suggest that it is highly improbable that two unrelated whales in the sample would display identical multilocus genotypes.

v. Microsatellite allele frequencies

(a) Associations of alleles within loci

Deviations from HWE were not detected with χ^2 tests of the number of homozygotes, nor with exact tests for Hardy-Weinberg proportions. This was the case

when all 80 samples were treated as one population or when groups were examined individually. Group A tended to show an excess of homozygotes at SW2 based on the Fisher exact test, but the test was marginally non-significant after using the Bonferroni procedure (*cf.* Weir 1990) to adjust for 5 multiple comparisons (estimated exact probability = 0.0157). The χ^2 test for SW2 within group A also had the lowest probability value of any of the comparisons ($\chi^2 = 3.78$ using a correction factor of 0.25 for continuity, $p \approx 0.05$).

(b) Associations of alleles between loci

Estimates of probability values for exact tests of genotypic disequilibria were non-significant for comparisons among the 80 whales ($p > 0.1$ for each pair of loci). This result was consistent with the assumption of unlinked markers.

(c) Allele frequencies within groups

Comparative visual inspection of group allele frequencies suggested that at most loci some alleles may have become enriched in different groups, as evidenced by peaks in the frequency distribution (Fig. 4.3, *e.g.* allele number 132 at SW19 in group A).

Results of Fisher's exact test for independence of group and allelic composition suggested that there was genetic differentiation of the three groups, A, B, and C (Table 4.2; combined test results for the 5 markers: $\chi^2 = 44.3$, 10 df; $p < 0.0001$). The most striking difference was at SW15, where the composition of alleles in group A was clearly different from that in the other groups (Fig. 4.3; Table 4.2). SW15 is X-linked and Group A contains a relatively high proportion of males, but no sex-related patterns could be discerned to explain the unique distribution.

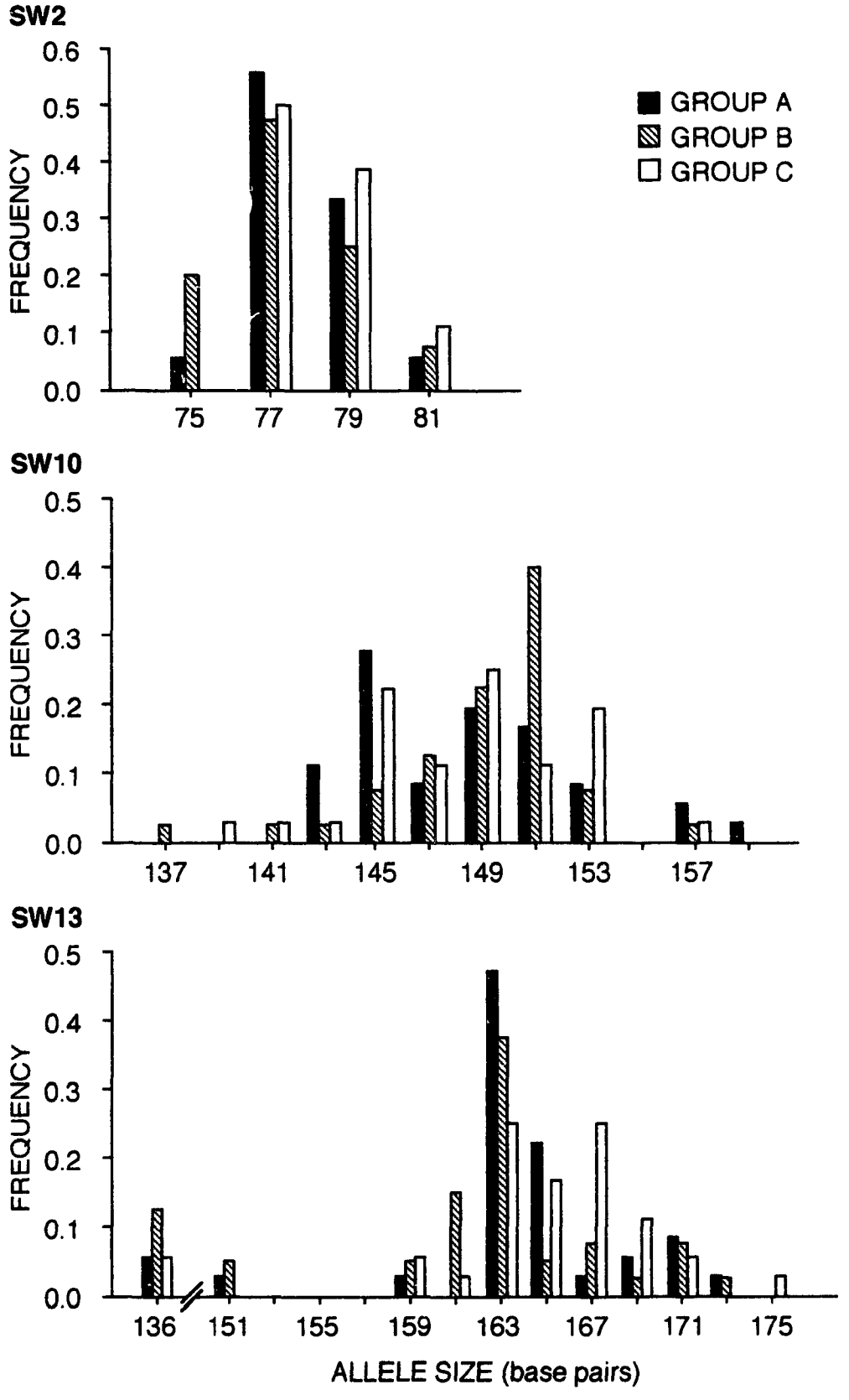

Fig. 4.3: continued on next page.

