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# Isolation and genomic analysis of the cetacean Y-chromosome

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# Abstract

The male-specific mammalian Y-chromosome represents a powerful tool for studying male-mediated gene flow and genome evolution. Here it was possible to identify 7 polymorphic microsatellites for the first time in an odontocete species, using a combination of cell culture, cytogenetics and molecular approaches. Initially, the development of an efficient and repeatable methodology for obtaining a growing lymphocyte culture that facilitated the isolation of individual chromosomes is described. Flow karyotypic characterization and isolation of individual chromosomes via flow sorting or microdissection is reported for the killer whale *(Orcinus orca)*. Microdissected Y-chromosomes from the killer whale and bottlenose dolphin *(Tursiops truncatus)* were screened for sequences containing microsatellite motifs. 15 and 10 male-specific microsatellites were identified from the killer whale and bottlenose dolphin, respectively. Additional microsatellite loci were identified from previously published fin whale Y-chromosome sequence. 6 markers designed from heterologous sequences amplified from sperm whales (*Physeter macrocephalus*), were also screened for variation. All 31 markers were monomorphic in the bottlenose dolphin, only 2 loci showed 2 variants in the killer whale and 7 were polymorphic in the sperm whale. In addition 162 anonymous regions of the Y-chromosome, isolated from the delphinid species were used to characterize the comparative composition of the 'Y' relative to the autosomes in these species. Characteristics are discussed in the context of the genome as a whole, species-specific history and with reference to the expected patterns of mammalian Y-chromosome evolution.

***Acknowledgements***

*Wow! Acknowledgments - where to start? There have been so many people who have contributed towards this thesis in myriad ways. From the people who taught me new techniques and provided facilities, to those who gave me a kind word, just when I needed it the most. Many people have given me physical, mental and emotional support for the five long years that it has taken me to put this work together. If I were to list everyone who deserves a mention, the acknowledgements would be as long as the thesis and for fear of omitting someone, I will just say this - thank you to all who have helped me get where I am today, you know who you are and I am forever in your debt. I can not thank you enough.*

*Nevertheless, there are a number of people and institutions I would like to acknowledge publicly: I am grateful to my funding body (the Natural Environmental Research Council – NERC) for their financial support and understanding of my circumstances that led this study to require an extra year. Thank you to The Cambridge Veterinarian School for the use of flow sorting, microdissection and FISH facilities; to the Wise Lab for sharing culturing techniques and to Durham and Newcastle Flow Cytometry group*

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*Thank you to all the people who have provided samples, I am only sorry that I was not able to realize the full potential of them all, whilst techniques were in the development stage - Castle Veterinarian Surgery, Barnard Castle; Marineland, Antibes; the Seal Sanctuary, Truro; The Marine Mammal Centre, Sausalito; the Veterinary Laboratories Agency, Defra; NOAA, California; St Andrews University, Isle of May; and "The seal research contingent", University of Durham.*

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*Finally, I hold a particular fondness for those whose advice I sought in the early days who said it could not be done –Against all odds! Carpe Diem!*

*An addendum acknowledgement is reserved for my examiners – I thoroughly enjoyed discussing my work with you and I am grateful for your comments.*

*Thank you one and all,*

*Vittoria Elliott*
*In the words of Mark Twain:*
*"I'm glad I did it, partly because it was worth it,*
*but mostly because I shall never have to do it again"*

## *Dedication*

*I dedicate this work to: my parents, Hayward and Susie,*

*Without whom it would not have been possible;*

*To "Daddy Hug", who welcomed me into his arms and his heart;*

*And to the memory of his sister, Anne*

*Who I'm sure would have achieved great things*

*Had she been given the opportunity.*

**List of Abbreviations**

DMY - Y-specific DM-domain **gene** required for **male development** in the **medaka** fish.

NRY - Non-recombining region of the Y-chromosome.

LTA - Lymphocyte transformation assay

MSY – Male-specific Y-chromosome

Ne – Effective population size

PAR – Pseudo autosomal region

SDF – Sex determining factor

SRY – Sex determining gene

ZFY – Zinc finger Y-chromosome protein

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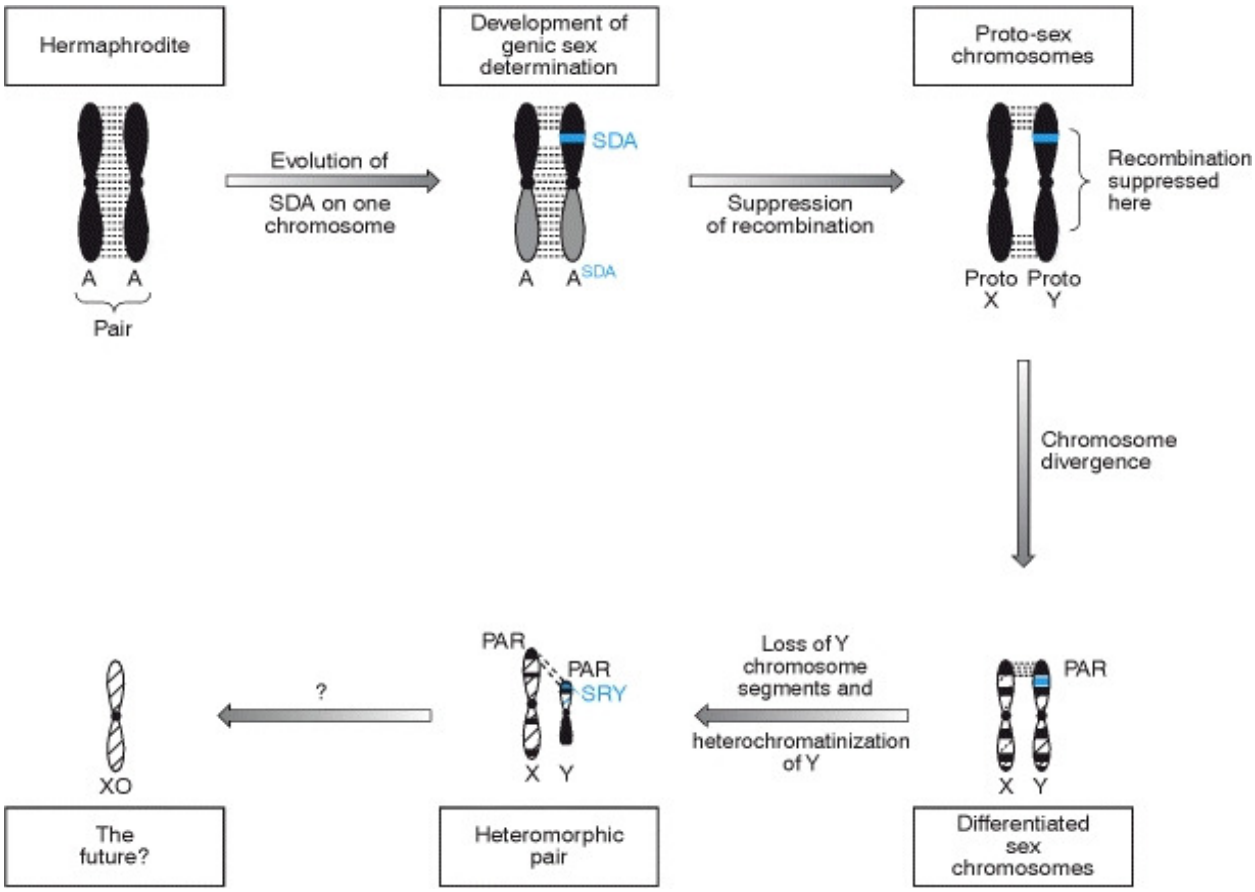
# Chapter 1: General Introduction

## 1.0 The Y-chromosome overview

Its haploid condition, the existence of a large non-recombining region, and its exclusively patrilineal transmission make the Y-chromosome a unique marker of general interest to understanding genomic evolutionary mechanisms. Moreover, these characteristics make it particularly valuable for addressing species-specific problems, such as population dynamics, functional significance, historic processes, migratory patterns, and sexual selection.

**Fig. 1.1.1 Taken from Strachan and Read, 1999.**
**Mammalian sex chromosomes most likely evolved from a pair of autosomes, one of which acquired a sex-determining allele, leading to recombination suppression and chromosome differentiation**
One of a homologous pair of autosomes in an ancestral genome is envisaged to have evolved a sex determining allele (SDA). Thereafter, the need to avoid exchange of the SDA and possibly the evolution of *sexually antagonistic genes* led to suppression of recombination between the two chromosomes, except in small regions, known as pseudoautosomal regions (PARs). Lack of exchange between the homologs led to chromosome divergence. Because most of the Y is not involved in any recombination events, it degenerated by a series of chromosomal deletions. Present day human X and Y chromosomes retain small regions of homology outside the PARs, partly as a result of very recent X-Y transpositions. Possibly the inexorable pressure to reduce the Y means that eventually it will be completely eliminated and a mechanism of sex determination will evolve which is based simply on X : autosome gene dosage.



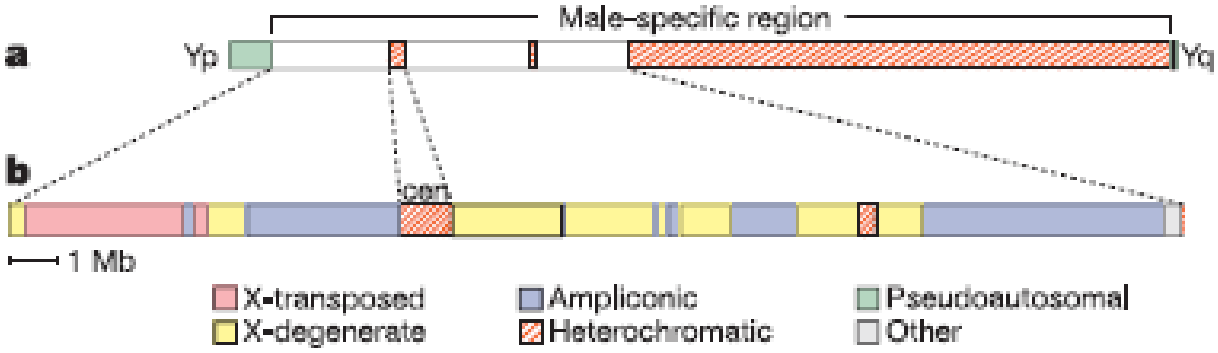
## 1.2 Evolution of Y-chromosomes

The first step in the evolution of differentiated sex chromosomes is the acquisition of a gene that determines 'maleness' (Rice, 1996), which results in the subsequent loss of recombination between former autosomes (see Fig. 1.1.1, taken from Strachan and Read, 1999, for explanation). This pattern of acquisition has occurred many times across different taxonomic groups within plants and animals. The theory that the seX-chromosomes were derived from a pair of autosomes, one of which has subsequently degraded, was proposed very early on (Muller, 1914; Ohno, 1967). It is now widely accepted that a pair of autosomes gained a sex determining function and gradually became heteromorphic (See Fig. 1.1.1) (Graves, 1998).

The evolution of sex chromosomes from an autosomal pair has occurred multiple times across different taxonomic groups (i.e. flies, mammals, birds, plants) by a generalized mechanism (Bakloushinskaya, 2009). The first step in the evolutionary process is the acquisition of a sex determining allele which results in genic sex determination. As a result of the sex-specificity of the chromosome with the newly acquired function, recombination is suppressed between the previously autosomal chromosomes and sex-specific genes start to accumulate on the sex-specific chromosome compounding the effects of recombination and increasing the isolation between the former autosomes (Strachan and Read, 1999). Subsequently, loss of chromosome segments, heterochromatinization and translocations from elsewhere in the genome aid in the break down of homology and the recruitment of sex specific genes to the heteromorphic chromosome (Strachan and Read, 1999). In some species the evolution of the sex chromosomes has continued to a point whereby the heteromorphic pair no longer share any gene content (dunnart) (Toder et al., 2000, Graves et al., 2002), whilst in others the heteromorphic chromosome has been lost all together (mole vole) (Just et al., 2007) (Fig. 1.1.1, taken from Strachan et al., 1999). As a consequence of the consistent evolutionary forces acting on the development of sex chromosomes there are some generalized features of mammalian Y-chromosomes (Fig. 1.1.2, taken from Skaletsky et al., 2003). These include the presence of ampliconic regions as a result of the palindromic hairloops; X-degenerate sequence;

X-transposed; autosomal transposed; a pseudo-autosomal region (PAR) and often large variably sized heterochromatic regions.

**Fig. 1.1.2 Taken from Skaletsky et al., 2003.** a) A schematic representation of the whole human Y-chromosome, including the PAR (green) and heterochromatic regions (red hashed). b, Enlarged view of a 24-Mb portion of the MSY, extending from the proximal boundary of the Yp pseudoautosomal region to the proximal boundary of the large heterochromatic region of Yq. Shown are three classes of euchromatic sequences, as well as heterochromatic sequences. A 1-Mb bar indicates the scale of the diagram.




The marsupial sex chromosomes are thought to include an ancestral form of the mammalian Y-chromosome, which has degenerated extensively from its former homology to the X. Although the minimal Y-chromosome of the dunnart *(Sminthopsis spp.)*, a marsupial species, apparently lacks any post-divergence additions (Graves, 2006), there is evidence of translocation onto the sex chromosomes throughout the mammalia. Whilst marsupials appear to have very limited additions, the monotremes; platypus *(Ornithorynchus anatinus)* and echidnas *(Zaglossus spp. And Tagyglossus spp.)*, which have ten relatively large Y-chromosomes, show evidence of large translocations (Toder et al., 1997; Rens et al., 2007) and additions to the sex chromosomes of eutherian mammals appear to have occurred several times during the evolution of different groups (Graves, 1995) .

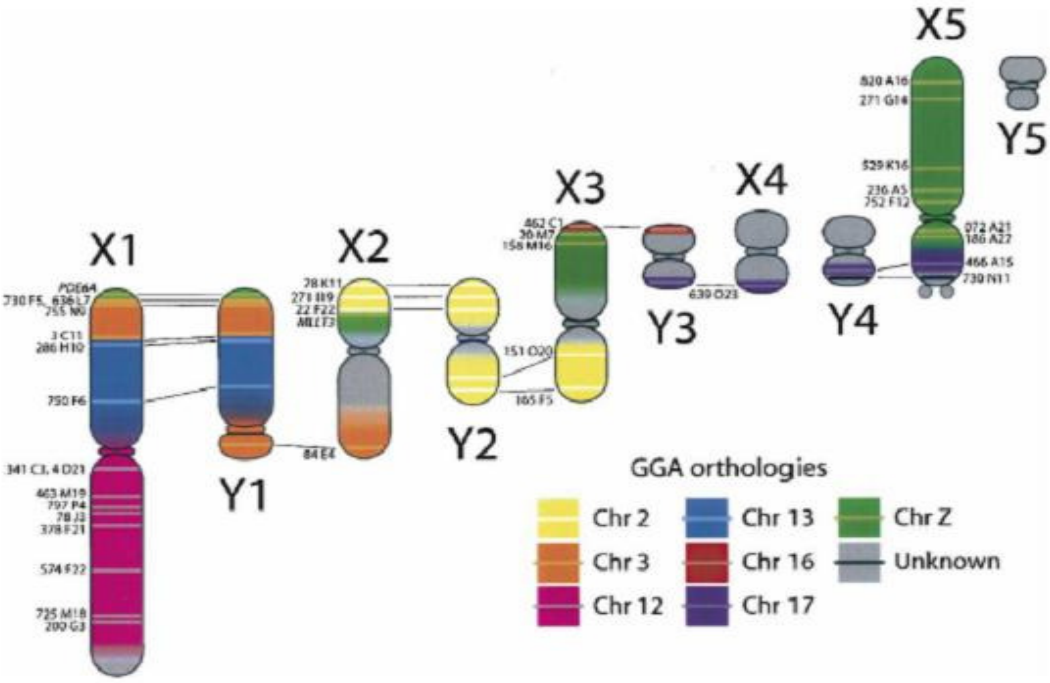
Genetic sex determination has evolved across many taxonomic groups showing some consistency in mechanism; with high levels of genic similarity unrelated to phylogeny. It would be expected that sex determination would remain fairly static but in reality genetic sex determining function spontaneously evolves and continues to evolve

quite rapidly (Veyrunes et al., 2008). There are striking differences between quite closely related species but also surprisingly similar mechanisms of evolution and male-specific gene content, with similar genes being conserved across distant taxonomic groups (Deuve et al., 2006; Rens et al., 2007).

Just within the mammalia there is a wide variety of sex determination systems, which still continue to evolve. The Y-chromosome of some species is becoming progressively smaller as it deteriorates (e.g. the dunnart) (Toder et al., 2000); some have accumulated translocations from elsewhere in the genome (e.g. human) (Waters et al., 2001; Skaletsky et al., 2003); whilst some species have lost the Y-chromosome all together; rather having a sex determining gene on one of the autosomes (mole vole) (Arakawa et al., 2002). This latter is likely a sex determination system at the start of its evolutionary process and potentially, as one of the autosome pair that has acquired the sex determining function is selected to accumulate male-beneficial genes the pair of autosomes may evolve into proto-sex chromosomes in this species.

The karyotype of the platypus (Fig. 1.1.3) shows a set of ten comparatively large sex chromosomes that form a chain during meiosis, carrying many of the same sex specific genes that are found on the Y-chromosome in therian mammals (Rens et al., 2004; Veyrunes et al., 2008).

**Fig. 1.1.3. Taken from Veyrunes et al., 2008**
The 10 sex chromosomes from male platypus with the location of the 32 BACs mapped in this study and four BACs/genes mapped in recent studies (colored bands). BAC numbers are indicated, and homology with chicken is represented by different colors. BACs that hybridized to both an X and a Y revealed pseudoautosomal regions and are connected by lines.




Five genes found on mouse (*Mus musculus*) and human Y-chromosome are also present on that of the wallaby (*Macropus spp*) and dunnart (Toder et al., 2000), indicating that the Y-chromosome evolved its male-specific function before the eutherian clade split off from marsupials ~130 million years ago (Toder et al., 2000). This suggests there is common ancestry for mammalian Y-chromosomes in contrast to the Y-chromosomes of plants and insects which are the result of convergent evolution (Bachtrog, 2005; Marais et al., 2008). In humans, cross-amplification of Y-chromosome heterochromatic regions hybridize to autosomes; in contrast, the dunnart and wallaby do not share any cross homology with the X-chromosome or autosomes (Toder et al., 2000). The dunnart has a ~10Mb minimal and completely differentiated marsupial Y-chromosome that does not recombine and does not share any homology with any autosomes as demonstrated by fluorescent in situ hybridization (FISH) (Toder et al., 2000; Veyrunes et al., 2008), whilst some mammals, such as the mole vole *(Ellobius talpinus)* (Just et al., 1995); and the Japanese spinous rat *(Tokudaia spp.)* (Kobayashi et al., 2007) are missing a Y-chromosome all together.

The Z and W sex chromosomes found in birds, reptiles and butterflies evolved by a superficially similar mechanism to the X-Y mammalian system, in that one pair of autosomes acquired a sex-determining gene, subsequently evolving in to a heterogametic pair, although in contrast to mammals the female is the heterogametic sex (Veyrunes et al., 2008). Aside from the sex determining factor (SDF); however, the systems share very few of the same genes, indicating that they evolved from a different autosomal pair to that of the X and Y (Veyrunes et al., 2008). The superficial similarity of the Z-W system demonstrates that the evolutionary patterning of genetic sex determination is consistent across taxa independent of phylogenetic relatedness (Graves, 1998; Marshall Graves, 2008; Wallis et al., 2008). This convergent evolution suggests that the degeneration of the non-recombining chromosome is an inevitable evolutionary process that is only contingent on the recruitment of a sex determining factor and commensurate with the acquisition of sex-specific function, independent of evolutionary history (Fraser et al., 2004). Although sex determination may originate through different mechanisms across the animal kingdom, there do appear to be certain consistent characters that prevail. Whilst many reptiles, fish and amphibians have a temperature-dependent sex determination system, many of the same genes associated with spermatogenesis in mammals, such as DMRT1 and dax1, are also associated with sex determination and differentiation across the animal kingdom - from flies and worms to humans and fish (Otori et al., 2006; McElreavey et al., 2007; Gribbins et al., 2008; Hoopfer et al., 2008; Yu et al., 2008; Chuma et al., 2009; Sambroni et al., 2009; Xu et al., 2009). For example, the DMRT1 gene, which is sex determining in birds plays an important role in spermatogenesis in mammals and is homologous to the double sex (dsx) and mab-3 transcription factors, which play an important role in male sexual development in flies and nematodes (Waters and Marshall Graves, 2009). The similarity of sex determination mechanisms with an apparent lack of phylogenetic association is suggestive of a strong selective drive coupled with an ease of mechanism (Takehana et al., 2007).

### 1.3 Y-chromosome structure and function within the mammalia

The mammalian Y-chromosome is a haploid largely non-recombining chromosome, characterized by a male sex determining gene (SRY), few other genes and large portions of heterochromatic repetitive DNA (Charlesworth, 2003). These unique characters are as a consequence of their distinct structure and function caused by their singular existence (Bachtrog, 2006). Whilst all other autosomal chromosomes in the genome have a life-long partner, the Y-chromosome has limited alignment with its X-chromosome counterpart across a short, pseudo-autosomal region (PAR), during meiosis (Ellis and Goodfellow, 1989). The location and length of this region varies across taxa, but most species have a reduced x-homologous region and a larger male-specific section, which does not recombine and therefore evolves as a haploid component exempt from the confounding effects of recombination (Toder et al., 2000). This freedom confers its exceptional utility as a genetic marker but it is not without its disadvantages.

The human Y-chromosome, of which the most is known of any Y, is made up of approximately 60Mbp of which 23Mbp is euchromatin and 40Mbp is heterochromatin. It contains 156 transcription units of 7 protein-coding genes, which form 27 protein units, including the SRY male-determining factor and 10 genes expressed exclusively in the testes, which are predicted to provide male-specific function (Bachtrog and Charlesworth, 2001; Sundqvist et al., 2001; Graves, 2002; Rozen et al., 2003; Skaletsky et al., 2003; Bachtrog, 2006).

In humans, aside from the (PAR), consisting of a 2.6Mb at the distal end of the short arm of the Y (Yp) and a second 0.4Mb region at the distal end of the long arm of the Y (Yq), the Y-chromosome does not recombine and as such is clonally inherited (Underhill et al., 2000). In some mammals the PAR consists of only one section; for example the mouse has a 5Mb region (Hillyard et al., 1993), whilst in others the PAR has been completely lost, as in the dunnart where the Y-chromosome does not undergo recombination with the X at all, segregating by filament attachment associated with the synaptonemal complex instead (de la Fuente et al., 2007). The size of the PAR also varies considerably between taxa, for example, the PAR is more extensive in the artiodactyls (specifically – sheep *Ovis aries*) than in humans (Toder et al., 1997).

## 1.4 Y-chromosome genes

Aside from the genes expressed in the PAR, there is a class of multicopy genes that are found exclusively on the Y in the male-specific region (MSY), and expressed solely in the testis (Lahn and Page, 1999). These genes are expected to have male-specific function related to spermatogenesis (Skaletsky et al., 2003).

Genes and their expression on the Y-chromosome are characterized by paucity and specialization. In stark contrast to the high levels of autosomal homology found between even distantly related species (O'Brien et al., 1999), the Y-chromosome exhibits poor conservation at relatively close taxonomic levels, with many genes lost or re-arranged from one species to another. Whilst the mouse and human share a copy of the UBE1 gene on the Y, most primates lack this gene, despite their closer evolutionary relationship (Mitchell et al., 1998). Many of the genes found on the Y-chromosome appear to have a retrotransposition origin as indicated by the presence of highly homologous autosomal sequences and a lack of introns (Toder et al., 1997; Toder et al., 1997; Lahn and Page, 1999). This acquisition of Y-chromosome sequence from the autosomes is supported by investigations in the medaka *(Oryzias latipes)* neo-y chromosome, which is not yet heteromorphic, suggesting a recent acquisition of the X-Y sex determination system in this species (Kondo et al., 2006). Evidence from the neo-y in Drosophila miranda and from the black muntjac *(Muntiacus crinifrons)*, supports the theories of sex chromosome patterns of evolution (Bachtrog et al., 2008; Zhou et al., 2008). Whilst the human Y appears to be at an advanced stage of evolution carrying several translocated regions (Skaletsky et al., 2003), the medaka is at an early stage of evolution, exhibiting comparatively little degeneration (Kondo et al., 2006).

For most species the sex determining region (SRY) and/or the DNA binding protein domain, zinc finger protein (ZFY) have been successfully used for sex testing (Moreira, 2002; Bryja and Konecny, 2003). Whilst in humans polymorphic Y-chromosome sequences have been used to study paternal inheritance and study evolutionary patterns (deKnijff et al., 1997;

Kayser et al., 1997; Roewer et al., 2001) in most other species these two genes represent a large proportion of the knowledge about the Y-chromosome (Palsboll et al., 1992; Berube and Palsboll, 1996; Olivier et al., 1999; Rosel, 2003; Crawford et al., 2008; Lindsay and Belant, 2008; Choi et al., 2009).

Recently a number of studies have started to look at cross-homology of Y-chromosome genes, both between the X and Y and to other species. These studies have informed the understanding of the Y-chromosome indicating differences in the mutation process associated with the male germline and the pattern of degeneration of Y-borne genes. Species comparisons have highlighted certain divergent patterns but also reinforce similarities in the process of Y-chromosome evolution independent of taxonomic group (Toder et al., 1997; Chowdhary et al., 1998; Murphy et al., 1999; O'Brien et al., 1999; Murphy et al., 2001; Raudsepp et al., 2002; Galtier, 2004; Raudsepp et al., 2004; Raudsepp et al., 2004; McFee et al., 2006; Murphy et al., 2006; Pearks Wilkerson et al., 2008; Waters and Marshall Graves, 2009).

The DMY gene that determines male sexual development in the medaka appears to have a conserved binding domain with a high similarity to the bird sex determining gene (DMRT1) (Smith et al., 2009); coding for proteins that are also involved in sex determination in *Drosophila* and *Caenorhabditis elegans* (Kondo et al., 2006). The platypus multiple XY chromosomes do not share homology with the single X and Y of eutherians, instead the platypus chromosome 6 has a number of homologues with the eutherian sex chromosomes, which suggests that the sex determining region was recruited to the present sex chromosomes after the monotreme – therian split (Wilson and Makova, 2009). There are however, homologues to the avian sex determining gene (DMRT1) on the X3 and X5 platypus sex chromosomes (Gruetzner et al., 2006; Daish and Grutzner, 2009), which suggests convergent evolution of Y-chromosome protein function across quite distant taxonomic groups. Despite the temperature dependent sex determination (TD) in most reptiles, studies have shown that the genes involved in the cascade of sexual differentiation notably include the DMRT1 gene, demonstrating extraordinary conservatism across vertebrates, through convergent mechanisms across the Z-

W, XY and TD sex determination methods (see Table 1.4.1). In contrast, whilst there is striking concordance between human and Drosophila repetitive elements for maintaining male fertility (Bachtrog, 2003), the Y-chromosome of two closely related Drosophila species are dissimilar in their structure and content (Carvalho and Clark, 2005) as a result of selective sweeps bringing about rapid changes, as described below. Palindromes on a single Y-chromosome contain complementary sequence that can form internal hairpin loops, which thus pair internally allowing recombination to take place (Repping et al., 2002; Rozen et al., 2003; Lange et al., 2009). This process of gene conversion allows a gene copy on one arm of the palindrome to non-reciprocally replace the homolog on the other arm (Rozen et al., 2003) and as such can result in an active gene copy replacing a mutant form and vice-versa (Graves, 2004; Graves, 2006). Recent evidence from chimpanzees has demonstrated that the gene conversion is biased towards more frequent "good" copies replacing deleteterious ones through a mechanism that conserves the older copy, which is more likely to be non-deleterious (Jobling, 2008) Although it is not known whether the existence of palindromes is a common feature of all Y-chromosomes, there is evidence for both genic and non-genic multi-copy loci across a number of taxa (Olivier et al., 1999; Raudsepp et al., 2004; Luo et al., 2007). The number and copy number of y-borne genes potentially resulting from palindromes varies extensively between species (Toder et al., 2000).

**Table 1** Details genes associated with spermatogenesis in humans and whether they are also found in on the Y-chromosome in other mammals

| Gene | Y-chromosomal genes, functions and Y-linked presence in other mammals |
|---|---|
| **SRY** | SRY is the sex determining gene for most mammals with the notable exceptions of the monotremes (Waters et al., 2009). |
| **ZFY** | The ZFY gene generally mapping to the Y-chromosome in mammalian studies thus far, including felids, canids (Murphy et al., 2006) bovids and equids (Raudsepp et al., 2004), was implicated as the sex determination gene but the discovery of the SRY gene relegated the ZFY gene to an unknown role in spermatogenesis. It shows high expression in the testis by also in other tissues. |
| **AMEL-Y** | The Amelogenin gene AMEL-Y is found on the Y-chromosome in mammals with a homolog on the X (AMEL-X). It is a gene that codes for tooth enamel and is not related to spermatogenesis in any species studied thus far including goats (Pidancer et al., 2006); cetaceans (Mace et al., 2008); felids ( Murphy et al., 2006); and Liu et al., 2002) . |
| **DMRT1** | DMRT1 the avian sex determining gene is a key regulator of sexual development in nematodes and flies; and is preferentially expressed in the testis |

| | |
|---|---|
| | of humans suggesting that it plays a role in spermatogenesis (Smith et al., 2009). |
| **TSPY** | TSPY gene is a testis-specific protein in humans (Chandley et al., 1995) and has homologs in other mammals including felids, canids (Murphy et al., 2006); bovids (Liu et al., 2002) and equids (Raudsepp et al., 2004). |
| **DAZ** | DAZ is a male sterility gene and is exclusively expressed in the testis. Although it maps to the Y-chromosome in closely related primates it has thus far been found to be autosomal in other mammals, for example; felids (Murphy et al., 2006), and equids (Raudsepp et al., 2004). |
| **SMCY** | The SMCY gene has been found exclusively in humans and mice and has been implicated for a role in spermatogenesis, although SMCY knock-out mice still have normal testis development (Agulnik et al., 2001). |
| **AZF** | Although the AZF gene is implicated in spermatogenesis, men that lack the gene still undergo normal spermatogenesis, so its exact role is unclear. Thus far it has been found to be lacking from the Y-chromosomes of other mammals, for example felids (Murphy et al., 2006), and equids (Raudsepp et al., 2004). |
| **USP9Y** | The USP9Y gene codes for a protein required for sperm production in humans but thus far it has not been reported from the Y-chromosome of other mammals. |
| **DFFRY** | The DFFRY gene found on the human and mouse Y-chromosome also maps to the felid Y-chromosome (Brown et al., 1998, Murphy et al., 2006). |

### 1.5 Mechanisms at work on the Y-chromosome and unique functional characters

Whilst the Y-chromosome is carried in all male cells, it is replicated exclusively in the spermatagonia of the male germ line and undergo many more cell divisions than do oocytes (e.g. in humans: 300-700 vs ~20) thus if each cell division confers the opportunity for mutation, it increases exponentially the possibility of spontaneous mutation of the Y-chromosome sequence (Bartosch-Harlid et al., 2003; Goetting-Minesky and Makova, 2006). Furthermore, being located in male germ cells of the testes the Y-chromosome encounters many more harmful mutagens than do the ovaries (Graves, 2004) and sperm itself is an oxidative environment lacking enzymes for DNA repair (Aitken and Graves, 2002). The rest of the genome must also pass through this environment, but unlike the Y-chromosome, the X and autosomes have the opportunity for repair out with the testes and sperm when passing a generation in a female host. (Wyckoff et al., 2002), found that in shared genes on the X and Y, the Y-borne copy of the gene is much more rapidly mutated and (Makova and Li, 2002) found that most *de novo* mutations are derived from the father rather than the mother, supporting the notion that there is a higher Y-specific rate of mutation.

It is widely accepted that there is a male-biased mutation rate with males transmitting more mutations than females to the offspring. A common explanation of this phenomenon is that sperm acquire more mutations than eggs because their progenitor cells undergo more cell divisions each generation and mutations typically result from errors during replication (Ellegren, 2007). Males produce sperm continuously throughout their reproductive lives, while females are born with the full complement of eggs that they will have throughout their lifespan resulting in this higher rate of cell division. This is the cell-division theory for male-biased mutation (sometimes called "male-driven evolution"). This pattern of male-biased mutation is evident in a wide range of species and although the cell-division theory is a logical explanation for this observed condition, it fails to account for why the male germ line would be more tolerant of mutation that are typically deleterious (Blumenstiel et al., 2007). A possible explanation is that if there is a trade-off between quantity of sperm and energetic expenditure to maintain the quality through a reduced mutation rate, sperm competition would select for the greater quantity of sperm; provided that deleterious mutations were not involved with spermatogenesis, as sexual selection would obviously select against those carriers (Blumenstiel, 2007). However, and importantly, the male mutation rate is biased in plants which do not have a similar pre-determination of state for sperm and eggs as do mammals, suggesting some additional or alternative mechanism is involved – in plants at least (Whittle and Johnston, 2002).

### 1.6 Y-chromosome mutation and mechanisms of degeneration

The lack of recombination across most of its length not only results in a loss of homology to the X, but also favours the accumulation of transposable elements from elsewhere in the genome and the incorporation of repetitive material (Graves, 2004). As such, the Y-chromosome contains an unusually high abundance of repetitive DNA (Charlesworth et al., 1994; Bachtrog et al., 2008). Furthermore, the male specific Y-chromosome (MSY) is inherited as a single unit and therefore has a high dependence on the quality of its neighbours, such that if positive selection pressures drive one gene into the next generation it may carry with it

deleterious ones as the two regins are inseperable. As a result of this co-inheritance, genomic processes, such as hitch-hiking and Muller's rRatchet will have extensive implications for the evolution of the y-chromosome (Bachtrog, 2008). Strong selection on one section of the Y-chromosome; therefore, will carry neutral and/or mildly deleterious regions in to the next generation as a result of the aforementioned processes whereby genes are inextricably linked in one inherited unit. In the absence of recombination; however, the effectiveness of selection is also reduced (Rice, 1996; Orr and Kim, 1998), which causes degeneration of genes that were active on the ancestral chromosome (Charlesworth, 1978; Charlesworth, 1991). On the other hand, genes that specifically benefit male fecundity appear to accumulate on the Y suggesting there is some as yet unknown mechanism driving the maintenance of male-specific beneficial genes (Rice, 1996; Charlesworth et al., 2005). Recent studies have indicated a role of palindromes and the multi-copy biased gene conversion process in the maintenance of beneficial male-specific Y-chromosome genes (Jobling, 2008).

The genes associated with spermatogenesis carried on the Y-chromosome may be under positive selection or may be retained as a result of negative selection against deleterious mutants (Engelstadter, 2008). Interpretations of drift due to population structure versus selection against infertility are complicated on the Y (Jobling et al., 1998) and therefore interpretation of markers needs to be cautionary.

Divergence is caused by hitchhiking during positive selection, which may cause 'poor' genes (provided they are not too deleterious) to be retained because they exist on a Y-chromosome variant with advantageous genes (e.g. genes essential for spermatogenesis) (Hughes et al., 2005) or by sexual selection (Roldan and Gomendio, 1999). Background selection may result in 'good' genes being lost as a result of the 'mediocrity' of the quality of the other genes that share their origin on the same Y-chromosome variant (Bachtrog, 2008). Background selection and hitchhiking will inevitably result in the loss of variation and potentially mildly advantageous genes. As a consequence of the lack of recombination the effects of 'Muller's Ratchet' will be compounded with the result that once a variant is lost, it is lost forever

(Gabriel and Burger, 2000). A high variation between individuals and the stabilizing effect of palindromic gene conversion will increase the influence of these processes because gene conversion will inevitably reduce the variation as one strand clonally replaces the other, further reducing the existence of intermediate Y-chromosome variants, (Graves, 2002, 2004, 2006).

### 1.7 Species differences

Graves (2005) suggests that all Y-chromosomes from different taxa are subject to the same forces of degradation as a result of a lack of recombination, increased mutation rate and low selective pressure. However, comparative studies between the human and chimpanzee (Hughes et al., 2005) led to the conclusion that processes can be driven by taxon-specific influences. The human Y-chromosome has been shown to be subject to higher levels of mutation, deletion and insertion than the rest of the genome by a factor of 4.8 (Graves, 2006), resulting in high levels of *de novo* genetic diseases from father to son (Makova and Li, 2002) which will inevitably effect the evolution of the Y-chromosome. At the same time, there is evidence in some species of near-identical palindromic regions that undergo gene conversion acting as a homogenizing force, further reducing the variation between Y-chromosomes (Repping et al., 2002).

Selective sweeps can often be facilitated by the demography and ecology of a particular species(Yi and Charlesworth, 2000). Low population sizes and/or high reproductive success of a few males within the population will accelerate the effects of the on-going processes of background selection, 'Muller's Ratchet' and hitchhiking, already acting on the Y-chromosome, as a consequence of its lack of recombination (Bachtrog, 2008). Thus the rate of degeneration is lineage-specific as it is influenced by the effective population size (Ne), generation time and mating behaviour (reproductive skew) (Orr and Kim, 1998; Charlesworth and Charlesworth, 2000; Steinemann and Steinemann, 2000; Bachtrog, 2006; Buschiazzo and Gemmell, 2006; Bachtrog, 2008; Engelstadter, 2008). For example, the mouse Y-chromosome would be expected to be more degraded and have a higher gene specialization as a result of its short

generation times driving the Y-chromosome to evolve much more quickly driving lethal deleterious mutations to extinction (Nachman, 1998; Toure et al., 2005). Reduced population sizes will increase the probability of the loss of gene variance by drift and therefore will increase the degradation process, for example, following a bottleneck or founder event (Wallner et al., 2004; Lopez-Giraldez et al., 2006). The increased degradation of the chimpanzee Y-chromosome in relation to that of the human is consistent with this expectation, as a result of high levels of skewed reproductive success in the former (Erler et al., 2004; Hughes et al., 2005).

Furthermore, selective breeding of domestic species led to the apparent fixation of few Y-chromosome variant in the horse (Wallner et al., 2004) and domestic dogs (Bannasch et al., 2005). Population bottlenecks, occurring in the wild or as a consequence of zoo-based breeding programs can have profound consequences on the variability of the Y-chromosome.

### 1.8 Y-chromosome in population genetics

Y-chromosomal markers represent a paternal counterpart to mitochondrial DNA (mtDNA) for evolutionary and population genetic studies. The use of y-chromosomal markers in non-model species has been hampered by a lack of sequence information and the difficulties associated with the Y-chromosome (Petit et al., 2002).

The majority of Y-chromosome microsatellites from non-human mammals have been shown to have very little variation and there are few non-model species for which it has been possible to isolate male-specific polymorphic microsatellite loci. Extensive efforts in the felids yielded only one polymorphic microsatellite locus (Luo et al., 2007); screening of BAC libraries in the horse (*Equus ferus caballus*), shrew (*Soricidae spp*) and bovids (*Bos spp*) yielded only two, one and four polymorphic male-specific microsatellites, respectively (Edwards et al., 2000; Wallner et al., 2004; Handley and Perrin, 2006). Even from the human Y-chromosome up until the late 1990s, the amount of Y-chromosome microsatellite variation that had been identified

was very limited, requiring an exhaustive survey to isolate the variable markers, eventually yielding over 200 markers (Kayser et al., 2004).

Y-chromosome microsatellite markers are expected to have similar mutation rates to nuclear loci as they are subject to the same repair processes. In humans, the Y-chromosome has been shown to carry the same types of polymorphic loci as autosomes (Jobling and Tylersmith, 1995; deKnijff et al., 1997; Underhill et al., 1997) with Y-chromosome microsatellite diversity expected to be roughly equivalent to that of autosomes (Roewer et al., 1992; Goldstein and Schlotterer, 1999). However, Y-chromosomes do not pass through female meiosis or mitosis. 200+ SNPs have been identified for the human Y-chromosome (Hammer et al., 1998; Shen et al., 2000; Karafet et al., 2008) and Underhill et al. (2000), describe reduced nucleotide diversity compared to non-Y-chromosome nuclear sequences (autosomes). By contrast, Shen et al. (2000) reported that in 65,120bp of coding and non-coding gene regions, there was a 5x higher mutation rate than corresponding autosomal genes. Despite this, the lack of Y-chromosome diversity is however, explained by a possible combination of drift, the ¼ Ne as a result of neutral theory reducing the genetic variation (Nei, 1987); male reproductive success; and potentially natural selection acting on spermatogenesis genes (Hammer et al., 1995).

While many polymorphic microsatellite markers are now available for human studies, this is largely due to the genomic sequence available for the human Y-chromosome. Prior to the availability of this sequence that made the comprehensive survey of human Y-chromosome microsatellite loci of Kayser et al. (2004) possible, only 53 human Y-chromosome Microsatellites had been identified. Of the 53, several of the polymorphic markers were multicopy loci (12), and thus not suitable for population genetic analysis (Butler et al., 2005). The two main issues of multicopy loci are that not much is known of their evolutionary processes and therefore they potentially violate the expectations and/or assumptions of population genetics principles. Secondly, the scoring process is problematic as it is not possible to be certain of the allocation of alleles to the correct loci. The cautionary guidance is therefore to avoid their use.

It would be desirable to have paternally inherited and polymorphic Y-chromosomal markers in cetaceans for addressing male-specific questions, such as male relatedness in multi-male groups; male reproductive skew; paternity testing; and to compare male and female histories with social structure, in addition to male versus female dispersal and/or migration patterns. A search for Y-chromosome microsatellites in the fin whale, a mysticete species, identified five polymorphic loci (Hatch, 2004). However, to date there are no male-specific microsatellite markers available for odontocete species.

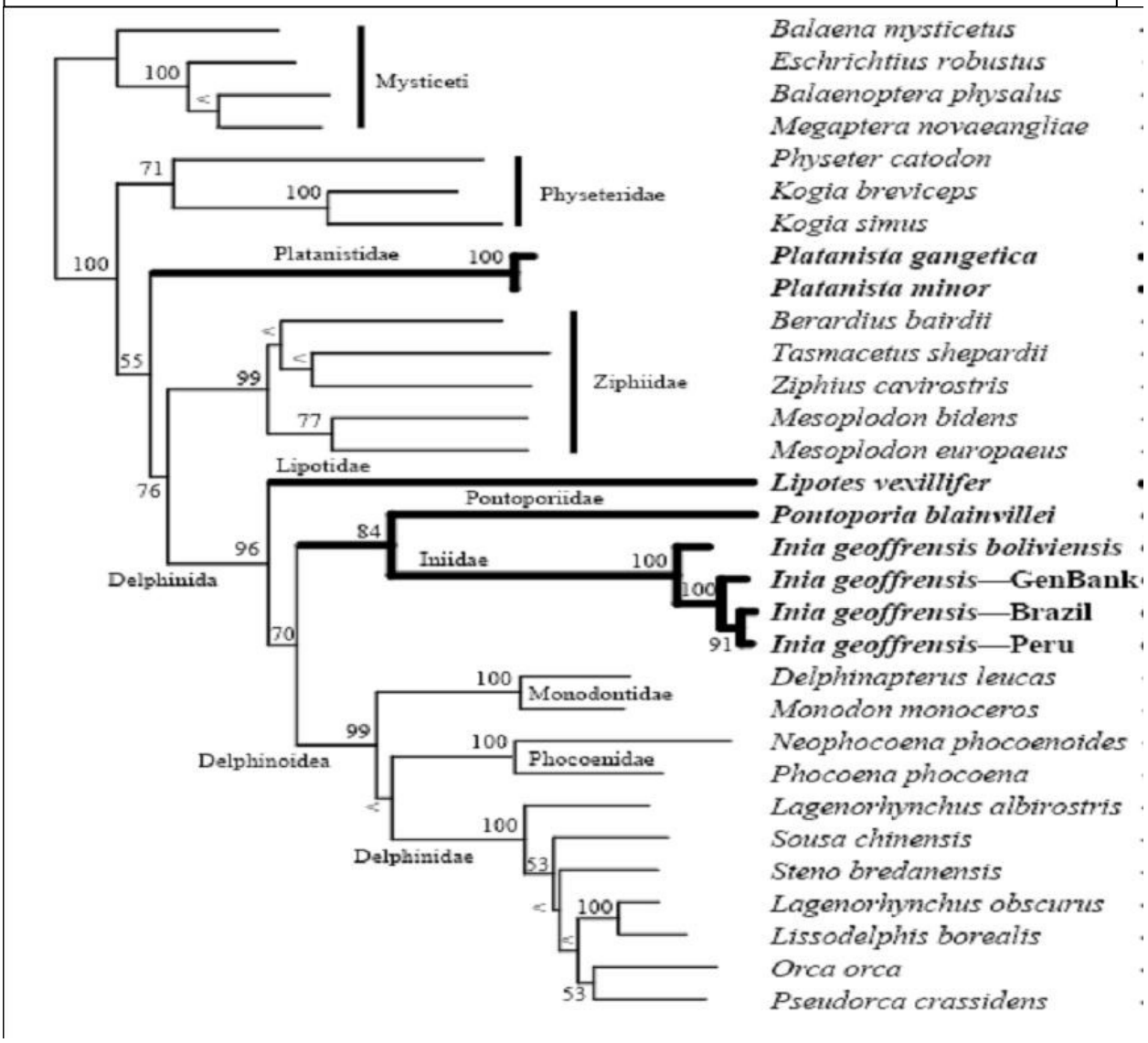
### 1.9 An Introduction to cetacean Taxonomy

The cetacea (whales and dolphins) diverged from the artiodactyla (even-toed ungulates) into two clades; the mysticetes (baleen whales) and odontocetes (toothed-whales). Although the relationships between the odontocetes has not been definitively determined, independent of methodology there are certain placements that are consistent (see Fig 1.9.1, and Appendix 1 taken from Mc Gowen et al., 2009). There is striking concordance between most phylogenies for the placement of the Physeteridae; the genus including the sperm whale (*P. macrocephalus)* basal to the odontocetes (McGowen et al., 2009). The beaked dolphins (Ziphidae) are generally thought to have evolved relatively soon after the Physeteridae (Hamilton et al., 2001), providing a phylogenetic explanation for some of the similarities in karyotype and morphology (O'Brien et al., 2006).

Although the exact evolutionary relationships are not consistent most phylogenies also place the Delphinidae; containing the bottlenose dolphin *(Tursiops truncatus)* in a more derived position (Hamilton et al., 2001). The killer whale *(Orcinus orca)* presents more difficulties, being variously placed as a very recently derived member of the Delphinidae family (Hamilton et al., 2001); the outgroup to the Super family Delphinoidea, which includes the beluga, narwhal and porpoises (Caballero et al., 2008); basal to the Delphinidae family, which just includes the oceanic dolphins; and as part of the subfamily orcininae as a sister taxa to the genus *Orcaella*

(the irrawady dolphin); or *Grampus griseus* (Risso's dolphin); or the false killer whale *(Pseudorca crassidens)* (William et al., 2009).

**Figure 1.9.1 taken from Hamilton et al., 2001**
A maximum likelihood tree of the phylogenetic relationships within the cetacea to the evolution of oceanic and river dolphins. *Physeter catadon* – Sperm whale; *Orcinus orca* – Killer whale; Delphinidae – Oceanic dolphins; Delphinoidea – Super family including Beluga and Narwahl; Delphinida – the dolphins. The maximum-likelihood tree was obtained by carrying out 20 replicate heuristic searches, assuming the HKY85 model of nucleotide evolution with a transition to transversion ratio of 6.0 and a gamma shape parameter of 0.2. Bootstrap values (dervived from 1000 replicates of neighbour-joining searches using Jukes-Cantor corrected distances) are shown in the nodes. Values less than 50 are denoted by '<'.




## 1.10 Y-chromosome utility

The Y-chromosome has been variously used in population genetics to investigate species origins, (e.g.Hammer et al., 1998; Hurles and Jobling, 2001; Mace and Crouau-Roy, 2008); population histories (e.g. Zerjal et al., 1997; Michael et al., 2001; Karafet et al., 2002);

sex-biased admixture (e.g. Hurles et al., 1998; Handley et al., 2006); male to female differences in migration/sex-biased dispersal (e.g. Seielstad et al., 1998; Eriksson et al., 2006); and sex related behavioural differences (e.g. Poloni et al., 1997). Furthermore, the paternal transmission of the Y-chromosome is especially informative for the detection of interbreeding between species (e.g. wolf – dog introgression Vila et al., 2003). Aside from its utility for tracing both male-specific and species history, the unique inheritance of the Y-chromosome can help elucidate particular patterns and processes of genomic evolution (Galtier, 2004). Studying the MSY and Y-chromosomal characters can provide additional insight into the biological significance of recombination and the potential mechanism of evolution and significance of tandem repeats.

This study aims to use a comparative investigation of the cetacean Y-chromosome to add to our understanding of Y-chromosome evolution and male-specific population genetics of cetaceans. The work presented here details the process from obtaining a fresh marine mammal sample for cell culture through the development of Y-specific markers to their application to investigate male-specific history. Chapter 2 explains the development and standardization of a set of methods for obtaining fresh viable samples to produce a growing culture. Chapter 3 describes how the cultures developed in Chapter 2 were put to good use for the isolation of the Y-chromosome, also demonstrating the utility of flow cytometry and producing some interesting by-products and discoveries in the process. Chapter 4 applies the Y-chromosome sequence fragments obtained from Chapter 3 to questions of the similarities and differences of the cetacean Y to the autosomes and to the Y of other species. Finally, in Chapter 5 the development of microsatellite markers enables the investigation of male-specific population genetics in odontocetes, in particular; the sperm whale.

# Chapter 2: Isolation and Culture of Cells from Marine Mammals

## 2.1.0 Introduction

In order to obtain sufficient mitotic cells from which to isolate whole chromosomes for flow cytometry and microdissection, an actively proliferating culture of cells is required (Petit et al., 2002). Unlike model species, which are often available in animal house facilities, or humans from which samples can be readily obtained, tissue samples from marine mammals are logistically more difficult to acquire. Collection of samples relies on access to captive facilities (DeGuise et al., 1996) and/or opportunistic collection during large-scale collaborative efforts from wild caught animals (Beineke et al., 2004; Hall et al., 2006) and/or from strandings (Duffield et al., 1991). As such, it is important to identify optimal conditions to maximize the potential success. This chapter details the experimental evaluation of methodological variables that were assessed in order to identify the most efficient and effective conditions. The lymphocyte culture method was assessed with variable transport, storage and assay conditions, with the object of standardizing the protocol (as far as was possible) for obtaining longer-term viable cultures of lymphocyte cells. Corneal epithelial cell cultures were also established from stranded marine mammals, and were assessed for their growth characteristics. A particular aim of the lymphocyte assay was to achieve proliferation of the immunological system for the purpose of harvesting large numbers of cells for chromosome isolation regardless of contaminant load.

While research groups have been attempting to culture marine mammal cells since the 1960s, it is not until recently that greater success in routine culture has been possible, with many authors still noting particular difficulties in maintaining marine mammal cultures. In particular, studies have cited slow *in vitro* proliferation and long doubling times (Marsili et al., 2000), as well as a propensity to develop contamination at a relatively early stage, for example (Cecil and Nigrelli, 1970). Early attempts with Pacific white sided dolphin *(Lagenorhynchus obliquidens)* kidney primary cultures found that it took a week before cells started to proliferate (Cecil and Nigrelli, 1970), compared with the usual two to three days for human primary cultures (Mather

and Roberts, 1998). Marsili et al. (2000) also found that only after two to three weeks did their epithelial cell cultures start to form partial monolayers, which then took more than four weeks to reach confluence for passage. This is distinct from non-transformed human cells and model cell culture species, which tend to be passaged every two to three days at low passage number (Mather and Roberts, 1998). Similarly, more recently Marsili et al. (2000) derived common dolphin *(Delphinus delphis)* cultures from tissue taken by biopsy dart and found that cells also demonstrated reduced growth rates, noting that it took 7-21days to establish, only reaching confluence after 15-20 days.

Yu et al. (2005) produced a bottlenose dolphin *(Tursiops truncatus)* epithelial transformed cell line that grew more rapidly, showing a similar growth rate to that of non-transformed human cells of three to four days. In comparison, the non-transformed control cultures grew to confluence after six days in culture. Pine et al., (2004) report a similar increase in the rate of division of a bottlenose dolphin kidney epithelial cell line following transformation of a slow growing culture. Wang (2000) found significantly reduced growth rates from a spotted dolphin *(Stenella plagiodon)* kidney epithelial culture and a susceptibility to contamination. Although, Carvan et al. (1994) reported relatively short doubling times of 1.3 days at early passage numbers and seven days to grow to confluence for dolphin cell cultures derived from a premature calf, this is still longer than the normal 16 hour doubling times of human derived untransformed cultures (Vasiliev and Gelfand, 1981). In addition, they acknowledged that cetacean cells are usually difficult to grow and that their shorter times were unusual, citing the difficulties of rapid loss of viability and slow growth rate incurred by Andrews et al. (1973) and Smith et al. (1987), as more normal. The culture of Carvan et al. (1994) was initiated from neo-natal kidney, which is likely to have more proliferative potential than tissues derived from older animals that have been found stranded on a beach, (which may also have been diseased and/or dead for some time) and as such, may explain the greater degree of success that they achieved. Duffield et al (1991), described very rapid onset of cell growth at culture initiation from explants derived from corneal cells. Corneal tissue has regenerative properties and therefore has greater

potential to proliferate *in vitro* than tissues derived from other sources (Van Horn et al., 1977; Duffield et al., 1991). Although the majority of corneal cultures established by Duffield et al. (1991) did proliferate within 24 hours of initiation, outgrowth for certain specimens was delayed for two or three weeks, emphasizing the importance of age and condition of the initial tissue from which cultures are derived.

It has been found that cells derived from large, long-lived species tend to grow more slowly in culture; for example human cell cultures grow more slowly but complete more population doublings before effectively 'senescing', than cells derived from mice (Hornsby, 2003). A study by Rohme (1981), further demonstrated that there was a clear negative correlation between the longevity of a mammalian species and the *in vitro* growth rate and a positive interaction between longevity and the life spans of their cultures. As marine mammals are large, long-lived species, this may explain their slow *in vitro* growth rate.

The environmental differences and physiology associated with an aquatic existence may also impact the *in vitro* performance of cell cultures derived from marine animals. Several groups have attempted to incorporate these conditions into their lymphocyte culturing routine with variable success. Whilst the majority of marine mammal cells have been cultured in standard (37℃, 5% $C0_2$) culture conditions, for example, (DeGuise et al., 1996; Beineke et al., 2004); some groups have attempted to modify the culture temperature. For example, the National Marine Cell Line Library have varied their incubation temperatures between 35-37℃, to reflect differences in the marine environment, with varying success depending on the species, and tissue type (Wise and Wise, 2005; Wise et al., 2009) whereas Duffield et al., (1991) modified the incubation temperature to 36℃, with apparent increased performance of the assay. Kniazeff et al. (1967) required an increased L-Glutamine concentration in their culture medium; however later studies that used reduced standard doses demonstrated improved culturing conditions (Carvan et al., 1994; Marsili et al., 2000; Pine et al., 2004). Attempts in marine species to apply focal species serum in lieu of foetal bovine serum (FBS), have also shown variable success (DeGuise et al., 1996; Bertram et al., 1997; Keller et al., 2005) whereas Lahvis

et al. (1995) routinely cultured cells in 6-8% $C0_2$ in lieu of the standard 5%. Although attempts have been made to incorporate physiological differences into the cell culturing protocol for marine mammals and other species with differing body temperature (Keller et al., 2005), these studies have not demonstrated an improvement in the applied standard human culturing conditions. This may suggest that these conditions are best suited to the culture setting irrespective of the living environment of the species and that a reduced response in some species is a consequence of a slower *in vivo* rate of growth rather than an artifact of cell culture techniques having been conceptualized with other species and therefore requiring adaptation.

A further complication with marine mammal cell culture appears to be the propensity to develop contamination in the latter stages of growth. Cecil and Nigrelli (1970), established kidney fibroblast cultures from both seal and dolphin cells and observed high susceptibility to virus contamination, which they suggest may have already been present in the tissues used to initiate the culture. Given the culturing environment, they suggest that the virus was able to preferentially proliferate away from the immunological environment that kept their levels low *in vivo*. Andersen and Friedrich (1988), cultured lymphocytes from blood samples collected from the open vena jugularis of a long-finned pilot whale *(Globicephala melaena)*. Subsequently they had difficulties in establishing lymphocytes, partly as a result of high levels of contamination, which were likely caused by the method of collection (from an open wound as opposed to from a sterile venipuncture). High levels of contamination were also observed in primary cell cultures established by Pirtle and Kniazeff (1968) from dolphin kidney cells and Marsili et al. (2000) from biopsy samples. More recently, most marine mammal studies have generally kept their culture period to a minimum (1-6d) to avoid the pitfalls of contamination (Duffield et al., 1991; DeGuise et al., 1996; Beineke et al., 2004). However, some methodological approaches preclude the use of short-term cultures (for example, obtaining sufficient cells for flow sorting and cell age-related characterization). These various studies indicate that the potential to establish viable cell cultures from marine mammal tissue is fundamentally dependent on the condition and character of the starting material.

### 2.1.1 Advantages and disadvantages of lymphocyte and corneal cell culture

Most cell types have a limited *in vitro* life span as a result of the exhaustion of their proliferative capacity once they reach replicative senescence, unless they are transformed (Sourlingas et al., 1999) Studies of human T-lymphocyte cells indicate that they appear to die by apoptosis once they have reached their proliferative capacity (Perillo et al., 1995). Human T-lymphocyte cultures show a progressive prolongation in their cell cycle with increasing numbers of population doublings (Cristofalo and Pignolo, 1993; Perillo et al., 1995) but with no difference in cell size between late and early population doublings (Perillo et al., 1995), in contrast with human fibroblasts, which develop substantial heterogeneity as they continue through successive cell cycles. Furthermore, chromosome doublings and other chromosomal abnormalities have been noted in epithelial and fibroblast populations at higher passage number (Grubeckloebenstein et al., 1994). Evidence from marine mammals is consistent with findings from human cultures, several studies having reported a characteristic slowing in rate of growth at higher passage numbers and a finite *in vitro* life-span (Marsili et al., 2000; Pine et al., 2004; Yu et al., 2005). For example, Carvan et al., (1994) reported that kidney epithelial cells from dolphins had a finite life-span in culture of about 50 population doublings and showed characteristic slowing in growth rate, morphological changes of the cell, and the start of abnormal karyotype numbers (aneuploidy) at higher passage numbers. Similarly a fibroblast culture from dolphin tissue exhibited signs of senescence and cellular abnormalities after approximately four months in culture (Marsili et al., 2000).

Lymphocytes can be stimulated to proliferate rapidly and repeatedly with the application of mitogens. The lymphocyte assay represents a convenient and tractable source of cells from animals kept in captivity, from which aseptic blood samples can be collected. However, obtaining a blood sample from wild-caught animals can present particular logistic difficulties. Several studies have shown that the Lymphocyte-transformation-assay can successfully be applied to marine mammal species for short-term cultures of up to approximately six days for example, (Lahvis et al., 1995; Beineke et al., 2004) (Dimolfettolandon et al., 1995; Lahvis et al.,

1995; DeGuise et al., 1996; Beineke et al., 2004). Previous studies, however, have applied various methodologies with inconsistent conditions and results. It is yet to be revealed whether this methodology will provide sufficient cells for chromosome isolation and flow sorting.

On the other hand, adherent cell cultures can be established from tissues taken from stranded animals and/or from biopsy sampling. Unfortunately this latter source will only be available opportunistically and can be subject to an increased risk of contamination, often showing reduced *in vitro* potential. Cell cultures established from corneal cells however, are less likely to develop contamination, as the ocular cavity remains sealed from the environment post-mortem (Duffield et al., 1991). In addition, stem cells, which have been found to exist in various regions of the eye including the outer edges of the cornea, have been found to persist even into old age (Baker, 2008; Majo et al., 2008). Cultures derived from corneas from any age of animal will therefore, be more easily established than those from most other tissues; as the cornea provides a source of stem cells which will readily proliferate (Baker, 2008; Majo et al., 2008). If the eye remains intact, cells remain moist within the oculum and therefore are less likely to become dried and lyse over extended periods. It is therefore possible to establish adherent cell cultures from marine mammals but as mentioned above, care must be taken over the quality of the starting material and the risk of chromosomal abnormalities being expressed at later stages of culture**.**

Both lymphocyte cell cultures and corneal cell cultures have their advantages and disadvantages: lymphocytes can be readily isolated from fresh blood drawn by a minimally invasive procedure; corneas present a reasonably sterile environment in dead animals. Ultimately when relying on opportunistic availability, taking advantage of several options is wise. Therefore, given the nature of the two methods and the availability of materials, it was decided that this study would focus on the culture of mitogenically stimulated lymphocytes and corneal epithelial cells (when samples became available). However, due to the number of cells required to obtain chromosomes for further experimentation and the reduced growth rate and potential for contamination of cells derived from alternative sources (i.e. liver, kidney tissue, etc), the

aforementioned methods represent the best source of cells from live and captive animals, and from dead animals, respectively.

### 2.1.2 Collection Procedure

Accessing cells for *in vitro* culture from wildlife presents unique challenges regarding sample collection and storage, especially if studies are conducted in remote locations or there is a significant delay before samples can be removed from the field location or collection facility to the lab for analysis (Lavoie and Grasman, 2005). Several studies have noted a reduced proliferation following delay between collection and the establishment of cells in culture, for example (Lahvis et al., 1993; Nakata et al., 2002; Beineke et al., 2004). Previous studies using lymphocytes from cetaceans, e.g. (Lahvis et al., 1993; Nakata et al., 2002; Beineke et al., 2004) and other mammals e.g. (Franklin et al., 1994; Lavoie and Grasman, 2005) have noted a sharp decrease in cell viability when drawn into heparinized tubes and stored before cell separation and seeding the culture. Lahvis et al. (1993) noted that cell viability reduced over time, and demonstrated a substantial reduction in viability following a delay of more than 30 hours prior to seeding the culture.

The temperature at which the samples are kept until separation also appears to have a significant effect on the viability of the lymphocyte cell culture. Despite standard blood cell culture recommendations for sending blood from livestock at 4°C (McClure et al., 2000), Lahvis et al. (1993) stored and shipped blood samples at ambient temperature and viability was maintained for up to 30 hours whereas Ross et al., (1996) stored seal blood on ice until they were shipped at 4°C, and resulting lymphocyte viability was significantly reduced. A study using equine lymphocytes compared the proliferation capacity of fresh versus stored refrigerated lymphocytes, finding a reduced response of the latter (Witonsky et al., 2003) and also noted a decrease in cell viability of storing cells at 4°C for 24h. In human testing facilities there appears to be a shift towards a tendency to recommend shipment of blood samples at room temperature; however, the marine mammal community has not followed suit, with the majority detailing

methodological protocols including some degree of chilling of the sample, before isolating the lymphocytes (Dimolfettolandon et al., 1995; DeGuise et al., 1996; Beineke et al., 2004; Keller et al., 2005). Given the nature of their habitat, range and the likelihood of samples being collected opportunistically, it is important to identify and optimize methods of collection, storage and separation to maximize the cell culture viability from marine mammal blood samples.

Although some studies have noted a negative impact of storage, the procedure applied by different studies has been variable and the reduced efficiency of different approaches was not compared nor quantified. When a certain amount of storage is necessary prior to application of the Lymphocyte Transformation Assay (LTA), it is important to define and quantify the confines within which the assay can be successfully applied. Determining optimal methods of sample or cell preservation to maximize cell viability dependent on the circumstances of shipment will benefit the success of the assay. Initially this study assessed the cell viability over time under alternative storage conditions in an attempt to standardize the procedure and to determine the limits of condition variability. For subsequent collections the most effective conditions were applied to maximize the efficiency of the assay, resulting in large numbers of viable cells for effective lymphocyte proliferation.

### 2.2.3 The Lymphocyte Transformation Assay (LTA)

The aim of the study was to optimize conditions for the establishment of cell cultures and to maximize the number of cells available for subsequent cytological and molecular studies. The lymphocyte-transformation-assay is based on polyclonal stimulation of lymphocyte populations and is accepted as a technique to evaluate lymphocyte function; and this study exploited this assay to maximize the number of cells containing genetic material (i.e. metaphase chromosomes) available for subsequent cytological and molecular studies, including flow cytometry and metaphase preparations.

Leukocytes (or white blood cells), which are an integral component of the vertebrate immune system, are divided into several cell types, which include lymphocytes, (B-cells, T-cells,

and Natural Killer cells). Both are present in vertebrate blood, B-lymphocytes are responsible for humoral immunity, involving antigen recognition by antibodies; and T-lymphocytes are involved in cell-mediated immunity, which results in activation and release of cytokines in response to an antigen. Lectins, which are glycoproteins of non-immune origin, react with specific sugar residues on cell surfaces. By a process of agglutination of erythrocytes and leukocytes and/or precipitation of complex carbohydrates, lectins are useful mitogens that cause the proliferation of lymphocyte cells as a result of their activity. Acting as an antigen, lectins cause lymphocytes to divide and proliferate specifically by inducing mitosis in T-memory cells, which have a prolonged life-span in the blood and retain an immunological 'memory' of specific intruders thus recognizing them as such. The lymphocyte transformation assay relies on initial fractionation of the blood into its separate components of red blood cells, plasma, granulocytes and the all important mononuclear T- and B-cells.

Isolating and stimulating a proliferating population of lymphocytes for cell culture can be useful for a number of important applications, from immunological function assays (Keller et al., 2005), environmental contamination assessment (Lahvis et al., 1995) and population monitoring (Hall et al., 2006) to flow cytometric analysis of chromosomal characters (O'Brien et al., 1993), isolation of individual genes and cross-homology to focal species (Beineke et al., 2004) and molecular cloning of purified samples (Inoue et al., 2001). The ability to isolate and maintain cells for short and long-term lymphocyte studies has far-reaching applications. Although the Epstein Barr virus (EBV) has been used to immortalize lymphoblastoid cell lines in humans and various other species (Amoli et al., 2008)**,** the activity of the disease, aside from changing the character of the cell line into a carcinomic one, relies on species-specific virus recognition. So far there is no evidence that the EBV infects marine mammals and therefore can not be used to transform their cell lines.

*In vitro* mitogen-induced lymphocyte stimulation, which occurs in the LTA, mimics the immune response of antigen stimulation following an immunological challenge, *in vivo* (Kristensen et al., 1982). Mumford et al. (1975) conducted a preliminary investigation of the

immune response of peripheral blood lymphocytes (PBLs) from two dolphins, two killer whales and a pilot whale. However, prior knowledge of the species-specificity of the assay was unavailable and optimization was therefore not conducted to account for this. In particular, it was found that the response of the pilot whale was not consistent with that of the other two species. Andersen and Friedrich (1988) used PBLs to establish temporary cultures for karyotyping long-finned pilot whales but had great difficulties establishing viable cultures, demonstrating the need to assess species-specific parameters prior to conducting experimentation and taking care with the sterility of collection method. The majority of lymphocyte studies on cetaceans have focused on the bottlenose dolphin (*Tursiops truncatus)* reinforcing the potential utility of the assay and defining improved culture conditions, including identification of mitogen-specific responses (Colgrove, 1978; Lahvis et al., 1993). However, the conditions tested by the various studies were not comparable, and optimal conditions identified by each of the studies were considerably variable. For example, Beineke et al., (2004) developed a quantitative assay to optimize mitogen stimulation of porpoise lymphocytes, which produced different results to those acquired for dolphins, thus reinforcing the requirement of assay optimization on a species-by-species basis.

Specifically, and in contrast to other marine mammal studies, porpoise lymphocytes responded better to the mitogen Phytohaemagglutinin (PHA) than to Concanavalin-A (Con-A) (Beineke et al., 2004). Mumford et al. (1975) noted species-specific differences in the response and its magnitude indicating that comparative values may also be of interest. Lahvis et al., (1993) further noted that the variability in mitogen-specific response extended to the individual level, something that has also been determined in many other species, including belugas (DeGuise et al., 1996), turtles (Keller et al., 2005), porpoise (Beineke et al., 2004), ducks (Bertram et al., 1997), cattle (Franklin et al., 1994; Miniscalco et al., 2003) and chickens (Talebi et al., 1995). These studies also found that the experimental parameters of the assay can have a major impact on the outcome of the study. Results from previous studies with marine mammals have been equivocal in their stimulation responses to mitogen; however, they

highlight a need to identify optimal stimulation criteria on an individual basis. In particular, Mumford et al. (1975) found that maximum response to mitogen stimulation occurred after three days in culture, which might have subsequently reached a maximum with continued culture. The study found that killer whale cells responded well to PHA stimulation but the pilot whale cells did not. Mumford et al., (1975) used the B-cell mitogen poke weed mitogen (PWM) in addition to PHA, to stimulate bottlenose dolphin PBLs, and found that PWM gave a much higher stimulation index than did PHA, showing a 53-70% rate of stimulation. This result contrasts significantly to results obtained by a different group, using the same species, who found very little response to PWM (Lahvis et al., 1993). The increased response to Con-A over that of PWM of the latter study is more consistent with the results of studies in other species where Con-A consistently showed higher rates of stimulation (Beineke et al., 2004). In addition, differences in timing of cultures have been recognized. Maximal stimulation occurred at six days with Con-A and five days with PHA in bottlenose dolphin (BND) (Lahvis et al., 1993). Killer whale and bottlenose dolphin were maximally stimulated on day three with PHA, whilst pilot whale showed a poor response to stimulation after seven days in culture (Mumford et al., 1975).

In spite of their complexity, lymphocyte-transformation-assays have been exploited successfully for a number of subsequent investigations in marine mammals, including karyotyping (Arnason et al., 1980; Andersen and Friedrich, 1988), assessment of immuno-suppression caused by environmental contaminants (Lahvis et al., 1995; Ross et al., 1995; deSwart et al., 1996) and cytokine expression (Inoue et al., 2001; Beineke et al., 2004). In order to use lymphocytes for successive experimentation, it is necessary to develop and quantify reproducible methods for successful proliferation. By establishing base-line response values it will be possible to apply the conditions identified in this study for additional experimental purposes. For example, knowing the baseline variation resultant from the assay itself, it would be possible to draw more meaningful conclusions from variations observed after modifying experimental conditions.

The mitogen stimulation study was ultimately prompted by the lack of agreement between published methodologies for cetacean species. The only study to apply the LTA to killer whales (Mumford et al., 1975) did not determine optimal concentrations; furthermore, as with an early study of bottlenose dolphin lymphocytes, concentrations were not well defined (Colgrove, 1978). De Guise et al. (1996) applied the LTA to belugas and found optimal responses to Con-A between 0.5 and 2µg/ml were slightly greater than those to PHA, which maximally stimulated between 0.39 and 1.56µg/ml. In contrast, Lahvis et al. (1993) found maximal stimulation from 2.5-5µg/ml of Con-A and a highly reduced response to PHA, which maximally stimulated at 2µg/ml. However, in a later study from the same group, Con-A was found to stimulate maximally at 0.5 and 0.13µg/ml whilst PHA stimulated maximally at 0.5 and 2.0 µg/ml, in different individuals (Lahvis et al., 1995). Inoue et al. (2001) found a positive proliferative response in bottlenose dolphins using 7.5µg/ml of Con-A. Finally Beineke et al. (2004) applied the LTA to porpoise and found that PHA across a range of 2 to 10µg/ml performed marginally better than Con-A, which maximally stimulated at 2 to 5µg/ml and showed less variation. Likewise with harbour seals optimal stimulation methods appear to be equivocal. De Swart et al. (1993) found that Con-A elicited a maximal proliferative response at 5µg/ml. However, whilst Ross et al. (1993) applied the optimized concentration of 5µg/ml of Con-A, Neale et al. (2005) applied an optimal concentration of 20µg/ml PHA.

There appears to be a highly different optimal response between species in addition to highly variable responses between individuals observed by most studies. The range of mitogen concentrations utilized in this study included all the values found to elicit a reasonable response by the various studies in marine mammals. Previous studies have also maintained cells in culture only for short periods, up to five days before harvesting, whereas this study aimed to keep cells growing for longer periods in order to maximize the total cell numbers.

An assessment of the appropriate experimental parameters, including storage conditions, separation method, stimulant and time in culture was required to achieve ‘long-term’ stimulation

of lymphocyte cultures for bottlenose dolphin, killer whale and grey seal to obtain sufficient cells for chromosome isolation and flow sorting.

### 2.2.4 Aims

1. To identify “best practices” for storage of blood samples taken from marine mammals and outline the limitations
2. To elucidate repeatable and consistent mitogen stimulation conditions and extend the longevity of the lymphocyte stimulation assay (LSM)
3. To establish adherent cell cultures from marine mammal corneas for subsequent applications

## 2.2.0 Methods

### 2.2.1 Lymphocyte cell cultures

Several experimental procedures were trialed in order to identify the optimal conditions for lymphocyte proliferation. Time-delay and storage condition are important variables of the lymphocyte stimulation assay, especially when conducted with wildlife. Therefore, the effect of time delay and storage temperature on lymphocyte viability and proliferative capacity were tested. The collection procedure does not appear to be species-specific and blood samples from the focal marine mammals are limited. In order not to waste the precious focal material of this study therefore, time trials and storage conditions were initially tested with 3 surrogate species: ovine (n=64); bovine (n=8); and rat (n=8) prior to their application in the focal species. One sample from each individual of the focal species: grey seal; killer whale and bottlenose dolphin, was subsequently used to verify the validity of the surrogate system, prior to use in continued experimentation and application of the LTA. By studying the effect of time delay on viability it was therefore, possible to define the limiting period and conditions for successful isolation of viable lymphocytes.

#### 2.2.1.1 Collection Procedure (Surrogate Species)

Blood samples from livestock ovine (n=64), bovine (n=8) were collected opportunistically, into heparinized tubes whilst conducting routine veterinarian immunological blood collection procedures. Rat blood samples (n=8) were collected from individuals bred for unrelated experimentation, prior to their sacrifice.

***Experimental parameters***

A) Transportation Storage – two conditions: as whole blood or isolated lymphocytes; at three temperatures: body temperature (~37℃) or on ice/refrigerated (4℃). The effect of temperature and storage condition was assessed by cell viability analysis and subsequent stimulation index.

B) Pre-seeding time-delay – <4hrs, 8h, 12h, 24h, 28h, 32h, 36h, 44h, 48h

The effect of delay before seeding was determined by assessing the percentage cell viability at the given time periods. To ensure that cells that appeared viable were able to exhibit a proliferative response, isolated cells were seeded following counting.

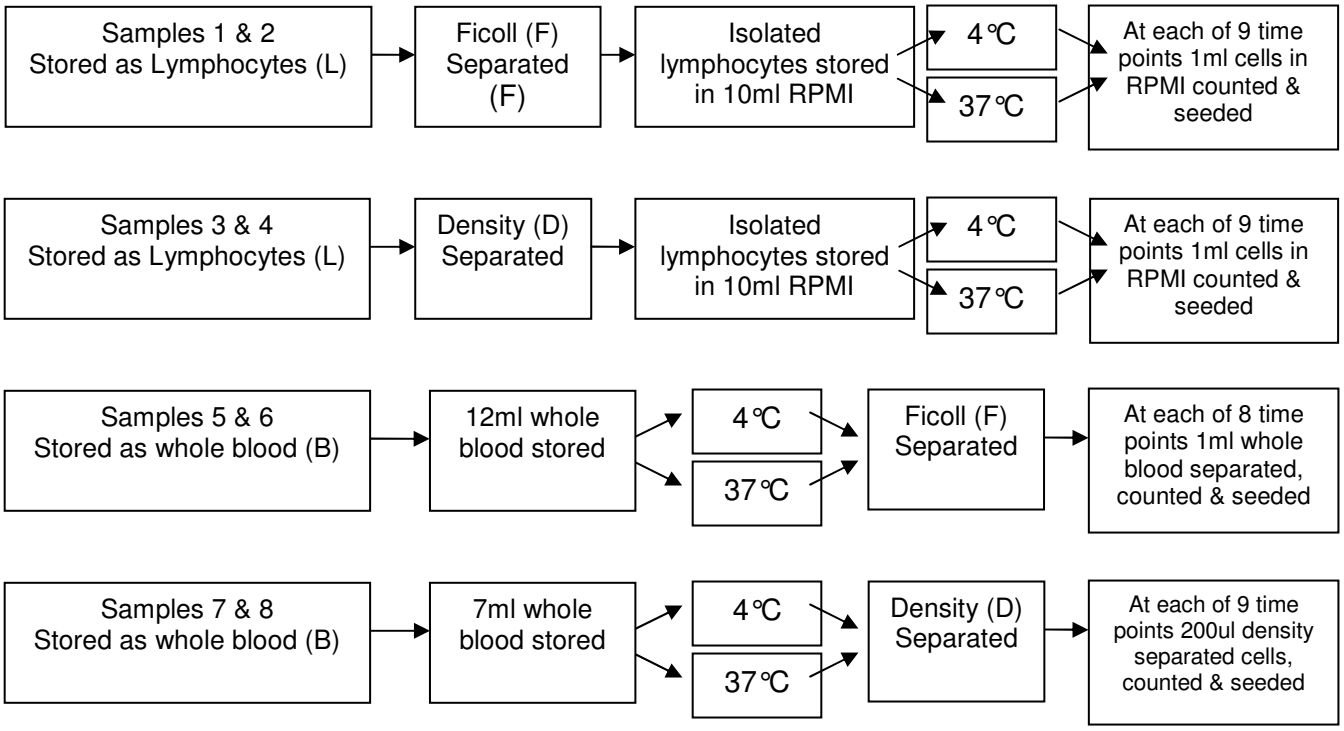
C) Lymphocyte separation – two methods: using a ficoll-density gradient with centrifugation (LSM), and density settlement (as detailed below).

D) It was also possible to assess whether there was an interaction between the storage condition, temperature and isolation method.

#### 2.2.1.2 Storage Conditions (Surrogate Species)

Samples were collected locally and delivered to the laboratory within one hour of venipuncture. Each set of eight conditions was repeated eight times (replicates = 8 for each condition). In each replicate, one sample was processed for each of eight conditions, as detailed in table 2.2-1, below.

**Table 2.2-1:** Shows the storage and processing conditions of for the eight replicates.



Each sample for a set of eight (one per storage condition) was drawn into a heparinized tube from a different animal, on the same date. Two 6 ml samples were taken from two of the eight individuals, each replicate because the 7 ml capacity of a single tube was insufficient to incrementally separate 1ml at each of 9 time points, as required by the whole blood/ficoll separation method (see below). The 8 treatments were repeated 8 times with samples from different animals collected on different dates, with the exception of sets 7 & 8, which were both collected on the same day. With the exception of samples 7 & 8, which were refrigerated prior to collection, all samples were drawn fresh at the time of collection.

At the first time point (T=0), the samples to be stored as separated lymphocytes suspended in medium were counted with trypan blue to establish the level of cell viability prior to storage. Samples to be stored as whole blood were not counted prior to storage at T=0 because the ficoll procedure requires 1ml of whole blood for lymphocytes to be isolated for counting, which would have further restricted the time points, therefore the viability from the lymphocyte stored samples was averaged and used for all T=0 values.

Samples to be isolated and stored suspended in Roswell Park Memorial Institute medium (RPMI), separated by the 'density settlement' method were processed after having isolated the cells to be stored in RPMI that were processed using the 'ficoll' separation method to allow the cells in the former preparation to settle out. It also resulted in all samples having been retained at room temperature for equal amounts of time prior to storage in their respective conditions. Assessment of cell numbers and viability was conducted at each of 9 time points (<4hrs, 8h, 12h, 24h, 28h, 32h, 36h, 44h, 48h), as follows:

- 200µl of buffy coat suspension was withdrawn from each of the two 'density settlement' tubes (one at 4°C, one at 37°C), counted and seeded.

- 1ml of whole blood from each of the two 'ficoll' tube (samples were inverted to resuspend lymphocytes that had settled out), was processed, counted and seeded.

- 1ml of cells suspended in RPMI (samples were inverted to resuspend lymphocytes), was withdrawn from each of the four tubes ('ficoll' 4℃/ 'ficoll' 37℃/ 'density' 4℃/ 'density' 37℃), counted and seeded.

### 2.2.4 Collection Procedure (Marine Mammal Species)

Cetacean blood samples were collected on two separate occasions from animals housed at Marineland Park, Antibes, France. Results from storage methodological trials and mitogen stimulation from the first collection were analyzed and informed the second collection group protocols to ensure that optimal conditions for cell stimulation and proliferation were used. Methodological trials included storage time effect on cell viability, storage temperature, storage condition (blood or lymphocytes) and cryopreservation. Cetacean blood samples were drawn by venipuncture from a vessel on the ventral aspect of the tail fluke with a 20-gauge needle directly into sterile heparinized tubes, at the time of collection. On the first occasion 3 x 7ml of blood was taken from each of five killer whales (Freya, Inouk, Sharkane, Wiki, and Valentin); and 2 x 7ml of blood was taken from each of seven bottlenose dolphins (Alize, Fenix, Lotty, Malou, Neo, Sharky and Silver), transported to Durham University at body temperature (~37℃) or on ice (4℃) within six hours of venipuncture. On the second occasion, 4 x 7ml of blood was taken from each of five killer whales, as above; and 3 x 7ml of blood was taken from each of only three individuals (Alize, Fenix, Silver), due to the increased logistic requirements of sampling from bottlenose dolphins. The second sampling group was transported at body temperature (~37℃) to the University of Cambridge within five hours of venipuncture. Grey seal blood was collected from animals recuperating at the Seal Sanctuary, Truro, by venipuncture from the jugular vein directly into sterile heparinized tubes, on two separate occasions. The first samples (n=2) were transported to Durham University by Royal Mail courier at ambient temperature on the day of venipuncture within 12 hours of collection. The second samples (n=2) were collected and transported at body temperature (~37℃) to the University of Cambridge.

#### 2.2.1.4 Storage Conditions (Marine Mammal Species)

Due to limited material, trials informed by the results of initial investigations with domestic species applied to the focal species were conducted with a more limited set of conditions. Results with the surrogate species indicated a negligible impact of separation method, therefore comparative isolation trials were not conducted with the focal species but rather the most appropriate method was selected for ease of processing following the various storage methods.

All stored whole blood samples were separated using the density settlement method, as a small volume (200µl) of lymphocytes could easily be removed at each of the five time points. Use of the 'Ficoll' method, aside from involving a greater number of processes, would have required a total higher volume of blood in order to isolate because even when using a small volume (~1ml) of LSM, the ficoll method still requires layering of sufficient quantities of blood to function effectively. However, as there is generally a higher total volume of cells obtained using the ficoll method and a greater purity of cells, the samples stored as lymphocytes were separated using the 'Ficoll' method.

For the bottlenose dolphin and grey seal, both samples were applied to the storage trials and subsequently the isolated cells were used for the mitogen stimulation experiments. For the killer whales, two samples from each animal were applied to the storage trials and one was retained for use in the mitogen stimulation experiments. Per animal, 7ml of whole blood was split between two tubes (3.5ml each) and stored at either 37℃ or 4℃ degrees. An additional 7ml was separated by ficoll centrifugation and stored with unsupplemented medium (RPMI) either at 37℃ or at 4℃ degrees.

For both stored blood and isolated lymphocytes, at each of five time-points, cells were counted and seeded as for the surrogate species. Count time-points for killer whale and dolphin samples were at 8hrs, 12hrs, 24hrs, 32hrs and 36hrs and for grey seal samples at 12hrs, 16hrs, 24hrs, 30hrs and 36hrs. It was not possible to count cells at T=0 because it took between 8 and 12 hours to reach the lab from the collection point; therefore, following results from the surrogate species, T=0 for marine mammals was assumed to equal approximately 100%. It also follows

that isolated cells for the cetaceans and grey seal were not isolated for storage until T=8 or T=12, respectively. Cells counted and seeded at the first time point were subsequently combined for each individual and applied to the stimulation experiments. Cells from successive time points were also used for stimulation experiments. At each time point 1ml of lymphocytes suspended in RPMI was removed from each of the two pre-separated tubes stored at either 4℃ or 37℃, with a sterile pipette. 100µl of buffy coat suspension was also withdrawn from each of the two tubes stored as whole blood at either 4℃ or 37℃, with a sterile 18g needle and syringe.

The remaining killer whale blood samples from each individual were separated using the ficoll separation method and the lymphocyte cells combined with those counted from the storage trials. Following counting and viability analysis of stored samples, cells were combined for each individual and redistributed equally according to the LTA conditions detailed below. Fortuitously, although only two samples were obtained for the bottlenose dolphin in lieu of the three from the killer whale, absolute cell numbers were roughly equivalent as 1ml of blood from the bottlenose dolphins yielded many more lymphocytes than did 1ml of killer whale blood.

#### 2.2.1.5 Methodologies

##### 2.2.1.5.1 Ficoll Separation of Lymphocytes

Lymphocyte separation with ficoll centrifugation using lymphocyte separation medium (LSM)

***Rationale:*** Cells are rapidly separated by centrifugation through a Ficoll-sodium diatrizoate solution, the main constituent of MP Biomedicals lymphocyte separation medium (LSM), which creates a ficoll-hypaque gradient. The ficoll gradient causes the erythrocytes and granulocytes to sediment to the bottom of the tube, whilst the mononuclear cells (lymphocytes) and platelets are separated to the interface between the upper two phases of LSM and plasma.