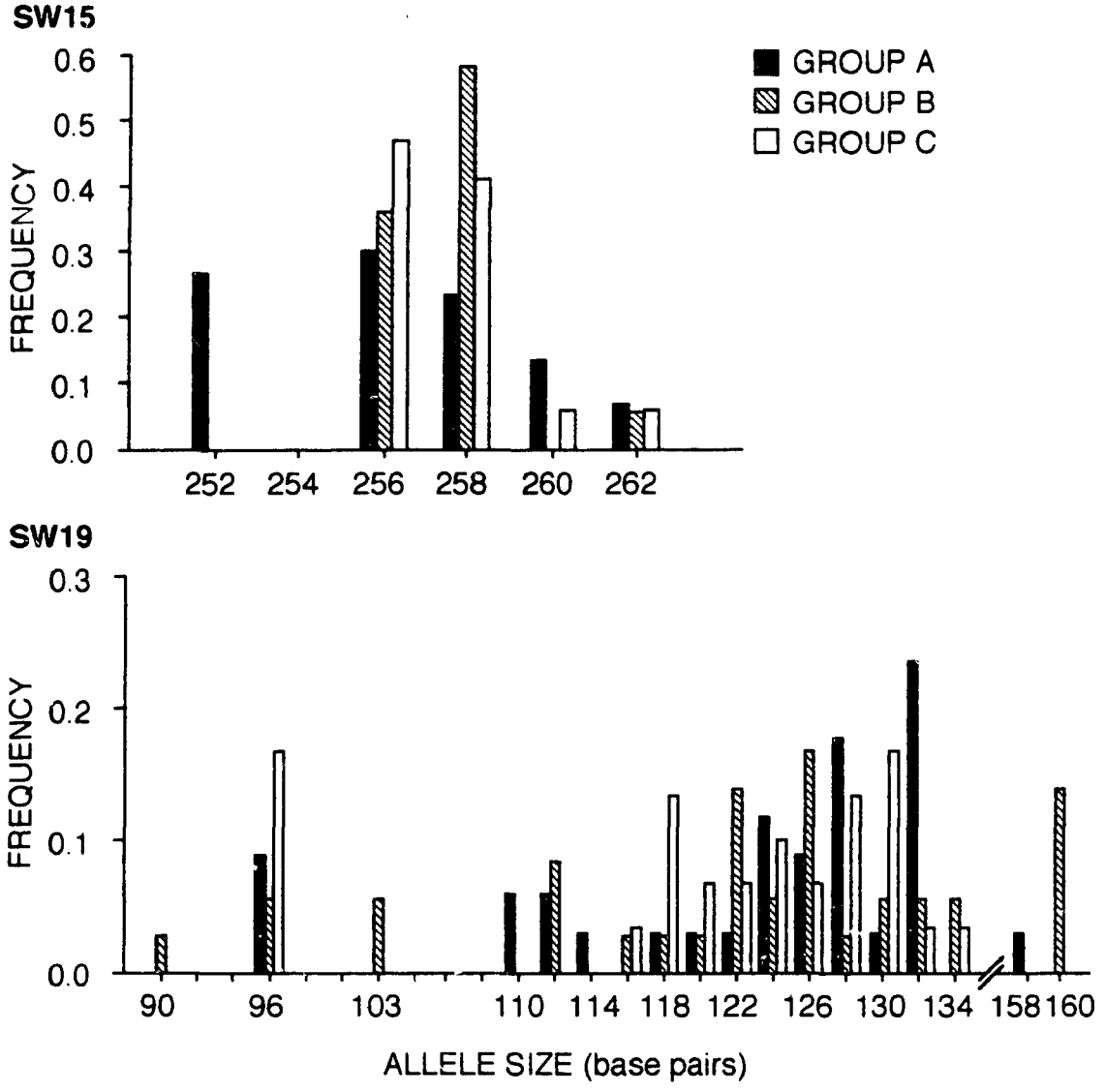

Fig. 4.3: Allele frequencies within 3 social groups (A, B and C) at 5 microsatellite markers (SW2, SW10, SW13, SW15, and SW19). Allele sizes correspond to the estimated sizes of PCR products. Genotypes for all individuals are presented in appendix E.

Table 4.2: Comparisons of allelic compositions in the different groups, A, B and C. The p values represent the estimated probabilities of falsely rejecting the null hypothesis that group and allelic composition are independent.

Locus	p value for comparison with all three groups	p value for comparison of each pair of groups	
SW2	0.07504	A vs B	0.28412
		A vs C	0.54938
		B vs C	0.02690
SW10	0.09522	A vs B	0.08094
		A vs C	0.61764
		B vs C	0.07310
SW13	0.02038	A vs B	0.10490
		A vs C	0.07200
		B vs C	0.05322
SW15	0.00014	A vs B	0.00010
		A vs C	0.00608
		B vs C	0.33594
SW19	0.01158	A vs B	0.02886
		A vs C	0.09968
		B vs C	0.07018

vi. Measures of microsatellite allele sharing

(a) Similarity of multilocus profiles

The similarity of multilocus profiles amongst all individuals (male and female) within the same group was higher than expected (s = 35.8%, p = 0.01, *i.e.* observed s was higher than 99/100 of the values of s calculated for the Monte Carlo permutations of the 80 multilocus profiles). Similarly, the observed ratio (1.13) of within-group s to between-group s was also significantly higher than expected ($p < 0.01$). However, when multilocus profiles were compared amongst whales in different groups, s was *lower* than expected based on the Monte Carlo test (s = 31.8%, p = 0.03).

The above results are consistent with kinship within groups. Moreover, the difference between the observed and Monte Carlo values are expected to be conservative since the permutations of the data do not represent truly random samples of population allele frequencies. The large majority of alleles were sampled from only three groups, and thus average similarity between any two individuals in the data set is likely to be higher than the average similarity of any two individuals in a data set that contains mostly only genetically unrelated whales.

As a summary value, s is unable to easily distinguish between simulated groups of different kinship structure, since allele sharing is still relatively high amongst random (and presumably unrelated) individuals. For example, the range of s values for simulated random groups overlaps significantly with the range of s values for simulated matrilines (Fig. 4.4).

(b) Frequency of allele sharing values

The distribution of allele sharing values for all pairwise comparisons within groups, A, B and C, was significantly different from the distribution of allele sharing values for all pairwise comparisons between groups, A, B and C, such that a greater proportion of high values (≥ 4/10 alleles shared) were consistently observed within groups

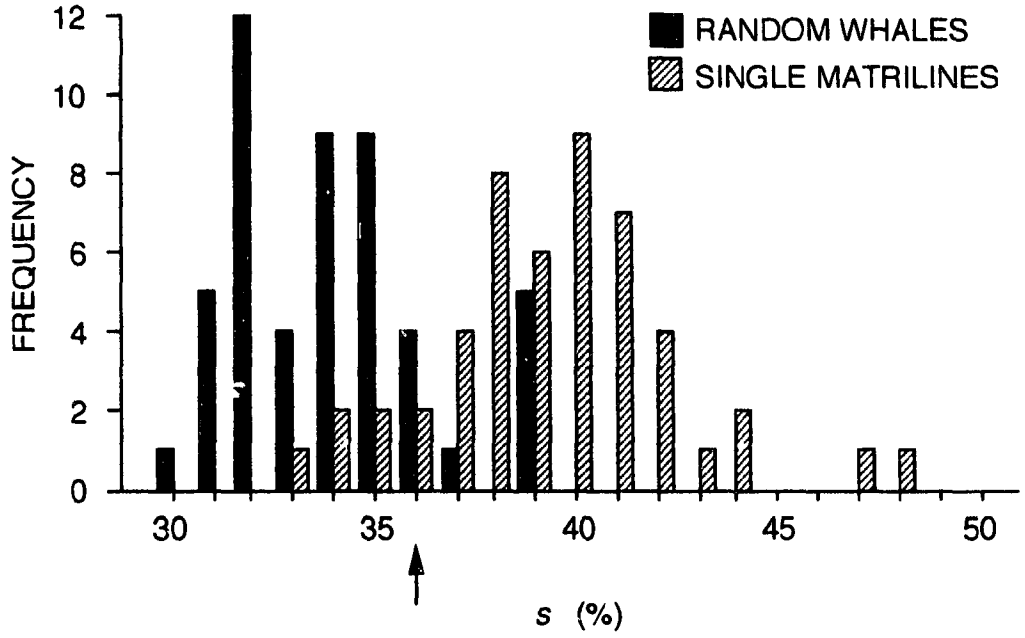

Fig. 4.4: Overall similarity of multilocus profiles, s, for simulated single matrilines and simulated random whales (n = 50). Arrow represents the observed value for the three groups, A, B and C, combined.

(Fig. 4.5). When the frequency distributions of allele sharing values within each group were individually examined (rather than combined into a single distribution as in Fig. 4.5), all three groups tended to have a greater proportion of high allele sharing values than observed in the between-group frequency distribution, but sample sizes were low for statistical analysis (data not shown).

Because comparison of males and females across all 5 markers is confounded by the X-linkage of SW15, the presented results include only female pairs. Because of the strong female basis to the groups, this may also be the most fitting comparison since it better emphasizes relationships amongst adult members of the groups (all male samples are from juveniles that will disperse). Nonetheless, similar patterns are observed when multilocus profiles are compared amongst all whales, male and female, unadjusted for X-linkage ($G = 32.1$, 7 df, $p < 0.001$).

The frequency distribution of allele sharing values within the observed groups of sperm whales (as depicted in Fig. 4.5) was also compared to frequency distributions of allele sharing values within simulated groups of five different kinship structures (Fig. 4.6). Allele sharing in the observed groups was most similar to allele sharing in groups comprised of single matrilines, but was also not statistically different from allele sharing in groups containing two distinct matrilineages. By contrast, allele sharing in the observed groups was markedly higher than in groups comprised of unrelated mothers each with their own offspring. As expected, allele sharing in the observed groups was also much higher than in groups of random individuals, but much lower than in groups of half-sibs.