***Protocol:*** Aliquots of 7ml whole blood drawn into sodium heparin tubes were diluted 1:1 with Hanks' Balanced Salt Solution (HBSS). An appropriate quantity of Lymphocyte separation medium (LSM) to achieve a ratio of 3:2ml with undiluted blood, was transferred to a 50ml Polypropylene centrifuge tube. The diluted blood was carefully layered over the LSM (SLI) containing the ficoll-hypaque gradient and centrifuged at 200g (~1,000 RPM) for 20 minutes at

room temperature. This step resulted in four distinct tiers of stratified blood components (see Fig. 2.2-2) separated out into the various components. The third layer up, above the red blood cell (RBC) layer and sitting immediately atop the medium (LSM) layer, the thicker lymphocyte cell layer was carefully 'lifted' off the medium using an 18-gauge needle attached to 20ml syringe, placed through the upper aqueous layers. The lymphocyte layer and the medium layer down to the RBC layer was carefully removed, assuring no red blood cells were included, and transferred into a 15ml collection tube. The solution was then diluted 1:1 with phosphate buffered saline (PBS), inverted and centrifuged at 140g for 10 min to wash the cells. The upper clear aqueous phase was transferred to a fresh 15ml tube and the wash repeated to remove the LSM and platelet contamination, which can interfere with cell growth. The final suspension was spun at 1,000 rpm for 10 min and the supernatant carefully removed. The washed lymphocytes were gently resuspended in 5 ml RPMI1650 with supplements as detailed in the LTA section below, at a seeding density of 100,000 cells/ml and transferred to 60mm plates or P25 flasks.

#### 2.2.1.5.2 Density separation of Lymphocytes

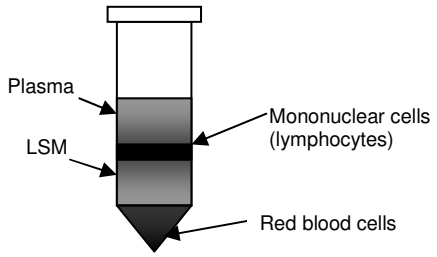
Lymphocyte separation by gravitational separation in vacutainers

***Rationale:*** Erythrocytes are denser than platelets and lymphocytes and therefore over time will settle to the bottom whilst the lymphocytes will go into suspension.

***Protocol:*** 7mls of whole blood was collected into rubber bunged heparinized tubes and kept upright for 4-8 hours with no agitation until a clear layer of cells was visible above the red blood cells. The leukocytes separate out to a suspended layer above the erythrocyte and granulocyte layer that settles to the bottom of the tube. An 18-gauge needle was pierced through the rubber bung and the leukocytes were aseptically aspirated into a 20ml syringe and then transferred to a 50ml polypropylene centrifuge tube by piercing through the lid and gently ejecting the cells. By separating the cells in this way, it is possible to keep the cells under sterile conditions without the need for a safety cabinet. Following isolation, lymphocyte cells were washed twice with PBS by centrifugation at 200g for 20 minutes at room temperature and then resuspended in 5ml of

RPMI1650, with supplements as detailed in the LTA section below, at a seeding density of 100,000 cells/ml and transferred to 60mm plates or P25 flasks.

**Figure 2.2-2:** shows the separate fractions resultant from the isolation medium




#### 2.2.1.5.3 Cell counting and viability assessment

The number of viable peripheral blood leukocytes (PBLs) was determined via trypan blue exclusion and counting of unstained cells using a haemocytometer. All counts were performed by the same individual and replicated three times for each count and the mean value used, to ensure consistency. Total numbers of PBLs in individual suspensions under different test conditions were determined using a haemocytometer and the resulting figures were employed to estimate cell proliferation in individual cultures. Cells were counted with a haemocytometer prior to seeding cultures, and at various time periods post-stimulation, to assess the level of proliferation. Trypan blue was also added to assess the viability of the cells.

To estimate cell concentration, post-centrifugation cells were dispersed by pipetting up and down. 50µl of the cell suspension was diluted 1:2 with PBS. An equal volume (100µl) of 0.4% w/v trypan blue was added to the cell suspension and mixed thoroughly. Approximately 20µl of cell/trypan blue suspension was transferred to each chamber of the haemocytometer (Neubauer, BDH). Both viable (unstained) and nonviable (stained blue) cells within each of the four corners and the centre square were counted under a light microscope and the average of the four corners was calculated. The concentration and cell viability was determined as follows:

$$\% \text{ viability} = 100 \times \frac{\text{Total \# viable cells}}{\text{Total \# of cells (viable + non viable)}}$$

Cells/ml = 50,000 x Total # viable cells in 5 squares x dilution factor

#### 2.2.1.5.4 Differential mitogen stimulation (LTA)

Following the storage and separation trials, cells from the surrogate species trials were seeded and stimulated in order to verify viability of isolated cells, following the standardized protocols for Sheep: 10µg/ml PHA (Stirtzinger et al., 1986); Cattle: 5µg/ml Con-A, PHA 2.5µg/ml (Miniscalco et al., 2003) and rat: Con-A 6µg/ml, PHA 5µg/ml (Kanda et al., 2004). Stimulation trials were conducted exclusively with the three focal species because previous studies have shown that the LTA is species-specific and therefore there was no benefit to using the surrogates.

Cells from the different storage/separation treatments for the three focal species were combined for each individual (five killer whales, three bottlenose dolphins, two grey seals). Combined lymphocytes from each individual were divided between the different stimulation treatments and seeded in duplicate or triplicate at a density of 100,000 cells/ml in 12-well culture plates. Two or three replicates for each stimulation treatment were seeded. Two mitogens (phytohaemmagglutinin (PHA) and Concanavalin-A (Con-A)) at eleven concentrations were used to stimulate lymphocytes into proliferation and the effect of each was assessed by cell counting at nine time points. The effect of mitogen type, concentration and time since seeding were all assessed with relative cell number. The effect of re-stimulation of cultures was evaluated qualitatively in a subset of cultures by assessing the cell recovery elicited by washing the cells and addition of fresh mitogen. The effect of cryopreservation was also evaluated qualitatively by determining the recovery and proliferative capacity of stimulation of cryopreserved cells.

#### 2.2.1.5.5 Mitogens used to stimulate lymphocytes:

***Phytohaemmagglutinin*** (PHA) is a lectin, isolated from the common bean *Phaseolus vulgaris*. PHA-P consists of two molecular species, an erythroagglutinin (PHA-E), which has low mitogenic activity and high erythroagglutinin activity, and leucoagglutinin (PHA-L), which has high mitogenic activity and high leucoagglutinating activity, but very low erythroagglutinin

activity. The combined effect of PHA-P, therefore, results in effective stimulation of mitosis in T-lymphocytes.

***Concanavalin-A*** (Con-A) is a lectin extracted from the jack bean *Canavalia ensiformis,* which binds specifically to glycoproteins and as such, has mitogenic activity on T-lymphocytes, encouraging them to commence cell division.

***Protocol:***

Number of viable cells in unstimulated (control) cultures maintained throughout stimulation trials, was used to determine the effect of test conditions (i.e. mitogen stimulation) on the cell yield by direct enumeration of viable cells in treated relative to control cultures at designated time intervals. Viable cell number was used to establish the relative cell proliferation rates to the different mitogen types and concentrations. Trypan blue exclusion was used to determine cell viability, as before. Cells from the different storage conditions were combined, per individual, and then applied to the LTA. Cultures were established in triplicate at a density of $1x10^5$cells/ml, for each of the treatment conditions and the cells were counted at two-day intervals. Stimulated cultures contained one of two mitogens: phytohaemmagglutinin (PHA: Sigma), Concanavalin A (Con-A, Sigma) at eleven concentrations (0, 0.5, 1, 2, 4, 6, 8, 10, 12, 16, 20 µg/ml). 0.1ml of culture from each replicate was sampled and assessed for number and viability, as before for storage trials, every second day, from 2 to 14 days.

The remaining cells from the storage treatments and from later time points that were not applied to the LTA longitudinal study, were either cryopreserved or seeded with low concentrations of mitogen (0.5µg/ml Con-A; 2µg/ml PHA) to maintain the cells until results from the trials could be applied. On day four, following counting procedures, cells were stimulated with concentrations observed to be eliciting a reasonable response for each individual. As stimulation experiments continued the results informed the maintenance of growing stocks for intended use in the subsequent flow sorting processes. This subset of cultures, when observed to be losing viability at later time points, were washed, provided with fresh medium and re-stimulated with fresh mitogen. The effect was qualitatively assessed by observations of

recovery as indicated by a reduction in the accumulation of debris and cells with vacuoles and an increase in cell number. All cultures were established in RPMI 1640 medium supplemented with 10% v/v FCS, 2% w/v L-Glu, 1% w/v essential amino acids, 1% w/v non-essential amino acids, 1% w/v antibiotic (100U penicillin/streptomycin) and antimycotic (Fungizone 2.5µg/ml). All reagents were from Serum Labs International (SLI), unless otherwise noted. Cultures were incubated at 37°C with 5% $CO_2$.

#### 2.2.1.5.6 Cryopreservation and resurrection

The effect of cryopreservation and recovery was assessed to indicate the post-freeze viability and re-stimulation capacity of lymphocytes following long-term storage for future experimentation. Cells were stored in ampoules (Greiner) at a cell density of $5x10^6$ or greater following re-suspension in freezing mix; containing 70% RPMI 1640: 20%FCS: 10% dimethyl sulfoxide (DMSO). Vials were wrapped in bubble-wrap in a polystyrene freezing container, and placed at -80°C overnight. Cryovials were subsequently stored long-term at -140°C.

Stocks of lymphocytes were recovered by transfer to 15ml Polypropylene centrifuge tubes with 5ml fresh 4°C medium and then warmed in a 37°C water bath to bring them rapidly back to culture temperature. Cells were spun at 1500rpm in a Beckman coulter centrifuge in order to remove the freezing mix, containing dimethyl sulphoxide (DMSO), before they were re-suspended in fresh medium (as before), and seeded at a density of $1x10^5$ cells/ml in culture plates or flasks (Gibco). Cultures were stimulated with mitogen and incubated at 37°C, 5% CO2, in a humidified incubator, as before. Cell viability and number were assessed by trypan blue counts with a haemocytometer. Cells were maintained in culture and their post-cryopreservation viability and stimulatory capacity was observed over time.

### 2.2.1.6 Statistical Analysis

A Chi-squared Fisher's exact test was conducted on the mitogen stimulation data replicates to ensure results were consistent between replicates. Because the data were not normally distributed, as to be expected from lymphocyte data, non-parametric tests were

required. The Wilcoxon test on paired data was used to assess the difference in median between isolation methods (Ficoll/Density), temperatures (4℃ /37℃), conditions (Blood/Lymphocyte) and mitogens (Con-A/PHA). The Spearman rank test was used to assess the degree of correlation between proliferation and time and concentration, respectively. The Kruskall-Wallis test was used to assess the analysis of variance of killer whale, bottlenose dolphin and grey seal samples, to determine whether the response was significantly different between species.

### 2.2.2 Corneal cell cultures

#### 2.2.2.1 Establishing culture

Corneal epithelial cells were established according to the methods detailed in (Duffield et al., 1991) with modifications from Wise et al. protocols, (Wise and Wise, 2005) using eyes removed from stranded animals. Eyes from six elephant seals were provided by the Marine Mammal Centre, Sausalito, California, from stranded animals that failed to respond to treatment and unfortunately died. Eyes from eight grey seals, and three porpoises were provided by the British stranding network. The eyes were removed, intact from the socket and shipped at room temperature in a dry plastic bag. They were then kept at 4℃ until culturing.

The intact eye was first rinsed with distilled water prior to making any incisions, to remove any gross contamination. The eye was always held with the cornea face up so that any contaminants washed away from the cornea. The use of any chloride-based solution to wash the eye was found to be too harsh a treatment and therefore, the antiseptic disinfectant, 'Savlon' rinse was used. Savlon contains a very dilute concentration (0.3g/100ml) of chlorhexide gluconate. Five ml of Savlon was diluted in 100ml of distilled water and the eye was placed, cornea face up, in the solution. The cornea was subsequently rinsed with distilled water to remove any traces of the disinfectant. The cornea, including the basement membrane, was removed from the front of the eye and then placed in a solution containing culture medium, Dulbecco's Modified Eagle Medium (DMEM), 10%FCS, L-Glu, and 10x normal concentration of

antibiotic (penicillin-streptomycin 100units/ml) and antimycotic (Fungizone 2.5µg/ml) solution and left for 1 hour at 4℃. Corneal samples were processed for culture by excising small pieces of the cornea and mincing with two scalpel blades into approximately 1mm squares. Approximately 20 pieces of tissue were transferred to each T25 culture flask to seed cultures; each piece of tissue was carefully placed on to the surface of the flask, using sterile forceps, to allow for outgrowth from the explants. 0.5ml medium was washed over the explants before inverting the flask, ensuring the tissue pieces remain affixed, and left overnight in the incubator at 37℃ with 5% $C0_2$. The following day 5ml of 37℃ medium was gently added to the plates, avoiding moving the explants and returned in the upright orientation to the incubator.

#### 2.2.2.2 Passaging primary cultures of corneal epithelial cells

When a culture reaches ~80% confluence, cells need to be 'passaged' in order to maintain their growth. It is important to split them before they reach confluence, otherwise contact inhibition will likely result in slowing or halting their further growth and potentially faster-growing, transformed phentoypes may be selected. For passaging, medium was aspirated from the culture flask and then washed gently with 5ml phosphate buffer saline (PBS) to remove traces of serum. Two to five ml 0.25% trypsin-EDTA solution was added to the flask and incubated at 37℃ for 3-5 min. The side of the flask was banged against the palm after 3 min to help dislodge the cells, as prolonged treatment can result in toxicity of the cell culture. If the cells had not lifted they were incubated further (up to a maximum of 5 min) and the banging repeated at 1minute intervals. An equal volume of medium to the trypsin solution was added to neutralize the trypsin activity. The cell suspension was washed over the culture surface several times to dislodge any stubborn cells remaining attached and then transferred to a 15ml tube. Cells were centrifuged at ~1000rpm for 5min. The supernatant was then aspirated from the cell pellet leaving ~100ul in which the pellet was subsequently re-suspended. Five ml of fresh medium containing supplements, as before, was then added to the suspension. Cells were then counted and re-seeded into two fresh flasks at the appropriate density for the size of flask (e.g.

T25 – 500 – 2000 cells in 5mls). (Seeding density was dependent on passage number and requirements, i.e. at higher passage numbers cells grew slower and therefore required higher seeding densities to stimulate growth.) If the tissue used to seed the explant was intact and still viable, it was removed from the flask using sterile forceps prior to trypsinization and then returned to the original flask following removal of the cells, for re-explanting. The sample procedure was then followed as with fresh explants.

#### 2.2.2.3 Cryopreservation and Resurrection

Adherent corneal cell cultures were trypsinized 3; however instead of re-suspending in culture medium, the trypsin was removed by centrifugation and the cell pellet was resuspended in freezing medium (containing 10% w/v DMSO, 20% w/v FCS and 70% w/v medium, as before). Cells were stored in ampoules at a cell density of $5x10^6$ or greater. Stocks of epithelial cells were recovered by transfer to 15ml Polypropylene centrifuge tubes with 5ml fresh 4°C medium and then warmed in a 37°C water bath to bring them rapidly back to culture temperature. Cells were spun at 1500rpm in a Beckman coulter centrifuge in order to remove the freezing mix, containing dimethyl sulphoxide (DMSO), before they were re-suspended in fresh medium (as before), and seeded at a density of $1x10^5$ in culture plates or flasks (Gibco).

## 2.3.0 Results

### 2.3.1 Storage and Transportation

Absolute number of lymphocytes was variable between individuals and species but was controlled for in the experimental design, as storage results were recorded as percentages and all mitogen stimulation experiments were seeded with the same volume of cells. To account for any inaccuracy in the cell counting, all cells were counted three times by the same individual and the average taken from the three counts. In a biological system it is not possible to control for all variables; however, the experimental design applied here attempted to account for as much variation as possible.

Samples with more than 50% viability were found to actively divide when stimulated following storage trials, as indicated by a rapid increase in cell numbers. Samples with between 30 and 50% viability proliferated effectively, however there was a noticeable time delay between seeding the cultures and a proliferative response. There was also an indication that their proliferative capacity was diminished, as indicated by a reduction in the cell increase. However, this was not quantified and could have been a function of the reduced numbers of viable cells rather than the capacity of the viable cells to divide. Samples showing a viability of less than 30% exhibited extreme delays in the onset of proliferation or failed to proliferate following seeding and stimulation.

### 2.3.2 Method of separation, storage condition and temperature

**Table 2.3-1: showing the sample sizes for the transport, storage and separation**

| | | | Surrogate species | | | Focal Species | | |
|---|---|---|---|---|---|---|---|---|
| **Condition** | **Temp** | **Separation** | **Sheep** | **Cattle** | **Rat** | **KW** | **BND** | **GS** |
| Blood | 37℃ | ficoll | 8 | 1 | 1 | X | X | X |
| Blood | 4℃ | ficoll | 8 | 1 | 1 | X | X | X |
| Blood | 37℃ | density | 8 | 1 | 1 | 5 | 7 | 2 |
| Blood | 4℃ | density | 8 | 1 | 1 | 5 | 7 | 2 |
| Lymphocytes | 37℃ | ficoll | 8 | 1 | 1 | 5 | 7 | 2 |
| Lymphocytes | 4℃ | ficoll | 8 | 1 | 1 | 5 | 7 | 2 |
| Lymphocytes | 37℃ | density | 8 | 1 | 1 | X | X | X |
| Lymphocytes | 4℃ | density | 8 | 1 | 1 | X | X | X |
| KW – Killer whale, BND – Bottlenose Dolphin, GS – Grey Seal | | | | | | | | |

### 2.3.3 Surrogate Species (Ovine, Rat, Bovine)

**Ovine**

Datasets 7 & 8 were drawn by the veterinary practice 3-4 hours prior to the arrival of the investigator and kept refrigerated at 4℃. Initial investigation of the data highlighted inconsistencies of ovine sample sets 7 & 8. Closer examination revealed that at time point T=0 the percentage viability of replicates 7 & 8 were substantially reduced (mean = 74% and 79%, respectively) in comparison to the other replicates (mean = 97%). The viabilities observed in replicates 7 & 8 more closely resemble those of bloods stored at 4℃ for four hours, as expected from the intitial storage period. Data are therefore presented with and without replicates 7 & 8. Figures 2.3.1 and 2.3.2clearly demonstrate that the majority of the variation at each time point for particular conditions was due to these two datasets. Analysis of the both the complete dataset (8 replicates) and the restricted data (6 replicates) revealed significant differences between conditions, as detailed below and in Table 2.3.1. Blood at 4℃ was significantly different from any other treatment combination ($p<0.05$) and only with the restricted dataset was the difference between lymphocytes at 37℃ and blood at 37℃ found to be significant ($p= 0.05$ and 0.001, in the latter). Irrespective of treatments all samples lost cell viability over time, therefore variation between groups indicates the difference in the rate of loss dependent on the treatments.

***Separation method***: As can be seen from figures 2.3.1 and 2.3.2 for ovine storage trials, with both complete and restricted datasets, the separation method did not make a significant difference to the viability of the cells stored as either blood or lymphocytes ($p>0.3$, for all combinations). The absolute number of cells isolated by the ficoll centrifugation technique was marginally greater than by the density settlement method but the cell viability and response to stimulation of both methods of separation was generally comparable.

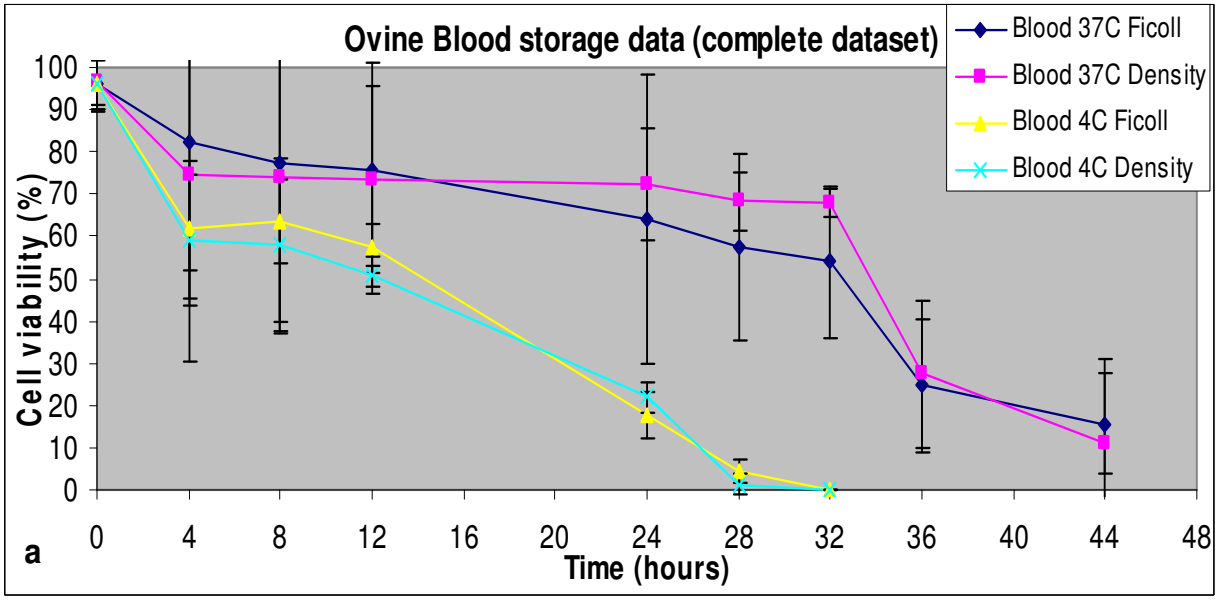
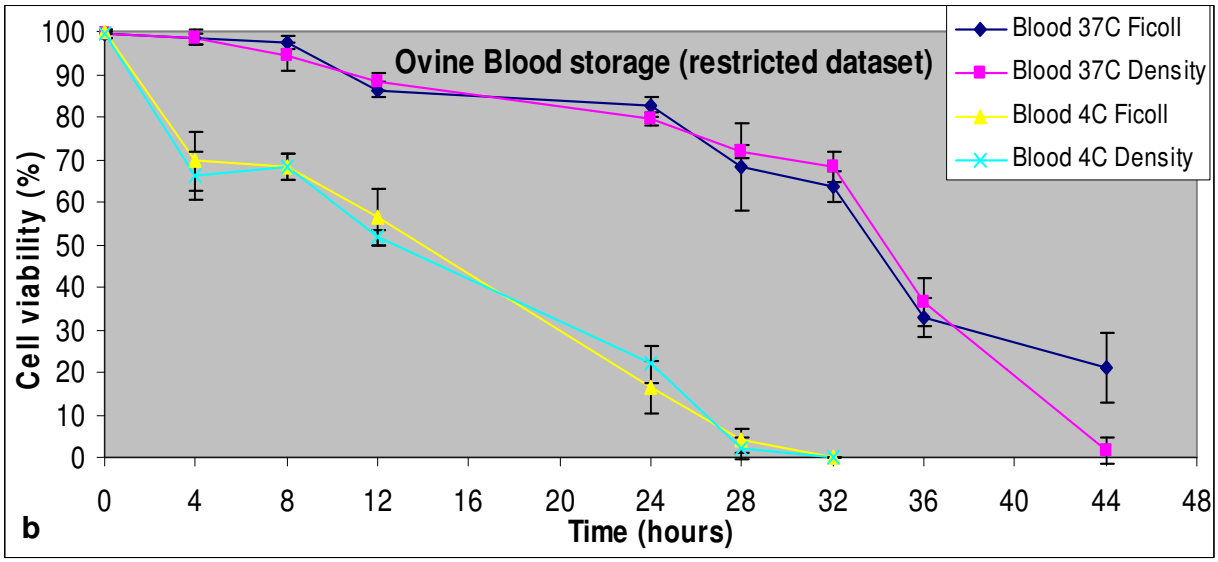



Figure 2.2.1 shows the effect of time and different storage conditions on the viability of ovine lymphocyte cells when stored as whole blood. Blood was either stored at 37℃ or 4℃ and separated either by ficoll separation medium or density settlement, as described in the text. a) shows the complete dataset across 8 replicates, b) shows the restricted dataset across 6 datasets as indicated int eh text. Error bars represent the standard deviation form the mean.



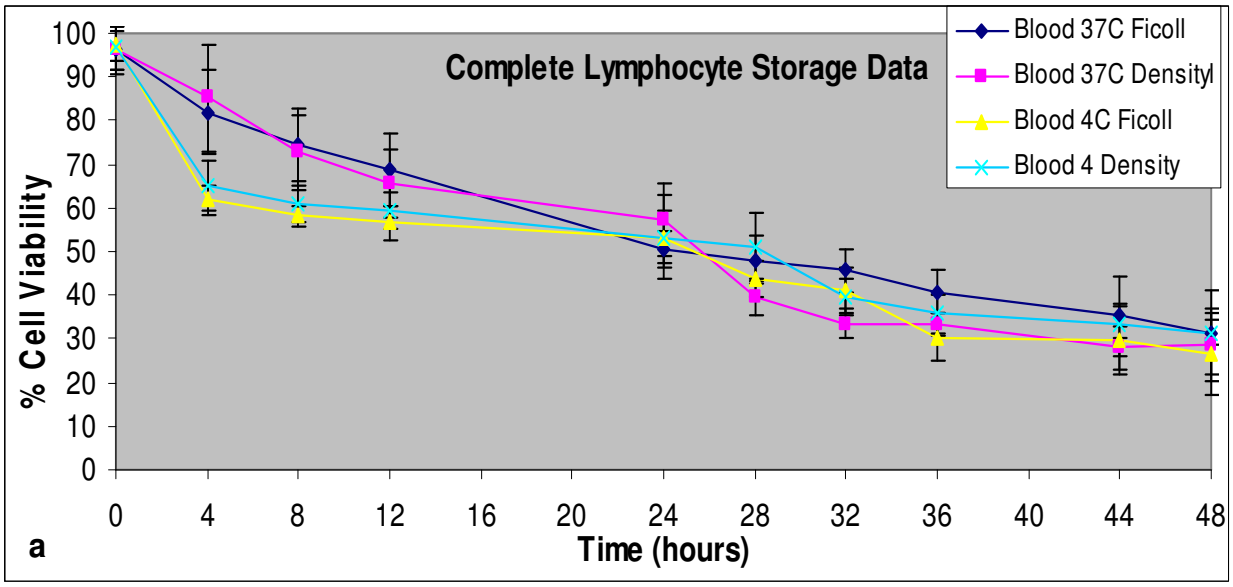



Figure 2.2.2 shows the effect of time and different storage conditions on the viability of ovine lymphocyte cells when stored as separated lymphocytes. Lymphocytes were separated either by ficoll separation medium or by density settlement and either stored at 37℃ or 4℃, as described in the text. a) shows the complete dataset across 8 replicates, b) shows the restricted dataset across 6 datasets as indicated int eh text. Error bars represent the standard deviation from the mean.

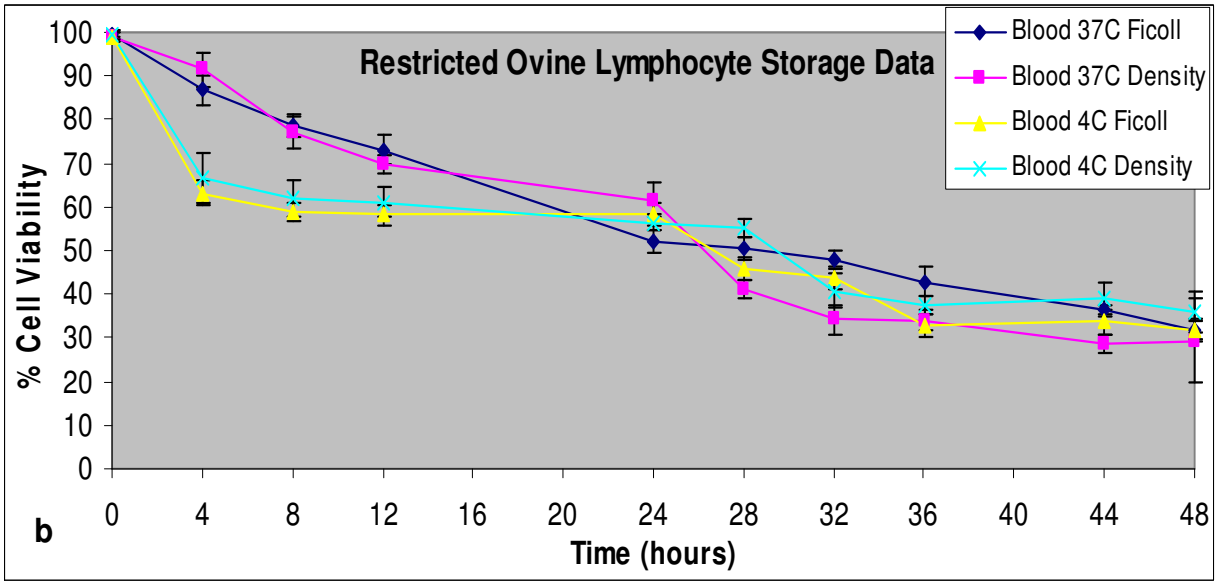


**Table 2.3.1** shows the p-values for analysis between storage treatments. Following analysis of Ficoll v.s density, data were combined across separation methods as there were no significant differences, as shown along the diagonal. Figures in italics and below the diagonal are for the restricted data set (without dataset 7&8, as detailed in the text). Above the diagonal are the complete dataset p-values. Values in **BOLD** are significantly

| **Table 2.3.1** | | **Ficoll** | | | |
|---|---|---|---|---|---|
| | | **Lymph 4°C** | **Blood 4°C** | **Lymph 37°C** | **Blood 37°C** |
| **Density** | **Lymph 4°C** | 0.32/*0.32* | **$6.25^{-13}$** | 0.05 | **0.0003** |
| | **Blood 4°C** | **0.002** | 0.74/*0.68* | **$2.78^{-5}$** | **$6.12^{-7}$** |
| | **Lymph 37°C** | 0.53 | **0.04** | 0.35/*0.35* | 0.98 |
| | **Blood 37°C** | **0.002** | **6.12^-07** | **0.038** | 0.68/*0.74* |

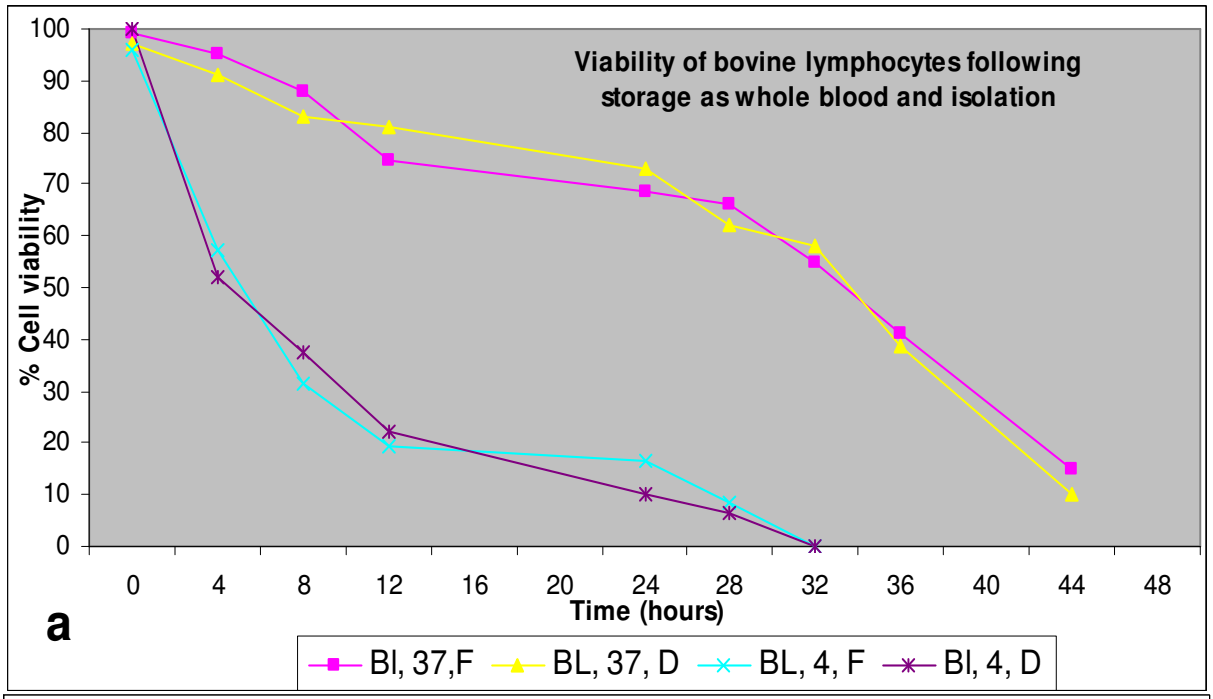
***Blood vs Lymphocytes:*** Figures 2.3.1 and 2.3.2 illustrate that samples showed improved retention of cell viability when stored as blood samples at 37°C compared with samples isolated immediately and stored as lymphocytes at 37°C. However, there was an interaction between the state (blood or lymphocyte) of the sample and the temperature, such that samples stored as whole blood at 4°C, retained less viability over time than did isolated lymphocytes. Blood samples stored at 4°C showed a major reduction in viability over time. Table 3 shows that there was a significant difference between blood and lymphocytes at 37°C (p=0.038); and between blood and lymphocytes at 4°C (p=0.002). The ovine storage figures also indicate that there was a difference in the profile of the two storage states. Although blood samples stored at 37°C initially retained better viability, after 32 hours the viability rapidly declined. In contrast, the cells stored as lymphocytes (both at 4° and 37°), showed a more regular decline, such that after the critical 32 hours, they retained higher cell viability (average of 33% and 37%, respectively) than did the samples stored at 37°C as bloods (average of 26%).

***Temperature:*** As mentioned above, temperature interacted with condition of storage of the blood sample, such that blood samples kept at 37℃ performed better ($p=6.12^{-7}$) than those stored at 4℃; however, when cells were isolated immediately and stored at either 37℃ or 4℃ they showed a similar rate of decline in viability and the viability was not significantly different between temperatures ($p=0.53$).

***Interaction:*** The interaction between state and temperature demonstrated that ovine blood samples retained the most viable lymphocytes under the following conditions: Blood at 37℃ > Lymphocytes at 4℃ = Lymphocytes at 37℃ > Blood at 4℃.

**Rat and Bovine** The trials with 6 replicates, for each set of conditions (8) with sheep blood samples were repeated using one set (8) each of rat blood samples and cattle blood samples. The results for rat and bovine samples shown in figures 2.3.4 and 2.3.5 are consistent with those obtained from the sheep samples detailed above, thus demonstrating their inter-specific applicability. The rat samples showed a less severe response to storage as whole blood at 4℃ (32%) compared with (17%) in ovines, whilst also maintaining greater than 30% viability in all other sample conditions at 36 hours. In contrast the bovine samples showed a more severe reaction to storage as whole blood at 4℃, compared with the ovine and rat samples, which maintained viability greater than 50% up to 12 hours after venipuncture, bovine samples had already lost more than 60% viability after 8 hours storage. Cell viability was generally comparable between the three species for cells stored as lymphocytes at both temperatures. Although samples stored at 37℃ as whole blood faired better than any cells stored following isolation, the increased performance of the former was less obvious for the rat samples. Trends were not tested statistically because the sample size of one replicate was not sufficient for statistical power but the main aim to test whether the trend was similar between species was fulfilled. Because the sample set represented only one replicate for each condition the consistency was not tested statistically but did demonstrate the cross-specific agreement in the trend.

**Figure 2.3.3** shows the effect of time and different storage conditions on the viability of bovine lymphocyte cells when stored as a) whole blood or b) separated lymphocytes. Whole blood was either stored at 37℃ or 4℃ and then separated by ficoll separation medium or density settlement, as indicated in the. Lymphocytes were separated and then stored at 4℃ or 37℃, as described in the text.



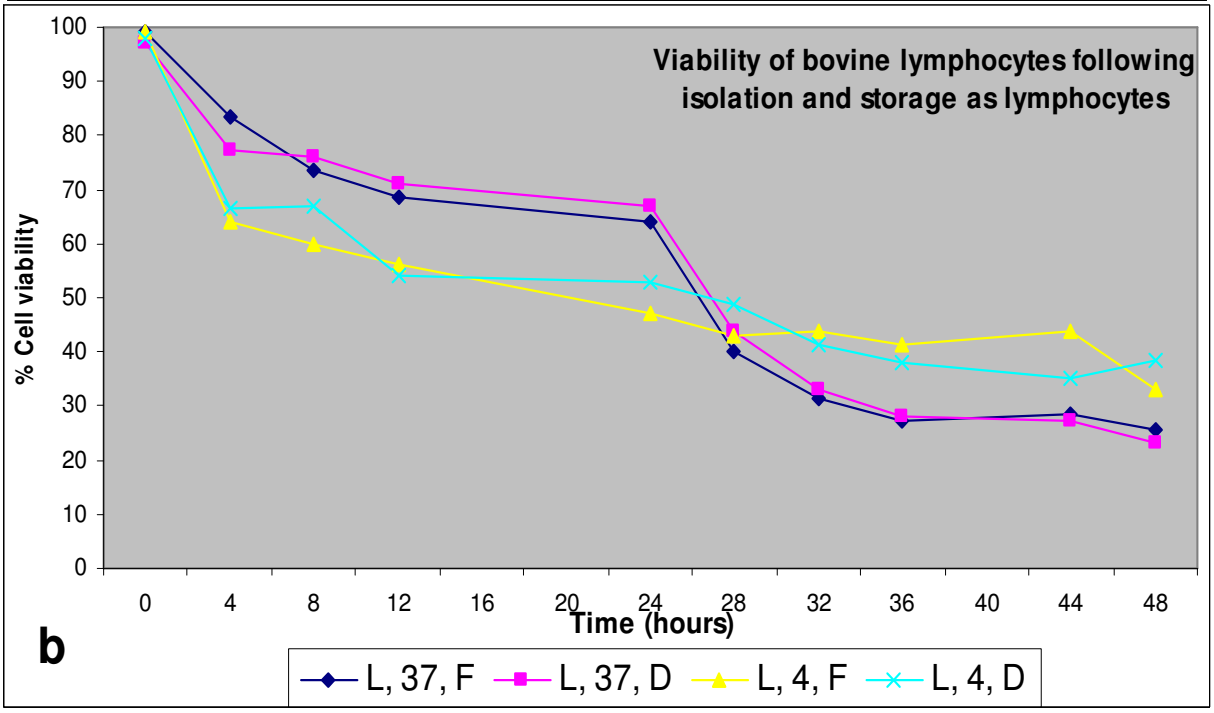
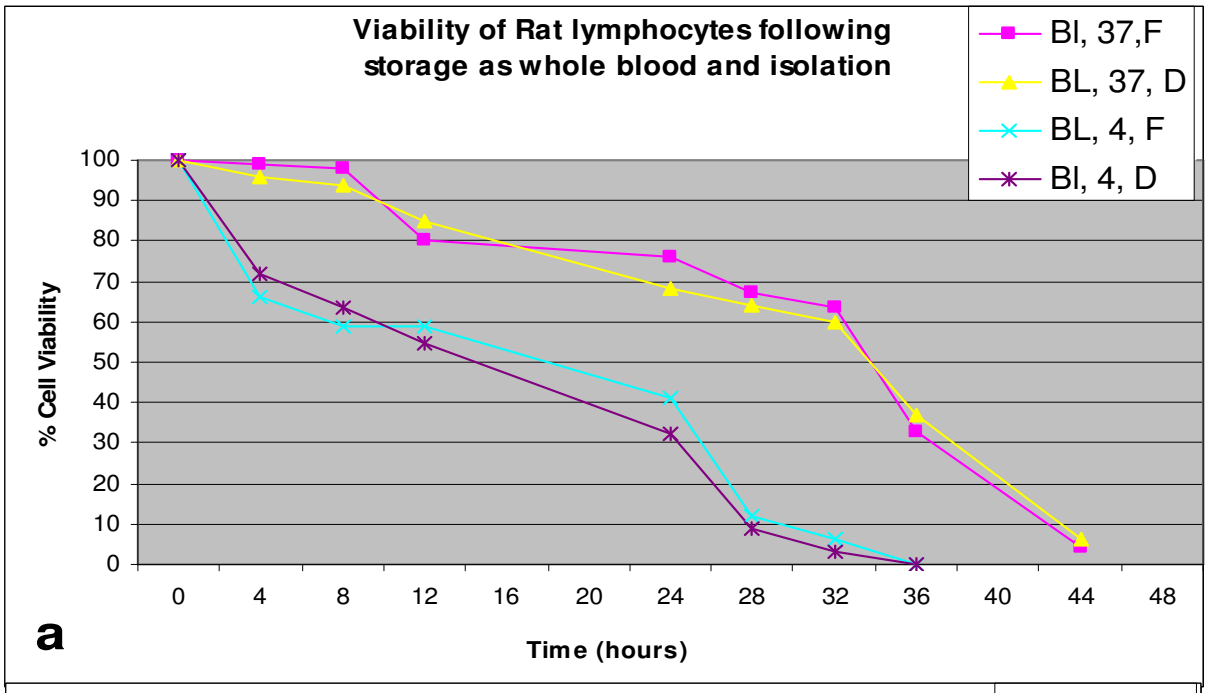
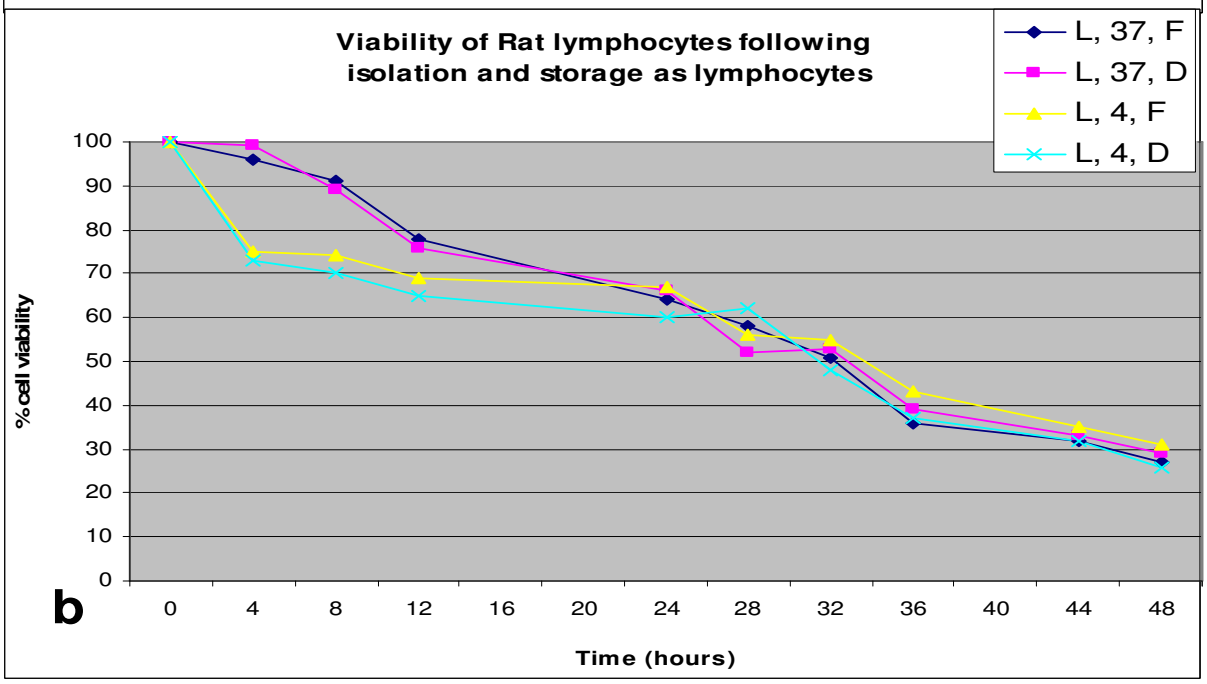



**Figure 2.3.4** shows the effect of time and different storage conditions on the viability of rat lymphocyte cells when stored as a) whole blood or b) separated lymphocytes. Whole blood was either stored at 37℃ or 4℃ and then separated by ficoll separation medium or density settlement, as indicated in the. Lymphocytes were separated and then stored at 4℃ or 37℃, as described in the text.



### 2.3.4 Marine Mammal Species (Killer Whale, Bottlenose Dolphin, Grey Seal)

Due to limited material, trials applied to the focal species were conducted with a limited set of conditions. The results from the surrogate species indicated that there was no significant difference between separation methods and therefore the most appropriate method was applied in each instance for the focal species. The cells were also counted at less frequent intervals, due to reduced sample volumes. As expected, consistent with the trend observed in the surrogate species, viability reduced concomitant with time since venipuncture, when applied to blood samples from the focal species (killer whale, bottlenose dolphin and grey seal), although the reduction for all treatments, was generally more extreme.

***Killer Whale:*** Similar to the ovine, results from five killer whale samples maintained as whole blood suffered decreased viability when stored at 4℃ (see Fig. 2.3.5). However, it is evident from Table 2.3.2 that contrary to results with the ovine blood samples, overall separation and storage of lymphocytes at either 37℃ or 4℃ was equally good at maintaining cell viability as whole blood at 37℃, (no significant difference between any of the three treatments, $p>0.05$). However, Figure 2.3.5 indicates that although non-significant across all time periods the profiles of the three treatments are distinct. Within the first 24 hours, samples maintained the highest number of viable cells when stored as whole blood at 37℃ (t24 = 56%). Following this apparently critical time point, cells stored in whole blood at 37℃ rapidly lost viability (t32 = 30%), whereas cells maintained as lymphocytes (either at 4℃ or 37℃) continued their steady decline, maintaining at least 48% viable cells at 32 hours. Under all three conditions more than 50% viability is maintained until approximately 24h of storage. Partitioning the data at 24 hours, revealed that although there was no significant difference between the three treatments prior to 24 hours ($p=0.49$); post-24h there was a significant difference between all three treatments ($p=6.89^{-7}$), such that (L4>L37>B37).

**Figure 2.3.5** shows the effect of time and different storage conditions on the viability of killer whale lymphocyte cells when stored as whole blood or isolated lymphocytes at either 4°C or 37°C. Error bars represent the standard deviation from the mean.

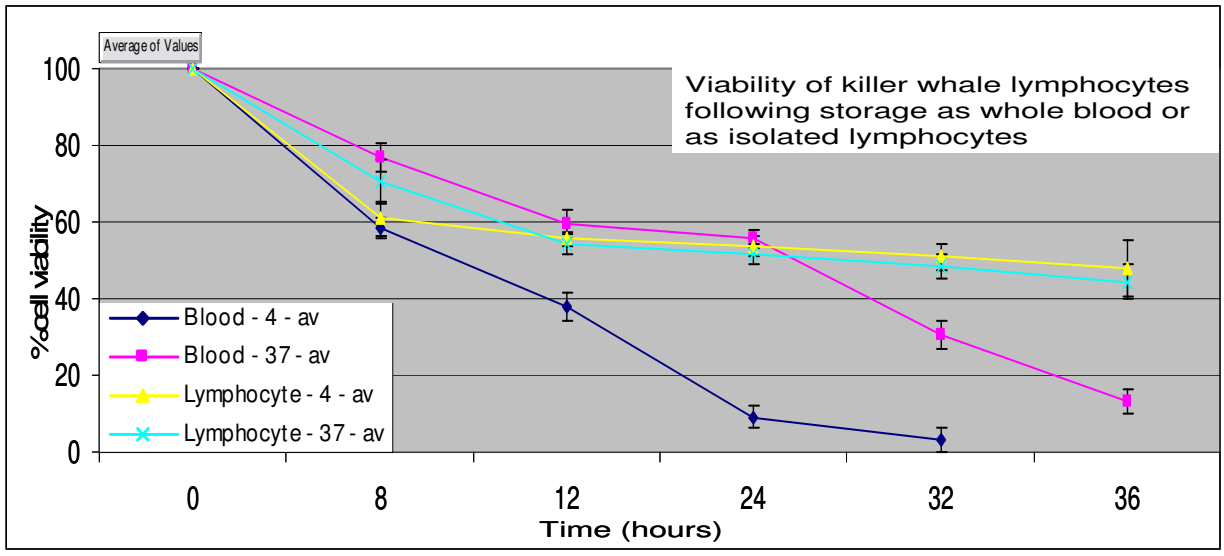



**Figure 2.3.6** shows the effect of time and different storage conditions on the viability of bottlenose dolphin lymphocyte cells when stored as whole blood or isolated lymphocytes at either 4°C or 37°C. Error bars represent the standard deviation from the mean

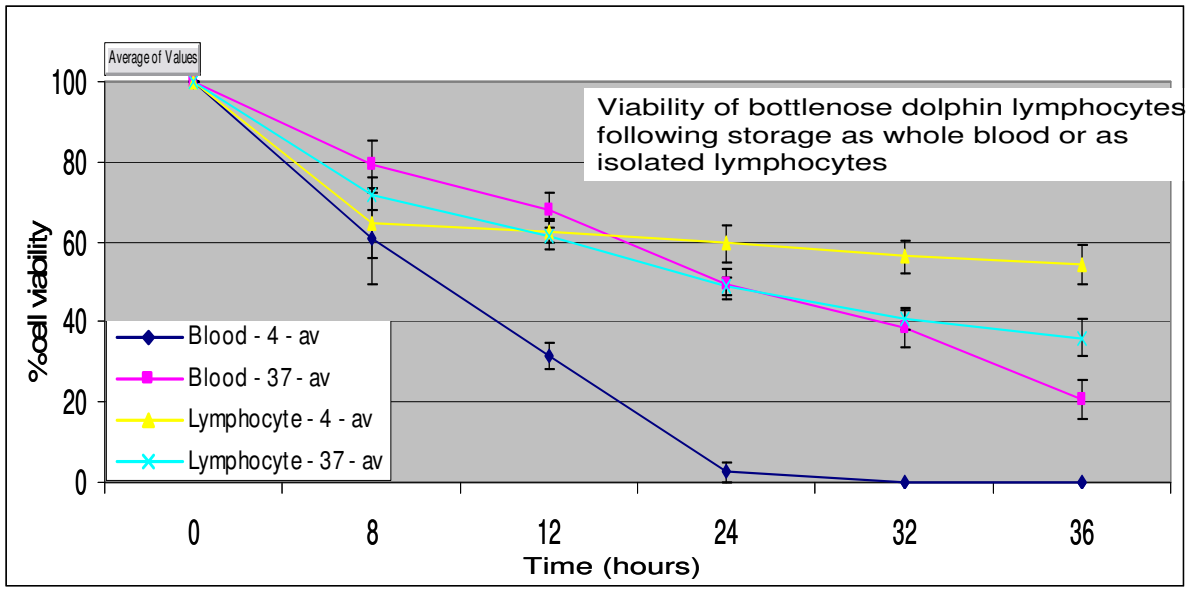
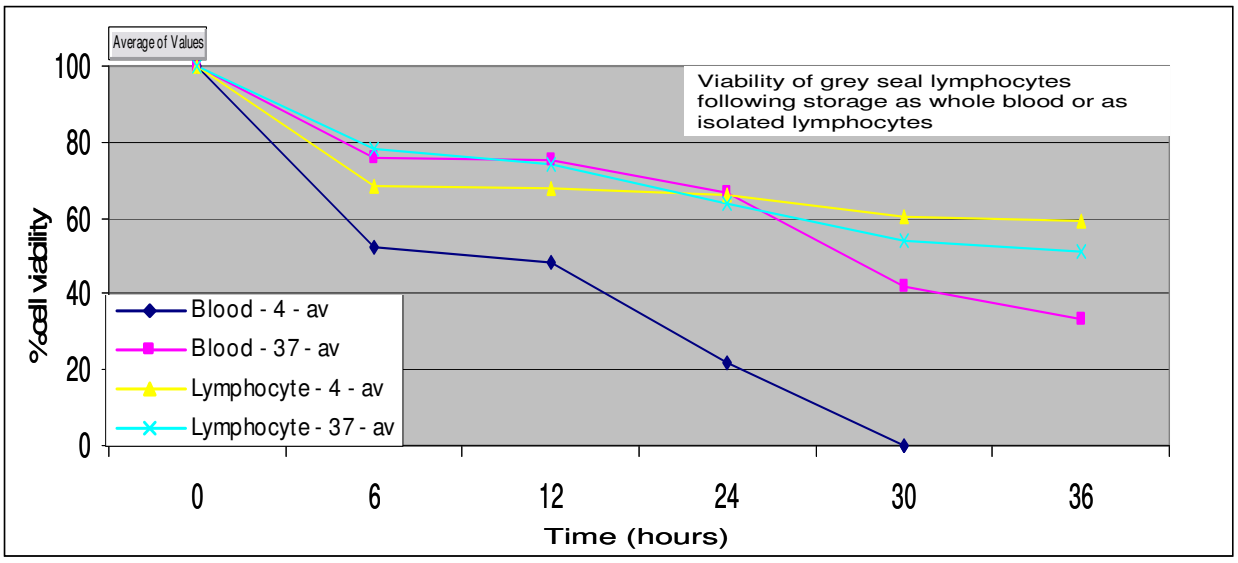


**Figure 2.3.7** shows the effect of time and different storage conditions on the viability of grey seal lymphocyte cells when stored as whole blood or isolated lymphocytes at either 4℃ or 37℃.




***Bottlenose Dolphin:*** Figure 2.3.6 for the bottlenose dolphin, shows that consistent with the other species, storage of whole blood at 4℃ was clearly detrimental to cell viability. Table 2.3.2 demonstrates that, in agreement with results from the killer whale, there was no significant difference between the cell viability maintained by storage as whole blood at 37℃ and that of isolated lymphocytes at either 4℃ or 37℃ ($p>0.05$) across the seven replicates. Similar to the killer whale though, the treatments followed a profile where viability was initially maintained best when whole blood was stored at 37℃ (t12 = 68%), but cells sustained viability more effectively over a prolonged time period with prior separation and storage of lymphocytes, either at 4℃ or 37℃ (L4°, t36 = 54%). Partitioning of the data showed that, as in the killer whale, there was no significant difference between the three more successful treatments prior to 24 hours ($p=0.3$). However, post-24 hours, there was a significant difference between lymphocytes at 4℃ and blood at 37℃ ($p=1.21^{-10}$); and lymphocytes at 37℃ ($p=7.88^{-15}$). Contrary to the results of the killer whale however, blood and lymphocytes stored at 37℃ were not significantly different ($p = 0.05$).

***Grey seal:*** Similar profiles to those of the killer whale were obtained from the grey seal (Fig. 2.3.7l), showing good maintenance of cell viability by storage either as whole blood at 37℃ or lymphocytes at both 4℃ and 37℃; whereas samples stored as whole blood at 4 degrees suffered a very rapid decline in viability. Cell viability of blood at 37℃ in the grey seal was also time-dependent, showing good viability up until 24 hours (66%), when they suffer a rapid decline (t30=42%), whereas a high level of viability is maintained throughout when stored as lymphocytes at either temperature (t36>50%). Trends in the grey seal were not tested statistically as the sample size of two was not sufficient to provide statistical significance.

**Overall:** Similar to the surrogate species, as can be seen from figures 2.3.5 – 2.3.7, all three focal species appeared to demonstrate an interaction between method of storage and the time delay, such that samples initially fare better at 37℃, stored as blood, but after a critical time point (between 24 and 32 hours, dependent on the species), the samples retained improved viability when stored as isolated lymphocytes. This longer-term maintenance of viability observed with lymphocyte separation prior to storage was more pronounced in the marine mammal species.

There was an apparent delay of response to mitogens when lymphocytes were stored at 4℃, however, the magnitude and degree of the delay was not quantified. Clearly storage of whole blood at 4℃ is detrimental to lymphocyte viability in all species studied herein.

**Table 2.3.2** shows the p-values from a Kruskal-Wallis test analysis between storage treatments for the bottlenose dolphin (BND) and killer whlae (KW). Values represent the combined average of the killer whale and bottlenose dolphin for each of the storage treatements. The diagonal represents the difference between killer whale and bottlenose dolphin. Values in **BOLD** are

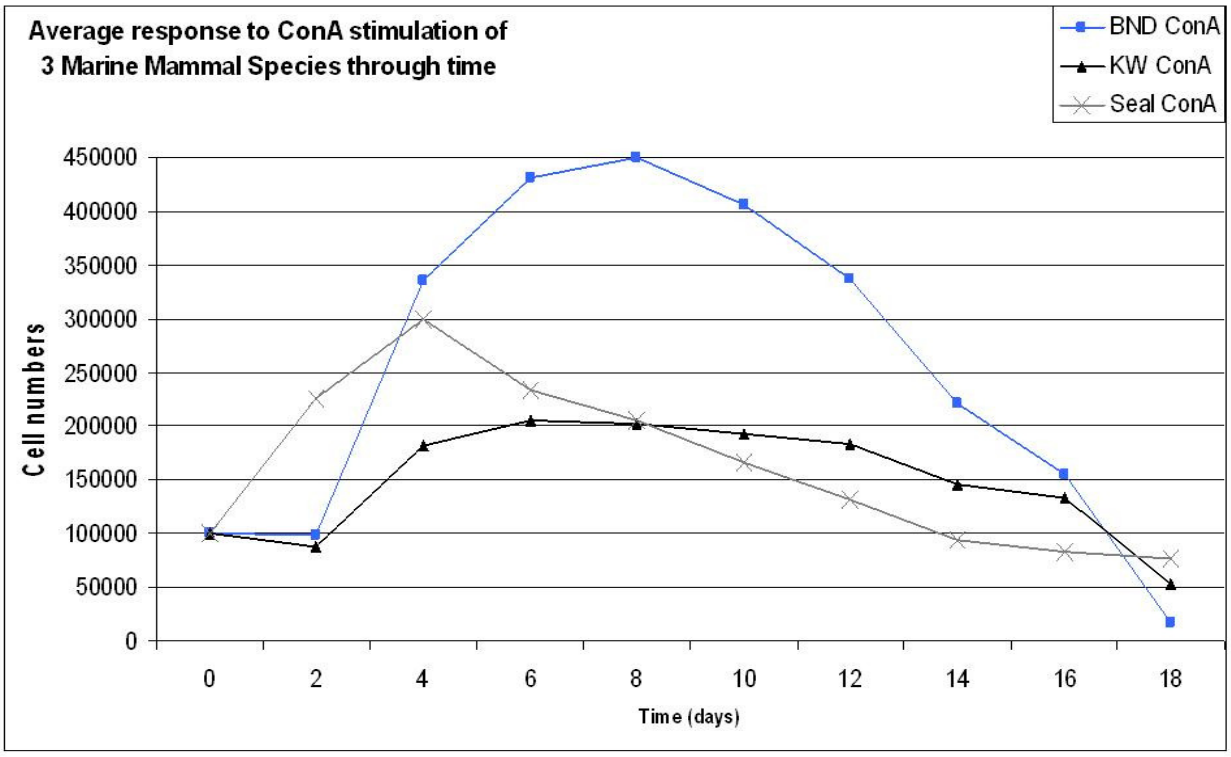
| **Table 2.3.2** | | **KW** | | | |
|---|---|---|---|---|---|
| | | **Lymph 4°C** | **Blood 4°C** | **Lymph 37°C** | **Blood 37°C** |
| **BND** | **Lymph 4°C** | 0.09 | **$1.49^{-5}$** | 0.99 | 0.89 |
| | **Blood 4°C** | **$2.49^{-8}$** | 0.93 | **0.001** | 0.002 |
| | **Lymph 37°C** | 0.08 | 0.21 | 0.81 | 0.28 |
| | **Blood 37°C** | **0.11** | **$4.92^{-5}$** | 0.91 | 0.79 |

### 2.3.5 Results of differential mitogen stimulation in marine mammals

Cell numbers represent the number of viable cells for the average of three replicates for each time point/concentration/mitogen combination, for six killer whales, three bottlenose dolphins and two grey seals, respectively. For six trials one replicate failed to amplify and therefore the response variable represents the mean of two rather than three. For sets where all three replicates failed to respond to stimulation, this was taken as a true response indicator and the result was recorded as a negative response to stimulation. No significant difference was seen between the replicates for each condition ($p>0.05$). Stimulation of lymphocyte cultures was found to be similar for the three marine mammal species with variations mainly observed in the magnitude and timing of the response between species and individuals.

The cellular response of the stimulation experiments indicated that the maximal stimulation response was achieved with Con-A for the bottlenose dolphin samples, but with PHA for the killer whale and grey seal. The concentration and day that elicited the maximal response differed between species and individuals. For Con-A, it was found that the dosage that stimulated the maximal response in one individual could give rise to a negative effect in other individuals of the same species in three out of the ten individuals tested across the three species. Although the absolute maximal stimulation response to PHA was lower than to Con-A in bottlenose dolphins, lymphocytes were also well stimulated by PHA. PHA however, stimulated the cultures effectively across a range of concentrations (2-10µg/ml) and maintained high cell numbers for several days (6-12d) prior to and subsequent to the day of maximal response, in all individuals; whereas the maximal concentration for Con-A varied greatly between individuals and was limited to an effective dose range of one or two concentrations and one or two days for each individual. Table 5 details the day and concentrations of PHA and Con-A for maximal stimulation for each individual and the range within which stimulation was effective.

**Figure 2.3.8** the average response to mitogen stimulation through time for 3 marine mammals shows the number of viable lymphocyte cells from the killer whale, bottlenose dolphin and grey seal in response to a) Con-A and b) PHA mitogen stimulation, in relation to time.



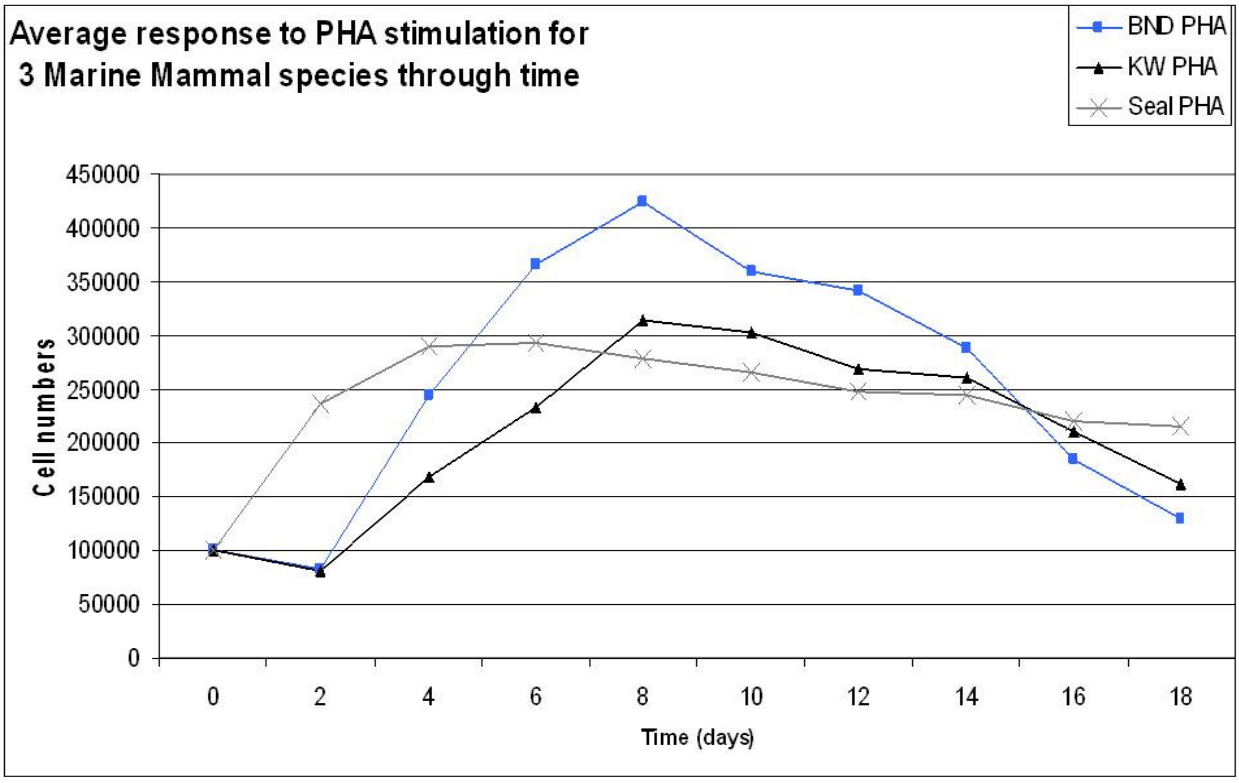



**Figure 2.3.9 shows the number of viable cells in lymphocyte cultures from three marine mammal species (killer whale, bottlenose dolphin and grey seal, in response to variable concentrations of Con-A and PHA mitogen stimulation.**

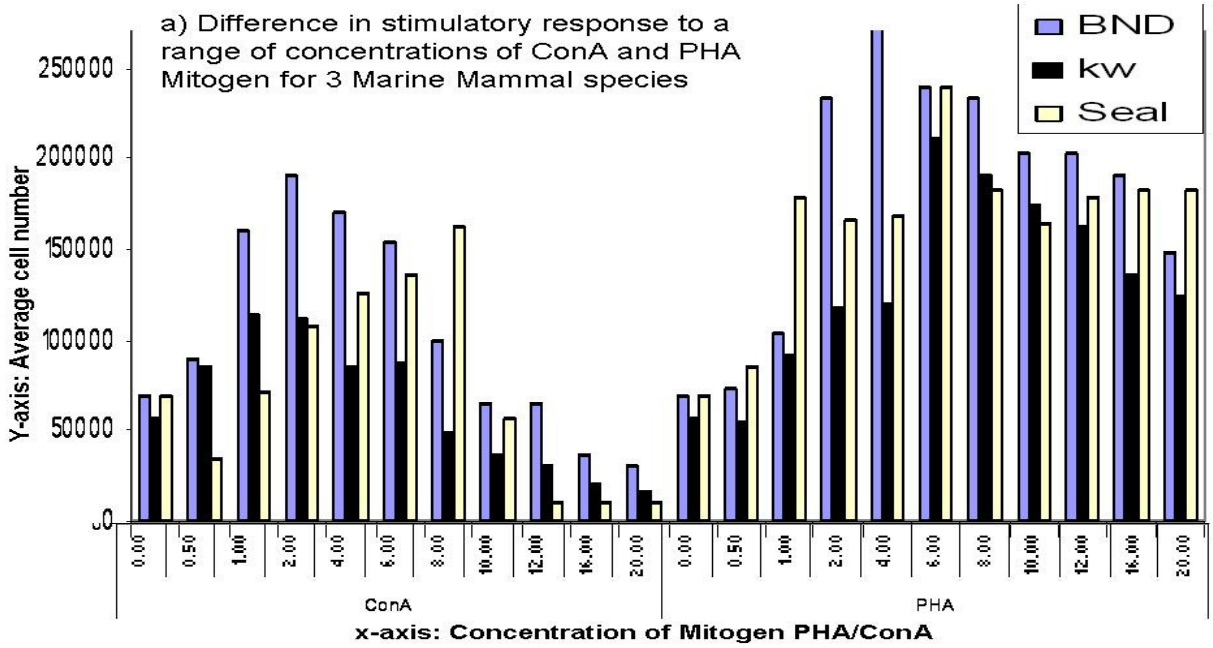



**Figure 2.3.10 shows the number of viable cells in lymphocyte cultures from killer whales, in response to variable concentrations of Con-A and PHA mitogen stimulation.**

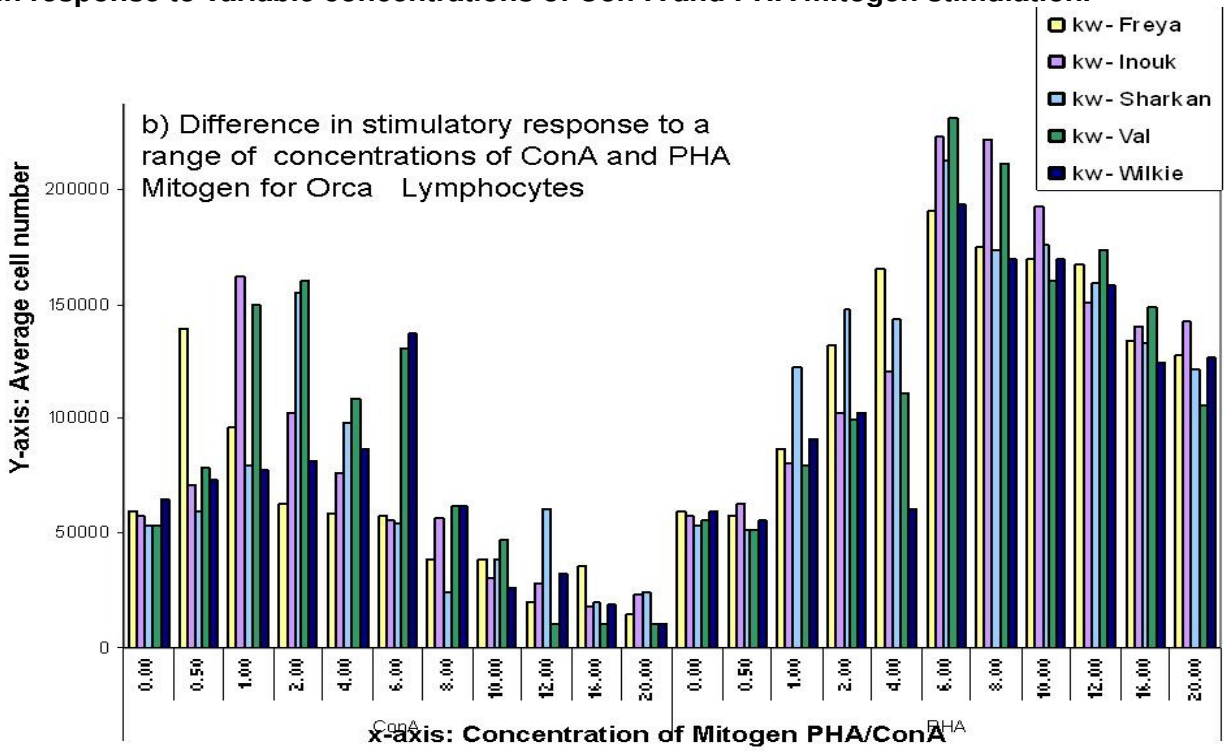


**Figure 2.3.11 shows the number of viable cells in lymphocyte cultures from bottlenose dolphin, in response to variable concentrations of Con-A and PHA mitogen stimulation.**

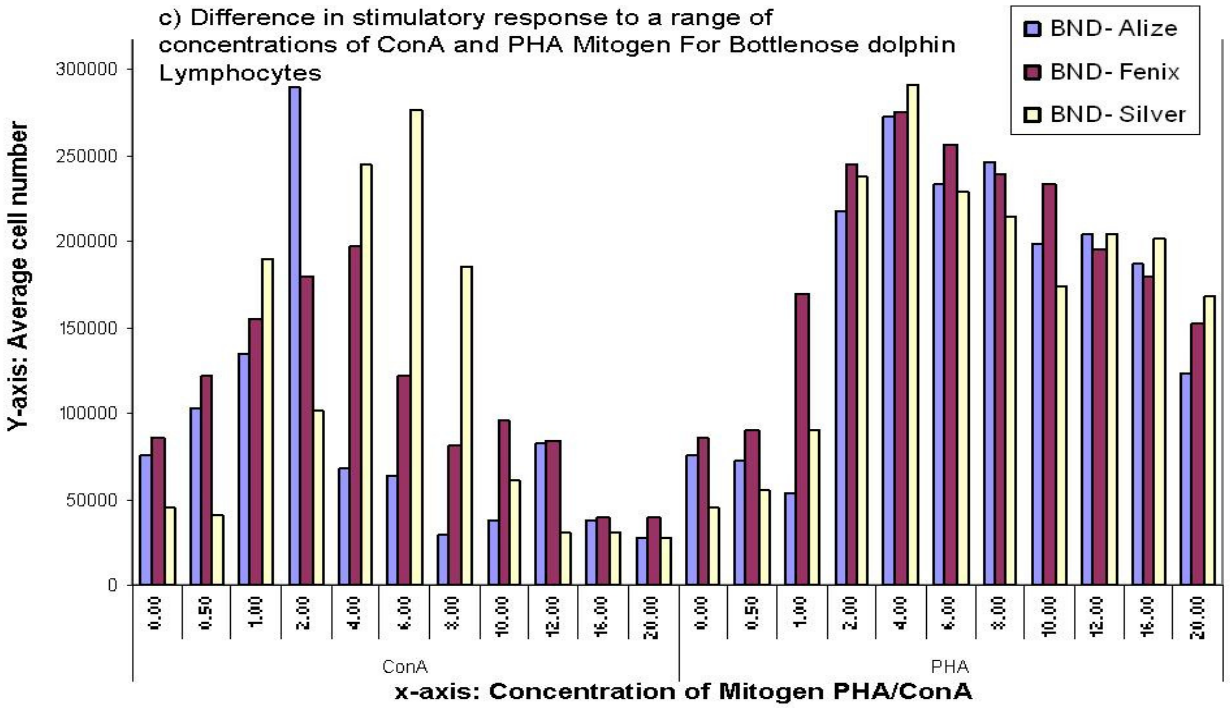



**Figure 2.3.12 shows the number of viable cells in lymphocyte cultures from grey seal, in response to variable concentrations of Con-A and PHA mitogen stimulation.**

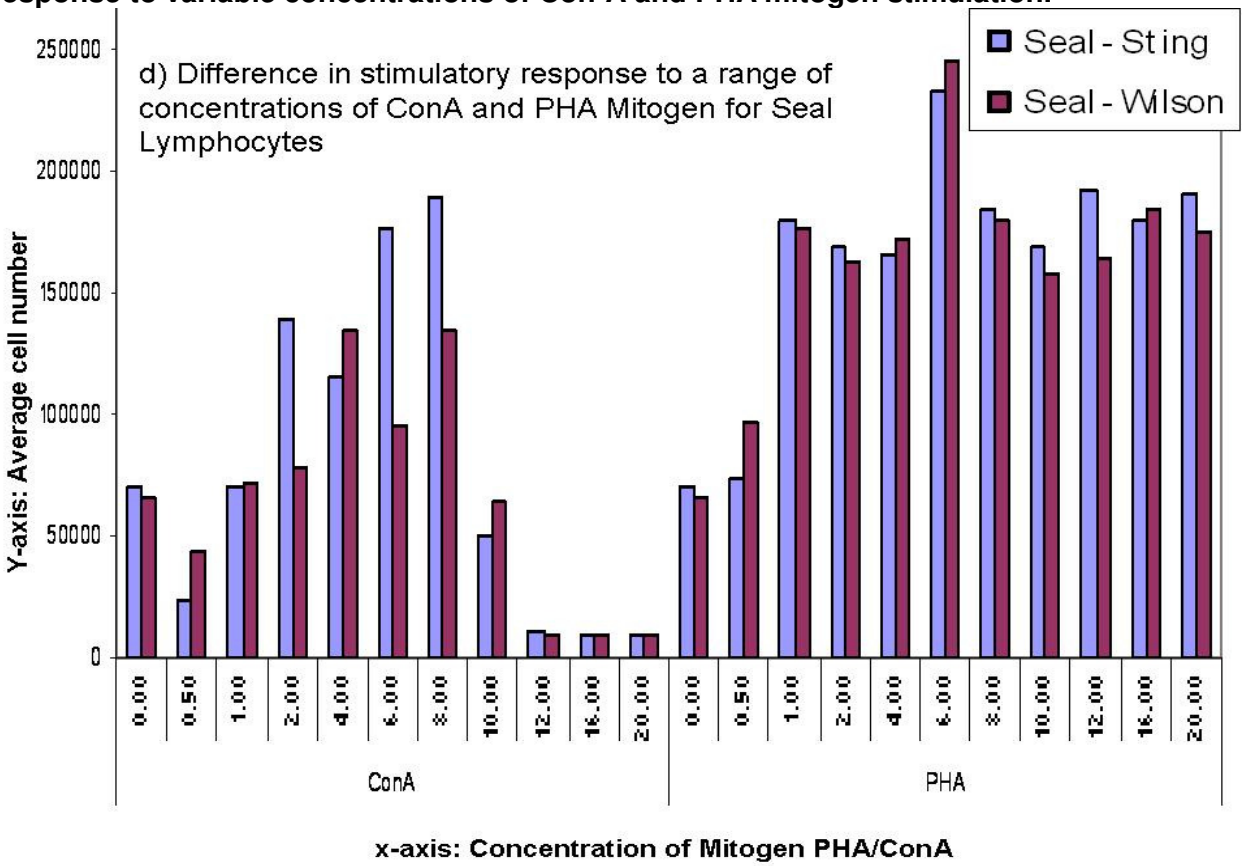


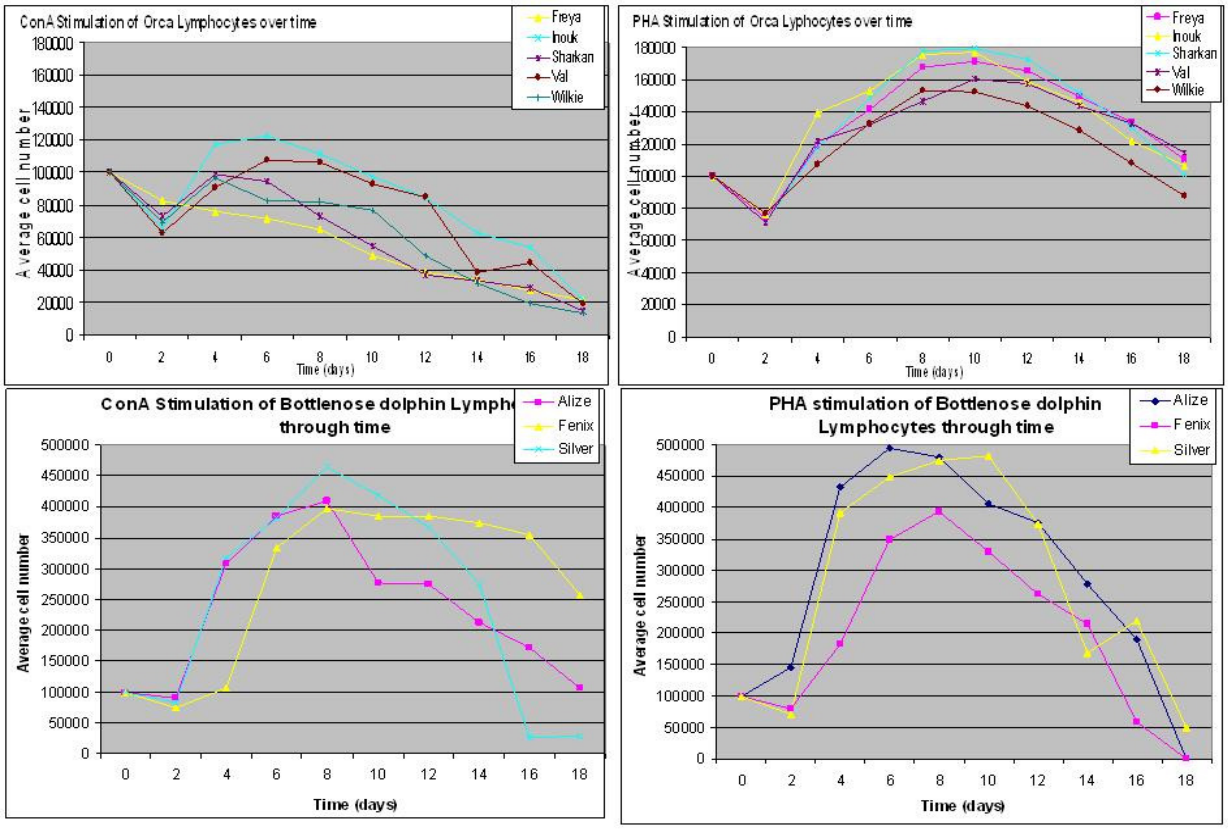
**Table 2.3.3** shows the day and concentration of maximal stimulation for each individual Grey highlighted squares inidicate which mitogen gave the maximal reponse observed for each individual. cRange = the range of different concentrations that also showed a proliferative reponse of at least 75% of the maximal value

| Species | Mitogen | Individual | Day | Conc | cRange |
|---|---|---|---|---|---|
| Orca | ConA | Freya | 4 | 0.5 | None |
| Orca | **PHA** | Freya | 8 | 6 | 4-12 |
| Orca | ConA | Inouk | 10 | 1 | None |
| Orca | **PHA** | Inouk | 8 | 6 | 4-10 |
| Orca | ConA | Shark | 6 | 2 | 1-2 |
| Orca | **PHA** | Shark | 10 | 6 | 4-10 |
| Orca | ConA | Val | 12 | 1 | 1-2 |
| Orca | **PHA** | Val | 12 | 8 | 4-12 |
| Orca | ConA | Wiki | 4 | 6 | 4-6 |
| Orca | PHA | Wiki | 8 | 6 | 4-12 |
| BND | **ConA** | Alize | 6 | 2 | 0.5-2 |
| BND | PHA | Alize | 8 | 4 | 2-10 |
| BND | **ConA** | Fenix | 6 | 4 | 2-4 |
| BND | PHA | Fenix | 8 | 4 | 2-12 |
| BND | **ConA** | Silver | 10 | 6 | 4-8 |
| BND | PHA | Silver | 8 | 4 | 4-12 |
| Seal | ConA | Sting | 2 | 6 | 4-6 |
| Seal | **PHA** | Sting | 6 | 6 | 1-20 |
| Seal | ConA | Wilson | 2 | 8 | 4-6 |
| Seal | **PHA** | Wilson | 4 | 6 | 1-20 |

The initial lymphocyte response to time was similar between the cetacean species, all individuals showing an initial decline in cell number between seeding and day two followed by a sharp recovery by day four to cell numbers at least 25% percent greater than the seeding density. In contrast the response of seal lymphocytes was more rapid and cell numbers were greater than seeding density by day two of culture. The overall response through time varied considerably within and between species and concentrations (Fig. 2.3.8 – 2.3.13). Bottlenose dolphin lymphocytes, on average across the three individuals, were maximally stimulated by Con-A (Fig. 2.3.11) however, there was no statistical significance ($p>0.05$) in the difference between the two mitogens. Analysis within and between individual responses however showed a significant difference between the response of individuals to the two mitogens (KW, $p<0.01$;

BND= <0.01). There was also a significant difference between the response of individual killer whales to stimulation with Con-A (p=0.002) but not to PHA (p=0.36), indicating a less variable response between individuals to the latter. The response of bottlenose dolphins to both mitogens was not significantly different between individuals (Con-A, p=0.32; PHA, P=0.87). Killer whales and seals, for each of the five and two individuals, respectively, were stimulated maximally by PHA. All individuals across species responded effectively to a range of dosages of PHA. Figure 2.3.13 indicates that following maximal stimulation to Con-A, cells rapidly died without additional stimulation (the latter indicated by subsequent observations with re-stimulation), whereas PHA stimulated lymphocytes remained viable in culture generally for a period of 2 to 6 days following their maximal proliferation.

**Figure 2.3.13** shows the number of viable cells in lymphocyte cell cultures from five killer whales (top); and three bottlenose dolphins (bottom) in response to Con-A (left) or PHA (right) mitogen stimulation as a function of time.



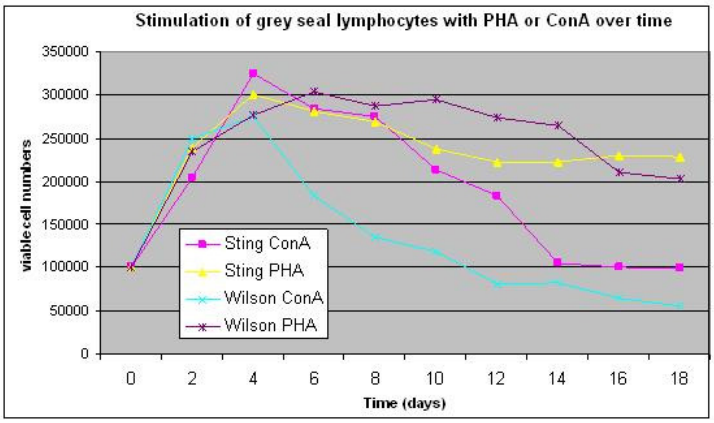

The magnitude of the response to both mitogens was consistently higher in dolphin lymphocytes than in killer whale and seals (Fig. 2.3.13). Killer whale lymphocytes were

stimulated by PHA, maximally on day six or eight at 6µg/ml across the five individuals. Although the maximal concentration and day of maximal stimulation varied between individuals, the difference was not significant for PHA ($p>0.05$). Con-A elicited a maximal response in killer whaless at 0.5, 1, 2 6µg/ml on day four, six, eight or ten for different individuals. As indicated in figure eight, some individuals were negatively affected by the concentration of Con-A that stimulated maximally for another. For example, the results for Freya suggest that there was a toxic reaction to stimulation with Con-A at 2µg/ml or above.

Bottlenose dolphin lymphocytes showed a consistently greater proliferative response to stimulation with both mitogens than did killer whales or seals (Fig. 2.3.13). Within the dolphins, there was less variability between individuals and differences were not significant ($p>0.05$) compared with the killer whale, however, the maximum response for the three dolphins was observed with different concentrations for the three individuals. All individuals were maximally stimulated with Con-A and PHA on day eight and six, respectively (Table 2.3.3 and Fig. 2.3.13). All three individuals were maximally stimulated with Con-A, although the magnitude of the response differed between concentrations. PHA did not elicit maximal stimulation for any of the three individuals; however, a range of concentrations elicited a response within 25% of the maximal stimulatory capacity achieved with Con-A.

For the grey seal, the response pattern was consistent between individuals for both mitogens (Fig. 2.3.14). Maximal stimulation occurred on day four or six for Con-A and PHA, respectively. For both seals, maximal stimulation was elicited with 6µg/ml of PHA, and the pattern of stimulation was very similar between the two individuals. However, the response to Con-A was inconsistent between the two individuals. Although the response was internally consistent between replicates for the two seals, one individual was maximally stimulated with 6µg/ml whereas the other demonstrated a generally reduced response to Con-A. Maximal stimulation occurred at 8µg/ml for the second individual (Fig. 2.3.14).

**Figure 2.3.14** shows the number fo viable cells in lymphocyte cell cultures from two grey seals in reponse to Con-A or PHA mitogen stimulation, as a function of time.




For all three marine mammal species, PHA showed the most consistent results, across concentration levels, time-scale and individual. Although Con-A had the strongest proliferative response at the appropriate concentration in the bottlenose dolphin, it also showed the most variability between individuals, ranging from being completely ineffectual to eliciting a toxic reaction, as indicated by the immediate and rapid reduction in cell numbers compared with controls.