It should be noted that the frequency distribution of allele sharing values for each of the five simulated kinship structures was significantly different from the frequency distribution for all other simulated structures ($p < 0.001$ in all cases). Furthermore, for each simulated kinship structure, the proportion of high allele sharing values was consistently greater within groups than between ($p < 0.001$ in all cases), except for the simulated groups of random individuals. For the random groups, the frequency

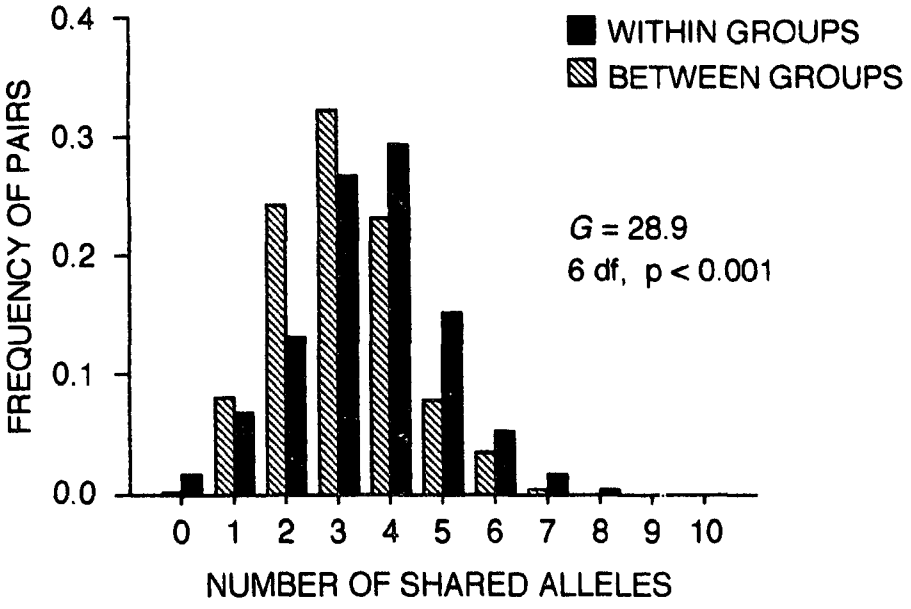

Fig. 4.5: Microsatellite allele sharing values within and between groups, A, B and C, for all females scored at 5 microsatellite markers.

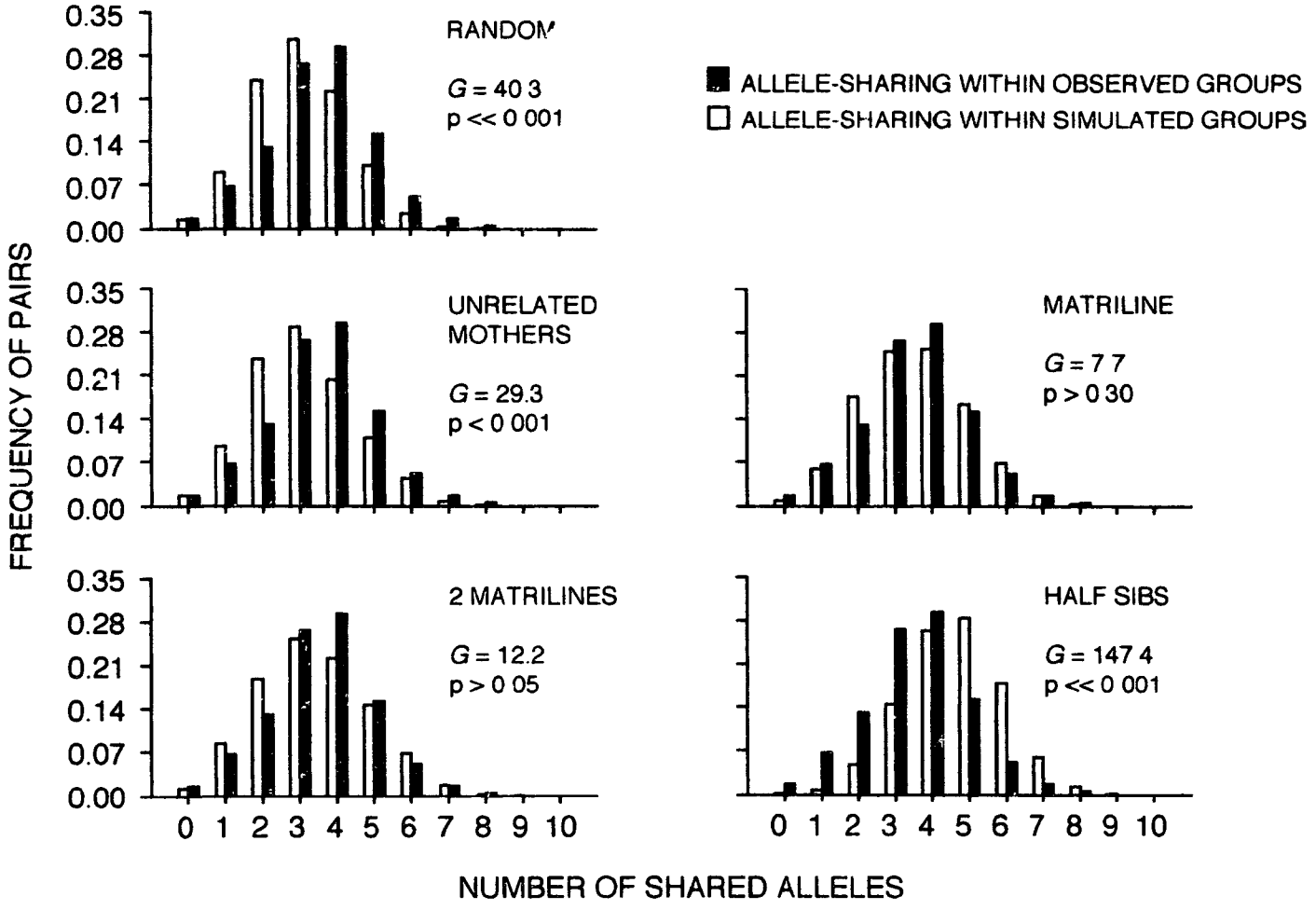

Fig. 4.6: Frequency distribution of allele sharing values for female pairs within observed groups, A, B and C, compared to distributions for female pairs within simulated groups of defined kinship structure. The *G*-statistic has 7 df for all comparisons.

distribution of allele sharing values was nearly identical within and between groups, as expected.

(c) Potential parent-offspring relationships

From an analysis of multilocus profiles, it was found that the observed proportion (0.917) of pairs that could be excluded from being potential parent-offspring within groups A, B and C was *less* than random expectations (Monte Carlo p = 0.02), suggesting that there were true parent-offspring pairs within the groups. A comparison between groups, A, B and C, found that the observed proportion (0.961) of pairs that could be excluded from being parent-offspring was *greater* than expected (p = 0.055).

When compared to simulated groups with different kinship structures (Table 4.3), the observed groups contained significantly fewer pairs that could be excluded from being parent-offspring pairs than did random groups, but significantly more than did groups of half-sibs. The proportion of pairs within groups, A, B and C, that could be excluded was not significantly different from the proportion within simulated matrilines, nor from the proportion within groups of unrelated adults and their offspring. However, when groups were examined individually, the proportion of pairs that could be excluded in groups A and B appeared too high when compared to simulated matrilines (Table 4.3).

vii. Coefficient of relatedness

Using the Queller and Goodnight (1989) estimator with the suite of five microsatellite markers presented here, average relatedness, *r*, within groups, A, B and C, was estimated as 0.0473. (The individual group values were 0.0347, 0.0659, and 0.0380 for A, B and C, respectively.) The jackknifed estimate was 0.0477, but the 95% confidence intervals (-0.0165 - 0.1118) were wide and overlapped with zero.

The Queller and Goodnight estimates appear imprecise when compared to known true *r* values for simulated groups. Estimated *r* values for the simulated matrilines ranged

Table 4.3: Proportion of pairs excluded from being parent-offspring in groups A, B and C, compared to simulated groups of different genetic structure. Values represent the percentage of simulated groups (n = 50) that contain a smaller proportion of excluded pairs than the observed groups. The proportion of pairs excluded from being parent-offspring in the observed groups is given in parentheses.

	A, B and C combined (0.917)	A (0.954)	B (0.926)	C (0.876)
Random	2	40	10	0
Unrelated mothers	72	100	84	6
2 matrilines	90	100	96	34
Matrilines	92	100	96	60
Half sibs	100	100	100	96

from -0.03 to 0.36, a much wider range than that seen in the true *r* values (0.06 - 0.19; Fig. 4.7). Estimated values of the 1000 random groups (true $r = 0$) ranged from -0.07 to 0.08 (mean = -0.0038 ± 0.0026 SD). Estimated *r* for group B (0.0659; the largest observed value) was greater than 98.8% of the simulated random groups, and greater than 23.3% of the simulated perfect matrilines. Estimated *r* for group A (0.0347; the smallest observed value) was greater than 91.7% of the simulated random groups, and greater than 8.8% of the simulated matrilines.

viii. Age of male dispersal

Male dispersal was estimated by the sex-ratio model to occur at age 5.7 yrs (95% c.i. = 2.6 - 10.9). The relative proportion of males to females amongst the 80 whales was 0.27, similar to the value presented by Best (1979) from whaling data (0.28). This proportion occurs at an age of 4-5 years according to Best's theoretical cumulative age composition model. This age approximates the mean age of male dispersal, providing that dispersal occurs at a fairly constant rate.