### 2.3.6 Overall patterns of stimulation from the three marine mammal species

Cell numbers declined in both control cultures and in stimulated cultures after two days in culture; however, the unstimulated cultures continued to steadily decline in cell numbers, whereas the stimulated plates started to increase resulting in >100% recovery to original seeding numbers by day four or five and continued growth thereafter in well stimulated cultures. Cultures took between four and twelve days to reach maximal proliferation across the different species (details in Table 2.3.3). After maximal stimulation, all cultures started to decline in cell number, although the reduction was generally more rapid in Con-A than PHA stimulated cultures (Fig. 2.3.8 - 11). The proliferative response was found to be recovered by the addition of fresh medium and mitogens, although the magnitude of the response was not quantified. As

expected, cell stocks lost viability following long-term (1 week to 12 months) cryopreservation and exhibited delays of up to seven days before stimulatory responses were observed, following reseeding. However, the length of cryoperiod did not appear to have a significant effect on the rate of recovery, although precise differences were not quantified. Optimal conditions varied between individuals but once established and reapplied to subsequent samples from the same individual consistently elicited strongly positive proliferation.

Whilst the response to PHA was lower than to Con-A in some individuals, it was also found to elicit a toxic reaction at relatively low concentrations in others. PHA consistently amplified lymphocytes without causing a toxic reaction and showed the least individual variation. For all species, the acceptable range of PHA concentration was at least +/-2.5µg/ml whereas Con-A had a narrower range (<2µg/ml) for all species. The average between species was not significantly difference in proliferative response to either PHA or Con-A mitogen stimulation (d.f – 5, $p=0.6$). In particular there was a strong Spearman rank correlation between killer whale and bottlenose dolphin ($R^2=81\%$, $p<0.05$); killer whale and grey seal ($R^2=72\%$, $p<0.05$); and bottlenose dolphin and grey seal ($R^2=69\%$, $p<0.05$) to stimulation at 4 and 6µg/ml PHA.

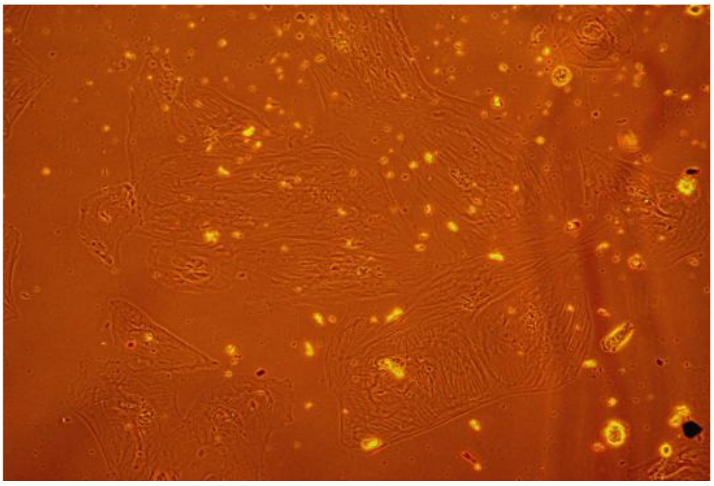
### 2.3.7 Characterization of corneal cell cultures from grey seals and elephant seals

Epithelial and fibroblast cultures were successfully established from the corneas from five of eight elephant seals and three of eight grey seals. In most cultures, within 24 hours, individual cells had attached to the surface of the culture flask exhibiting fibroblastic and epithelial characters. After three to five days it was possible to observe several colonies of cells forming. The majority of the cell colonies exhibited epithelial morphology and it became clear that epithelial cells were preferentially amplified with the applied culture conditions.

Cells initially exhibited large rounded morphology, however once the cells propagated they appeared to quickly demonstrate a more typical epithelial morphology as they reached confluence (long, parallel cell monolayers) as shown in Figure 2.3.15 of phase contrast images of the cells at confluence. Some tissue explants took two to three days to establish whilst others remained in culture for two to three weeks before cell proliferation was observed. Aside from differences in the rate of proliferation (between five days and two weeks to reach confluence), which was most likely associated with the age of the sample and its condition, there was no obvious disparity in the cell morphology, described above.

After eight weeks to four months (dependent on the sample), cells started to exhibit typical age-related changes in morphology including an increase in cell size, increased vacuolization and reduced growth rate (Figure 2.3.15), which was taken to indicate the cells were starting to senesce. Once culture flasks reached 80% confluence the cells were split 1:4, and half of the cells were used to seed two fresh flasks and the other half was cryopreserved to maintain cells at early passage number. At early passage numbers (1-8) cultures reached confluence after approximately six days, depending on the sample, after which cells started to show reduced growth rates (not reaching confluence for up to two weeks. For passages numbers over eight, for each pair of cultures from one individual, one flask was split 1:2 and the other was cryopreserved. The morphology of the majority of the cells is indicative of squamous epithelial cells; nuclei with prominent nucleoli are visible. Cells undergoing mitosis were apparent in early cultures, indicative of a substantial rate of proliferation.

**Figure 2.3.15** above shows the long thin morophology of the mixed population of corneal cells as they reached confluence and below at 4+months in culture reaching senescence, at 25x magnification



## 2.4.0 Discussion

### 2.4.1 Storage of blood samples

The results of the surrogate trials with sheep, cow and rat blood samples, would suggest that samples stored for less than 24 hours maintain the largest number of viable cells at body temperature as whole blood; however, if the samples need to be stored for longer than 32 hours, the most viable cells are retained by separating them first and storing them at 4℃ or 37℃ following either separation method. Although there were slight variations in the exact timings and relative maintenance of viability between the different species, the pattern that was observed for the surrogate species was replicated with marine mammal samples. The cell viability assessment corresponded to the proliferative capacity of the cell culture as indicated by the results of stimulation with both the surrogate and focal species. All samples with greater than 50% viability proliferated effectively following seeding and stimulation. Samples with a viability of 30-50% showed a delayed response in proliferation and samples with viability of less than 30% suffered extreme delays or failed to proliferate. As a result, it was determined that the success rate from blood storage was too low to exploit blood samples obtained from animals in distant localities and thus cell culture from elephant seals, for example, relied instead on opportunistic sampling from post-mortem specimens.

Extensive trials with bloods from the surrogate species supported by the literature (Mumford et al., 1975; Andersen and Friedrich, 1988; Lahvis et al., 1993; Ross et al., 1996; Raj et al., 1997; Chang et al., 2000; Miniscalco et al., 2003; Belloni et al., 2008) suggests that lymphocyte viability is gradually lost as time increases between venipuncture and seeding of lymphocyte cultures. This study is the first to demonstrate this effect quantitatively and to identify that blood samples stored as whole blood at body temperature (37℃) retained superior viability and had improved recovery over those stored at 4℃. Furthermore, in the short term it was shown to be more efficient to maintain the cells in whole blood rather than processing them at the collection point but at critical time points the different storage conditions changed their order of efficacy. After approximately 32 hours, cells retained viability more effectively having

been isolated compared with cells that were not. These same responses were also observed when storage trials were applied to the focal species.

These results indicate that storage at body temperature or 4℃ following isolation can improve the viability of the cells longer term. However, if cells are to be seeded within ~32 hours of venipuncture, samples are best stored as blood at room temperature. Therefore, if it is predetermined that it will not be possible to seed cells from blood samples within the critical 32 hour period following venipuncture, it is preferable to isolate the lymphocytes and store them at 4℃ or 37℃ in medium.

Following an initial decline in cell viability, samples isolated from whole blood and stored in medium may have shown a slower rate of decline through time because the cells would have been buffered by remaining in suspension. As the cells from samples stored as whole blood will naturally settle out over time (as per the rationale for the density settlement method of isolation), it is possible that they become dried and lysed as they are no longer buffered by the other elements of whole blood. The consistently pronounced initial decline seen when samples were stored at 4℃ suggests that the cells are maybe 'shocked' by the sudden temperature drop. However, the steady decline thereafter is probably as a result of the more stable temperature provided by refrigeration compared with maintenance at body temperature (37℃), which may fluctuate.

The LSM separation method did appear to yield a higher absolute number of viable cells; however, separation methods were generally comparable. Whilst LSM may be the preferable method when directly isolating cells in the lab shortly after venipuncture, it is important to note that density settlement is a successful 'low-tech' alternative, not requiring specialist equipment for separation. Furthermore, the density settlement method enables the cells to remain sterile during isolation without the need for a sterile cell culture laminar flow safety cabinet. It is therefore, feasible to use the density settlement method in the field where centrifugation is not available and when a sterile environment is not achievable. An extension to the density method was successfully applied with blood samples collected from grey seals in the field, subsequent

to the initial optimization pilot study detailed above. The settled out buffy coat layer was drawn into a syringe through the bung of the heparinized tube using an 18g needle, as before. It was subsequently transferred into 15ml polypropylene centrifuge tubes containing 7ml medium, pre-prepared under sterile conditions. The needle was pierced through the lid of the tube, removed from the syringe and then taped to the lid of the polypropylene centrifuge tube to maintain the sterile environment. Samples were maintained on ice until transported to a lab where cultures could be seeded. This method was found to be very effective at maintaining sufficient viable cells for samples obtained from remote field locations, where there was no access to a centrifuge or safety cabinet and demonstrated the comparable utility of this low-tech methodology.

It would be useful to extend these methodologies further to establish whether the addition of supplements to the storage medium extends the viable time period. It would also be worth investigating whether the sterile storage environment of the polypropylene centrifuge tube could be used to initiate cultures prior to reaching a laboratory facility, if it were possible to maintain the cells at 37℃ in the field. A recent study detailed improved conditions with the addition of mitogens to the storage medium (Belloni et al., 2008) and it would be interesting to establish whether this would improve the performance of the assay detailed herewith within the same context. Furthermore, combining these storage methods with the use of a conservative dose of mitogen that has been shown to elicit a proliferative response to a reasonable degree from all species and individuals (4µg/ml PHA) would maximize the potential for success, under conditions where assessment of the individual optimal dose is not possible, due to a lack of sterile conditions.

The detailed quantification and assessment of the limitations of the various storage conditions informed the subsequent collection of bloods for experimental application within this study. Furthermore, it should permit other investigators to collect samples in an informed context based on the known limitations of sample retrieval.

### 2.4.2 Lymphocyte Assay

As the results from previous lymphocyte studies in marine mammals have been inconsistent it was important to identify effective and replicable conditions to ensure maximal stimulation from lymphocyte cell cultures for the subsequent investigations. Consistent with the results of Lahvis et al. (1995), Con-A does appear to be the most powerful mitogen for PBLs in dolphins. However, this study has shown the response to PHA is more consistent across species and individuals as Con-A also demonstrated a toxic effect at high concentrations and for some, even at low concentrations. This toxicity is not nearly as pronounced with PHA, even at quite high doses. PHA has been found by this study and those with other marine mammal species, for example, beluga, porpoise and harbor seal (Dimolfettolandon et al., 1995; DeGuise et al., 1996; Beineke et al., 2004) to be an effective lymphocyte stimulant, albeit to a lesser extent than the optimal responses obtained with Con-A, in certain individuals. Most marine mammal studies have shown comparable results for Con-A and PHA, with the exception of Lahvis et al., (1993) where Con-A appeared to be a much more effective stimulant for bottlenose dolphin cells.

The results here are in agreement with Lahvis et al. (1995) with regard to the response of bottlenose dolphin lymphocytes but demonstrate that PHA is an effective alternative with a lower risk of toxicity. This study has confirmed species-specific differences but suggests a consistent approach to obtaining an efficient replicable proliferation of lymphocytes from a range of species.

With bottlenose dolphin lymphocytes maintained in culture for up to six days, Lahvis et al. (1995) found cells to be maximally stimulated at the end of their trial period. This study extended the length of time the cells were kept in culture and found that they can be maintained well for longer periods with no significant loss in cell viability with the use of PHA. Indeed at some concentrations for certain individuals they did not reach their proliferative peak until after six days in culture. The application of fresh medium and mitogens also demonstrated that the proliferative response could be recovered to prolong the culture up to several months. The

study of Lahvis et al. (1993) suggested the maximal conditions to be the optimal conditions; here a more conservative approach has been adopted in accord with Keller et al., (2005), whereby despite maximal proliferative responses for some individuals at high doses, the conditions that were most consistent across all individuals were adopted and applied in subsequent stimulation assays.

Bertram et al. (1997) showed that individual variation in the duck lymphocyte assay was associated with the level of monocytes in the culture which was dependent on the method of isolation. Monocytes act as antigen-presenting cells and therefore mediate the lymphocyte response to challenge. Consistent with the findings of this study, Bertram et al. (1997) found that there was a less variable response to PHA stimulation than to Con-A, which, given that it appears the Con-A response is mediated more by monocytes than PHA, could explain the greater individual variability in proliferative response with Con-A, as the response would be dependent on the homogeneity of the cell population.

Lahvis et al. (1995) found that the immunological response to Con-A and PHA was negatively correlated with the levels of various environmental contaminants measured in the blood of bottlenose dolphins. However, this study has also shown that individuals that performed poorly with low doses of mitogen did respond to higher doses of PHA, which may indicate that the effect of environmental contaminants may be overcome with increased stimulation.

The differential characteristics of the mitogens noted above may explain the variable response elicited with Con-A when compared to that of PHA stimulation. Furthermore, if the cells can still mount an immunological response to a mitogen, the cells have not lost their immunological capacity, which would arguably suggest that the lymphocytes themselves have not been affected by the environmental contaminants. Because the response of Con-A is thought to be monocyte mediated, the reduced response with Con-A stimulation could be a result of the environmental contaminants acting on the monocyte population, which does have as great an affect on PHA's propensity to stimulate T-cells in an antigen-dependent manner.

There may also be an interaction between the mitogen and the contaminants rather than the cells themselves.

During prolonged culture of the lymphocytes, culture of lymphocytes in DMEM rather than the usual RPMI culture medium resulted in an abundance of adherent cell phenotypes in the culture environment. Considering the subsequent processes of these investigations, which required a high volume of mitotic cells, the adherent cell phenotype may be an advantage. In adherent cultures mitotic cells can be harvested preferentially as a result of their propensity to become loosely attached to the substrate at mitosis and therefore easily separated by agitation of the flask and removal of the cells in suspension. A future development of this assay would be to ascertain whether sufficient adherent lymphocytes with normal characteristics (e.g. karyotype number) could be obtained by application of DMEM. A consideration is the space limitation of adherent cells due to their generally larger size and the one-dimensional surface area in comparison to the density of cells from suspended lymphocyte cultures.

Theoretically the increase of this adherent phenotype could simply be related to the differences in culture medium. Recent work has shown that both lymphocytes and monocytes are required for blastogenesis and the LSM is a mixed cellular environment. Adherent cells have been shown to be mainly derived from the monocytes, which act to mediate the cell response present in blood cultures, therefore the use of DMEM could be selectively amplifying the monocyte cell population.

Many studies, both with marine mammals and with other species have commented on the need to optimize culture conditions prior to commencing experimentation. For example, Keller et al. (2005) showed that there was significant individual variation in loggerhead sea turtles. It has been noted by several authors that there is a degree of individual variation in response, which must be accounted for in the experimental design. Talebi et al. (1995) noted that there was a heterogeneous response of individuals to stimulation with all three mitogens tested. In addition, substantial variation was noted in the proliferative response to Con-A but not to PHA, as observed in bovines (Miniscalco et al., 2003) and pigs (Koch et al., 1991). However,

this study has demonstrated that there are inconsistencies in the response of different individuals, which can be exploited to ensure a reliable proliferative response across species and individuals, irrespective of individual variation in immunological response.

### 2.4.3 Conclusions

It is widely accepted that the lymphocyte assay is dynamic and species-specific and therefore it is essential that optimization be conducted on a species-by-species basis. This study assessed both the storage conditions and the required parameters of the lymphocyte assay for three marine mammal species. The results of the storage component indicate the optimal conditions, as well as the options available to maximize the efficacy of the assay under various scenarios. The results of the lymphocyte assay underline the differences between species and individuals in their response but demonstrate that there are also consistencies in the individual response, which can be profited from to reproducibly elicit a lymphocyte proliferative response.

It was possible to outline a range within which the assay will work efficiently regardless of the individual. It should, therefore be possible to apply these suggested parameters to samples from unknown individuals using a limited range of conditions without the need of further optimization. Use of 4 or 6µg/ml of PHA should provide effective proliferation of lymphocytes from all individuals with the main variable then being reduced to which day the maximal stimulation will occur. Consistently, cultures that did not show a rapid proliferative response with 4µg/ml of PHA demonstrated a requirement of a higher concentration of PHA. This could inform stimulation assays to practise the use of 4µg/ml, followed by an increase to 6µg/ml if a rapid proliferative response is not observed by the fourth day of culture. Further to the study of Lahvis et al. (1993), which identified the utility of the lymphocytes assay in the bottlenose dolphin, here it was possible to standardize the assay across a range of marine mammal species. It has also demonstrated the importance of storage conditions to the quantity and quality of cells capable of mounting a successful proliferative response to stimulation.

Previous studies with marine mammals have independently been conducted with a number of different marine mammal species; however, this represents the first study in which the conditions have been quantitatively assessed across a range of species concurrently and the comparative limits of the optimal response have been identified. It has been possible to identify a set of cautionary methods of storage conditions and mitogen stimulation that could be profited from for a range of species and individuals to maximize the successful application of the LSM to marine mammals.

# Chapter 3: Cytogenetic Analysis of the Killer whale and Bottlenose Dolphin

## 3.1.0 Introduction

Variability in the length and intensity of certain chromosome regions has been noted by cytogeneticists in banded metaphase spreads. Heteromorphisms have been described for most species and are thought to reflect individual variance in the nature and copy number of tandemly repeated, transcriptionally inactive heterochromatic regions, consisting largely of satellite DNA at telomeres and centromeres or interstitial heterochromatin (Jacobs, 1977; McKay et al., 1978; Higgins et al., 1985). Figure 3.1.1 and 3.1.2 show examples of the particularly heteromorphic Y-chromsomes and and killer whale karyotypic heteromorphism, which is wide spread. In humans the frequency of some variants has been linked with population associations (Lubs et al., 1977; Bhasin, 2005). Variation of this sort has traditionally been identified from banded metaphase karyotyping studies (e.g. Arnason, 1974; 1981; Arnason, 1981; 1985); however, flow cytometry has also been used to characterize chromosome variants effectively (e.g. Trask et al., 1989a; 1989b).

**Figure 3.1.1** taken from Paresque et al., 2006 shows a) the variation of chromosome 3, 4 and 8. H: Heteromorphic, M: metacentric. and b) interindividual variability of the sex chromosomes in *Oligoryzomys nigripes*, the black-footed pygmy rice rat.

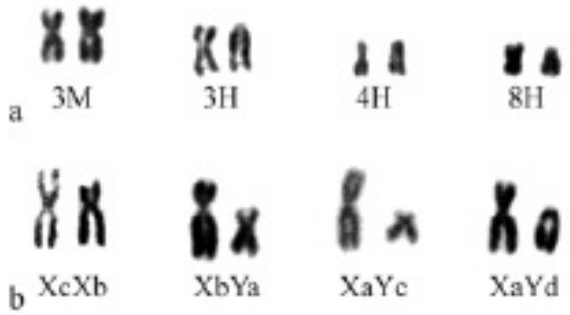
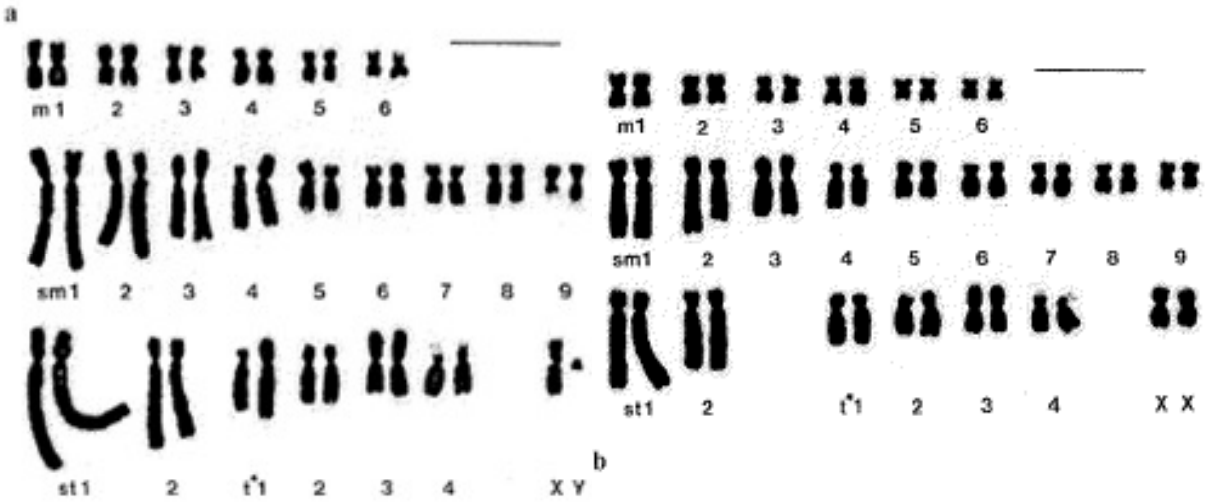


**Figure 3.1.2** taken from Arnason Lutley, 1980 shows the conventionally stained karyotypes of the Killer whale *Orcinus orca,* (2n=44) a) ♂1; b) ♀1. Size heteromorphisms occur in both karyotypes. Bar: 10µm.



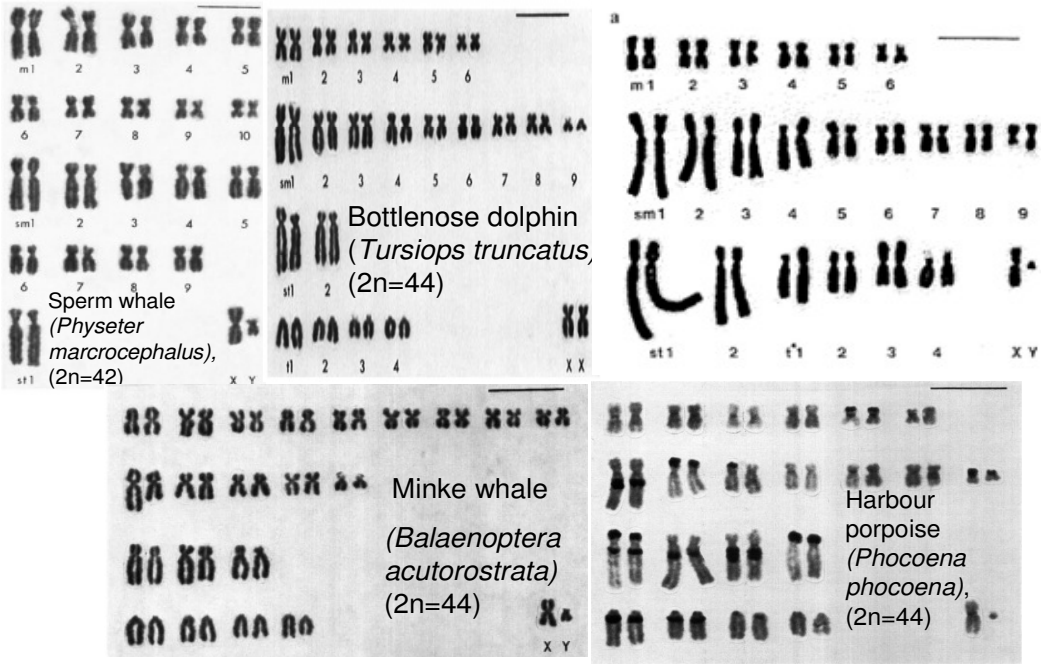

Flow cytometry has been used in human research to quantify DNA content differences between mitotic chromosomes and to study individual chromosome variability (Green et al., 1984; Harris et al., 1985; Gray and Langlois, 1986; Bhasin, 2005; Kuriki, 2005). Quantitative measures of relative base composition and total DNA content are obtained by staining the chromosomes with the DNA-specific fluorochromes Hoechst (HO) and chromomycin A3 (CA-3) that preferentially bind AT- and GC-rich DNA, respectively (Gray et al., 1979). A flow karyotype is produced from the relative fluorescence intensity of the chromosomes, which indicates the comparative profile of each population of chromosomes. It is subsequently possible to identify individual chromosomes on the basis of their peak position. The distance from the origin reflects the relative total DNA content whilst the distance from a slope where x=y, indicates the ratio of GC:AT of the chromosome.

The flow karyotype can be very useful for characterizing the number, size and basepair composition of metaphase chromosomes. Differences between flow karyotype profiles can indicate species-specific differences (Schmitz et al., 1992; Rabbitts et al., 1995; Schmitz et al., 1995; Langford et al., 1996; Rens et al., 1999; Rens et al., 2004), individual differences (Trask et al., 1989; Trask et al., 1989) and chromosomal abnormalities (Rens et al., 1994); whilst composition of each chromosome when compared to a standard karyotype can demonstrate an

imbalance in base composition, indicating accumulation of heterochromatin, for example (Trask et al., 1989; Trask et al., 1989). Flow karyotyping also facilitates the production of chromosome-specific libraries by the identification of individual chromosome peaks and flow sorting.

Individual differences in chromosome composition in terms of DNA content changes such as deletions, insertions or translocations have been identified with the use of flow karyotypes (Van Den Engh et al., 1985; Hausmann et al., 1991; Boschman et al., 1992; Hausmann et al., 1993; Rens et al., 1994; Gygi et al., 2002; Bhasin, 2005). Detection of loss or gain of DNA content and character in humans has been facilitated by flow karyotyping and by extension has been used to quantify heteromorphism of chromosome populations within and between individuals (Trask et al., 1989; Metezeau et al., 1993; Mefford et al., 1997). The degree of heteromorphism has been shown to be largely associated with the relative accumulation of heterochromatin, composed of transposable-element-derived and satellite DNA (Kurek et al., 2000; Bachtrog, 2003; Wyandt and Tonk, 2004; Dimitri et al., 2009). Satellite characters, which are largely species-specific, appear to be inherited according to Mendelian segregation within populations (McKenzie and Lubs, 1975) and exhibit concerted evolution leading to inter-specific molecular homogeneity (Pons and Gillespie, 2004). Using flow karyotyping, Trask et al. (1989b), demonstrated the consistency of inheritance of heteromorphism in families confirming the assumption that, although there are exceptions due to novel translocations or repeat extensions, heteromorphisms are generally inherited traits (Wyandt and Tonk, 2004).

**Figure 3.1.3** Marine Mammal karyotypes from the **sperm whale** (taken from Arnason et al., 1980); the **bottlenose dolphin** (taken from Arnason, 1974); the **killer whale** (taken from Arnason and Bernischke, 1973); the minke whale and the **Harbour porpoise** (taken from Arnason 1974). Showing the uniform 2n=44 chromosomes in all mysticetes and **odontocetes** (in bold) aside from two odontocetes – the sperm whale and beaked whales (not shown), which are 2n=42.




Most cetacean species have a very conserved karyotype of 22 pairs (2n=44). This typical karyotype is found in both mysticetes and odontocetes with only a few exceptions in each group having 21 pairs. Within the odontocetes the sperm whale and the ziphidae (beaked whales) have 2n=42 chromosomes. The ziphidae have lost one chromosome pair by the fusion of the two pairs, similar to a number of cases of 2n=42 among the mysticete species. In contrast, the sperm whale karyotype appears to have been formed by an unknown mechanism along with a number of additional distinctive characters, including the large size of some chromosomes.

The bottlenose dolphin conforms to the typical delphinid karyotype with 7 metacentric pairs, 8 sub-metacentric pairs, 2 sub-telocentric, and 4 telocentric chromosomes, plus a metacentric X-chromosome and a telocentric Y-chromosome (see Fig. 3.1.3, above) (Bielec et al., 1997). Although there is a limited amount of heteromorphism, these characteristics are

largely conserved across the delphinidae, with the exception of the killer whale (Kulu et al., 1971; Arnason et al., 1980).

The killer whale (orca), has particularly heteromorphic chromosomes (see Fig.3.1.2, above) , not only in terms of the differences between sister chromatids but also from one cell to the next and from one individual to the next (Arnason, 1974; Arnason, 1982). The heteromorphism is largely attributable to variation in heterochromatic regions found at the centromere and telomeres. Most species exhibit some degree of heteromorphism, but the accumulation in the killer whale is striking, particularly since the other species within the cetacea are far more conserved both within and between species. As a result of the heterochromatin the four delphinid telocentric chromosomes take on a subtelocentric character but for ease of interpretation and comparison they retain the traditional delphinid nomenclature. 'Killer whale' in fact is a misnomer and orcas are actually a member of the delphinidae, a large rapidly radiating family within the odontoceti, with fossil representatives dating back to the Middle Miocene (Barnes, 1976).

The striking uniformity in karyotype number over the 53 MY since the split of cetaceans from their most recent common terrestrial ancestor, the hippopotamus *(Hippopotamus amphibius)*, contrasts with the variation found in some shallower lineages. The gibbon-hominidae split occurred only16-18 MYA (Arnason et al., 1998); however, the more than 20 species within the group have karyotype numbers ranging from 2n = 38 to 52 (O'Brien et al., 2006). Although the karyotype number is mostly uniform (2n = 36 or 38) across the 38 extant species of felidae, which radiated very rapidly 10-15MYA (see Fig. 3.1.4) (O'Brien et al., 2006), the canidae by contrast, which radiated to the extant 36 species between 0.3 and 12MYA (Bardeleben et al., 2005), have karyotype numbers ranging from 2n = 32 to 2n = 78 (see Fig. 3.1.5) (O'Brien et al., 2006). Genomic arrangement clearly does not follow a "molecular clock" mutation rate as proposed for genomic mutation rates. The greater time frame for cetaceans suggests that there may be some mechanism of conservation in the genomic organization of

cetaceans, and the rapid radiation of species has been proposed as a causal factor (Chowdhary et al., 1998; Murphy et al., 2001).

**Figure 3.1.4 taken from (Pecon Slattery and O'Brien, 1998)**showing the phylogeny of the Felidae with chromosome numbers for the 8 lineages. Phylogenetic reconstruction of the final intron of Zfy in 34 species of Felidae using MP. Shown is the consensus tree of 16 trees of equivalent length and topology generated by 50% majority rule. The numbers on limbs are the number of steps/number of homoplasies. Values in italics are MP (above line) and NJ (below line) bootstrap proportions in support of adjacent nodes .50%. Asterisks denote significant nodes (*P ,* 0.05) derived in ML analysis. The arrow indicates the position of an additional node present in the NJ analysis only. Trees are rooted by midpoint rooting.

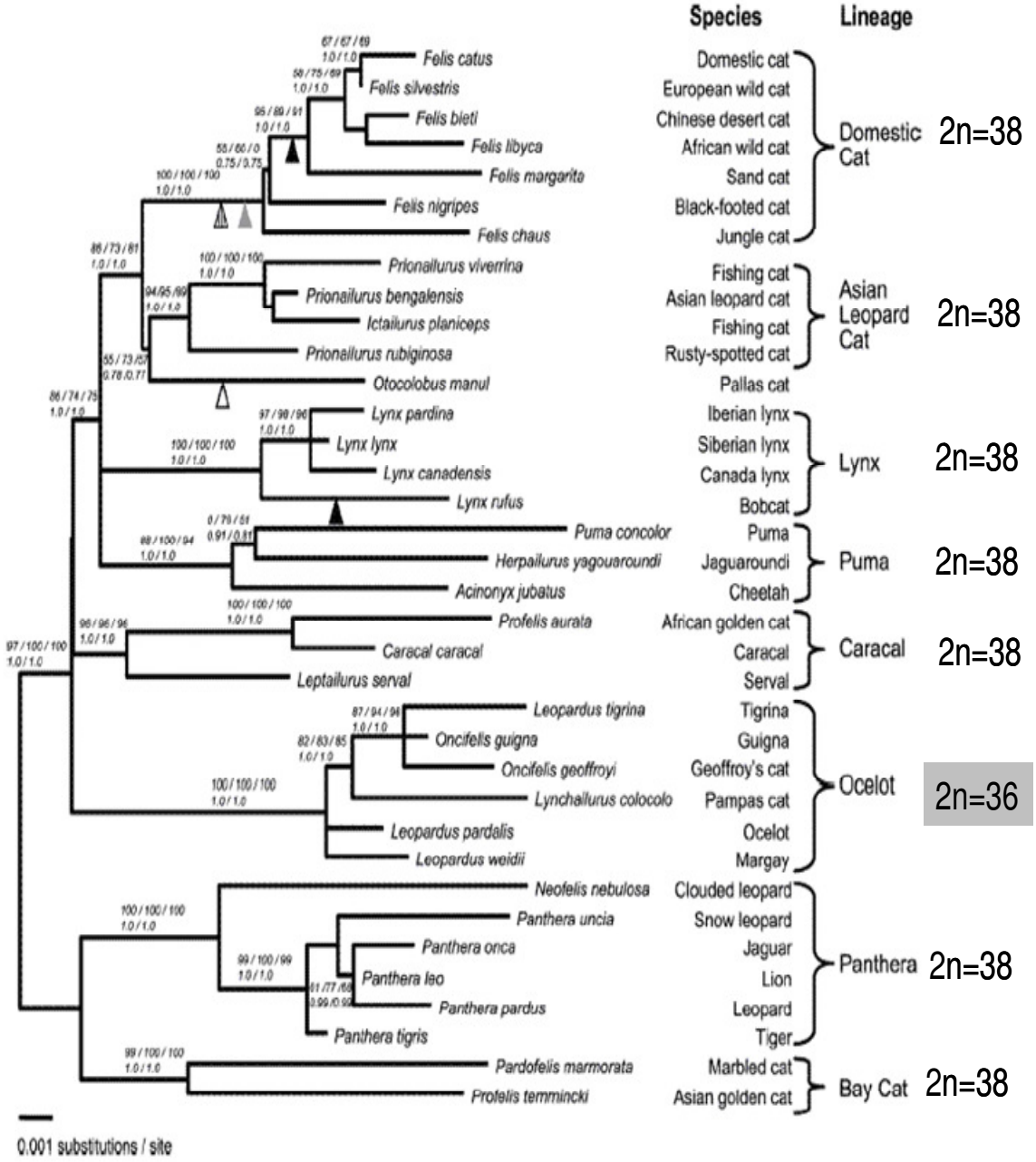
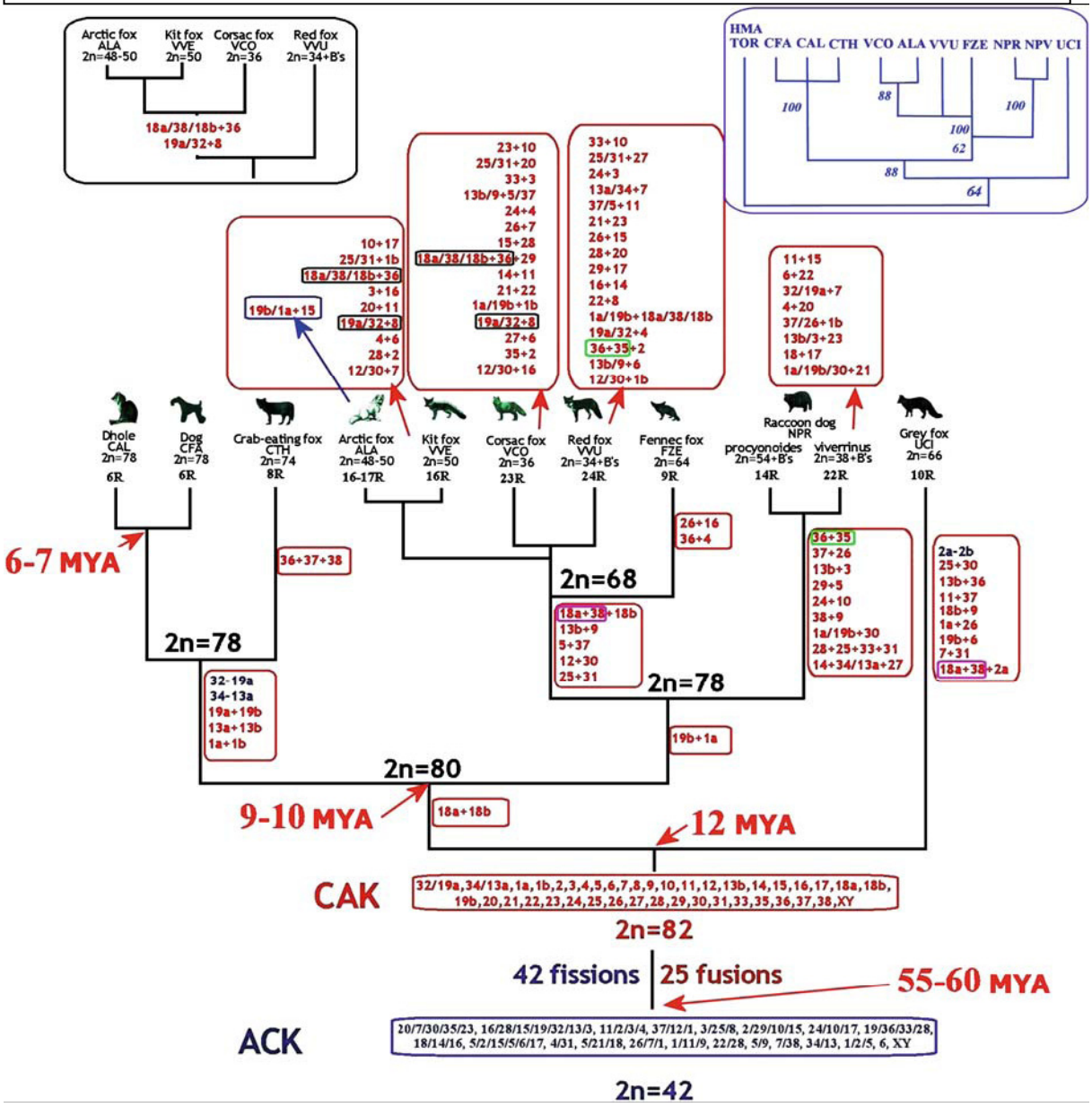


**Figure 3.1.5** taken from Grphodatsky et al., 2008, shows the phylogeny of the Canidae demonstrating the divergence in karyotype number. Karyotypic relationships of the Canidae. Chromosome structural changes defined by chromosome painting and banding (see explanation in the text) mapped to the canids phylogenetic tree proposed by Lindblad-Toh et al. (2005). Each chromosome (in brackets) of the Ancestral Carnivora Karyotype (ACK, 2n = 42; Murphy et al. 2001); at the bottom of the figure in a dark blue frame is shown as a set of domestic dog chromosomes. Above in a red frame are shown the chromosomes of the Canidae Ancestral Karyotype (CAK, 2n = 82), formed from ACK as a result of 42 fissions and 25 fusions. Chromosomal signatures CFA19a/32 and 13a/34 are shown through slashes. Above on branches of the tree, in a red frame, chromosomal fusions (+) and fissions (j) are indicated. At the branching points the most probable numbers of chromosomes are shown. In red frames above animal pictograms the chromosomal rearrangements are shown that took place during the formation of the chromosomal complements of the Japanese raccoon dog, the red fox, corsac fox, and also arctic fox plus kit fox. The dark blue arrow indicates the rearrangement of chromosomes 19b/1a + 15 that determines the polymorphism in the arctic fox, 2n = 48Y50. Chromosomal fusions that partly contradict this phylogeny are shown in light red, green, blue and yellow frames (see Discussion). The most probable phylogeny of arctic fox, kit fox, corsac fox and red fox is shown in the black frame. The R numbers indicate the number of chromosomal fusions/fissions separating each lineage from CAK. The divergence time, as millions of years ago (MYa), is indicated for sometree nodes as discussed in Lindblad-Toh et al. (2005). One of the most parsimonious trees retrieved from cladistic analysis of chromosomal characters identified in our study is shown in the blue frame (see Results, Supplementary Figure S2, and Supplementary Table S1). Bootstrap values are indicated in italic.



If rapid speciation occurred within the cetacea, as for the felidae, within a short space of evolutionary time the slow processes associated with chromosomal rearrangements such as translocations and inversions, which can cause large changes to the genome but do not generally occur rapidly, would not have had time to accumulate, though this couldn't explain the chromosomal variation among the recently radiated canid species. The nuclear DNA mutation rate of cetaceans has been suggested to be low compared to other mammals (Ohland et al., 1995; Bininda-Emonds, 2007), though it isn't clear how this might relate to the conservation of chromosome number. In addition to the rapid radiation of species within an order (Chowdhary et al., 1998; Murphy et al., 2001), the ecology and demography of a species have also been proposed as causal factors in the relative variability or uniformity of karyotype number across species (Wilson et al., 1975; Stanyon et al., 1988; Volker et al., 2008). However, despite recent studies demonstrating the mechanisms and biomolecular processes involved in the increased susceptibility of a species to rearrangement (Carbone et al., 2009), the underlying species-specific causes remain elusive.

The conservation of gross karyotype characters whilst developing high levels of heteromorphism characterized by heterochromatin in the killer whale is an interesting juxtaposition, which poses questions regarding the genomic evolution of the killer whale compared with other cetacean species. The heterochromatic regions of the killer whale were found to include a 1,579bp repetitive element that characterizes all delphinids. Furthermore, this element shows high homology to a similar repetitive unit that is found in all other cetacean families (Widegren et al., 1985). The repeat unit was estimated to comprise ~15% of the killer whale genome and maps to the chromosomal regions previously identified as being heterochromatic (Arnason et al., 1980; Arnason et al., 1984; Widegren et al., 1985; Gretarsdottir and Arnason, 1992; Gretarsdottir and Arnason, 1993).

It is evident from karyotype studies that the Y-chromosome of cetaceans in general is much smaller than those of other mammals. In particular, the killer whale Y-chromosome is one of the smallest within the delphindae and almost half the size of the average bottlenose dolphin

Y-chromosome (Arnason et al., 1980; Bielec et al., 1997). Previous studies of the killer whale karyotype have shown that the Y-chromosome constitutes leass than 0.5% of the killer whale genome (Arnason et al., 1980). In contrast, in other mammalian species the Y-chromosome constitutes a greater proportion of the genome, for example in the human it constitues on average 2%, in the minke whale it constitutes 1.7% (Arnason et al., 1974), in the domestic cat ~1.5% (Weinberg et al., 1997). In their study of bottlenose dolphin to human cross-homology, Carvan et al. (1994) produced an ideogram of the bottlenose dolphin karyotype and painted human chromosome-specific paints on to dolphin metaphase spreads. The level of conservation of chromosome regions between these taxa was shown with particular emphasis on the reciprocal regions of the bottlenose dolphin with respect to their organization within the human genome (Bielec et al., 1997; Bielec et al., 1998). They were not however, able to elucidate any reciprocal relationship with respect to the Y-chromosome because they used the karyotype of a female.