Discussion

i. Kinship in social groups of sperm whales

The non-random distribution of microsatellite allele variation within social groups provided direct evidence for kinship in sperm whales. Thus, multilocus profiles were more similar overall amongst group members than amongst members of different groups. On a finer scale, high values of allele sharing (≥ 4/10 alleles shared) were more frequent amongst group members than amongst whales from different groups. The number of group members sharing at least one allele at every marker (consistent with parent-offspring relationships) was also higher than random expectations. Actual estimates of *r* for the observed groups were imprecise, but when compared to estimates of *r* for simulated groups, also suggested that there was genetic relatedness amongst group members.

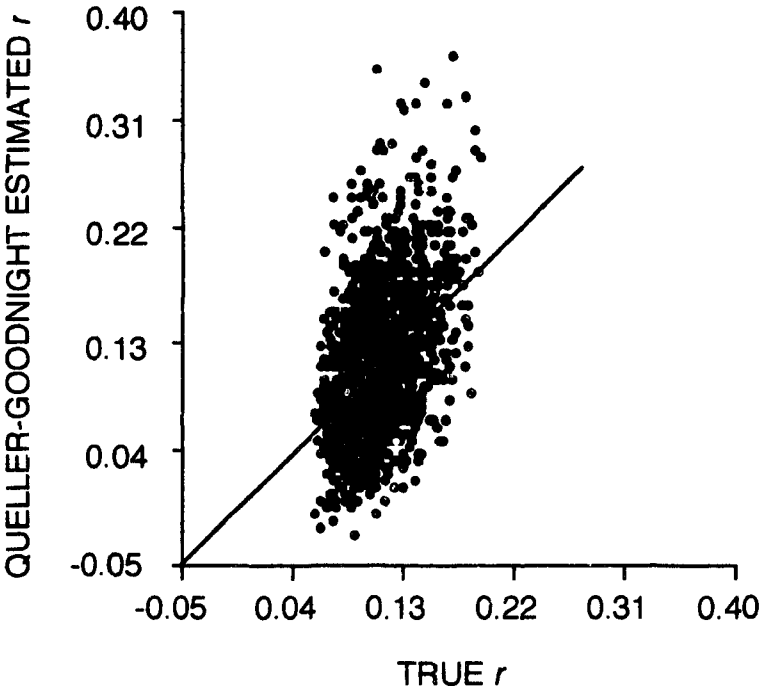

Fig. 4.7: Comparison of the Queller-Goodnight estimated coefficient of relatedness to the true coefficient of relatedness for simulated matrilines (n = 1565). The diagonal line gives the expectation if the Queller-Goodnight r perfectly estimates true r .

The above indices of kinship, although non-independent, were consistent in clearly showing greater genetic similarity within groups than between. This is indicative of a family structure. Within each 'family', there was also evidence for enrichment of alleles, seen as unique peaks in the allele frequency distribution of a locus, or as statistical differences in allelic compositions of groups. Observations of allele enrichment are consistent with restricted dispersal of juvenile females, *i.e.* the enriched alleles are expected to be those that have been inherited from the oldest females and thus transmitted to the most offspring (Amos 1993). Genetic differentiation of groups reflects the changing allelic balance within different groups. This allelic balance changes continuously over time, depending on both the survival of different allele lineages and the stability of groups. Different allelic balances are consistent with the results of tests indicating that the similarity of multilocus profiles (s) and the number of possible parent-offspring pairs between groups were lower than expected, *i.e.* enrichment of different alleles in different groups could magnify differences between groups compared to random expectations, thereby making it less likely that whales between groups would share alleles by chance alone.

ii. Kinship structure

Molecular genetic analysis of relatedness in wild populations of social mammals has previously proven to be most insightful when allele sharing can be calibrated against known kinship or when additional information, such as maternity and age-class, are available for some individuals (*e.g.* Packer *et al.* 1991; Lehman *et al.* 1992; Amos *et al.* 1993; Morin *et al.* 1994). Unfortunately, this type of data is extremely difficult to obtain for many species, including sperm whales. As an indirect test of possible kinship patterns, I compared the observed data to simulated groups of five different, but well-defined, genetic structures.

The simulations included groups of random individuals, expected to show relatively little allele sharing, and groups of half-sibs, expected to show relatively very

high allele sharing. The pattern of allele sharing in the observed groups fell somewhere in the middle of these extremes and was clearly distinct from the pattern seen in either model.

The remaining three simulated kinship structures represented the principal *a priori* predictions of group structure. Differences between these simulated str' :tures arise mainly from varying degrees of female dispersal from natal groups. Thus, it was expected that the observed social structure was likely to be similar to either:

(1) a matriline (with no dispersal of females);

(2) two matrilines (as suggested by the model of units described in Whitehead *et al.* (1991), refer to Chapter 1);

(3) a group of unrelated mothers with their offspring (resulting from dispersal of both sexes so that the only genetic relatives in a group would be mother-offspring and half-sib pairs).

Allele sharing in the observed groups was similar to allele sharing in simulated groups containing either single matrilines or two distinct matrilines. However, in the simulated groups of unrelated mothers, the proportion of high allele sharing values was too low when compared to the observed data. (Moreover, this latter simulation probably allowed for an overly generous amount of high allele-sharing values, such that the difference between the observed groups and a structure based on unrelated mothers should be even more pronounced. In the simulation, every female was given two offspring. This increased the number of high allele sharing values due to the addition of extra parent-offspring and half-sib relationships, but based on the long calving intervals (~4-5 years) observed in sperm whales, it is unlikely that each female would be accompanied by as many as two offspring if dispersal of all juveniles does indeed occur.)

Taken together, the comparisons of data from observed groups to data from simulation models showed that female dispersal was limited and clearly suggested a matrilineal basis for sperm whale family structure. Assuming a model of matrilines, the

sex ratio data further suggested that males disperse from the family groups at about age six years.

iii. Additional comments about the kinship analysis

(a) Hardy-Weinberg Equilibrium

No deviations from HWE were detected using both the goodness-of-fit χ^2 test and the exact test for Hardy-Weinberg proportions. The χ^2 test pooled all homozygous individuals and all heterozygous individuals because of the small sample sizes and the high polymorphism observed at each locus (Lessios 1992).

The meaning of a non-significant result in tests for HWE is generally difficult to interpret (Lessios 1992). Deviations from HWE may have been expected because of the non-random structure in the data. However, it is also possible that factors leading to deviations from HWE can work in opposite directions such that they cancel each other out (reviewed in Lessios 1992). Moreover, even if all females travel with their mothers, real deviations from HWE may actually be slight because of high gene flow through the males (*cf.* Melnick and Hoelzer 1992). It is clear that all males disperse over wide distances (see Chapter 1), and the influx of alleles from several different males into any given group is probably very high since individual males are likely to father only one calf per group (Best 1979; Whitehead and Kahn 1992; Whitehead 1993).

(b) Allelic variation within different groups

Different levels of allele sharing within the three different groups may have been due simply to chance sampling events. However, differences may suggest real variation in group kinship patterns. Differences might also be indicative of inaccuracies in the delineation of social groups. Group memberships could not be determined very precisely, for several reasons. Some group members were not sampled genetically (Table 4.1) and some were not photographically-identified during the time of observation (especially in

group C). As well, because of the fluid associations amongst different groups and the possibility of transient individuals (Whitehead and Waters 1990; Whitehead and Kahn 1992), some of the samples may have been collected from whales which were not actually long-term members of the core group. However, even if not entirely closed in membership, the three groups recognized as distinct were sufficiently well-defined that they would be expected to include several genetic relatives if a family structure exists in sperm whale social groups.