By creating a flow karyotype from the killer whale it is possible to identify heteromorphism of separate chromosome populations and elucidate the comparative characters of the genome both within species and between them. By creating chromosome-specific paints from the killer whale it will be possible to probe bacterial artificial chromosome (BAC) libraries to identify chromosome specific clones. In addition, the resource can subsequently be used to reciprocally paint the cetacean chromosomes on to metaphases of other species and thereby identify conserved regions of the species with respect to their organization in the killer whale. Reciprocal FISH painting studies have been used to identify conserved regions across taxa and to establish the extent of conservation of chromosome organization (Telenius et al., 1993; Graves, 1998), which can help to build an understanding of evolutionary patterns and processes and indicate phylogenetic relationships at the level of the whole genome. FISH studies are also used to identify the locality of specific gene regions, which are known in one species and have retained homology in less well-studied species (for example, Langford et al., 1996; Chowdhary et al., 1998; Tian et al., 2004; Mao et al., 2007; Trifonov et al., 2008). This study aimed to

produce a flow karyotype of the killer whale and to isolate chromosome-specific DNA to create chromosome-specific paints. In particular, the killer whale Y-chromosome was isolated to create a Y-chromosome-specific library for genomic and population genetic applications, as detailed in Chapter 4 and 5.

**Aims**

1. To isolate the killer whale and bottlenose dolphin Y-chromosome from growing cultures and create Y-specific WGA DOP-PCR libraries for screening
2. To identify the utility and information provided from flow karyotyping and produce the first bi-variate flow karyotype for a cetacean
3. To produce chromosome-specific fluorescent paints for future ZOO-FISH studies

### 3.2.0 Methods

#### 3.2.1 Basic principles of fluorescence activated flow cytometry

Flow cytometry was originally used for the assessment of physical and chemical characters of cells as they flow in a liquid stream through a measuring point surrounded by various detectors (Cram et al., 1990). Following the discovery that by using a finer nozzle, smaller particles could be carried in single droplet through the stream and in combination with the development of methods to isolate cellular particles, it became possible to assess not only the characters of whole cells but also their components (Langlois et al., 1980).

Typically at the measuring point the particles intercept a beam of light from a laser and when the light hits the droplet, the particle inside will scatter the light, the profile of which is collected by an array of detectors positioned at various angles around the measuring point. The amount of light that is scattered by a particle is a combination of its size, shape and refractive index. With preferential particle associated fluorescent labelling it is also possible to detect the amount of fluorescence of each particle, and thus identify the composition of the labelled fraction. The combination of fluorescent stains selective for nucleic acids and the development of methods of chromosome isolation (Crandall, 1987), Langlois, (1989) has made it possible to apply the principles of flow cytometry to individual chromosome characterization. Furthermore, by coupling the flow cytometer with a cell sorter, it is subsequently possible to isolate and collect the fractions of interest.

Following chromosome isolation (detailed in the methods section), the preparation can be labelled with fluorescent stains that are taken up preferential by DNA as a whole (propidium iodide), or preferentially by 'GC'-rich DNA (chromomycin A3), or 'AT'-rich DNA (bisbenzimidazole). The fluorescent antibiotic Chromomycin-A3 (C-A3) shows an affinity for the 2-amino group of guanine in DNA, and hence is specific for GC-rich DNA (Crissman and Tobey, 1990). The bisbenzimidazole (Hoechst, HO) binds non-intercalatively to AT-rich regions of DNA (Langois and Jensen, 1990). The GC-rich specificity of C-A3 combined with the AT-rich

specificity of Hoechst dye has been exploited to determine the basepair composition of individual chromosome populations (Langlois, 1989).

Fluorescently labelled chromosomes are passed singly through the beams of two lasers that excite at the appropriate ranges for the fluorochromes (HO - UV: 375 nm; C-A3 - 405 nm). The fluorescence intensity of each chromosome is then quantified as it passes through each beam in turn and plotted to yield a bivariate flow karyotype, based on the profile of the relative basepair composition of each chromosome. The sample is passed through the centre of a channel in an isotonic saline liquid. Each droplet contains a single chromosome, which passes in turn through the beam of an argon laser exciting the fluorescence of the C-A3, and Hoechst fluorescent stains bound to the DNA. The scattered fluorescent light generated as the chromosomes pass through the beam is collected by the photodetectors. Dichromic mirrors at right angle to the beam of light reflect wavelengths of light on to the detectors, which is converted into an electric signal that is recorded (Ormerod, 2000). The Fluorescent Activated Cell Sorter (FACS) measures five parameters; forward light scatter (FSC), Side light scatter (SSC), and three fluorescence intensity parameters (FL1, FL2 and FL3), which build an individual fluorescence profile of each chromosome population. By instructing the sorter to collect any droplet fluorescing at the wavelength of interest, any chromosome that matches the parameters will be collected. Droplets containing individual chromosomes pass through the laser and if they are not targeted for collection will pass into a waste container and be lost. It is possible to target three different populations of chromosomes at any collection phase but any chromosomes that are not being targeted will still pass through the beam and be lost. It is, therefore, necessary to have a substantial preparation of individual chromosomes, in order to collect sufficient for subsequent molecular applications.

A Dakocytomation FACS Flow cytometer in combination with C-A3 and Hoechst was used in this study to obtain a profile of the AT-CG ratio of DNA content, enabling the identification of a flow karyotype for the focal species. In order to isolate individual

chromosomes by flow sorting it was first necessary to accumulate a high proportion of cells at metaphase, as detailed in chapter 2.

### 3.2.2 Cell culture and Chromosome Isolation

A growing culture of primary lymphocyte cells (see Chapter 2) from a male killer whale was synchronized by the application of a thymidine block followed by colcemid treatment. Addition of thymidine produces a chemical imbalance that blocks the cell cycle at the G1 interval and therefore cells accumulate at the G1/S boundary. Once the block is released the cells then continue their cell cycle synchronously (Thomas and Lingwood, 1975). Following release from the thymidine block by the addition of fresh medium, the cells are synchronized when they reach metaphase. The subsequent addition of colecmid, which prevents the formation of the spindle, introduces a second block between metaphase and anaphase resulting in a higher percentage of cells at metaphase, at which point sister chromatids are present as individual chromosomes. This in turn facilitates the use of cells for metaphase spreads, as well as chromosome isolation for flow sorting. By the use of the double block, colcemid treatment (which becomes toxic to the cells when used for prolonged periods) can be reduced to a minimum time of ~20 minutes. A large proportion of cells are consequently at early metaphase and therefore this technique produces a produce a more homogeneous preparation of longer, better quality chromosomes (Clouston, 2001). Thymidine (15mg/ml) in phosphate buffer saline (PBS) was added to the growing culture the day before harvest for ~16 hours, to block the cells from continuing through the cell cycle, the thymidine block was released by centrifuging the cells at 200g for 10 min and resuspending them in fresh medium for ~4 hours before the addition of colcemid (10µg/ml) for 20 minutes, prior to harvesting the cells. Cells were again centrifuged at 200g for 10 minutes, the supernatant was removed and the pellet placed on ice until chromosome isolation. The final stage is the addition of a hypotonic solution, potassium chloride (KCl), which swells the cells facilitating the lysis and isolation of chromosomes.

### 3.2.3 Flow Cytometry for flow karyotyping and Cell Sorting

The cell preparation was harvested as before by bang harvest, as detailed in the microdissection cell culture section. The supernatant was poured off and the cell pellet was resuspended in fresh ice-cold complete medium. The suspension was then centrifuged at 180g for 10 min thereby removing dead cells and debris. After pouring off the supernatant the cells were resuspended in hypotonic 0.075M KCl solution to swell the cells (using 10mls for every $10^7$ cells). This was then incubated for 15 min at 37℃ before centrifuging at 180g for 5 min. All further steps were carried out at 4℃. The supernatant was poured off and the pellet was resuspended in cold polyamine buffer +digitonin (15 mM Tris, 0.2mM spermine, 0.5mm spermidine, 2mM EDTA, 0.5mM EGTA, 80 mM KCl, 20mM NaCl, 14 mM (0.1% v/v), beta mercaptoethanol adjusted to pH 7.2 before adding), at a concentration of 1ml/$10^7$ of cells. Following vigorous vortexing for 30-60 sec to break cell walls the cell lysis was monitored with fluorescence microscopy by placing a drop of cell suspension on a slide previously spread with a drop of a fluorochrome (ethidium bromide), a cover slip was placed over the suspension and examined; N.B. most chromosomes were free and in suspension after 60 secs of vortexing. Nuclei were removed before cell sorting by spinning down the nuclei at 180g for 10 min and transferring the supernatant to second tube. 1ml of B2 buffer was added to the pellet and resuspended by a 5 sec vortex. The centrifugation at 180g for 5 min was repeated and the supernatant removed and added to the first supernatant. The presence of nuclei was verified by assessing the preparation, as detailed before for microdissection, with an ethidium bromide stained slide. The suspension can be stored for 2 weeks at 4℃ with little loss of cells for flow cytometry. Immediately prior to using the preparation for flow cytometry, 40mg/ml chromomycin A3 in1mM MgCl2 and 0.5mg/ml Hoechst 33258 was added to the preparation. The suspension was then left for at 2 hours, for the fluorochromes to equilibrate before sorting. Fifteen minutes prior to analysis, sodium sulphite and sodium citrate were added at 25µm and 10µm, respectively. Chromosomes were analyzed and sorted according to Telenius et al., (1992). Chromosomes were passed in suspension through a (Dakocytomation flow sorter) FACS in

which two lasers were set to excite the dyes separately, to create a bi-variate flow karyotype, indicating chromosome size and base-pair composition. The individual peaks were identified in the resulting flow karyotype and clear peaks were isolated into separate collection tubes, pre-prepared with PCR water. Approximately 500 chromosomes were collected for each peak isolated. Using the sorted chromosomes as template a PCR reaction was carried out using DOP-PCR primers (5'-CCGACTCGAGNNNNNNATGTGG-3'). The PCR was conducted in a final volume of 50µl containing 4µl of 25mM MgCl, 5µl of 10xPCR buffer (500mM KCl, 100mM Tris-HCl, pH8.0), 2µl of 5mM dNTP's, 5ul of 17µM primer 6MW (as above), 0.5µl of 2.5U Taq polymerase (Supertaq, Promega). The following PCR conditions were used: 33 cycles of 1min at 92℃, 2 min at 56℃, and 2 min at 72℃, followed by 5 min final extension at 72℃. Fluorescent In-situ Hybridization (FISH) was used to create chromosome-specific paints from the flow-sorted and microdissected chromosomes; see 'FISH' section below.

### 3.2.4 Microdissection

Following lymphocyte cell growth and synchronization, as detailed above, cells were harvested and resuspended in 10ml hypotonic potassium chloride (0.075M KCl) for 10 min on ice. The cell suspension was centrifuged at 200g for 10 min and then fixed in a solution of methanol: glacial acetic acid (3:1). This suspension was then kept at 4℃ until required for metaphase preparations. To assess the quantity of preparation required for evenly spread metaphases suitable for microdissection, cells in a range of dilutions (1 in 5-15µl) were dropped onto slides and viewed under a phase microscope. The metaphase preparation was then suspended in the appropriate volume of 3:1 fixative so that 7µl of preparation could be used per slide. Coverslips prepared in 10% w/v SDS were removed from storage and rinsed under running water. Two to three drops of sterile water were dropped on to the centre of the slide ensuring that water remains in the centre. Seven microlitres of metaphase preparation was dropped using a pipetttor horizontally from a height of ~15cm, in order to maximize the spread, onto the centre of the slide followed immediately by 7µl of fresh 3:1 fixative and then allowed to

air dry until completely dry (which required approximately 5 min). Metaphase preparations were subsequently Giemsa stained by processing them through the following solutions for the critical times noted. The coverslips were first washed for 1min in 35mls of 1xPBS, then digested for 30 sec in trypsin-EDTA solution (35mls of 1xPBS with 5mls of 10x trypsin-EDTA), washed again for 1 min in a fresh 35 ml tube of 1xPBS, stained for 4 min with Giemsa solution (3ml filtered giemsa: 35ml 1xPBS) and finally rinsed in 30-40ml of sterile distilled water for 1 min. Coverslips were again left to air dry for approximately 5 min. Stained metaphase preparation coverslips were kept refrigerated until ready to microdissect.

Prior to microdissection, solutions for the collection buffer were prepared and the collection pipette and needles were prepared as follows. The collection buffer consisted of 6µl salt solution (20µl 100mM NaCl, 20µl 100mM Tris H-Cl pH7.5, 20µl 10mM EDTA ph 7.8-8, 20µl 1% w/v SDS, 20µl 1% v/v Triton-X, 20µl sterile distilled water to a final volume of 120µl) 3µl of glycerol, and 1µl of proteinase-K (15mg proteinase-K powder, 800µl sterile water, 200µl glycerol). Glass needles with a diameter of approximately 1µm were produced from a 1mm diameter borosilicate glass rod (Harvard part #30-0085) using a pipette puller. Collection pipettes were produced from disposable glass pasteur pipettes using a pipette puller. The very end of the needlepoint of the pipette was removed to produce a smooth opening with a diameter of approximately 5µm by tapping vertically against an iron disc. In order to strengthen the tip of the collection tube the end was carefully dipped into 2mM EDTA followed by dimethylsilane and then baked at 100℃ for 3 hours.

To remove individual chromosomes from a coverslip, a glass needle was placed into the clamp of an eppendorf micromanipulator 1571 mounted on one slide of Leica DRIMB microscope, the collection pipette was fixed on the other side in a pipette stand and the slide was placed on a moveable slide manipulator over the inverted microscope. Individual metaphases were located by sequential identification with 10x to 150x objectives. The collection pipette, prior to mounting, was dipped into the collection buffer and ~0.1µl was aspirated by capillary action. An individual chromosome was scraped from the surface of the coverslip onto

the tip of the needle and then manoeuvered inside the collection pipette where the chromosome was removed from the needle into the end of the pipette. The pipette was then removed from the stand and placed in a humidifier at 60°C for 1-2 hours.

Following incubation the pipette tip was broken off into a tube containing 'Sequenase' pcr reaction mix (USB, Ohio), containing 1µl Sequenase buffer, 0.5µl of 0.2mM dNTPs, 1.25µl of 20µM 6MW (DOP-PCR) primer (5-'CCGACTCGAGNNNNNNATGTGG-3') (Telenius et al., 1992), 2.25µl $dH_20$. The mix was subsequently vortexed and stored at -20°C until ready for PCR reaction. DOP-PCR was used to universally amplify the microdissected chromosome DNA in a PTC200 thermo cycler (MJ research). The procedure followed Telenius et al. (1992). A primary PCR was conducted with the reaction as follows. The reaction was first denatured for 5 min at 92°C to inactivate the proteinase-K, followed by 8 low temperature cycles, as follows: 1 min at 90°C, 2.50 min at 25°C and 2 min at 35°C, during which time, 0.25µl of 1:7 sequenase mix (1 sequenase: 7 sequenase dilution buffer) was added during the 25°C temperature step of each cycle. Following these 8 cycles 50µl of 'standard' pcr reaction mix was added, containing 10µl Buffer D, dNTPs, 250mM primer 6MW, Super Taq, W1 and $dH_20$. The PCR was then restarted and continued through 33 "high temperature cycles", followed by an extension period of 5 min. The 'standard' PCR was conducted in a final volume of 50µl containing 4µl of 25mM MgCl, 5µl of 10xPCR buffer (500mM KCl, 100mM Tris-HCl, pH8.0), 2µl of 5mM dNTPs, 5µl of 17µM primer 6MW (as above), 0.5µl of 2.5U Taq polymerase (Supertaq, Promega). The following PCR conditions were used: 33 cycles of 1 min at 92°C, 2 min at 56°C, and 2 min at 72°C, followed by 5 min final extension at 72°C.

### 3.2.5 Fluorescent In-situ Hybridization (FISH)

FISH was used to assess the degree of enrichment of chromosome sequence in the flow-sorted and/or microdissected material, and to determine the degree of contamination from other chromosomes or chromosome fragments in the various preparations. One microlitre of the DOP-PCR library was used as an amplification substrate for preparing a FISH painting probe by

conducting a secondary PCR with the primary PCR product as the template to create a chromosome-specific paint. The probe was subsequently hybridized to a metaphase spread created from the same individual from which the library was created.

#### 3.2.5.1 FISH ethanol series

A drop of methanol/acetic acid suspended metaphase was dropped onto a pre-cleaned slide. The slide was then passed through an ethanol series as follows: twice in 70% w/v ethanol for 2 min, each, twice in 90% w/v ethanol for 2 min each, 100% w/v ethanol for 4 min, and then left to air dry. Subsequently, the slide was incubated at 65℃ for one hour to age the slides, during which time the fluorescent probes (2µl of secondary PCR in 12µl of hybridization buffer) were denatured at 65℃ on a heat block for 10 min and then 30 min at 37℃. The slide was then denatured in 70ml of 70% w/v formamide in standard saline citrate (SSC) for 1 min 30sec, to pre-anneal the probe. The slide was then placed into ice-cold 70% w/v ethanol for 4 min and subsequently taken through the ethanol series as before and left to air dry. The denatured fluorescent probe (suspended in 10µl hybridization buffer*) was applied to the denatured slide, and covered with a coverslip, sealed with FISH fixative and incubated in a humidity chamber overnight at 37℃. The following day, the coverslip was removed and the slide was passed through a series of washes at 45℃, as follows: twice through stringency wash (Formamide/SSC) for 5 min, twice through 1xSSC for 5 min each, then once in 4xT (0.5% w/v Tween20 detergent/4xSSC solution for 4 min). 0.2µl of the antibody (Cy-3) in 100ul of 4xT, was immediately applied to the slide, which was covered with parafilm to prevent dehydration and incubated at 37℃ for 20 min. Following incubation the slide was rinsed 3 times in 4xT for 4 min each. The slide was then drained to remove excess fluid and covered with mounting medium (DAPI II) and a coverslip. The coverslip was sealed with FISH mounting sealant and viewed under a fluorescent microscope (Zeiss LSM 700).

### 3.2.6 G-banded metaphase preparations

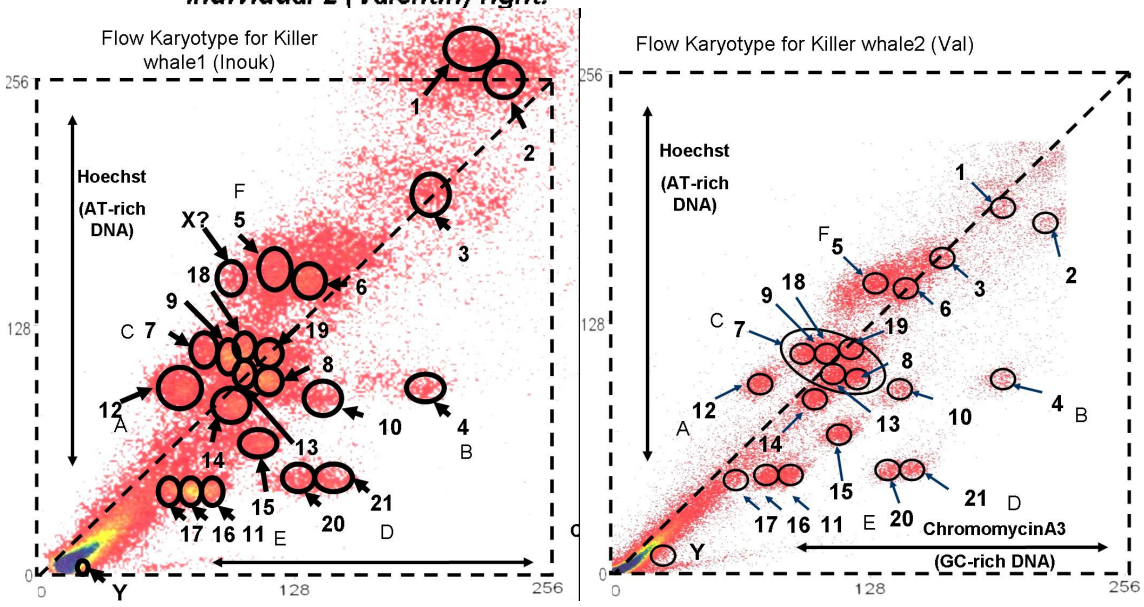
G-banded metaphase preparations were prepared as for microdissection coverslips with the following modifications. Metaphase were prepared on 1 cm thick slides, instead of coverslips and the trypsin digestion and giemsa staining periods were modified to optimize for banding patterns. The slides were aged for up to 2 weeks or by incubation at 60℃ for 45 min. Trypsin-EDTA digestion was optimal at 15 sec and Giemsa staining was optimal at 3 min. G-banded karyotypes were created following photography with a PALM microbeam microdissection microscope and camera system (Carl Zeiss Ltd., GmbH, Welwyn Garden City) and arranged using Photoshop CS2 (Adobe). The digital scale bar from the Zeiss system acted as an internal standard to allow the direct measurement of each chromosome, which were measured in pixel units and converted to a micrometer scale using the internal digital scale. The orientation and spread of the chromosomes will affect the absolute length but averages of 10 metaphase spreads were used to create a table of chromosome sizes and the corresponding basic ideogram. The relative length was also calculated and used to assign a nucleotide number estimate to each chromosome derived from an estimated total genome DNA content for odontoncetes, taken from Du and Wang (2006). Using a total genome size in *T. truncatus* of 3.07pg and a range of five odontocetes of 3.07 to 3.68pg (Du and Wang, 2006), the calculation of picogram to base pair genome size (based on 1pg = 978Mb, Dolezel et al., 2003) results in genome size range of 3.0 – 3.6Gb (which encompasses the 3.09Gb size of the human genome) and an average estimate of 3.2Gb for the odontocetes. It is therefore possible to estimate the base pair content of an individual chromosome by combining the relative length information (expressed as a percentage of the total combined lengths of all chromosomes) with the estimated total genome size.

### 3.3.0 Results

The bivariate flow karyotype of two male killer whales is presented in Figure 1. Peak identification is based on a combination of the final flow karyotype image and the observed accumulation. As it is a dynamic process, the final images do not always represent the event accumulation because early images will not account for all chromosome peaks if by chance some do not pass through the nozzle, whilst later images will have accumulated greater degrees of chromosomal fragments and thus the peaks will be obscured by dots representing non-specific fragments.

**Figure 1:**

***Flow karyotype – figure one shows 2 flow karyotype images, both from male Killer whales, individual 1 (Inouk) left and Individual 2 (Valentin) right.***




Although it was not possible to unequivocally identify all 21 autosomal pairs, it was possible to distinguish 21 peaks and a putative assignment of the X- and Y-chromosomes for individual one. The numbers in Figure 1 are for representation purposes only to indicate the identification of the full karyotype complement. The numbers assigned to the various peaks were based on the likely identity as indicated by the relative size and informed by the position of

the flow sorted/FISH painted chromosomes, which were identified. The labelling system used corresponds to that generally used for the classic cetacean karyotype, with the specific numbering scheme taken from Bielec et al. (1998). Aside from the FISH-painted chromosomes, it was not possible to unequivocally assign an identity to all chromosomes, particularly those of similar size, however chromosomes that are clearly not similar in size and character to others can be assumed to have been correctly identified and labelled. The Y-chromosome appears to occur within the swathe of small fragments from other chromosomes and therefore could not be clearly identified or collected. Due to their only be one quarter of X-chromosomes in the genome of a male relative to the autosomes, the X-chromosome 'events' through the flow sorter will be rare and although there is an indication of chromosomal 'events' in a similar region to that observed for the X in individual one, it was not possible to identify a peak that would correspond to the X in individual two.

The more condensed peak pattern of individual two meant that it was not possible to identify an individual peak for all 21 peaks, instead showing a cluster of the six similarly sized chromosomes, which is likely to correspond to the chromosomes assigned in Figure 1, for individual one. The majority of the cluster of six peaks can be subdivided but the assignment of all chromosomes therein was not possible. This cluster most likely represents the group of similarly sized metacentric chromosomes of the killer whale, which are difficult to assign using traditional karyotyping studies. Comparison between the flow karyotypes from the two individuals clearly suggests variability between their profiles and closer examination and the overlay of the peak profiles in Figure 2, indicates relative positional differences of specific chromosomes. In particular, chromosomes 9, 10 and 11 for individual one, appear to carry more total DNA compared with individual two, as indicated by their position further away from the origin as well as indicating a more AT-rich composition by their position biased towards the AT-rich region of the flow karyotype. Chromosomes 7 and 8 appear to be more AT-rich and most peaks appear to have a more dispersed peak pattern, suggestive of a heteromorphic population of chromosomes in individual one compared with individual two. In particular**,** the chromosomes

that likely represent the heterochromatic ‘t’-chromosomes (telomeric chromosomes, which are subtelomeric in the killer whale) appear to have dual peak populations.

***Figure 2*** *shows the relative chromosomal positions of the flow karyotype for the two orcas, Individual one is in blue and individual two in black.*

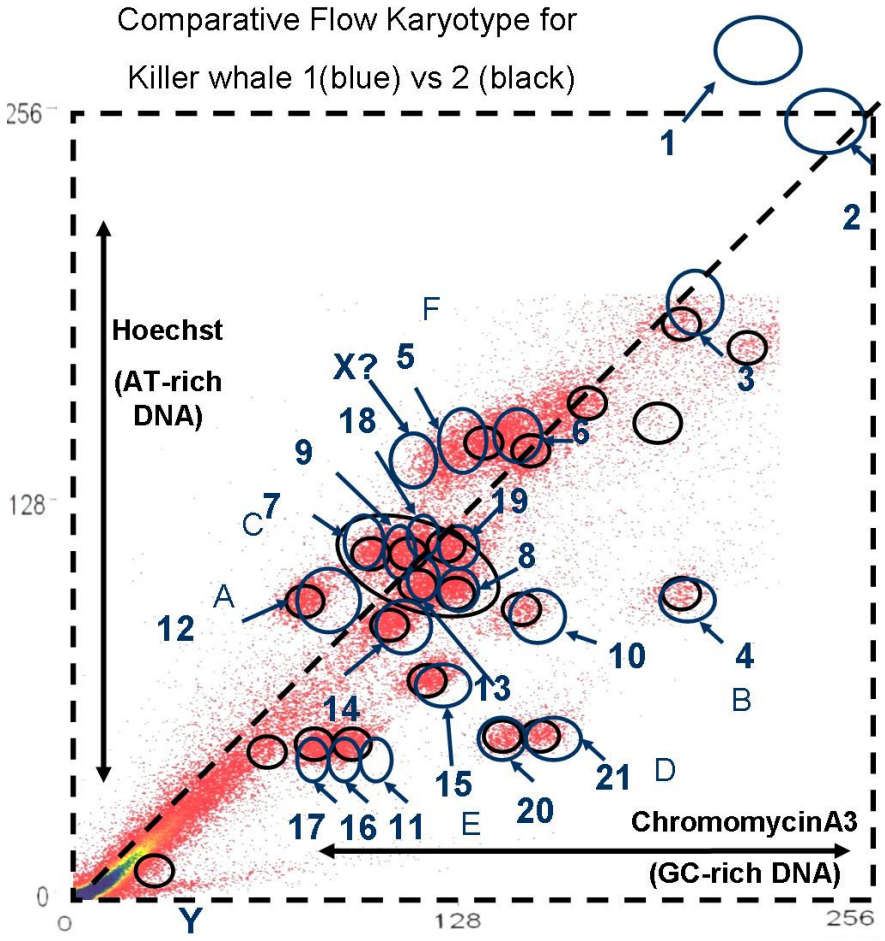


***Figure 3*** *shows the chromosomes that were flow sorted and and subsequently fluorescently labeled with cy-3 to create chromosome-specific paints. The images a-f correspond to the FISH images for the respective collected peaks. The flow karyotype was taken from individual two.*
*A) Shows a clean preparation, collection of the target chromosome with no non-specific selection in the flow sorting and no cross hybridization caused by cross-homology.*
*B) Shows a clean preparation, collection of the target chromosome with no non-specific selection in the flow sorting and no cross hybridization caused by cross-homology.*
*C) Shows a clean preparation, collection of the target chromosome with no non-specific selection in the flow sorting and no cross hybridization caused by cross-homology.*
*D) Shows a clean preparation, collection of the target chromosome with likely cross-homology to the p-arm of a second chromosome (yellow circles).*
*E) Shows a clean preparation, collection of the target chromosome with no non-specific selection in the flow sorting and no cross hybridization caused by cross-homology.*
*F) Shows collection of the target chromosome but also a lot of other fragments likely to be caused by contamination of the material during sorting.*

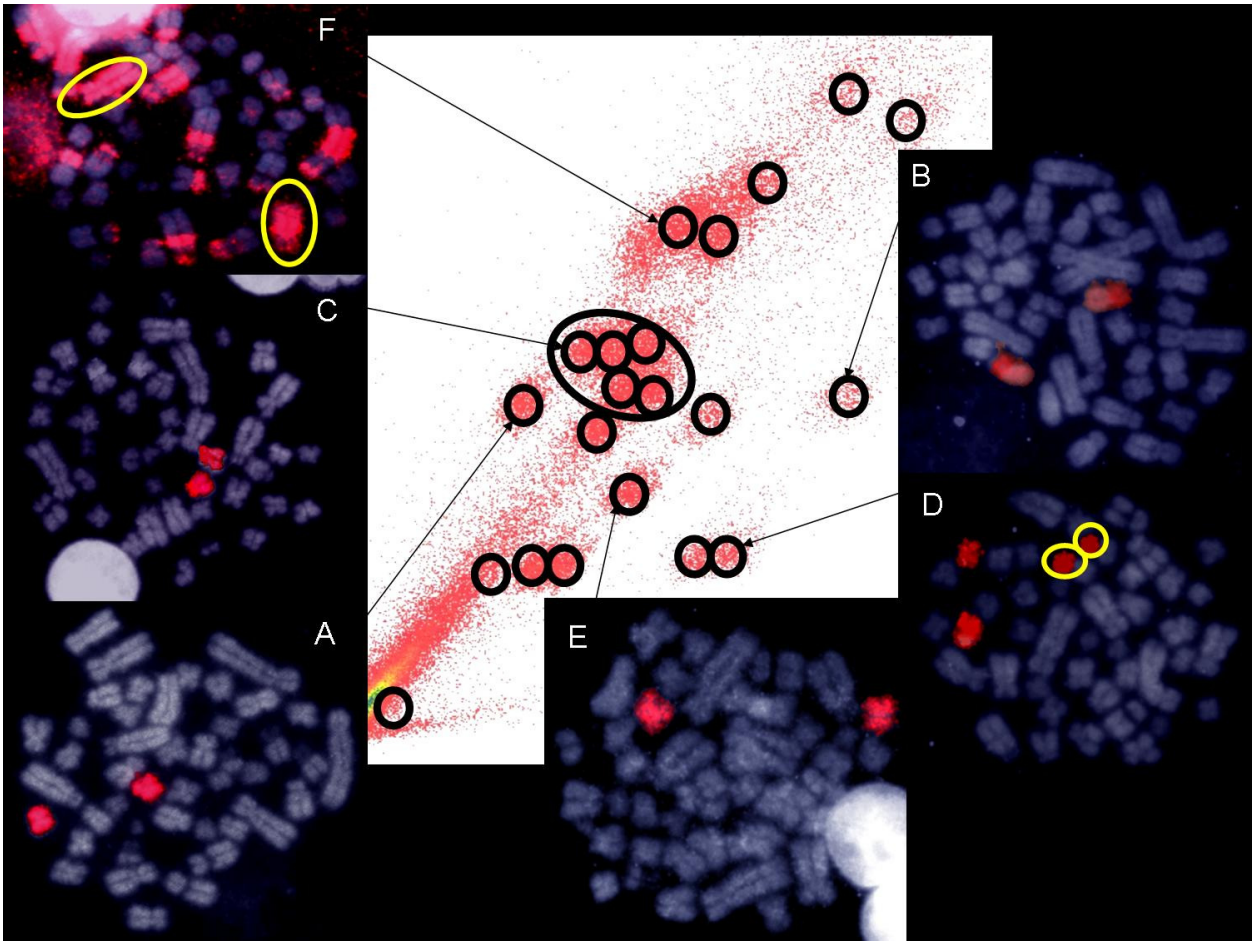
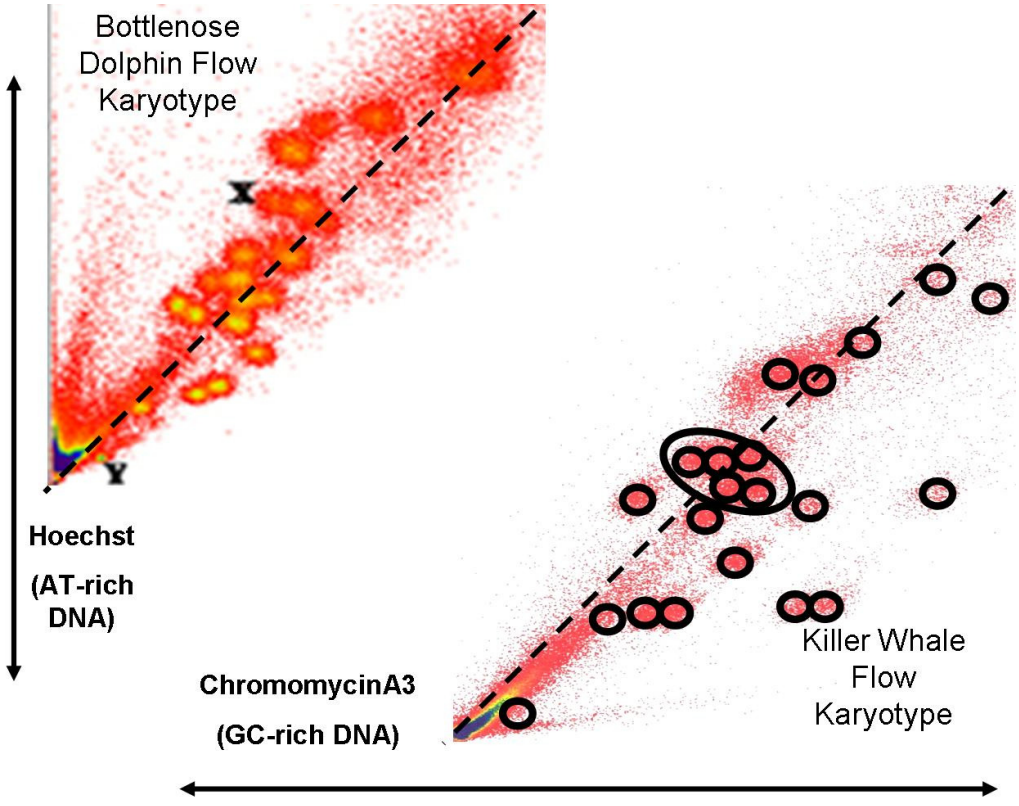


Figure 3 shows the chromosomes that were flow sorted from individual two, and subsequently fluorescently labelled with cy-3 to create chromosome-specific paints, which were arbitrarily assigned letters A-F, as shown in figure 3. The fluorescent pattern indicates complete chromosomal painting of all chromosomes that were flow sorted. Although all six probes show complete paint coverage, it appears that for chromosome 'F', there was contamination of additional chromosomal material from the debris that was collected in the same 'gating' selection for that sort, as the paint has partially labelled several of the larger chromosomes, supporting the concept that a proportion of the collected material contained contamination from broken parts of these larger chromosomes. Although the paint was contaminated and therefore will have limited utility as a chromosome-specific library, the complete painting of chromosome five confirms the location of this chromosome on the flow karyotype and indicates that a narrower selection field would be required to create a specific paint for this chromosome.

The fluorescence of chromosome 'E' corresponds to the third largest metacentric chromosome (chromosomes 15). Chromosome 'D' paint corresponds to the smallest 't' chromosome (chromosome 21), which has a subtelocentric appearance in the killer whale. This chromosome is one of four such chromosomes, which exhibit heteromorphic p-arms. There is however additional fluorescence indicated on the p-arm of chromosome 19, which is one of the two largest of the 't' chromosomes. The complete labelling of one chromosome pair with additional labeling of the p-arm of a second chromosome supports the notion that there was genuine cross-hybridization of the collected material to this region of the other chromosome. With reference to figure one, the chromosome represented by peak 'D', is adjacent to a second chromosome, which offers the potential for contamination. It is unlikely however, that contamination would result in the exclusive painting of one arm of the chromosome that is fairly distant, thus supporting the interpretation that the fluorescence is a result of cross-homology. Paint 'C' was from chromosome 8, as indicated by its relative size and submetacentric character. Its position on the flow karyotype to the left of the central line indicates that this chromosome is AT-rich comapartive to the other chromosomes of similar size. The chromosome collected in

peak ‘B’ corresponds to chromosome 4, as indicated by its submetacentric character and relative size to the other submetacentric chromosomes. It location on the flow karyotype identifies this chromosome as being particularly GC-rich. Finally, paint ‘A’ was identified as containing chromosome 12 as it hybridized to the largest metacentric pair. From the FISH painting it was possible to identify the chromosomal identity of all of the sorted and collected preparations and it was found that their identities corresponded well to the a priori assignment of chromosome numbers from the flow karyotype.

Figure 4 shows the Killer whale flow karyotype in comparison to that of the bottlenose dolphin taken from (Greminger et al., 2007).



Additional characters of the killer whale and bottlenose dolphin genomes are indicated by the comparative flow karyotypes (shown in Figure 4), not least the small size of the Y-chromosome (as indicated by its sorting with the debris) in comparison to the other chromosomes and in relation to the Y-chromosome of the bottlenose dolphin, which was large enough to isolate. This is consistent with the pattern observed from published killer whale and bottlenose dolphin karyotypes, which indicate the Y-chromosome of the latter species to be larger. Although direct quantitative comparison to the published bottlenose dolphin karyotype is not possible due to potential differences in label concentrations, because the methodology is interdependent and therefore internally relative, the width of the swathe of chromosomes is independent of differences in methodology. The dolphin karyotype clearly occupies a more linear space concentrated around the slope of x = y, where there is an equal ratio of AT: GC-DNA content. In contrast the killer whale karyotype deviates to a greater degree from an equal content with several of the chromosomes showing particularly GC-rich composition.

**Figure 5** (a) shows a flow karyotype for the bottlenose dolphin and (b) the corresponding FISH to a bottlenose dolphin metaphase spread, taken from (Greminger et al., 2007). (c) shows the killer whale microdissected y-chromosome, which was too small to flow sort, and (d) the corresponding fluorescent in situ hybridization of the y-specific paint to a metaphase spread of the same individual.

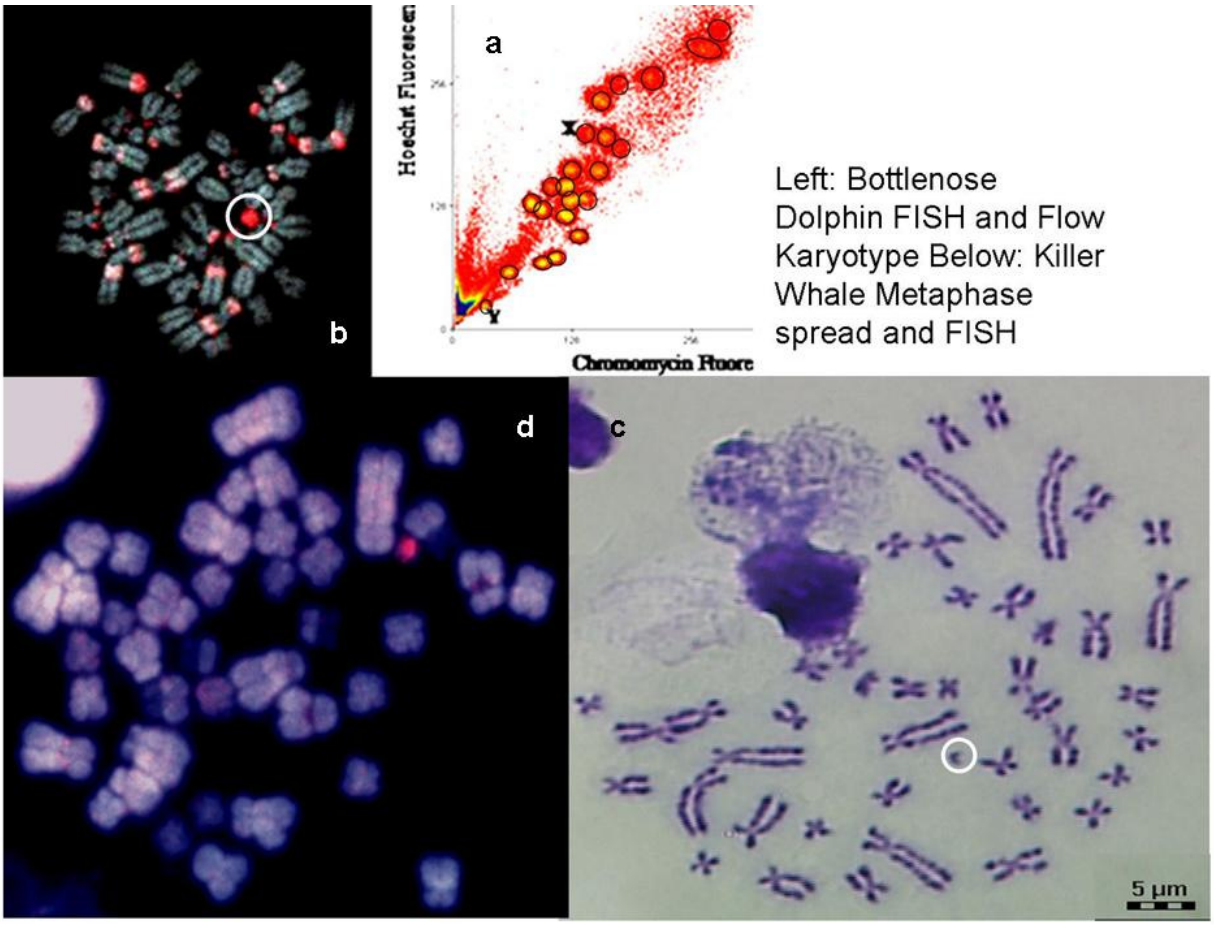
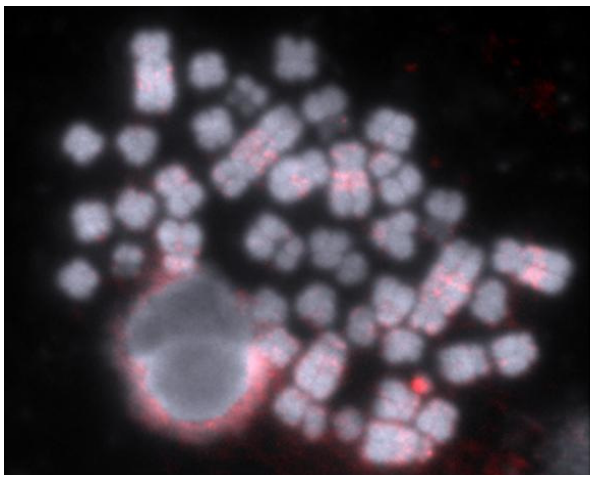


**Figure 6** shows the FISH image from microdissected y-chromosome specific paint from this study. Note the cross-hybridization/background fluorescence on the autosomes in comparison to the microdissected orca image in figure five.