Interactions amongst the members of group C were much more lively than is normally observed. A large number of whales were seen around the time samples were collected from group C, and it may be that the animated behaviours resulted from the meeting of related kin groups, much as has been described for the meeting of related families within clans of elephants (Payne 1989). (The kinship structure described here for sperm whales is very reminiscent of the structure observed in elephants. Other striking similarities between the social organisations of these two species have been described in detail elsewhere (Weilgart *et al.* in press).) Genetic relatedness amongst whales from different, but genetically-related, kin groups would still be relatively high, and the kinship analysis would not necessarily have distinguished such structure from a single family structure. In the present analysis, many indices of allele sharing actually suggested greater similarity within group C than in the other groups.

(c) Low numbers of potential parent-offspring pairs

It should be noted that whales who share an allele at every locus are not necessarily true parent-offspring pairs. However, if more individuals share at least one allele at every locus than expected by chance, it is probable that some whales are true parent-offspring pairs or other first order relatives.

Overall, the proportion of potential parent-offspring pairs observed within groups seemed low compared to the simulated matrilineal structures (see Table 4.3). Possibly the

observed low proportion of potential parent-offspring pairs was simply due to chance. Within group A, for instance, there were no female multilocus profiles that could possibly be mothers for five of the six males (all juvenile); the absent mothers may have been amongst the whales that were not sampled from the group. The relatively low proportion of potential parent-offspring pairs may also have been due to undetected errors in the scoring of allelic variation (including experimental artifact and non-Mendelian inheritance of markers, see Chapter 2), accidental inclusion of non-group members in the sampling, or biases in the collection of samples. The parent-offspring analysis is much more sensitive to errors than are the other allele-sharing analyses since one mis-scored allele could incorrectly exclude a pair from being parent-offspring, while overall sharing still remains generally high.

Alternatively, the relatively low number of potential parent-offspring pairs could be biologically significant. The studied whales may have been subject to heavy whaling, as recently as twelve years ago (Ramirez and Urquizo 1985). The removal of individuals from groups would disrupt a matrilineal structure and might reduce the number of observed parent-offspring pairs more than it might reduce the overall sharing within groups. The relatively low numbers of potential parent-offspring pairs could also suggest that elements of the matrilineal model are inaccurate. For instance, a mortality rate that was "U"-shaped with age, similar to that observed in killer whales (Olesiuk *et al.* 1990), may result in fewer older mothers. A reduction of parent-offspring pairs might also be seen in groups that contain mostly individuals from single matrilines, but that also display partial dispersal of females counterbalanced by the immigration of unrelated females from other groups (*cf.* Best 1979). Further study with additional genetic markers may resolve some of these questions.

(d) Coefficient of relatedness

Although a number of methods exist for calculating population averages of within-group relatedness values (*cf.* Bennett 1987; Pamilo 1989, 1990), the estimator of Queller and Goodnight (1989) also has the potential to provide estimates of individual relatedness values, such as for a single group or a single pair of individuals. Since the absolute genetic similarity of unrelated individuals within a species is likely to be high, it is important when measuring relationships to differentiate between alleles that are identical-by-descent (*i.e.* inherited from a recent common ancestor) and those that are identical-by-state (Grafen 1985). This is why for the Queller and Goodnight estimator the similarity of allele frequencies between individuals is compared relative to a random sampling of the gene pool, *i.e.* the relatedness estimator tries to determine the proportion of alleles that are identical-by-descent by measuring in different individuals departures from the population allele frequency, since the population frequency is an estimate of the expected frequency of alleles that are identical-by-state (*cf.* Grafen 1985; Queller and Goodnight 1989; Pamilo 1989). Thus, better estimates of population allele frequencies off Ecuador should increase the accuracy of the relatedness values. Estimates of relatedness would also be improved with sampling of more groups (although this assumes that all groups are similar) and the use of additional loci (which might allow estimates of r for single groups with only small sampling errors).

iv. A few comments about the collection of data

(a) Description of social bonds

For studies of wild mammal populations, the general approach employed to describe social interactions is to develop field methods for recognizing individuals, in order to make detailed, longitudinal observations of associations and behaviours (see Hammond *et al.* (1990) for a compilation of papers detailing such study in a variety of cetacean species). Associations between individuals are usually measured by how close the

individuals are seen together in space or in time (*e.g.* Cairns and Schwager 1987). At present, it is very difficult to record more precise or descriptive characterizations of social relationships in free-living cetaceans.

Long-term patterns of temporal association amongst individual sperm whales have been constructed by looking at how often different whales were photographed close together in time (Whitehead *et al.* 1991), but group membership is best defined when photographic records are available over extended periods. In a preliminary field season near the Galapagos Islands, I attempted to better characterize specific interactions between individuals by considering sequences of behaviour, approach/avoidance movements, and relative positions of whales on the surface (K. Richard unpublished data). None of these methods were very successful, mainly because: (1) the frequency of most surface behaviours, other than breathing/resting is relatively very rare, thus making it difficult to interpret "interactions"; (2) the most interesting interactions usually occur during group socializing periods at which time tail-flukes (required for individual identification) are seldom shown to the camera; (3) it was often very difficult to visually keep track of all the individuals involved in an interaction; and (4) the effort required to search for sloughed skin samples generally prevented opportunities to obtain identification photographs from all interactants. Some of these problems might be overcome by the use of a more dynamic method of recording behavioural interactions, such as with the use of a video camera or a wide-angle camera positioned on the mast, or perhaps with the use of radiotelemetric tags placed on individual whales. However, neither of these technologies addresses the fundamental difficulty of trying to study the behaviour of underwater animals from a surface platform. As well, one also needs to obtain genetic samples linked directly to each of the behavioural interactants.

(b) Genetic tools

Under favourable conditions, the sloughed skin collection method allows for the collection of an impressive number of genetic samples. Disadvantages of this method are that sampled whales are generally unidentified (although this is also the case with biopsy dart samples), and that samples are of low-grade quality. This substantially increases the time and resources required to analyze individuals within a group. Nonetheless, the genetic variation revealed by the array of five microsatellite markers presented here was sufficient to distinguish most, and probably all, individuals.

The microsatellite markers were also sufficiently powerful to reveal kinship within groups. It was not possible, however, to resolve individual relationships with any degree of certainty. Although additional markers should improve estimates of the average degree of relatedness within groups, recent work by Brookfield and Parkin (1993) suggests that an unrealistically large number of very polymorphic markers may be required in order to distinguish third-degree or even second-degree relatives from genetically unrelated individuals. In this context, the development of additional microsatellite markers for sperm whales may provide diminishing returns, particularly in light of the difficulties already encountered in the present PCR analyses. In any event, microsatellites were the obvious genetic markers of choice for this study. Indeed, half of the microsatellite primer sets developed from sperm whales produced strong PCR products that were highly polymorphic, with three of the markers being exceptionally polymorphic (>10 alleles). Moreover, the markers could be reliably scored even at samples that were very small and partially degraded. The great disadvantage of microsatellites is that development can be time-consuming and costly, and this proved to be the case here. However, development of further microsatellite markers for sperm whales may prove easier than in this study. Microsatellites have now become a commonly used tool in several molecular laboratories, and consequently several improvements to protocols for developing markers have been suggested in the literature.

v. Comparison to other species

The genetic ecology of most cetacean species has yet to be elucidated. In general, mysticetes (baleen whales) do not appear to form long-term social bonds, but many odontocetes (dolphins and toothed whales, including the sperm whale) are highly social (Tyack 1986). Kinship within social groups has been well studied only in three species of odontocetes (namely *Orcinus orca*, *Globicephala melas*, and *Tursiops truncatus*). The most complete interpretations have arisen from dedicated, long-term studies of killer whales, *O. orca*, near British Columbia (Bigg *et al.* 1987, 1990), and bottlenose dolphins, *T. truncatus*, on the west coast of Florida (Wells *et al.* 1980; Scott *et al.* 1990). Putative relationships were determined from behavioural patterns constructed over several years of observation, but genetic analyses were required in order to confirm some relationships and determine others (especially paternity) that were not easily revealed by behavioural interactions (Duffield and Wells 1991). Social structure in pilot whales (*G. melas*) has also been described, based on a molecular genetic analysis of two complete pods killed in the Faroes Islands fishery (Amos and Dover 1990; Amos *et al.* 1991, 1993). Remarkably, in both killer whales and pilot whales, both sexes appear to be philopatric; groups are thus very stable. However, social groups are much more fluid in the smaller dolphin species, and social bonds appear to form largely amongst "friends" rather than amongst genetic relatives (Connor and Norris 1982; Evans 1987).