The results of FISH from the microdissected killer whale chromosomes are shown in Figure 5. The image shows that the Y-chromosome is intensely fluorescent and specifically painted whilst no other chromosome or chromosome fragment in the metaphase preparation is fluorescing. From this it could be assumed that the DOP-PCR library was enriched for Y-specific sequences from throughout the chromosome and that it contained no other intact chromosome or large chromosome fragments. In contrast, the FISH image (taken from Greminger, 2007; 2009) of the bottlenose dolphin flow sorted Y-chromosome shows complete painting of the relevant chromosome but also additional fluorescence on a number of additional chromosome regions. This additional hybridization could indicate cross-homology of the dolphin Y-chromosome material to other chromosomes within the bottlenose dolphin genome. However it is more likely a by-product of the methodological acquisition of the Y-chromosome by Greminger et al. (2007). From the published flow karyotype image of the bottlenose dolphin it can be seen that although the Y-chromosome is separated from the main region of chromosomal debris, there is potential for contamination from small fragments of other chromosomes. Thus, the additional painting of several chromosomes could be a result of non-specific collection of chromosomal material during flow sorting. In contrast, the method of microdissection whilst yielding reduced quantities of material, is a more precise method for obtaining chromosome-specific DNA, particularly from small chromosomes that have a similar profile to other

chromosomes or chromosomal material. If the additional fluorescence does indicate cross-homology, it would be consistent with the relative sizes, which may indicate a more recent acquisition in the dolphin Y-chromosome. Evident in Figure 5, is the relative size difference of the bottlenose dolphin Y, the dolphin chromosome being larger than that of the killer whale, which may account for the difference. However, FISH from microdissected bottlenose dolphin Y-chromosomes (Figure 6) also indicated chromosome-specific painting of the Y-chromosome with additional painting elsewhere in the karyotype, although the pattern of chromosomal hybridization to the autosomes was different from that shown by the flow-sorted paint.

Comparison of the characters of the killer whale and bottlenose dolphin flow karyotypes in Figure 5 highlights some quite noticeable distinctions between these two delphinids. In general, there is a higher degree of resolution of the bottlenose dolphin karyotype than of the killer whale karyotype, largely due to the six similarly sized metacentric chromosomes of the killer whale. The bottlenose dolphin in contrast has a cluster of nine similarly sized chromosomes, which do not appear to overlap in their distribution. As can be seen from Figure 6, the killer whale G-banded karyotype indicates the presence of six very similar sized metacentric chromosomes, which are likely to correspond to the cluster of six chromosomes that were not able to be unequivocally separated on the flow karyotype. In contrast, the metacentric chromosomes of the bottlenose dolphin form a more obvious size gradient. The chromosomes of the two species also appear to have different ratios of 'AT' and 'GC' content, as indicated by the difference in their position relative to the x- and y-axis. All of the bottlenose dolphin chromosomes have a fairly even ratio of GC- to AT-bases, as indicated by their position close to the line where x=y (i.e. GC=AT). The killer whale on the other hand has a number of chromosomes (~6 or 7) that sit further away from the origin on the x-axis, indicating a bias towards GC-rich content.

These patterns are consistent with the banding karyotype shown in Figure 7, which shows the darkly stained heterochromatic regions are more abundant and prominent in the killer whale karyotype. Variation in the area and spacing of the peaks for the respective

chromosomes between the two species is also suggested by Figure 5. Aside from the aforementioned cluster of six chromosomes, the peaks of the killer whale karyotype have a larger diameter than those of the bottlenose dolphin, particularly considering that the karyotype represented in Figure 3 is taken from individual two, the less heteromorphic of the killer whaless. Of particular interest to this study, the Y-chromosome in the bottlenose dolphin appears to be larger and more GC-rich than that of the killer whale, although both species show a biased GC-content for this chromosome.

Table 1 shows the relative sizes of the killer whale chromosomes based on the G-banded karyotype and the results of calculation of their relative size in nucleotides, which facilitated the identification of total DNA-content used for calculations in Chapter 4.

**Figure 7:** Shows the G-banded karyotype of the orca from which the chromosome sizes were derived. Scale bar =5micrometers.

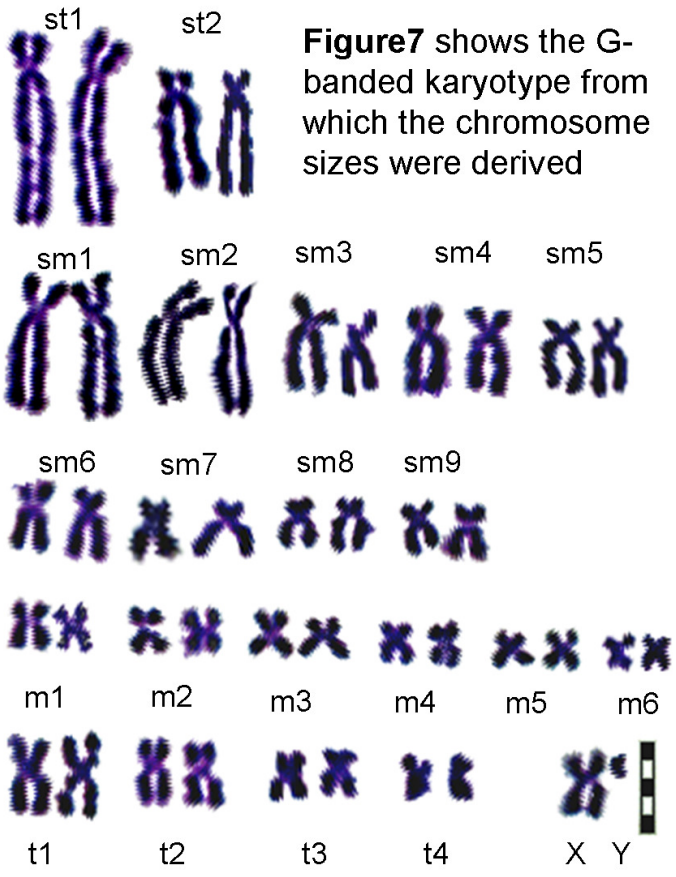


**Table 1:** shows the relative sizes in micrometers and basepairs of the orca chromosome complement. Chr ID – is the nomenclature applied to orca karyotypes by Arnason et al. (1980) and the Chr no. – is the nomenclature used in Bielec et al. (1998), which corresponds to comparative karyotyping protocol across species. Chromosome length was measured in micrometers as detailed in the text and then translated into approximate basepair compositions based on their relative percentage of the whole genome.

| Chr ID | Chr No. | Chro length (um) | Relative length (%) | ~size Gb |
|---|---|---|---|---|
| st1 | 1 | 16 | 13.0 | 415 |
| st2 | 2 | 11 | 8.9 | 285 |
| sm1 | 3 | 9.5 | 7.7 | 246 |
| sm2 | 4 | 8.5 | 6.9 | 221 |
| sm3 | 5 | 8 | 6.5 | 208 |
| sm4 | 6 | 6 | 4.9 | 156 |
| sm5 | 7 | 5 | 4.1 | 130 |
| sm6 | 8 | 4 | 3.2 | 104 |
| sm7 | 9 | 4 | 3.2 | 104 |
| sm8 | 10 | 3.5 | 2.8 | 91 |
| sm9 | 11 | 3 | 2.4 | 78 |
| m1 | 12 | 4 | 3.2 | 104 |
| m2 | 13 | 4 | 3.2 | 104 |
| m3 | 14 | 3 | 2.4 | 78 |
| m4 | 15 | 3 | 2.4 | 78 |
| m5 | 16 | 2 | 1.6 | 52 |
| m6 | 17 | 1.5 | 1.2 | 39 |
| t1 | 18 | 6 | 4.9 | 156 |
| t2 | 19 | 6 | 4.9 | 156 |
| t3 | 20 | 5.5 | 4.5 | 143 |
| t4 | 21 | 4 | 3.2 | 104 |
| X | X | 5 | 4.1 | 130 |
| Y | Y | 0.6 | 0.5 | 16 |

### 3.4.0 Discussion

Although identical in karyotype number and within the same Family, the flow karyotype (which indicates relative DNA content) implies genomic distinctions between the killer whale and bottlenose dolphin. Initial cetacean karyotypes were produced when cytogenetics was in its infancy and studies focused on the surprisingly striking similarities between members of the cetacea (Arnason, 1982), compared to those of the primates and artiodactyla (O'Brien and Stanyon, 1999; O'Brien et al., 2006). However, some studies suggested differences in the killer whale genome in particular. For example, Arnason et al. (1980) suggested a higher degree of heterochromatin within the killer whale karyotypes, though the methods available at the time were imprecise. Kulu et al. (1971) had earlier noted a unique karyotypic pattern in the killer whale superficially more similar to that of the Physeteridae (sperm whales – dwarf, pygmy), though later phylogenetic studies illustrated that this was not because the lineages shared close ancestry (e.g. McGowen et al., 2008; Ayoub et al., 2009). Here the FACS data show that the killer whale has a distinct profile, and a higher level of divergence between the chromosomes than seen for the bottlenose dolphin, consistent with inference from the earlier studies. A possible explanation would be the greater accumulation and diversity of heterochromatic regions in the killer whale genome.

Heterochromatic regions are dominated by non-coding DNA, which in turn is dominated by repetitive regions of various origins. The evolution of heterochromatin and accumulation of transposons and satellite sequence has been proposed to be associated with the age of the lineage (Tartof and Bremer, 1990; Lohe and Hilliker, 1995; Robles et al., 2004; Plohl et al., 2008), which may explain the superficial similarity of the killer whale karyotype to that of the sperm whale (a more ancient lineage) compared with that of the bottlenose dolphin (likely part of a younger lineage). Indeed, a look at baleen whales and the respective heterochromatin load of various species (Arnason, 1972; Arnason, 1974; Arnason, 1982; 1984a; 1984b) appears to support a correlation with divergence times (Sasaki et al., 2005) . The apparent age of the three primary study species, the bottlenose dolphin in a recent radiation, the killer whale early among

the delphinids, and the sperm whale relatively early within the broader cetacean radiation (see Fig. 1.9.1, in the Introduction) (Nikaido et al., 2001; Sasaki et al., 2005; Ayoub et al., 2009), is roughly consistent with their relative accumulation of heterochromatin, though this is not a precise measure. Further, as the accumulation of repetitive regions is a stochastic process (e.g. Dover, 1982), the degree of heteromorphism among chromosomes is apparently a consequence of the greater accumulation of these elements (Kurek et al., 2000; Bachtrog, 2003; Wyandt and Tonk, 2004; Dimitri et al., 2005; Dimitri et al., 2009, for review) and thus the greater degree of heteromorphism seen in the killer whale compared to the bottlenose dolphin is expected. The relative increase in DNA content of particular chromosomes of one of the killer whales could be an indication of individual variation in heterochromatin content. The pattern of chromosomal heterogeneity is consistent with findings from the G-banded karyotype and a study of six killer whale banded karyotypes by Arnason (1980) who found large regions of constitutive heterochromatin that was heteromorphic within and between individuals.

A component of heterochromatin in the killer whale genome is a 1,579bp satellite representing 15% of the killer whale genome (Widegren et al., 1985), and it is slightly AT-rich (GC = 44%). It is therefore possible that the karyotypic positional difference found for chromosomes 1 & 2 in individual 1 is influenced by the accumulation of this repeat, as they occur further from the origin but also further towards the AT-rich region of the karyotype. Chromosomes 1, 5, 9, 12 and 18, which are all found on the AT-biased portion of the flow karyotype have in fact been shown to carry a proportionally high load of the AT-rich satellite repeat in an earlier study (Widegren et al., 1985). It is clear from a comparison of the two killer whales studied here that the composition of chromosomes (likely due to the differential accumulation of repetitive elements) will vary among individuals, and this is most noticeable from the disposition of the chromosomes numbered 1&2 in Figure 1. Investigations of trends in heteromorphism inheritance have demonstrated the importance of differential parental chromatids (Trask et al., 1989). Analysis of mechanisms for heterochromatin accumulation has pointed to the loss of heteromorphism during ageing (Howard, 1996; Pritham, 2009), which can

result in homogeneity among chromosomes. Among the two killer whales studied, it was in fact the younger killer whale that apparently had the greater amount of hetermorphism, though of course this sample is too small to provide much relevant inference. The capture of chromosomal material from the flow sort and subsequent hybridization using FISH back to chromosomal spreads allows further investigation of the composition of chromosomes. The cross-hybridization of chromosome 21 paints to the p-arm of chromosome 19 but not to the other heterochromatic 't' chromosomes nor those of the large subtelocentric chromosomes. This suggests that although all of these regions may contain some of the same satellite repeat sequences, the composition and organization within chromosome 21 and 19 are similar to each other, and not to other chromosomes of this kind. The fact that the chromosome labelled 21 is quite distant from the one labelled 19 in the flow sort adds to the credibility of this interpretation (as nearby sorts may share material by misallocation). This pattern is suggestive of a translocation of the composite of the satellite region from 21 to 19, or vice versa or very little divergence since the origin on these chromosomes. In contrast, the lack of homology to other satellite containing regions suggests that the satellite region translocated to or evolved on these chromosomes independently. It has been suggested that exchange of genetic material and transposon activity may act in particular locations (Junakovic et al., 1998). In particular it has been proposed that exchange with telocentric chromosomes occur more readily than between other chromosome types promoting variant repeat distribution followed by homogenization through concerted evolution (Dod et al., 1989; Liao, 2000). This pattern may explain the pattern of homology between the p-arm of chromosome 19 and 21 but not with that of the other heterochromatic chromosomes. The p-arms of these two chromosomes are known to carry prominent satellite sequences (Arnason et al., 1980; Widegren et al., 1985), which supports the notion that there would be cross-homology at this location. Due to the nature of the collected material, which is subsequently fragmented by the DOP amplification process, individual fragments may hybridize to matching regions throughout the genome, irrespective of their origin.

As a group, cetaceans generally have small Y-chromosomes, indeed the comparative difference between the size of the X-chromosome and the Y is one of the most extreme within the mammalia (Toder et al., 2000; Graves, 2004). The results based on microdissection and FISH demonstrate a lack of cross-homology between the killer whale Y to the X-chromosome or any of the autosomes. In comparison to the heterochromatic human Y-chromosome which paints to proportions of the X and autosomes (Gvozdev et al., 2005), and the results of FISH with the microdissected Y-chromosome of the bottlenose dolphin, the killer whale Y-chromosome appears to be comparatively exclusive. The indicated cross-homology of the bottlenose dolphin Y-chromosome was also found by a recent study with FISH from flow sorted Y-chromosomes, which showed hybridization to several autosomes (Greminger, 2007; Greminger et al., 2007; 2009; Greminger et al., In Press).

Y-chromosome studies suggest that the size of the Y-chromosome may be related to the age of the species (Graves, 1998) but it is complicated by the great quantity of translocations in heterochromatic regions, found in abundance on most Y-chromosomes. The size of the Y-chromosome may be related to the age of the species in that the evolutionary process of the Y-chromosome follows a course of degradation over time. Therefore, all things being equal, you would expect that the older a lineage is the smaller the Y-chromosome would be. However, the expectation of time-dependent degeneration is complicated by two confounding variables. Firstly, the Y-chromosome does not only degrade but also receives translocations, often substantial (e.g. the human Y) (Waters et al. 2007) and secondly Y-chromosomes also accumulate large amounts of heterochromatin in some species but not in others (e.g. Drosophila melanogaster (Pisano et al., 1993)). Because evolution is primarily stochastic, the time to most recent common ancestor (TMRCA) does not appear to play a role in the evolution of the Y, the ecology of the species, however, will act as a driver for the process (Bachtrog et la., 2004 and van Hooft et al., 2007). Although chromosome painting studies and comparative gene mapping have shown distinct similarities in gene and protein content of mammalian Y-chromosomes (e.g. Murphy et al., 1999; Raudsepp et al., 1999) there are also distinct differences between Y-

chromosomes of different species (e.g. Murphy et al., 2006). Evolution of sex chromosomes do not appear to evolve in a time-dependent manner with large and divergent changes occurring between closely related species, whilst sexual selection for sex-specific proteins has resulted in striking concordance between quite distant taxa, by a presumably convergent mechanism of recruitment to the sex-specific chromosome.

The human Y is much larger than expected according to a lineage-based relationship as the result of a traceable translocation event from one of the autosomes (Waters et al., 2001). In contrast the marsupial Y chromosome, which does not show evidence of translocation events, follows a strictly age related association with size (Toder et al., 2000). It is therefore possible that the relative size of the sperm whale, killer whale and bottlenose dolphin Y are the result of a translocation event in the much larger sperm whale Y-chromosome, and related to age to a greater extent for the latter two species. The much smaller size of the killer whale Y-chromosome relative to that of the bottlenose dolphin, is consistent with this expectation, assuming the previously discussed relative phylogenetic positions are correct. The sperm whale is generally agreed to be a more ancient lineage than either of the other two species (Harlin-Cognato and Honeycutt, 2006) has a much larger (~2um) Y-chromosome than either the killer whale or bottlenose dolphin. A possible explanation is that although Y-chromosome theories suggest the steady disintegration of the Y-chromosome, there is a strong stochastic element, with chance amplification of repetitive regions or translocations increasing the size of the Y in some cases. This would be consistent with the relatively large size of the sperm whale Y-chromosome, but may also be relevant to the size of the bottlenose dolphin Y-chromosome, given evidence for homology to other chromosomes in this species.

### 3.4.1 Utility of chromosome products and flow karyotypic output

While the focus of this study is on the cetacean Y-chromosome, part of the work reported in this Chapter will have utility for future studies beyond the scope of this thesis. For example, the various chromosome-specific libraries will be useful as painting probes, in sexing cells from

mixed populations (such as chimeras and grafts), and for verifying the presence of the Y-chromosome in embryonic stem cells (Bergstrom et al., 1998). Human derived chromosome-specific paints were successfully applied to bottlenose dolphin metaphases to identify homologous regions between the species (Bielec et al., 1998).

The paints derived from this study will be useful for identification and characterization of other cetacean karyotypes. The potential of chromosome-specific painting to elucidate the exact relationships between the cetacean karyotypes was demonstrated by the cross-homology between two chromosomes that apparently share a satellite repeat. This demonstrates that there may be as yet unrealized compositional differences. The mechanism of divergence therefore to the subtelocentric physical appearance of the assumed 't' chromosomes in the killer whale may not be a simple as previously assumed.

In order to provide a size standard it requires the inclusion of a chromosome of known size i.e. the human (e.g. Bergstrom et al., 1998). Therefore, although it was not possible to 'quantify' the absolute values for DNA content from the flow karyotype (because no internal size standard was included due to the contamination that it would have caused), the flow karyotype does provide a more accurate and consistent qualitative evaluation of the DNA content.

It also illustrates the composition of chromosomes compared with previous banding studies, where sizes are dependent on the condensation of the chromosome preparation (Trask et al., 1989). Furthermore, it demonstrated that it would be relatively easy to apply this technique with the incorporation of a size standard to obtain exact profiles, if required. The relatively large size in some individuals of the biggest cetacean chromosomes has been previously noted, but the respective extent of the differences evident from the flow karyotype was not appreciated. The high-resolution flow karyotypes and the chromosome paints will facilitate further standardization of the cetacean karyotype.

# Chapter 4: Characterisation of the Cetaccean Y-chromosome

"Until recently, the Y Chromosome seemed to fulfill the role of juvenile delinquent among human chromosomes rich in junk, poor in useful attributes, reluctant to socialize with its neighbors and with an inescapable tendency to degenerate." *Jobling and Tyler-Smith, 2003*

## 4.0 Introduction

The Y-chromosome is comprised of one large (95% of the chromosome in humans) haploid and male-specific region that is flanked on one (most species) or two sides (in humans) by pseudoautosomal regions (PAR), where X-Y crossing over occurs during male meiosis (Skaletsky et al., 2003). The male-specific portion of the Y-chromosome represents the largest non-recombining region of the mammalian (nuclear) genome and, like mtDNA, has an effective population size one quarter that of autosomal DNA under neutral expectations (Hurles and Jobling, 2001).

The mammalian Y is almost entirely excluded from recombination with the X, which has resulted in the degradation of the male-specific region to a relic containing few active genes among large stretches of repetitive sequence (Charlesworth and Charlesworth, 2000; Graves, 2004; Graves, 2006). As a result of the origin of the sex chromosomes from an ancestral autosomal pair, Y-chromosome genes are largely formed from X-homologs or subsequently acquired as the result of translocations from elsewhere in the genome, that have mutated and taken on specialised male-specific functions (Graves, 2002). Comparative studies have shown that Y-chromosomes from different mammalian taxa have degraded to different extents; some retaining more of the PAR, some evolving different sets of genes, and some accumulating translocations (Graves et al., 1998). This gives rise to a considerable amount of variation, which is compounded by the effects of a lack of recombination. Y-chromosomes from different taxa range in size despite similar evolutionary processes, although they are all comparatively small in comparison to the X.

Variation in the male-specific region of the Y (MSY) escapes recombination and therefore passes from generation to generation in a clonal fashion. As a result, mutation processes acting on the Y produce lower sequence diversity than is observed on autosomes. However, it is highly susceptible to genetic drift, which results in a sweep of changes in one generation producing accelerated differentiation between the Y of different species. Furthermore, mutations appear to occur more rapidly in the male germline and as a consequence of the larger number of cell divisions, which gives rise to an increased mutation rate through continuing divisions of spermatogenic stem cells. The Y-chromosome thus suffers a consequence of this higher mutation rate in the male germline without correction through recombination (Blumenstiel, 2007). The human Y is rich in low-copy-number repeated sequences (i.e. few repeats of short microsatellite motifs) and a combination of gene conversion between paralogs and high base substitution in the different classes of sequence (Skaletsky et al., 2003) results in a mosaic of mutation rate varying across the Y chromosome (Heyer et al., 1997; Kayser et al., 2000; Rozen et al., 2003). Moreover, because of the lack of recombination, any selection will affect the entire chromosome and produce an increase in the frequency of mutatants more rapidly than would be expected by drift. Assessment of mutation rates at tri- and tetra-nucleotide microsatellites has demonstrated few mutation events, whilst other chromosomes have high mutation rates. The Y-chromosome is particularly divergent in marker and allele-specific differences in mutation rates (Heyer et al., 1997). The human Y-chromosome has been shown to be completely devoid of polymorphic GC-rich minisatellites (DNA that consists of a short series of bases 10–60bp), supporting the theory that these loci are largely the by-product of recombination (Jeffreys et al., 1998) whilst the only highly polymorphic Y-specific minisatellite found on the human Y is an atypical AT-rich locus (Jobling et al., 1998).

The human Y amounts to about 65 Mb of DNA (about 2% of the haploid genome) and the X is about 160Mb (about 5%). The X and Y are non-homologous over most of their length but pair across the PAR a tiny fraction, 2.6 Mb on the short arm and sometimes 0.4 Mb at the distal end of the long arm (Graves et al., 1998). A 35-60Mbp region of the long arm of the

human Y is heterochromatic and composed of highly repetitive non-coding DNA and repetitive palindromes (Skaletsky et al., 2003). On the human Y-chromosome there are high sequence copy numbers as a result of the palindromes and there is evidence of recent and rapid amplification of several genes on the Y, for example the RBMY-gene has 30 copies on human and rat (Zhang et al., 1992; Toure et al., 2005).

The PAR boundary separates the non-recombining region of the MSY from the high recombination region of the PAR (Galtier, 2004). Recombination is a function of chromosome size, with higher recombination rates being observed in the smaller chromosomes (Galtier et al., 2001), therefore the 2.6Mb PAR of the human Y represents a recombination hotspot (Yi and Li, 2005). Several studies have identified regions of GC-rich content, which appear to be 'hotspots' for recombination (Duret and Arndt, 2008; Arnheim and Calabrese, 2009; Duret and Galtier, 2009; Freudenberg et al., 2009; Jeffreys and Neumann, 2009), suggesting various mechanisms for their maintenance. The GC-content of the PAR is high in both humans and mice but understanding its mechanism and the implications are complicated by the asymmetric nature of forces driving GC content evolution. The PAR in artiodactyls is more extensive than the human PAR (Toder et al., 1997) and the marsupial Y does not appear to include a PAR (Sharp, 1982).

Uniquely in the genome, the Y is poorly conserved between species having very little cross-homology, which contrasts with the high degree of conservation of the X (O'Brien et al., 1999; Raudsepp et al., 2004) and autosomes between species; for example ZOO-FISH (Kulemzina et al., 2009) and comparative mapping (Nonneman and Rohrer, 2004) have shown high conservation of autosomes across all mammalian species but not of the Y-chromosome (O'Brien et al., 1999; Fronicke et al., 2003) The bottlenose dolphin karyotype has also been shown to contain very few rearrangements compared with the human karyotype (Bielec et al., 1998).

The small Y-chromosome of the marsupial (~10Mb) has been suggested to contain fewer repetitive sequences than the human Y (Toder et al., 2000). It has also been suggested to represent a minimalist form of the mammalian Y-chromosome, derived from an ancient region of

the mammalian proto-Y from before the eutherian-marsupial split (Graves, 1998) reduced in size as a result of the lack of a recent large translocation onto the human Y (Saxena et al., 1996; Skaletsky et al., 2003). There is evidence that palindromes that give rise to multi-copy loci are found across primates (Kirsch et al., 2009); however, evidence of these loci is lacking from other species. Despite the apparently reduced level of repetitive sequence on the marsupial Y, there have been indications that Y-chromosomes of eutherians may also carry an abundance of repetitive sequence by the presence of multicopy microsatellite loci (Sundqvist et al., 2001; Luo et al., 2007).

Comparisons between the human Y and those of other mammalian species have been particularly informative. However, the Y-chromosome has been problematic for sequencing efforts as a result of the abundance of repetitive sequences (Skaletsky et al., 2003) and therefore females have mostly been chosen for genome sequencing projects (NCBI). This has inevitably hampered investigations and comparative analysis of the Y-chromosome severely lags behind that of other genome regions, despite the extraordinary properties of the Y-chromosome in terms of its evolution**,** sequence composition and function which make it such a powerful resource for understanding genome evolution. The availability of the almost complete human genome euchromatic Y-chromosome has seen the coming of age of the Y-chromosome as a tool for numerous molecular investigations including evolutionary history and processes of genome evolution, for example, (Hurles and Jobling, 2001; Jobling and Tyler-Smith, 2003; Rozen et al., 2003; Skaletsky et al., 2003; Bosch et al., 2004; Jobling et al., 2007; Jobling, 2008).

Comparisons of the Y-chromosomes of different species have been important for the understanding of the function as well as the evolution of the Y. Recent comparative studies between marsupials and hominids have shown of the Y-chromosomes of these two groups bear certain similarities but also many differences in their arrangement and content, confirming the complex origin and evolution of y-chromosomal content (Toder et al., 2000). Furthermore, in some species the Y-chromosome carries many genes with male-specific functions (Murphy et

al., 2006), whereas in others there are a very limited number of genes on the Y, retaining them on autosomes instead e.g. the rat (Disteche et al., 1992; Ellison et al., 1996). The marsupial species, the dunnart, carries particularly few genes, confirming the hypothesis that the male determining region is the only essential element of the Y-chromosome, across taxa. These great differences indicate the disparity in the evolutionary histories of Y-chromosomes, for example, the human Y-chromosome, which evolved 150MYA has subsequently (80-130 MYA) received a large autosomal translocation (Waters and Marshall Graves, 2009), which is not apparent in other species; and the carnivore Y has acquired several novel genes (Murphy et al., 2006). The chimpanzee Y has recently been largely sequenced (Hughes et al., 2005), as has the rat Y (Gregory et al., 2002). For other mammals it remains necessary to rely on the amplification of fragments (Sundqvist et al., 2001), FISH-based studies (Toder et al., 2000) and comparative mapping of gene loci (Murphy et al., 2006) to provide these comparative data.

Microsatellites (MS), aka short tandem repeats (STRs) or short sequence repeats (SSR) are polymorphic loci consisting of repeating units of between 1 and 6bp. They are thought to accumulate by DNA slippage and mispairing during recombination and replication, which, have been shown to be linked to the locus length and simplicity of the repeat motif (Lai and Sun, 2003). Point mutations, which interrupt continuous microsatellites, result in their degeneration by eroding the tandem arrangement of their sequence, which produces two or more shorter repeat regions, thus reducing the accumulation of slippage (Kruglyak et al., 1998). Once microsatellites become interrupted it is thought that they will continue to degenerate, nevertheless leaving an indication of their former presence in the form of 'cryptic simplicity' (Tautz et al., 1986). The nature and rate of base mutations are influenced by local context (Varela et al., 2008). The majority of microsatellites evolve by increases or decreases in repeat number as a result of the slippage mutation rate (Goldstein and Schlotterer, 1999; Schlotterer, 2000).

The microsatellite DNA mutation rate is estimated for human genomes as approximately $10^{-3}$ per locus per gamete per generation (Dib et al., 1996), which is orders of magnitude higher

than the point mutation rate of $10^{-8}$ (Li, 1997). Webster and Hagberg (2007), argue that microsatellites promote biased substitution in flanking regions by their very nature, as a result of the slippage mutation process, with base substitutions tending to occur towards the end of the microsatellite arrays (Brohede and Ellegren, 1999). Most slippage events within coding DNA will introduce unsustainable mutations as they will likely result in frame-shift mutations, which will interrupt the code and result in deleterious chages and are therefore removed by repair mechanisms (Witte et al., 2001). By association microsatellites linked to gene regions may also be repaired by gene conversion, maintaining the integrity of the locus. Through time, microsatellites acquire more interruptions and degrade (Varela et al., 2008), therefore if microsatellites are 'hotspots' for point mutations and in the absence of any protection or selection afforded by linkage to gene loci, erosion of microsatellite loci will become a function of the rate of mutation (Brohede and Ellegren, 1999).

Studies from autosomal microsatellites have shown that there appears to be an unexplained stabilizing process acting on the interior of microsatellite loci (Sibly et al., 2003), which may be related to the recombination rate. It is thought that microsatellites might be generated and driven by recombination as a result of unequal crossing-over producing an initial tandem repeat, which continues to accumulate with cycles of recombination and subsequent slippage (Balaresque, 2007). If this is the case, then there would be a bias towards erosion of microsatellites on the Y-chromosome compared with autosomes. Microsatellites have been shown to be associated with selection, GC-content, local recombination rate (Metzgar et al., 2000), transposable elements and transcribed regions (Balaresque et al., 2003). In addition, demographic process and life history traits such as sex and age have been shown to influence their mutation process (MacDonald et al., 2006). For example, associations with gene regions may result in mismatch repair stringency acting to correct microsatellite mismatches (Balaresque et al., 2007).

In general, the typical mammalian life-history traits of high variance in male reproductive success and male biased dispersal (Greenwood, 1980), will inevitably reduce the effective

population size of the Y-chromosome in relation to maternally and biparentally inherited regions**,** and thereby impact its diversity (Chesser and Baker, 1996; Charlesworth, 2001; Laporte and Charlesworth, 2002). Results from investigations in domestic species, which have highly skewed ratios of breeding individuals e.g. cattle, (Gotherstrom et al., 2005; MacEachern et al., 2009), giving rise to extremely low levels of Y-chromosome variation, Hellborg and Ellegren, (2004) highlight the importance of variance in male reproductive success. In addition, the number of founding individuals of the domestic species has been shown to contribute significantly, as illustrated by the evidence of very few male contributors to the horse domestic stock (Lindgren et al., 2004; Lau et al., 2009). Sex-biased dispersal may also impact the relative variance as it can lead to greater diversity within a population at markers specific to the dispersing sex compared to a marker that is specific to the philopatric sex (Chesser and Baker, 1996; Laporte and Charlesworth, 2002). The small effective population size of the Y (⅓ and ¼ that of the X and autosomes, respectively) is also expected to reduce the rate at which the Y-chromosome will recover variation following a bottleneck. Demographic effects may therefore contribute considerably to reducing variation on the Y chromosome.

This study attempts to test the hypothesis that the specific characteristics associated with the haploid genome of the Y-chromosome in combination with species-specific characteristics of the cetaceans studied will leave a trace of the evolutionary processes in the genetic sequence. The minute size of the cetacean Y-chromosome, as shown in Chapter 3, may compound the effects observed in the Y-chromosomes of other species, which are also generally small and therefore have reduced variation as a consequence. Studies of the PAR boundary in humans and mice (Galtier et al., 2001), have suggested that GC-content is associated with high levels of recombination and therefore the expectation would be for the majority of Y-chromosome sequences to exhibit low GC-content as a result of the lack of recombination in the majority. However, the high recombination rate in the PAR would give rise to sequences with high GC-content, so the relative composition of the Y-chromosome should reflect this expectation. The lack of recombination on the Y-chromosome removes one of the two mechanisms thought to

contribute to slippage. This is counter-balanced by a higher number of replications in the male germline. In combination with the high rate of degeneration and the lack of conserved gene regions, it would be expected therefore that the Y-chromosome, which is largely lacking in genes and exhibits a mutation bias associated with the male germline, would show high levels of microsatellite interruption commensurate with the age of the locus, and consequently high levels of cryptic simplicity.

**Hypotheses**

1. The killer whale Y-chromosome should be a mosaic of sequence classes, being predominantly 'simplistic' and repetitive; carrying few genes and thus be AT-rich
2. The higher mutation rate as a consequence of exclusive transmission through the male germline should result in a higher incidence of sequence interruptions e.g. in microsatellite motifs and/or genes
3. There should be evidence of degeneration of the Y-chromosome as indicated by a lack of genes and an abundance of 'simple' sequence
4. There should be a low representation of high GC-rich fragments derived from the PAR because the PAR should represent a small proportion of the sequences
5. The size of the respective Y-chromosomes should result in size-dependent variation such that the sperm whale > bottlenose dolphin > killer whale

## 4.2 Methods

### 4.2.1 Cloning

The Y-specific library was created as described in Chapter 3. Briefly, individual microdissected Y-chromosomes were PCR amplified using a whole genome amplification method (WGA): degenerate oligonucleotide primer – polymerase chain reaction (DOP-PCR) (6MW: 5'-CCG ACT CGA G$(N)_{24}$ AT GTG). The DOP-PCR product was used as the starting material for subsequent cloning. 10µl of DOP-PCR product was loaded onto an agarose gel in 1xTBE buffer along with a 1kb ladder. Fractions between 400-1500bp were cut from the gel over a UV box using a razor blade. Pieces of gel containing the DNA were then extracted from the gel following the protocol for the 'QIAquick gel extraction kit', with a final suspension in 50µl $dH_2O$. Using 10µl gel-purified DOP-PCR product, linkers were added to both ends of the fragments. First two separate linkers A and B (Linker-A: 5'GCGGTACCCGGGAAGCTTGG3', Linker-B: P-5'GATCCCAAGCTTCCCGGGTACCGC3') were ligated together as follows: 50µM of each linker were combined together in an equal ratios (e.g. 5µl each) to make a final concentration of 25µM each and annealed together at 68°C for 5 min. The combined linker A-B was then ligated to the fractionated DOP-PCR DNA in the following reaction: 7.0µl sterile, deionized water; 1.5µl T4 DNA ligase buffer; 0.5µl annealed linkers; 10.0µl gel extracted DNA, 1µg/µl; 1.0µl T4 DNA Ligase enzyme for a final volume of 20µl.

Following ligation of the linkers the DNA product was PCR amplified prior to ligation into the pGem T-Easy (Promega) cloning vector. Using 1µl of linker ligated purified product and linker-A as the primer, a PCR reaction with an annealing temperature of 68.6°C was used to amplify the fragments to create a chromosome-specific PCR-amplified library, with the following reaction conditions: 96°C for 2 min; 30 cycles of 94°C for 40 secs, 68.6°C for 40 sec and 72°C for 1 min; then elongation at 72°C for 10 min. The PCR product was purified following the Qiagen PCR purification kit protocol with a final resuspension in 50µl of $dH_2O$.

Prior to transformation of the PCR product in to the pGem T-Easy vector (Promega), competent XL-1 Blue bacteria cells were made competent following the Hanahan protocol

(Sambrook, 2001). 1µl of purified microsatellite-enriched PCR product was ligated into a P-Gem T-Easy vector (Promega, A1360) overnight according to the vector instructions, as follows: 5µl 2xRapid Ligation buffer; 1.0µl pGem-T Easy vector (50ng); 1.0µl (~25ng) PCR product; 1.0µl T4 DNA ligase (units/µl), deionized water to a final volume of 10µl. The ligation was left at 4°C overnight and then transformed into competent 'XL-1 Blue' bacteria cells, according to the vector instructions. 100µl of 0.1M IPTG and 20µl of 50mg/ml X-Gal were spread on to the surface of the LB-amp agar cloning plates and allowed to absorb for at least 30 min at 37°C prior to use. A 100µl aliquot of frozen Hanahan competent 'XL-1 Blue' cells per ligation reaction was removed from storage at -80°C and placed in an ice bath until just thawed (~5 min). 5µl of the ligation was transferred into the tube containing the competent cells, gently flicked and placed on ice for ~20 min. The cells were 'heat-shocked' for exactly 45 sec in a water bath at exactly 42°C and then immediately placed on ice for ~2 min. Cells were streaked out on to the prepared plates and incubated overnight at 37°C. Positive colonies were identified by blue-white screening followed by PCR screening as follows:

Colonies containing an insert (white) were picked with a pipette tip and transferred to fresh plates for overnight re-growth. Each tip was also dipped into a PCR reaction containing vector and microsatellite-specific primers to verify the presence of a microsatellite insert. Each 10µl reaction contained the following conditions: 1x PCR buffer containing 10 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 50 mM KCl, 2 mM $MgSO_4$, 0.1 % v/v Triton X-100, pH 8.8, 250µM of each of the four dNTPs, 0.4U of Taq polymerase, 2pmole each primer SP6: 5'-CAT TTA GGT GAC ACT ATA G-3'; T7: 5'-TAA TAC GAC TCA CTA TAG GG-3') and $dH_2O$ to a final volume of 10µl. The amplification protocol had a denaturation time of 10 min at 95°C, 30 cycles of 95°C for 30 sec, 54°C for 1 min 30, 72°C for 50 sec, followed by an elongation at 72°C for 10 min. Clones containing inserts produced a single distinct band larger than 150bp (the size of the product produced by the vector with no insert) when run out on an agarose gel. Bands larger than 300bp were chosen and the corresponding re-streak colony was grown-up overnight in LB-amp broth for miniprep using a Fermentas Miniprep Kit. Following miniprep, according to the

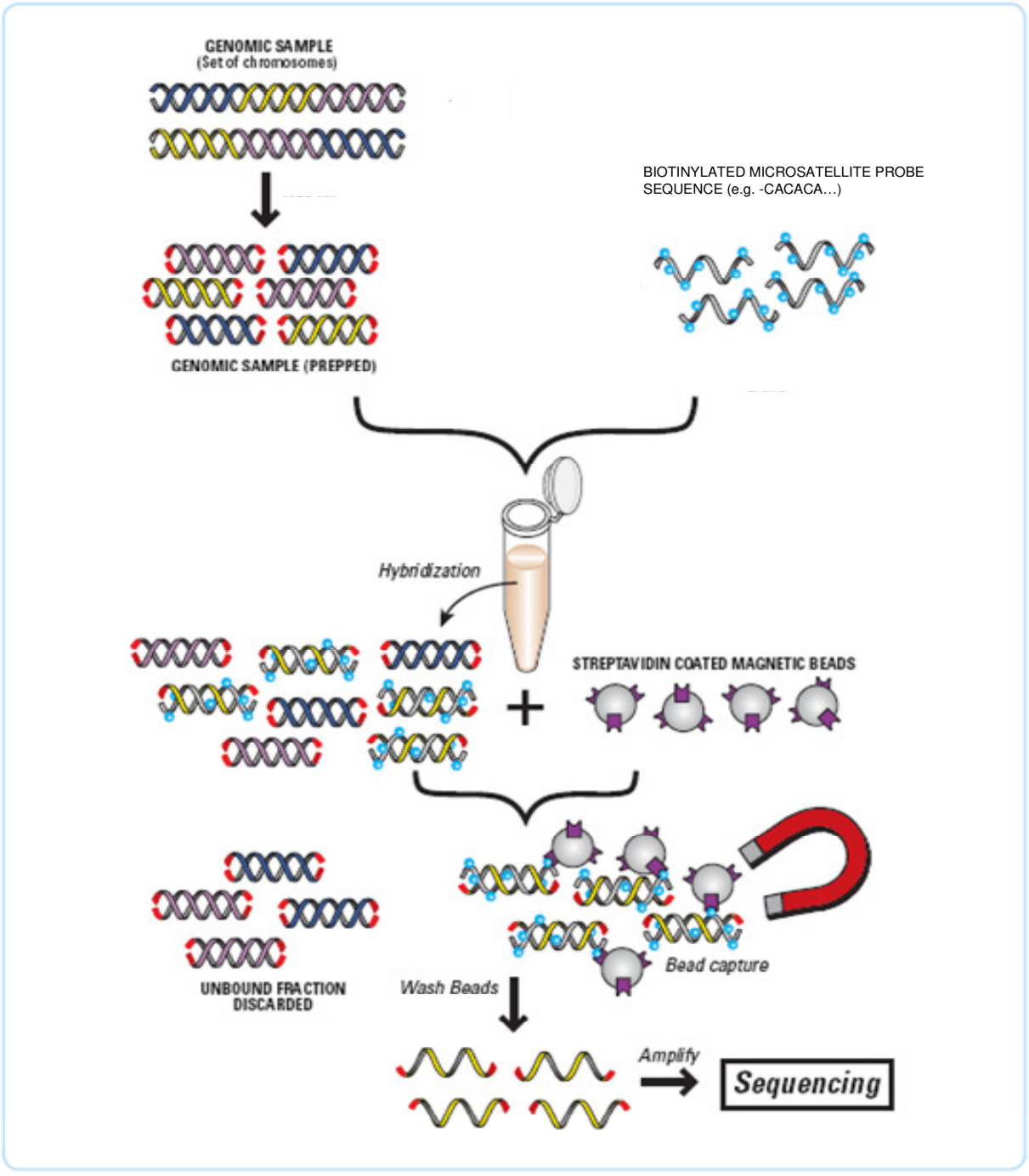
manufacturers' instructions, clones were verified for insert size by restriction digest with *EcoR1*, which cuts either side of the vector insert site producing a ~25bp fragments without an insert. The protocol followed manufacturers' instructions using 0.5µg of DNA in a 10µl reaction. Positive clones were sequenced with M13F/R vector primers on an ABI 3730 Capillary DNA Analyzer (Applied Biosystems, Warrington, UK).

In addition to non-selected libraries, 18 libraries were also created from enrichment probing using microsatellite motifs as follows: CA-MS: 5'- ATA GAA TAT $(CA)_{16}$-3'; GCC-MS: ATA GAA TAT $(GCC)_{12}$'; 5'- ATA GAA TAT $(GATA)_{16}$-3'; CA3'dideoxy: 5'Biotin-ATA GAA TAT $(CA)_{16}$-3'2',3'-dideoxyC. Microsatellite probing was carried out in order to enrich for the microsatellite motifs for use in population studies, detailed in Chapter 5. The CA, GCC and GATA motifs were chosen because they have previously been shown to be in relatively high abundance in mammalian genomes (Tautz et al., 1994). The 3'dideoxy group prevents the recombinant amplification artifacts that can occur as a result of the remaining microsatellite probe from the hybridization technique remaining as a substrate for PCR (Fisher et al., 1996; Koblizkova et al., 1998; Nater et al., 2008). The addition of the chemical modification blocks the extension of the oligonucleotide and prevents it acting as a primer so increases the efficiency of acquiring genuine microsatellite sequences. The principle of microsatellite probing is based on hybridization techniques (see Fig. 4.2.1, for explanation).