The highly social species of cetacean often display cooperative foraging or group defense (Evans 1987). However, in most species the young generally remain in constant contact with their mothers, and group members do not display daily communal care behaviours like those described for sperm whales. Sperm whales may have been under unusual selective pressures for 'group-rearing' of the young because of their deep ocean habitat which necessitates separation of the calf and mother during foraging.

Male sperm whales are unique among cetacean species studied to date in terms of the way they range across ocean-wide distances, roving singly between social groups of

females. The delayed sociological maturity of males and the extreme sexual dimorphism are also exceptional.

vi. Social evolution in sperm whales

The evidence for kinship described here indicates that the apparent altruism and tight social cohesion observed in sperm whales may to some extent be a consequence of kin selection. However, many important details of social evolution in sperm whales still remain intractable to critical study, partly because the animals are difficult to work with, but also because current technology is still limiting. For instance, the costs and benefits to fitness of social behaviour remain subject to conjecture, and as such any consideration of kin selection necessarily has to emphasize the observed degree of relatedness (r). It seems likely that the benefit/cost ratio for alloparental care could be high, which would result in favourable conditions for kin selection within matrilines. However, the potential for reciprocal altruism in sperm whales is also suggested by the long-term associations that occur among some individuals (Whitehead *et al.* 1991). Reciprocity amongst relatives could lead to greater altruism than that expected by kin selection alone. Detailed evaluation of the relative importance of kin selection and reciprocity requires additional progress in the behavioural and genetic study of sperm whale groups. The next step is to collect several samples from individuals with extensive sighting histories and known long-term associations. Longitudinal studies allowing estimation of relative ages of individuals within groups could prove especially profitable for elucidation of genetic relationships.

Many of the sloughed skin samples collected in this study were obtained from single, photographically-identified individuals, but the total sample set did not actually include samples from any two whales who were known to have long-term associations with each other. Only ten of the 194 different whales photographically identified during the study period had prior sighting histories (Dufault and Whitehead 1993). This precluded a comparison amongst specific individuals with definite long-term social

connections, and my analysis necessarily had to focus on determining the overall kinship pattern of whales that were encountered together in groups, rather than considering more specific relationships. With the continuation of field work at the same site in different years, future analyses may be able to examine allele sharing amongst whales known with certainty to share long-term social bonds. At present, there are several known long-term associations for whales studied off the Galapagos Islands, but most of these whales were studied in years when skin samples were not collected.

Detailed data like those described above may enable comparison of kinship patterns between areas with different geography and with different histories of whaling. Group sizes do vary between areas (Whitehead and Kahn 1992) and this may be reflected in somewhat different kinship structures. Samples from groups of juveniles would also be valuable for kinship analyses. Continued collection of samples with individual-specific data, as well as more extensive genetic typing, may ultimately allow the question of male reproductive success to be tackled. As well, development of polymorphic male-specific markers could prove useful for assessment of male gene flow in groups containing several juvenile males.

vii. Concluding remarks

My thesis combines genetic analyses with intensive, non-invasive field studies to reveal social structure in sperm whales. The results of this work demonstrate that a relatively small number of genetic markers can prove highly informative even when the study animals are not amenable to detailed data collection. Methods for precise characterisations of social bonds and genetic relationships remain elusive, however, and a complete understanding of social organisation is thus only likely to emerge from a process of successive approximations. This thesis advances this process by providing direct genetic evidence for matrilineal kinship within social groups of sperm whales.

APPENDIX A

SLOUGHED SKIN AS A SOURCE OF DNA

Description of sloughed skin

In total, 331 samples of sloughed tissue were collected during the Ecuador field study. Samples ranged in size from about 0.1 cm^2 to 100 cm^2. Most samples were very thin and 'sheet-like' pieces of transparent epidermal skin, appearing light grey in colour when bunched up. One quarter of the samples displayed very different qualities. These samples were texturally different and darker in colour. Most were thicker, stringy, slimy, and often carried an unpleasant odour. Possibly this type of tissue lines the inside of a whale's mouth, or is intestinal or rectal in origin, since faecal or food material is often also seen floating at the water surface (including squid beaks which are sharp, and probably able to tear sperm whale tissue). I could confirm that DNA from sloughed skin was sperm whale in origin by comparison to DNA from 5 stranded sperm whales.

Organic extraction of DNA from sperm whale tissue

Genomic DNA was extracted from skin using a protocol modified from that of Amos and Dover (1991). A sample of approximately 15 cm^2 (50-100 mg) was soaked and rinsed thoroughly in 0.85 M NaCl (note that repeated rinsing was critical to the success of the extraction if samples were preserved in a saturated salt solution), damp-dried and ground to a powder with mortar and pestle in liquid nitrogen. The sample was added to 500 µl of digestion solution (100 mM NaCl, 50 mM Tris, pH 8.0, 20 mM EDTA, 1% SDS, and 1-2 mg/ml Proteinase K), in a 1.5 ml microfuge tube, stirred with a drawn out pasteur pipette, and incubated at 65 °C for ~20 hours. The tube was then cooled to room temperature and one volume of phenol added to the digestion solution. The mix was gently inverted with a mechanized rotator for 10-20 minutes (until an emulsion formed), allowed to stand for one hour, microcentrifuged at 16 000 x g for 5-8 minutes, and then

the upper aqueous layer removed to a new tube. This was followed by a 'back-extraction', *i.e.* 150 µl of TE (10 mM Tris, 1 mM EDTA) was added to the original tube, mixed, centrifuged briefly, and the supernatant combined with the previously transferred aliquot. The phenol extraction was repeated if the supernatant appeared dirty or discoloured. Traces of phenol were removed from the supernatant by adding one volume of chloroform:isoamylalcohol (24:1). The tube was mixed gently to disperse the chloroform through the supernatant and allowed to stand for a few minutes before being centrifuged for 5 minutes. The supernatant was combined with an equal volume of 5 M LiCl in order to remove additional impurities, left at -20 °C for 20-30 minutes and then centrifuged for 5 minutes. The supernatant was again removed to fresh tubes such that each tube contained a maximum of 400 µl. Each aliquot of supernatant was mixed with one-tenth volume of 5 M Ammonium Acetate and 2.5 volumes of cold ethanol, and then stored overnight at -20 °C. The tubes were then centrifuged at 16 000 x g for 15-20 minutes. The ethanol was decanted and the remaining pellet washed twice in 750 µl of 70-80% ethanol, *i.e.* the tube was vortexed vigorously to loosen the pellet, allowed to stand for 30 minutes (in order to leech out salts), centrifuged for 5 minutes and then the ethanol decanted. The pellet was vacuum dried in a SpeedVac microfuge for about 15 minutes, resuspended in 100-150 µl TE and stored at -20 °C. Samples frequently required a day or two to be taken up completely into solution. Typical extractions yielded ~1-2 µg of DNA per mg of tissue.

DNA quality

When size fractionated on an agarose gel, most of the DNA extracted from sloughed tissue appeared as a smear, presumably due to degradation, but a significant quantity of high molecular weight DNA was also observed (Fig. A.1). DNA extracted from dead, stranded whales was also degraded but apparently to a lesser extent than DNA from the sloughed samples.

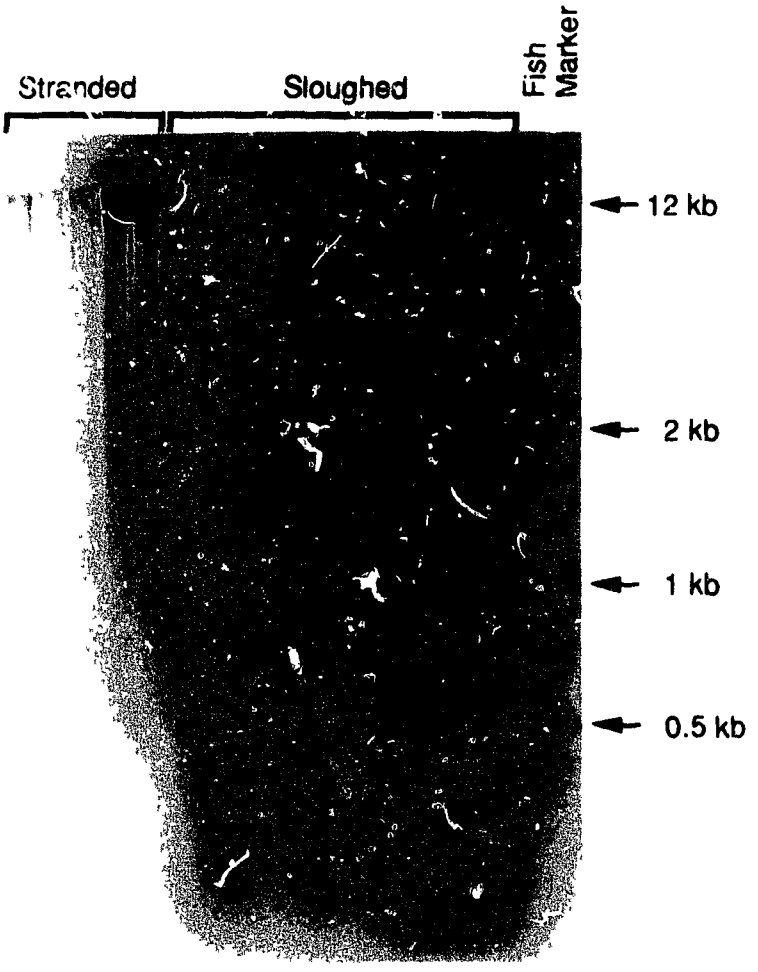

Fig. A.1: Quality of DNA extracted from sperm whale tissue. DNA samples are from dead, stranded whales or sloughed skin. The fish DNA was extracted from fresh *Tilapia* blood. Marker is the 1 kb ladder.

APPENDIX B

ADDITIONAL SEQUENCE DATA

The following sequences were cloned from the six microsatellites *not* used in the construction of multilocus profiles. Underlined sequences indicate target sites for PCR primers. For microsatellite SW17, a total of four oligonucleotides were designed, allowing for four different primer pair combinations. Two of the primer sites are underlined; the other two are italized. For each of the six microsatellites, PCR reactions with the designed primers failed to amplify the target DNA or generated several non-specific fragments that made the results non-interpretable. "N" denotes an ambiguous nucleotide in the sequence information. "??????????" denotes an unknown (but probably short) length of DNA in the middle of the SW7 clone that could not be sequenced.

SW7

5'-AGCTCACAAA.CCAACCACCC AATCACTTTG ACTCCTCTGT GCATGCTGCT
AGAGAGAGAG AGAGAGAGAG AGAGAGAGAG AGAGAGAGAG AGAGAGAGAG
AGAGAGATGA TGATAGAGAG AGAGAGCAGC AGCAGCAGCA GCAGAGGAGA
GATAGTGAT? ?????????? GCGAGTGAGA GAGAGAGAGA TTATGAGAAA
GAGAGAGAGA GAAAAACAGC AAGATGAAGA GCACCCACTT AAAGCT-3'

SW9

5'-AGCTTAATAT TCAAGAAACA AACAACCCAA TCCAAAAATG GGCAGAAGAC
CTAAATAGAC ATTTCTCCAA AGAAGACATA CAGATGGTCA AGAAGCACAT
GAAAGGCTGC TCAACAGCAC TAATTATCAG AGAAATGCAA ATCAAAACTA
CAATGAGGTA TCACCTCACA CCAATTAGAA TGGGCATCAT CAGAAAATCT
ACAAACAACA AATGCTGGAG AGGGTGTGGA GAAAAGGGAA CCCTCTTGCA
CTTGTTGTGG GAATTGAAAT TGATACAGCC ACTATGGAGA CAGTATGGAG
GTCTTGTCAC GGGAAAGGGA AGGGAGTGTG TCTTGGAGAG ATGACATGCT
GACAGAGACT GCAGACACTC TGATCTCTCT GATCTCTTCC AATACCTTTC
CTTTCCCTTT TTCTCTCTCT GTGTGTGTGT GTGTGTGTGT GTGTGTGTGT
GTGTGTGTGT GTGTGTGTGT GTGTGTGTGT GTGTGCGTGC GTTACATCTG
GCAGCT-3'

SW14

5'-AGCTCAGAAA CCAACGAGAG CCTCACTCCA GGTTCATGGT GTAGGAGATG
GAGGTGTGAG TGTCGGGGGT GTGGACACAT CTAAGAATGT AGGTACACGT
GAATGTGGAT GCATGCGTGC ACACATATGT GTCGATGTCT TAATGCATGC
ATACACCTCT TTCCTGAATC ACCAGGACCG AGCACGGGGC AGGCAGCTTC
CAAACAAAGG AGAAAAACGG CAACGAGAGG GGTCATGACA GCAAGAGTGA
GAGAGGGAGA GAAAGCAAGG TAGGCTGAGT GTGTGTGTGT GTGTGTGTGT
GTGTGTGTGT GTGTGTGTGT GTGTGTGTGT GTGTGTGTGT GTGTGTGTGT
GTGTGTCTGC ACATGCGCAC TAGGAGAATT TACT-3'

SW16

5'-AGCTGGTGTG TGTGTGTGTG TGTGTGTGTG TGTGTGTGTG TGTGTGTGTG
TGTGTGTGTG TAGCACCTGT GTATTACACT GTGTTAGCCT TCCCTCTTAA
AAAAAACAGA TTTTTTTTCC CCATATGCCT GCTTTTGCAA AAACCTTCTC
AAGGGAGTAA AATCCAAGCA GCCTTTGTAC AATAATGGTG TTGTTACTCT
CACAG-3'

SW17

5'-AGCTAGAAGG CTGTGTGGGG GAGGATTTTC ATCCCTGGTC CACATGCCTC
TATGAATAAA AGAAGCCTGA TGCATCTTTG ATATTTAATT CTTAAAATTA
TCCCCGGGCT TCCCTGGTGG CGCTGTGGTT GCGCGTCCGC CTGCCGATGC
AGGGAACCGG GTTCGCGCCC CGGTCTGGGG GATCCACATG CCGCGGANNN
GGGCTCAGTT GGCTAACCCA GTCAGTGGCA TGCTGGAAAC CTGGGTTTGA
ACCAGTCAGT CCAAATACTA ATGATA*CTCA TACATTCTGT GTCTGCCTAT*
CAGTGTGTGT GTGTGTGTGT GTGTGTGTGT GTGTGTGTGT GTGTGTGTGT
GTGTGTGTGT GTGTGTGTGT GTGTGTGTGT GTGTGTGTGT GTGTGTGTGT
GTGTGTGTGT GTGTCTGTGT CTGTGTCTGT G*TCTGTGTGC GTGTAGTTAG*
CT-3'

SW22

5'-CTGGAGATGA AGGGTGGGGG GGGAGAGAGA GAGAGAGAGA GAGAGAGAGA
GAGAGAGAGA GAGAGAGAGA GAGAGAGATT CTCTAGTTCT CATGANNNNN
TCTATTTCCC CTTGGTTCTC CGAGTTGCCC CGCATCTTAA AATATTAGGT
TGAATTATAT GAAATTGTTG TTTTTATAGG TCAAAAAGGT CAATAGCAGC
AATTATGTAT GCTGTAACCT AGTAAG-3'

APPENDIX C

ADDITIONAL PRIMERS TESTED FOR MICROSATELLITE CLONES

The following primer combinations were designed for microsatellite SW10:

SW10

Immediately adjacent to repetitive array:

5'-CCTCATTAGAAGACCAGA-3'

5'-AGGATGAGGGAGAAGGAA-3'

30 mer primers, overlapping the original primers in Fig. 2.2:

5'-TGCTAACCTAAGGATGGAGATGATACACAT-3'

5'-CCTGTCGAAATGATCAAGTTTTATGCATAC-3'

Both combinations successfully amplified the target sequence.

The following primers failed to be useful for PCR amplifications (including when paired with an appropriate good primer shown in Fig. 2.2):

SW13

5'-CTGTCTTAATGAAATTC-3'	(overlaps with good primer at 5' end of clone in Fig. 2.2, but is 4 bp shorter)
5'-CTGAATGTTACTTCCTTGAGA-3'	(immediately flanks 3' end of GT array)

SW19

5'-CTCTCATGACTAAAATAGTT-3'	(targets 5' end of clone)
5'-TATTATTATCCCTTCCC-3'	(targets 3' end of GT array between base pairs 120 and 136 of clone in Fig. 2.2)

APPENDIX D

SCORING OF GENOTYPES

The PCR-amplification products for most of the primer sets included "shadow", or "stutter", fragments, as seen in nearly all other studies of dinucleotide microsatellites (*e.g.* Schlötterer and Tautz 1992). Shadow fragments were stuttered in 2 bp intervals, and typically appeared as 1-3 bands that were smaller than the band representing the putative true allele. At locus SW15, there was often also a single, much fainter band that was 1-2 bp larger than the putative true allele. At locus SW2, there were occasionally irregular bands produced that were smaller than the putative true allele (*e.g.* lanes 1, 10, 11 in Fig. 2.3). The shadow fragments did not appear to affect the scoring of the gels, but did make less straightforward the differences between homozygous genotypes and heterozygous genotypes displaying two similar-sized alleles. Individuals were scored as heterozygous with two alleles separated by only one repeat unit if the smaller allele was more intense on the autorad than the larger allele (*e.g.* lane #1 in Fig. 2.5). The smaller allele was assumed to be more intense because of co-migration with the shadow fragment of the larger allele. In individuals scored as homozygous, the largest fragment (putative homozygous, co-migrating alleles) was clearly more intense than the smaller shadow fragment (*e.g.* lane #10 in Fig. 2.6). These patterns of differential intensity were always consistent between replicate analyses of a sample.

To illustrate the scoring of each locus, the genotypes for each sample in Figs. 2.3-2.7 are presented below:

Lane #	SW2 (Fig. 2.3)	SW10 (Fig. 2.4)	SW13 (Fig. 2.5)	SW15 (Fig. 2.6)	SW19 (Fig. 2.7)
1	77,79	143,155	163,165	258,258	096,132
2	77,79	143,151	failed	258,260	096,132
3	77,79	151,159	151,161	258,258	096,132
4	failed	145,157	151,161	256,262	126,126
5	77,77	145,153	136,171	256,256	134,136
6	79,79	145,149	136,171	256,258	120,124
7	79,79	not scorable	136,171	258,258	120,124
8	77,81	145,153	161,171	256,258	120,124
9	79,79	145,149	163,165	256,258	120,124
10	77,77	145,149	161,171	256,256	096,132
11	79,79			256,258	120,124
12					120,128
13					096,128
14					096,128
15					122,130
16					096,128

APPENDIX E

ALL MULTILOCUS GENOTYPES IN THE DATA SET

Genotypes at each microsatellite locus for all 80 unique profiles (typed at 4-5 markers), based on 164 samples of sloughed skin collected from sperm whales in Ecuador.

Genetic I.D.	Sex	SW10	SW13	SW15	SW19	SW2
GROUP A						
E91A01	F	143,151	163,165	252,260	130,132	077,079
E91A02	F	143,149	136,163	252,260	096,128	077,077
E91A03	F	145,153	165,165	256,258	110,132	079,079
E91A04	F	147,151	159,163	252,260	128,132	079,079
E91A05	M	147,149	163,167	252	124,132	077,081
E91A06	M	145,149	163,163	256	120,128	079,079
E91A07	M	145,149	163,171	258	128,158	077,077
E91A08	F	143,145	165,169	258,258	112,126	079,079
E91A09	F	151,151	163,163	252,256	096,118	075,077
E91A10	F	145,149	151,163	256,256	126,132	077,081
E91A11	M	145,149	136,163	252	096,124	077,077
E91A12	F	151,153	165,169	252,256	128,132	077,077
E91A13	F	151,159	163,163	252,258	---,---	075,077
E91A15	M	145,157	163,163	256	114,122	077,077
E91A16	F	145,149	165,173	256,258	110,124	077,077
E91A17	F	145,153	165,165	260,262	126,132	077,079
E91A18	F	143,147	163,171	256,262	128,132	079,079
E91A19	M	145,157	163,171	258	112,124	077,077
GROUP B						
E91B01	F	149,157	163,165	256,256	130,160	077,079
E91B03	F	145,147	136,163	256,258	124,160	075,079
E91B04	F	149,153	163,163	258,258	118,122	077,079
E91B05	F	147,151	163,165	258,262	096,126	075,075
E91B06	F	143,151	167,173	258,258	126,134	077,081
E91B07	F	149,151	163,163	258,258	126,160	077,079
E91B08	F	137,149	163,171	256,262	112,160	079,081
E91B10	F	147,147	159,163	256,258	116,132	075,077
E91B11	M	151,151	151,161	256	103,124	077,077
E91B12	F	147,151	136,171	258,258	112,122	075,077
E91B13	F	149,151	161,171	256,258	122,134	075,079
E91B14	M	149,151	136,163	258	096,112	075,079
E91B15	F	149,153	161,163	256,258	126,126	077,079
E91B17	M	151,151	151,161	256	103,128	077,077
E91B18	F	149,151	163,163	258,258	090,120	077,077
E91B19	F	145,151	136,161	256,256	130,160	079,081
E91B20	M	145,151	167,169	258	---,---	077,079
E91B21	F	141,151	161,167	256,258	---,---	075,077
E91B22	F	151,151	136,159	258,258	126,132	077,077
E91B23	F	149,153	163,163	256,258	122,122	077,077

Genetic I.D.	Sex	SW10	SW13	SW15	SW19	SW2
GROUP C						
E91I01	F	145,153	163,171	258,258	096,134	079,079
E91I02	F	149,151	163,167	256,258	116,128	077,079
E91I03	F	145,147	163,167	256,262	---,---	077,079
E91I04	F	145,151	159,165	258,260	128,130	077,079
E91I05	F	149,153	163,167	256,258	118,128	077,079
E91I06	F	147,153	163,175	256,258	122,124	077,079
E91I07	F	143,151	163,167	256,258	096,124	077,081
E91I08	F	145,145	136,159	256,258	122,124	077,077
E91I09	F	145,145	136,163	256,258	096,118	077,079
E91I10	F	149,149	167,169	256,262	096,118	077,077
E91I11	F	149,153	167,167	256,258	118,120	079,079
E91I12	F	139,147	163,165	256,256	120,130	077,081
E91I13	M	149,153	167,169	258	---,---	077,079
E91I14	F	147,149	165,171	256,258	096,126	077,079
E91I15	M	151,153	165,169	258	130,130	077,081
E91I16	F	145,149	161,167	256,256	---,---	079,081
E91I17	F	141,157	165,169	256,258	130,132	077,079
E91I18	F	149,153	163,165	256,260	126,128	077,077
UNASSIGNED WHALES						
E91P01	M	141,149	165,169	258	---,---	077,079
E91P02	F	143,147	163,163	258,260	---,---	077,079
E91P03	F	145,151	163,165	258,258	---,---	077,077
E91P04	F	147,153	167,171	256,262	116,126	077,077
E91P05	F	145,147	161,175	256,258	096,128	077,081
E91P07	F	153,153	163,163	256,258	096,130	077,077
E91P08	F	149,151	161,165	256,262	---,---	077,079
E91P09	M	149,153	159,173	256	118,128	077,077
E91P10	F	151,153	167,169	258,262	---,---	077,081
E91P11	F	145,151	163,165	258,262	---,---	077,077
E91P12	F	147,149	171,175	252,258	---,---	079,081
E91P13	F	145,145	163,165	256,258	126,128	077,079
E91P14	F	151,155	165,169	256,260	---,---	077,077
E91P16	M	149,151	165,167	258	---,---	077,077
E91P19	F	149,151	163,167	258,258	090,096	077,077
E91P20	F	145,145	136,159	256,258	---,---	075,075
E91P21	F	145,145	161,163	256,258	103,118	073,077
E91P22	F	149,151	161,163	256,258	---,---	075,077
E91P25	F	147,147	163,163	252,260	---,---	077,077
E91P27	F	155,157	159,163	252,256	---,---	077,077
E91P28	F	149,151	163,165	258,262	---,---	077,079
E91P29	F	143,155	161,171	256,258	126,134	077,077
E91P30	M	145,153	163,167	256	---,---	077,077
E91P31	M	145,149	151,159	256	---,---	077,081

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