**Figure 4.2.1**

1. A biotinylated probe containing a microsatellite motif of choice hybridizes to strands of DNA in the sample containing a complementary sequence.
2. The biotinylated probe binds to the streptavidin-coated magnetic beads.
3. A magnet holds the magnetic beads to the side whilst fragments in the sample that do not contain the motif are washed away.
4. The remaining fragments contain the microsatellite motif of interest and therefore the sample is enriched for the microsatellites.

(image adapted from chemagilent catalogue:
http://www.chem.agilent.com/en-US /Products/consumables/reagents/
sureselecttargetenrichment/PublishingImages/SystemWorkflow600B.gif



### 4.2.2 Characteristics assessed and analysis

All sequences were compared and aligned in clustal-X to assess sequence similarity; 12 clones were not unique and showed an identical or complementary sequence during alignment and were therefore excluded from further analysis. Y-specific cetacean sequences were derived from two sources: libraries that had been enriched for microsatellite loci (microsatellite loci) and those from non-enriched libraries (anonymous loci). Anonymous sequences were compared with the National Center for Biotechnology Information (NCBI) nucleotide genomic sequence database by use of nr/nt BLAST (Web site: http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify loci that matched other sequences on the Y-chromosome or elsewhere in the genomes of other species. These sequences were also used as input for the program Tandem Repeats Finder (TRF Website: http://tandem.bu.edu/trf/trf.html Benson, 1999) to assess the number of simple and complex microsatellites. It was also used to assess the relative incidence of microsatellite containing sequences with mismatches and indels (a signature of developing or decaying microsatellite loci). 100bp of flanking sequence was also assessed for the incidence of repetitive sequence. Subsequently the flanking sequences were aligned to identify the similarity of flanking sequences between loci. The sequences were searched for inverted repeat and secondary structure to give an indication of the possibility of palindromic regions. Cryptic simplicity was assessed with the program Simple (Hancock and Simon, 2005), to give an indication of the relative simplicity of the sequence. The program also produces base pair ratios, which were used to assess the relative and cumulative GC content of the various sequences.

### 4.2.3 Identifying the characters for comparison

When comparing the sequence of a small proportion of one tiny chromosome to that of a similar amount from the remainder of the genome, there are some obvious implications for selection bias in the comparative autosomal sequence. In order to reduce the bias as much as possible, several methods of acquisition were used with randomization and internal comparison between replicate datasets applied to acquire an equivalent quantity to match the combined

killer whale and bottlenose dolphin Y-chromosome datasets of 33*kb* of sequence. A direct comparison of 33*kb* of autosomal sequence could potentially be unrepresentative of autosomal character in general. The 33*kb* cetacean Y-chromosome sequence represents approximately 2-4% of the delphinid Y-chromosome. The equivalent percentage of the autosomal sequence would equate to 6.5Mbp; however, the comparative volume of sequence data of this size and the variability between regions could also result in an equivalent bias. Therefore, the resolution employed herein was to obtain 33*kb* (to match the size of the cetacean Y-chromosome dataset) of anonymous autosomal bottlenose dolphin sequence, randomly chosen 50 times from the autosomal bottlenose dolphin genomic contigs.

The 50 (33Kb) datasets of anonymous bottlenose dolphin sequence were downloaded from NCBI *Tursiops truncatus* Genome project draft assembly of a female bottlenose dolphin (Project ID: 20367) (Website: http://www.ncbi.nlm.nih.gov). The random sequences were chosen using the following formula in excel '=INT(RAND()*102325)' which produced a page number to select from the 102,325 pages of 100 sequences of autosomal data. One sequence was selected from each page according to a number generated from 1-100, using the following formula in excel '=INT(RAND()*101)'. The average autosomal bottlenose dolphin sequence from the assembly was ~800bp long therefore 40 pages were required to obtain a 33*kb* dataset for comparison. This process was repeated fifty times to obtain fifty individual datasets for comparison. The same sequence characters were assessed for each autosomal BND (aBND) dataset as for the Y-chromosome sequence. The aBND data was assessed for significant differences between datasets before being used in comparison to the combined killer whale and bottlenose dolphin Y-chromosome dataset, as detailed below. The aBND were combined and an average of the 50 datasets was used in comparison to the Y-chromosome data. All aBND sequences were first searched for homology to gene sequence in the NCBI Blast database as described above.

### 4.2.4 Comparisons (for each characteristic):

All assessments were conducted by standardizing the sequence length to 200bp by dividing by the actual sequence length and then multiplying by 200. To account for differences in the number of fragments between datasets the relative frequency was used by dividing by the total number of fragments. Comparative frequency of simplicity ratios across sequences were analyzed with the Kruskal-Wallis (K-W) statistic. GC-ratio was also standardized for 200bp sequence length and for fragment number as above and assessed with K-W statistical analysis.

The program 'SIMPLE' (Hancock and Simon, 2005) was used to evaluate the amount of simplicity found in a given sequence by assessing the base composition and order in a 32bp window of nucleotides; it then compares this value to a number (user defined – 1,000 in this study) of randomly generated sequences composed of the same nucleotides. The relative 'simplicity factor' (RSF) is a measure of the relative simplicity between the test sequence and the randomly generated sequences, which produces a ratio indicating the probability of the sequence existing by chance. Any sequence with a 'simplicity factor' greater than 1 has a greater than random level of simplicity, and suggests the operation of DNA slippage along that sequence, (see Tautz et al., (1986) for detailed explanation). The program allows assessment of differential weighting of motifs (mono-tetra). The following weightings were assessed for their effect, each motif was weighted high (5) in turn, equally weighted (3 each) and according to the program default values (0-mono, 0-di, 1-tri, 3-tetra), and the datasets were assessed for significant differences between weighting criteria.

Killer whale Y-chromosome versus bottlenose dolphin Y-chromosome was assessed for each characteristic as follows: 1) the percentage of 'Simple' sequences calculated from each dataset were compared; 2) sequence fragments were 'binned' according to 'simplicity ratios' (as detailed in the output of SIMPLE (Hancock and Simon, 2005) which were then divided by the total number of standardized 200bp sequences to provide relative simplicity ratios. Sequences were 'binned' as follows: <1 (ratio not significant) and at each number change to one decimal

place to a maximum of 3.3 above which there were fewer than 2 in each category. The relationship of significant to not significant sequence was also assessed with K-W analysis. Ratios of GC-content were 'binned' into 11 groups as follows: <20%; 20-25; 26-30; 31-35; 36-40; 41-45; 46-50; 51-55; 56-60; 61-65; 66-70. Similarly the GC-content analysis was conducted on the relative frequency of standardized sequences 'binned' according to AT-GC ratios. The frequency of AT-rich (fragments in 'bins' from 0-50) versus GC-rich (fragments in 'bins' from 55-70) sequence was compared. Differences were analyzed using the K-W test for significance. There was no significant difference between killer whale and bottlenose dolphin sequence ($p=0.86$ simple ratios and $p=0.58$ GC ratio), therefore the two datasets were combined for comparison to the autosomal bottlenose dolphin sequence.

Comparison between aBND and combined Y delphinid sequence was conducted as for the killer whale and bottlenose dolphin Y-chromosome sequences, using the mean of the 50 replicates. Descriptive statistics were reported for totals as well as the mean of the 50 replicates

### 4.3.0 Results

A total of 133 enriched and 162 non-enriched clones were sequenced from 10 (CA, GCC and GATA) enriched libraries, 8 (dideoxy-CA) enriched libraries and 20 non-enriched libraries. The majority (98%) of the enriched clones that contained a microsatellite contained the motif 'CA'. Although the addition of the 'dideoxy' group increased the efficiency of the probe, the libraries without the 'CA' modification produced clones containing the motifs at a lesser efficiency. In contrast the 'GCC' and 'GATA' probes failed to produce clones containing the respective motifs.

The bottlenose dolphin sequences comprised 13,427 basepairs of enriched sequences across 60 fragments and 15,110 basepairs of anonymous sequence across 83 fragments. The killer whale sequences comprised 14,039 basepairs of enriched sequence from 73 fragments and 17,772 basepairs from 79 anonymous fragments. In total 133 clones from microsatellite-enriched libraries gave rise to 27,466 basepairs of microsatellite-enriched delphinid Y-chromosome sequence and 162 clones from non-enriched libraries gave rise to 32,882 basepairs of anonymous delphinid Y-chromosome sequence. The 33*kb* anonymous sequences were used for comparative analysis, detailed in this chapter. The microsatellite-enriched sequences were used to design primers for population screening detailed in Chapter 5.

Characters were initially compared between the killer whale and bottlenose dolphin Y-chromosome sequence for the non-enriched sequences, separately. All delphinid Y-chromosome sequences were subsequently combined for comparison with the bottlenose dolphin autosomal sequences.

**Table1:** Table one shows the number of repetitive sequences for the various sequences analyzed. yKW – killer whale Y-chromosome, yBND – bottlenose dolphin Y-chromosome, Delph Y-chr – Y-chromosome sequence for both killer whale and bottlenose dolphin Y-chromosome sequences, aBND – autosomal bottlenose dolphin sequence

| | Number of Sequences with Repeat | Total Sequence (Kb) | Copy # of largest continuous repeat | | Copy # including mismatches | | Mis-match | Percent Flanking |
|---|---|---|---|---|---|---|---|---|
| | | | Average | Range | Average | Range | | |
| **yKW** | 16 | 17.77 | 8 | 4 to 15 | 23 | 6 to 37 | 4 | 65% |
| **yBND** | 12 | 15.11 | 8 | 6 to 12 | 16 | 7 to 40 | 3 | 73% |
| **Delph Y-Chr** | 28 | 32.88 | 8 | 4 to 15 | 24 | 7 to 49 | 4 | 68% |
| **aBND total*** | 52 | 1600 | 22 | 6 to 70 | 26 | 6 to 64 | 0.6 | 0.06% |
| **aBND av**** | 1.04 | 32 | 0.44 | | 0.52 | | 0.012 | $1.2E^{-5}$ |

*BND total represents the total values across 50 replicate comparable datasets (= 1600Kb of sequence); **BND av represents the average of the 50 replicates representative for one comparable 32Kb dataset. Flanking refers to the percentage of the identified repeat sequences with a repetitive motif within the 100bp flanking region, defined as described in Lopez-Giraldez et al (2007).

### 4.3.1 Number of repeat fragments and characteristics

Tandem Repeat Finder identified 16 and 12 microsatellite loci with a motif ranging from mononucleotide to hexanucleotide from the 17.8Kb killer whale and 15.1Kb bottlenose dolphin anonymous clone Y-chromosome sequences, respectively - approximately one every 9*kb*. In contrast the autosomal sequences yielded an average of 1 microsatellite locus per 33Kb dataset with a range across the fifty datasets of 0 to 6 microsatellites.

The Y-chromosome sequences clearly have a proportionally greater number of microsatellite loci per unit of sequence than do the autosomal sequences (Table 1). They also show that the repeat number (number of repeated elements, e.g. CA) in the longest continuous repetitive sequences for the Y-chromosome are much fewer (8) than for those from the autosomes (22). Although, with reference to Chapter 5, among the sequences obtained via enrichment the longest continuous repeat was 23; however, it was not male-specific and when primers were designed to flank the microsatellite a PCR product was amplified in both males

and females. The repeat number in anonymous sequences increased substantially with the allowance of mismatches in the region surrounding the largest continuous motif, for the Y-chromosome sequence (24) but not for the autosomal (26) sequences. A second indication of this pattern is that the percentage of sequences with additional copies of the same motif found in their flanking sequence (as indicated by Tandem Repeat Finder) was greater for the Y (68% of microsatellite loci) than for the autosomal sequences (0.06%). The autosomal sequence was also more likely to contain two different repeat motifs adjacent or to have an interruption of continuous non-repetitive DNA followed by a continuous stretch of a second or the same motif.

The greater number of anonymous Y-chromosome sequences containing a repeat could potentially be a function of interruptions resulting in one interrupted microsatellite being identified as multiple instances rather than a single locus; however, the same number of microsatellites was identified when interruptions were included. Only two Y-chromosome fragments had more than one microsatellite identified from the one contiguous sequence and in both cases they were isolated from one another by a region of non-repetitive DNA and would not have been identified as part of a single locus.

Autosomal sequences, which had a microsatellite motif in the flanking region were more likely to have a secondary continuous repeat with a different motif rather than fragmented repetitive regions, for example: $(CA)_{12}$, TGCTA $(AT)_{15}$) from an autosomal sequence compared with $(CA)_2TGAC(TG)_3TA(TG)_6CA(TG)C(TG)A(TG)_4C(TG)_2$) from a Y-chromosome sequence. The Microsatellites found in the autosomal sequence were more likely to contain uninterrupted sequences greater than 6bp and the total motif was less likely to contain interruptions (as measured by mismatch and indels) than the repeat elements on the Y-chromosome.

**Table 2: Repeat motifs** shows the proportion of different microsatellite motifs (mono-, di- , tri- and tetra-nucleotide) from the sequences analyzed. The total number of each motif , the range in copy number andRelative numbers of microsatellites and of copy number for the different motifs. The most common motif is also detailed yKW – killer whale Y-chromosome, yBND – bottlenose dolphin Y-chromosome, Delphinid Y-chr – Y-chromosome sequence for both killer whale and bottlenose dolphincombined, aBND total - shows the total across all replicates for autosomal bottlenose dolphin sequence, a BND av - shows the average across the replicates for the autosomal bottlenose dolphin sequence.

| | **Most common motif** | **Mononucleotide** | | | |
|---|---|---|---|---|---|
| | | **Total** | **Copy#range** | **A or T** | **G or C** |
| **yKW** | CA | 3 | 7 to 15 | 3 | 0 |
| **yBND** | CA | 0 | | 0 | 0 |
| **Delphinid Y-Chr** | CA | 3 | 7 to 15 | 3 | 0 |
| **aBND total** | CA | 12 | 25 to 31 | 6 | 6 |
| **aBND av** | CA | 0.12 | | 0.12 | 0.12 |

| | **Dinucleotide** | | | | | | **Tri-*** | **Tetra**** | **Penta***** |
|---|---|---|---|---|---|---|---|---|---|
| | **All** | **Copy#range** | **AT or TA** | **GC or CG** | **ACorCA GTorTG** | **TCorCT GAorAG** | **Total** | **Total** | **Total** |
| **yKW** | 11 | 4 to 15 | 0 | 0 | 10 | 1 | 0 | 3 | 0 |
| **yBND** | 11 | 6 to 12 | 0 | 0 | 8 | 3 | 0 | 0 | 0 |
| **Delph Y-Chr** | 22 | 4 to 15 | 0 | 0 | 18 | 4 | 0 | 3 | 0 |
| **aBND total** | 27 | 13-30 | 10 | 0 | 12 | 5 | 1 | 10 | 6 |
| **aBND av** | 0.54 | | 0.2 | 0 | 0.24 | 0.1 | 0.02 | 0.2 | 0.12 |

*Trinucleotide repeat motifs for aBND 1x AGT
**Tetranucleotide motifs for: yKW one each of ATTG, TCCT, TCTT; aBND 1x AAAG, 1xAAGG, 1xAAGA, 2xAATA, 2xATAA, 3xAAAT,
***Pentanucleotide motif for aBND 1xCTTTT, 2xCCCTA, 3xTTTCA

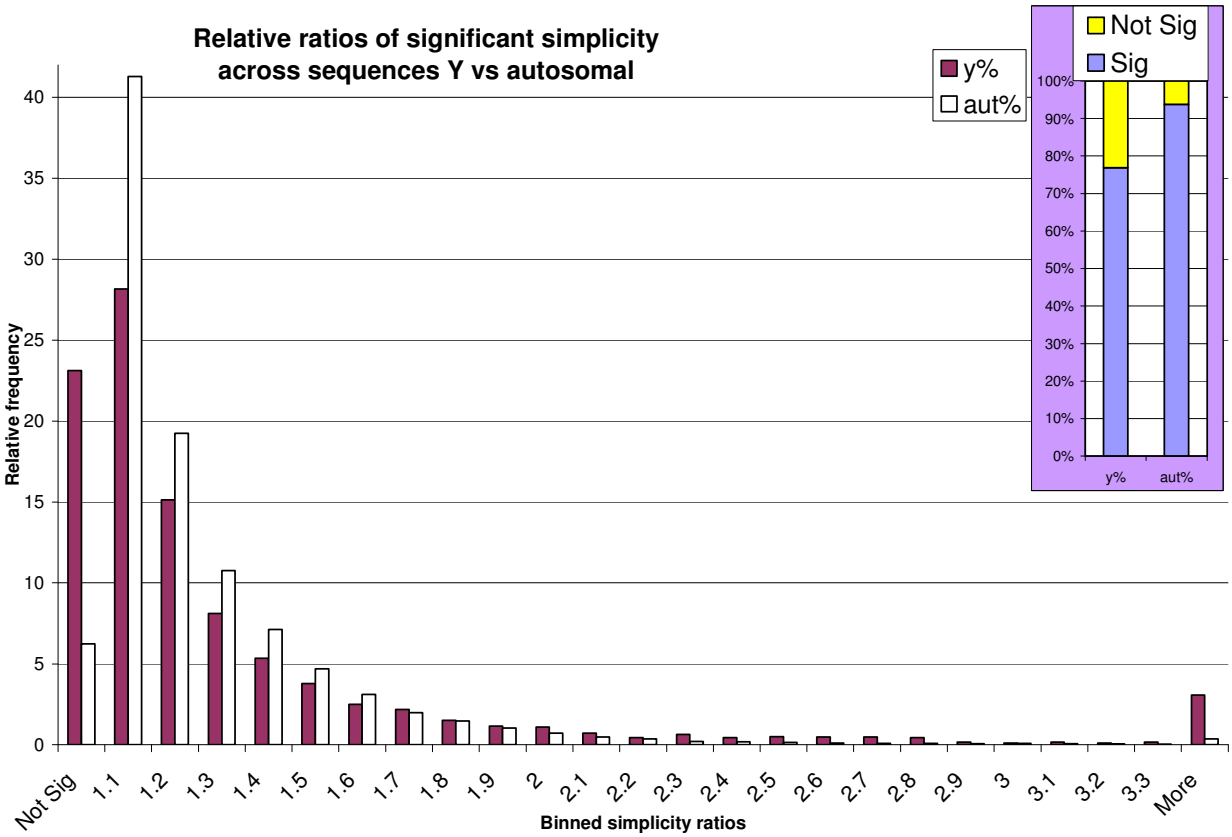



**Figure1**: shows the relative ratio of simplicity across standardized sequence lengths for the dolphin Y-chromosome and autosomal sequence
Inset: the percentage of sequences showing significant simplicity for the autosomal (aut) and Y-chromosome (y) sequence

The most common repeat motif for all sequences from both the aBND and anonymous Y-chromosome sequence was the dinucleotide 'CA/TG' and generally dinucleotides were far more frequent than any other motif size. As expected there was an absence of 'GC/CG' microsatellites and generally fewer microsatellites with increasing motif size. In contrast to the autosomal sequences, the Y-chromosome showed very few instances of any alternative motifs to the 'CA', including only three mononucleotide loci and no tetranucleotide repeats. The autosomal sequence had a relatively high proportion of tetranucleotides (10) and 'AT' dinucleotides (10), in contrast to three and zero on the Y-chromosome, respectively. Sample sizes were however too small to test whether the difference was significant.

### 4.3.2 Analysis of Cryptic Simplicity

The majority of sequences for both autosomal and Y-chromosome sequences were significantly 'simple' p>0.01 according to the output of 'SIMPLE'. More of the autosomal sequences had relative simplicity factor (RSF) values greater than 1 than for the Y-chromosome sequences (93% and 76%, respectively; Fig.1). The number of sequences with RSF > 1 did vary slightly depending on the weighting of different motifs, however the difference between analyses with different motif weighting was not significant (K-W, d.f. =5, p> 0.1 between all group weighting) for both Y-chromosome and autosomal sequences. Although more of the autosomal sequences had RSF values greater than 1, more of the Y-chromosome sequences showed higher RSF values (Fig. 1). The majority (67%) of sequences were in the range of 1-1.2 for both autosomal and Y-chromosome sequences. However, 85% and 79% were between 1-1.4 for the autosomal and Y-chromosome sequence, respectively and 95% of autosomal and 89% of Y-chromosome sequences fell in the 1-1.9 range. 10% of the Y-chromosome sequences therefore had a ratio greater than 1.9, compared with 5% of autosomal sequences. The difference in the relative frequency of ratios between the autosomal and Y-chromosome sequences, across all sequences was not significantly different across all 25 'bins' (K-W, d.f. = 24, p=0.49). However, there was a significant difference (K-W, d.f. 13, p=0.02) between the top 14 bins with ratios greater than two.

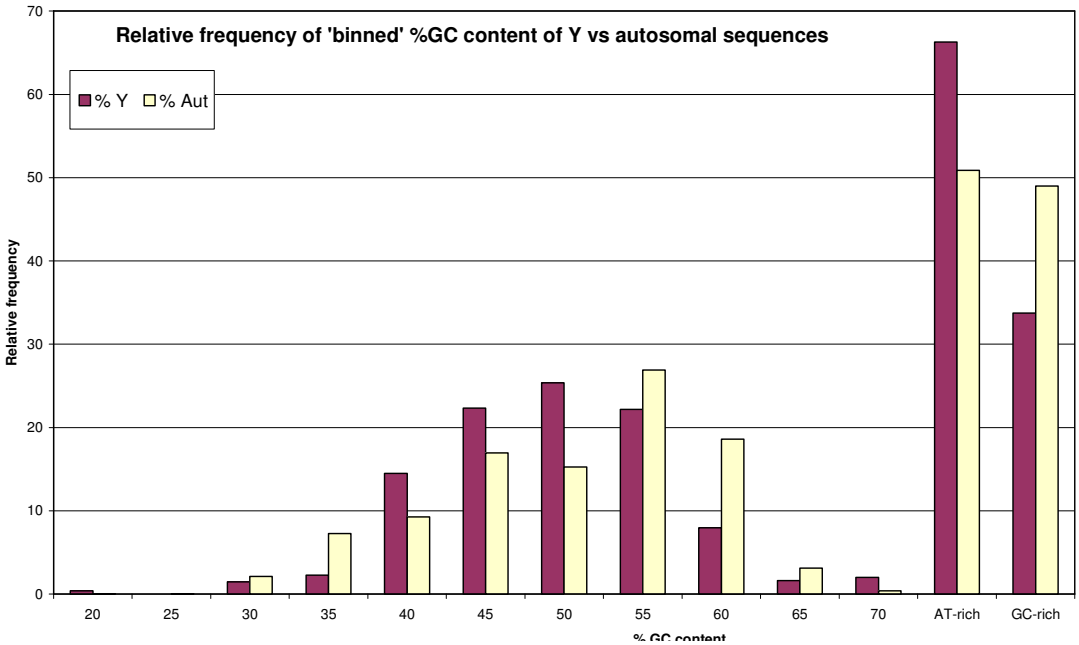



Figure 2: shows the relative frequency of GC-content across standardized delphinid autosomal and Y-chromosome sequences. Far left of histogram shows the relative cumulative ratio of GC- to AT-rich sequence

### 4.3.3 GC-AT ratio

As indicated by Figure 2, a greater number of Y-chromosome sequences were AT-rich 66% compared with 51% of autosomal sequences. There was a significant difference between the AT: GC ratio between the 11 bins (K-W, d.f. =10, p=0.04). The majority of Y-chromosome sequences had an even ratio of basepair composition compared with the autosomal sequences, the majority of which were 55% GC-rich. Only 32% of Y-chromosome sequences were GC-rich; however, there were 2% that were particularly GC-rich.

Overall, Y-chromosome fragments showed short, low variation microsatellites with high levels of interruption, very few tri- and tetra- repeats and low GC-content. By contrast, autosomal fragments showed long, highly variable microsatellites with few imperfect or interrupted and comparatively high GC-content.

## 4.4.0 Discussion

### 4.4.1 Microsatellite DNA Evolution

The genomic distribution of microsatellites is not regular across chromosomes or chromosomal regions (Oliveira et al., 2006). Types and patterns of microsatellite loci have also been shown to vary between genomic regions, with no wholly consistent pattern detected across all loci, but rather to vary according to the influences of local sequence context (Goodman, 1998; Marra and Schar, 1999; Zhang and Gerstein, 2003; Arndt and Hwa, 2005).

This study has suggested fewer than expected e.g. (Kelkar et al., 2008) long, perfect microsatellite repeats in anonymous Y-chromosome sequences compared to the autosomal samples, coupled with a high instance of long tracts of imperfect repeat sequence surrounding microsatellite regions. This pattern was also observed when sequences were enriched for repetitive DNA, with relatively low repeat numbers and all identified microsatellite loci being flanked by further repetitive tracts (see Chapter 5).

Almeida & Penha-Goncalves (2004) and others, point out that long (8<), uninterrupted repeats are relatively rare in eukaryote genomes. Microsatellite mutability was shown by Kelkar et al (2008), to be frequently higher on the human Y-chromosome, with the strongest effect seen for mononucleotides (Kelkar et al., 2008). This trend is consistent with higher numbers of replications in the male compared with the female germline (Ellegren, 2004), and possibly with replication slippage being more important for mononucleotide repeats (Schloetterer and Tautz, 1992). At the same time, point mutation degrades these repeats by interrupting the potential for slippage. If the Y-chromosome of the cetacean genome is relatively ancient and has not received any large additions from elsewhere in the genome, (as suggested by its small size; see Chapter 3), the long-term degeneration of repeats due to the accumulation of point mutations may explain the apparently lower frequency of these long repeats in the microsatellite sequences investigated here, though this was not an exhaustive survey.

Several recent studies have considered non-stochastic mechanisms for microsatellite evolution. Some reports suggest that microsatellites may have a functional significance, for

example, in the regulation of genes (Biggin and Tjian, 1988; Odonnell et al., 1994; Kashi and Soller, 1999), or acting as recombination hotspots (Treco and Arnheim, 1986; Wahls et al., 1990; Rosenberg et al., 1994; Bailey et al., 1998). Such mechanisms may suggest that loci with these functions were derived from autosomal origin. However, the processes are just as likely to be stochastic, based on replication error and the subsequent amplification of simple, repetitive arrays (Goldstein and Schlotterer, 1999).

Degradation of genes and loss of large chunks of the Y-chromosome is evident for all Y-chromosomes studied and is believed to be related to the lack of recombination and degree of redundancy of the genetic material of which it is composed (Charlesworth and Charlesworth, 2000; Engelstadter, 2008; Carvalho et al., 2009). This is in contrast to the lack of degeneration and preservation of the integrity of autosomal DNA as a result of purifying selection and recombination (Thacker, 1999; Yoshida et al., 2003; Gerrard and Filatov, 2005). While such mechanisms would not be expected to preserve neutral loci such as repetitive arrays, the turn-over mechanisms operate at a faster rate than point mutational change and therefore young arrays can be maintained.

There is evidence that the evolution of some microsatellites is linked to their proximity to coding regions but coding regions are inherently low in microsatellite density because of their consequent risk of loss of functionality (Toth et al., 2000). It has also been suggested that there is a negative correlation between microsatellites and heterochromatin (Lin et al., 1999), which would be consistent with an under representation of microsatellites on the heterochromatic Y, assuming heterochromatin is common to the Y of all species. However, Nadir et al. (1996) suggested a causal relationship between transposons, which are often at high density in heterochromatin (Gabrielrobez and Rumpler, 1992; Edelman and Lin, 2001) and microsatellites, which contradicts the expectations of their low density in heterochromatin.

The general ratio of perfect to imperfect microsatellites does not appear to be consistent across taxa, (Almeida and Penha-Goncalves, 2004; Lopez-Giraldez et al., 2007) and the relative number of complex and imperfect microsatellites reported by population studies using

microsatellites may be a function of ascertainment. For example, Lopez-Giraldez, (2007) suggests that the higher reporting of complex microsatellite loci in badgers was a result of the inability to identify the preferred simple loci, while other studies may have identified high numbers of complex loci but not reported them as they had sufficient simple loci to report. Thus, it rather suggests a low incidence of simple microsatellites rather than a high instance of complex ones. This is consistent with evidence from Almeida et al., (2004) who found 30 to 50 fold fewer long perfect repeats relative to imperfect microsatellites across a range of taxa. Generally, the number of complex microsatellites is reportedly high in delphinids (Buchanan et al., 1996; Valsecchi and Amos, 1996; Palsboll et al., 1997; Caldwell et al., 2002; Mirimin et al., 2006), which may suggest that they are relatively depauperate in simple microsatellites as a group (Lopez-Giraldez et al., 2006; Lopez-Giraldez et al., 2007). The contrasting evidence of high repeat numbers from the human Y-chromosome (Kayser et al., 2004), may be related to the more recent acquisition of a translocated region onto the Y in this species (Waters et al., 2001). As such, a relative lack of variability (as discussed in Chapter 5) for the Y-chromosome of the focal species in addition to that of others, may simply be a function of the relative lack of long simple loci.

In general, microsatellite length is thought to be a function of strand slippage resulting in the loss or gain of repeats (Levinson and Gutman, 1987; Tautz and Schloetterer, 1994). Microsatellites may be generated and extended during recombination of DNA strands during meiosis as a result of unequal crossing-over (Smith and Haigh, 1974; Blouin et al., 1996). The microsatellite mutation rate is a balance between repeat length and point mutations (Kruglyak et al., 1998), which suggests that either the former is reduced or the latter is increased on the Y-chromosome to produce an imbalance of interruptions of the microsatellite motif. Point mutations appear to limit the expansion of microsatellites by degrading the perfect repeat sequence. Various explanations have been put forward and a range of mechanisms used to describe microsatellite mutation processes. Santibanez-Koref et al. (2001) showed that degeneration was associated with the local mutation rate, which would suggest that it is high on

the Y-chromosome. However, rates of microsatellite slippage and SNP mutation rates have been shown to be comparable to those of autosomes (Roewer et al., 1992; Goldstein and Schlotterer, 1999; Kayser et al., 2000; Jobling, 2001; Roewer et al., 2001). Dupuy et al. (2004) even showed a bias towards an increase in allele length of human Y-chromosome microsatellites.

Although there has been a recent increase in the study of mutational bias acting around microsatellites, clear mechanisms of variability and complexity are still not well understood, (Wilder and Hollocher, 2001; Vowles and Amos, 2004; Lopez-Giraldez et al., 2006; Lopez-Giraldez et al., 2007; Webster and Hagberg, 2007). The distribution of microsatellites within genomes has been shown to be associated with particular regions and with each other, (Bachtrog et al., 1999). Microsatellite flanking sequence also has a tendency to be associated with an increased mutation rate, conserved sequence, cryptic simplicity and interruptions of the same motif (Wilder and Hollocher, 2001; Lopez-Giraldez et al., 2006). For example, there has been a high association of AT-rich sequence flanking microsatellites; Matula and Kypr (1999), and Wilder and Hollocher (2001) identified an abundance of one particular microsatellite with highly similar flanking regions throughout the genome.

The genesis of microsatellites is also thought to be related to transposable elements, for example A-rich tails of LINE and SINE elements (Arcot et al., 1995), followed by point mutation and slippage. Regions with high cryptic simplicity (Tautz et al., 1986); recombination hotspots (Benet et al., 2000; Guo et al., 2009); SINE insertion and genomic rearrangement hotspots have also been implicated in the genesis or evolution of microsatellites (Bailey and Shen, 1993; Thalamuthu et al., 2005).

It has been demonstrated that regions of increased likelihood or ‘hotspots’ exist for various genomic mechanisms including recombination, translocation, substitution, indels and chromosome breakage and that these are associated with the genesis of microsatellites and/or microsatellite insertion (Brandstrom et al., 2008; Costantini and Bernardi, 2009). There are also areas of the genome, which are conversely ‘coldspots’ for these mechanisms and it follows

therefore that there is a driving force for, or protection from, certain types of mutation associated with particular genomic regions (Varela and Amos, 2009). The lack of recombination and the reduced efficiency of mismatch repair mechanisms may yield a greater opportunity for the degeneration of microsatellites on the Y-chromosome.

### 4.4.2 Analysis of Cryptic Simplicity

Cryptic simplicity reflects DNA sequence that is composed of repetitive elements that are dispersed. These can either form the basis for the genesis of longer arrays of repetitive sequence (e.g. microsatellite loci), or reflect the degeneration of simple repeats impacted by point mutational change, and the consequent decay of the array (Lopez-Giraldez et al., 2006). Tautz et al. (1986) showed that cryptic simplicity was abundant in the genomes of most species, representing the majority of sequences tested. The vast majority of the autosomal bottlenose dolphin sequences were characterized by a high incidence of cryptically simple sequences suggesting a cryptic patterning to the majority of the sequences. The removal of any genic loci may have resulted in a larger representation of cryptic regions. However, only two sequences were rejected across the 50 datasets and the relative GC-content of the autosomal sequence was evenly distributed as expected. Although the majority of the Y-chromosome sequences were cryptically simple, there were fewer than found on the autosomes, despite strong evidence for microsatellite degeneration on the Y-chromosome. However, the strength of evidence for cryptic simplicity (high RSF values) was stronger on the Y-chromosome. Both the genesis and degeneration of microsatellites are indicated by the presence of cryptic simplicity (Wilder and Hollocher, 2001; Lopez-Giraldez et al., 2006), but the rate of decay of this signal is not well understood. Therefore, the age of the Y-chromosome may again be relevant to the proportion of sequences showing evidence of simplicity.

### 4.4.3 GC-content

GC-rich regions tend to be, according to the biased gene conversion hypothesis, a result of recombination hotspots (Galtier et al., 2001). Because of the reduced size of the PAR, it is believed that it is a hotspot of recombination in mammals, thus the per nucleotide recombination rate in the PAR is much higher than in autosomes, (Eisenbarth et al., 2000; Galtier et al., 2001) in contrast to the complete lack of recombination in the MSY. Therefore the evidence of a higher percentage of Y-chromosome sequences being AT–rich with a subset exhibiting particularly high GC content is consistent with fragments derived from the MSY and PAR, respectively.

Removal of any sequences from the autosomal dataset that aligned to coding regions, would have resulted in inadvertent 'selection' within the study for the more simplistic non-genic regions; however, because of the paucity of genes and the lack of cross-homology of the Y-chromosome, regions that were derived from the PAR could not be identified and would have remained in the sample set for the Y-chromosome. Although not all coding genes, presence of gene sequence within a high recombination PAR region may result in reduced simplicity because of gene conversion potentially conserving the sequence character and gene regions (Galtier et al., 2001; Skaletsky et al., 2003; Galtier, 2004).

If the character of the cetacean Y-chromosome is similar to that of the human, it would be expected to be made up of very divergent classes of sequence (Skaletsky et al., 2003). The evidence of uneven distribution of character sets of both GC-content and simplicity found in the dataset are consistent with this expectation. The delphinid Y-chromosome is small relative to that of the human and as such may have lost some regions present in hominids. The rat Y-chromosome is also much larger than the delphinid Y and is consistent with the character of the human (Bergstrom et al., 2003). The minimal Y-chromosome in the marsupial is both ancient and minute but may inform observed differences between the larger human and rat Y and that of the delphinid (Toder et al., 2000). The marsupial Y lacks much of the repetition found in mice and humans and also lacks the PAR region. These characters may be linked to the absence of the recent translocation on to the human Y-chromosome and/or the divergent demographic

history of the species. The indication of regions of high GC-content that do not contain known gene sequence would suggest that the cetacean Y-chromosome does not lack a PAR region and Toder et al. (1997b) reported an apparently more extensive PAR in artiodactyls than the human. However, the relatively low incidence of multi-copy loci observed in this study, (as indicated in Chapter 5) and the absence of evidence for palindromic regions could suggest that there is reduced repetition on the delphinid Y-chromosome, which is also consistent with reduced number of microsatellite loci, as detailed above.

### 4.4.4 Conclusions

Although the interpretation is limited due to the quantity and identity of the fragments isolated herein, the sequence characters of the delphinid Y-chromosome are consistent with those from the Y-chromosomes of other species. Overall, Y-chromosome fragments showed relatively short microsatellites with high levels of interruption, very few tri- and tetra- repeats and low GC-content. By contrast, autosomal fragments showed long, highly variable microsatellites with few imperfect or interrupted repeat arrays and comparatively high GC-content. Low GC-content on the Y-chromosome was consistent with other studies and with expectations given the low level of recombination. Recombination is restricted to the PAR segment of the Y-chromosome, and this may not have been sampled among the screened fragments, although higher GC-content in some clones suggests the possibility of PAR origin. Cryptic simplicity was found throughout both Y-chromosome and autosomal sequences, but the segments with the highest values were more likely to be found on the Y-chromosome. The apparent difference in the character of the microsatellite DNA loci on the Y-chromosome as compared to the autosomal sequences possibly relates to the age and process of degeneration of the Y-chromosome. Perfect, long repeat arrays are probably relatively young, generated by the comparatively fast mechanism of DNA slippage before point mutational change has had an opportunity to interrupt that process.

# Chapter 5: The Y-chromosome in population genetics

## 5.1.0 Introduction

As discussed in earlier chapters, the mammalian Y-chromosome consists of a small pseudoautosomal region (PAR) and a large male-specific region (MSY), which is clonally inherited as a haploid unit and consequently does not undergo crossing-over during meiosis (Skaletsky et al., 2003). As a result of these characteristics male-specific Y-chromosome (MSY) markers, which have an effective population size one quarter that of autosomal markers (Hurles and Jobling, 2001) can be used as a counterpart to mitochondrial markers. Whilst mitochondrial markers have been extensively used in population genetic studies (Avise and Hamrick, 1996; Avise et al., 2002), until recently Y-chromosome markers have only been available for humans.

Although initial screening appeared to indicate a paucity of genetic variation on the mammalian Y (Dorit et al., 1995; Hellborg and Ellegren, 2003), more recent investigations in humans subsequent to the almost complete sequencing of the human Y-chromosome have identified a variety of Y-specific polymorphisms, including SNPs and Microsatellites (Kayser et al., 2004). These markers have been used to estimate male-mediated gene flow (Jobling and Tyler-Smith, 2003), to investigate human phylogeography (Hammer et al., 1998), to resolve recent human population history (Hill et al., 2000; Bolnick et al., 2006) and to investigate relative rates of genomic evolution between closely related species (Kelkar et al., 2008). Rates of evolution at human Y-chromosome microsatellite loci are estimated to be approximately equal to or faster than autosomal tandem repeat loci (Kayser et al., 2000; Dupuy et al., 2004; Zhivotovsky, 2004) In contrast, Hellborg and Ellegren (2004), studied the variation across mammalian taxa using conserved intron regions of six different Y-chromosome genes and found nucleotide diversity to be significantly lower than theoretical expectations, compared with intron regions of autosomes.

Recent publication and comparison of the near-complete sequence of human and chimpanzee Y-chromosomes (Rozen et al., 2003; Skaletsky et al., 2003; Hughes et al., 2005;

Kuroki et al., 2006) reveals much of its structure and evolution, and provides a basis for surveys of Y-chromosome polymorphism in other mammalian species.

Despite the on-going degeneration of Y-chromosomes there has been some evidence that suggests that the rate of gene decay is slowing down (Charlesworth and Charlesworth, 2000). Recently Hughes et al. (2005), following the near-complete sequencing of the chimpanzee Y-chromosome, tested the rate of gene degeneration of the chimpanzee versus human Y. They found that none of the inactivated pseudogenes had been inactivated since the chimpanzee-human split, 6MYA, suggesting a reduced rate of degeneration. Furthermore, it was found that the chimpanzee Y-chromosome, which is subject to high levels of sperm competition compared to the human, appears to be subject to positive selection for spermatogenesis genes that, as a result of hitch-hiking, results in higher levels of non-functional gene decay (Hughes et al., 2005). In addition, there is some indication that purifying selection for Y-chromosomes carrying genes beneficial to spermatogenesis, acts on the Y-chromosome, with a greater influence in chimpanzees than in humans because of the reduced promiscuity in the latter resulting in less sperm competition (Kuroki et al., 2006). These recent studies on human versus chimpanzee Y-chromosome sequences, in addition to previous empirical and theoretical studies in other species (Lugon-Moulin and Hausser, 2002; Eriksson et al., 2006; Lawson Handley and Perrin, 2007) demonstrate the importance of the ecology of a species to the character of the Y-chromosome, underlining the extent of the divergence that results.

Although there is evident cross-homology between the closely related hominoid species, the utility of these markers for distantly related mammalian species may be limited, particularly since the MSY can be very dynamic with respect to size and gene content (Delbridge and Graves, 1999; Murphy et al., 2006)

Surveys of Y-chromosome polymorphisms in natural populations of non-human mammals have been hampered by a lack of Y-specific markers as a result of a reduced effective population size, haplotypic evolution and associated technical difficulties (Petit et al., 2002). As described in Chapter 1, the Y-chromosome is subject to selective sweeps, ‘Muller’s Ratchet’ and

'background selection'. In addition, the human Y-chromosome shows evidence of a high occurrence of gene conversion in the euchromatic-male-specific-y (eMSY) and degeneration and repetitive sequence, particularly in the non-recombining-Y (NRY) heterochromatic region, which is yet to be fully sequenced (Rozen et al., 2003; Skaletsky et al., 2003).

Y-specific variation has been identified and used effectively to elucidate sex-specific patterns in several species including, mice *Mus musculus* (Boissinot and Boursot, 1997); macaques *Macaca spp.*(Tosi et al., 2002); baboons *Papio hamadryas* (Handley et al., 2006); bovids *Bos spp.* (Edwards et al., 2000; Hanotte et al., 2000); buffalo (Van Hooft et al., 2002); ovids *Ovis spp.* (Meadows et al., 2004); horse *Equus spp.* (Wallner et al., 2004) deer *Odocoileus spp.* (Cathey et al., 1998); canids *Canus spp.* (Sundqvist et al., 2001; Vila et al., 2003); field voles *Microtus agrestis* (Jaarola et al., 1997); common shrews *Sorex araneus* (Balloux et al., 2000; Lugon-Moulin and Hausser, 2002); and white-toothed shrew *Crocidura russula* (Handley et al., 2006). The limitation of most of these studies was that markers were suitable for addressing inter-specific phylogenetic questions**,** but were not sufficiently variable to investigate intra-specific questions.

Microsatellites and SNPs from the extensive human Y-chromosome marker set have facilitated the study of population and community based differences in the evolution and diversity of Y-chromosome markers (Li et al., 2007). For example, Kayser et al. (2003) observed low levels of Y-chromosome diversity in West Papua New Guinea male populations in comparison to high mtDNA diversity, which suggests there has been a history of patrilocality and/or polygyny (skewed male reproductive success). Investigation of north European Y-chromosome distribution, identified divergence patterns associated with both geography and language (Zerjal et al., 2001) whilst several studies have identified differential origins between males and females within communities (Perez-Lezaun et al., 1999; Silva et al., 2006). In addition to markers developed from the human sequenced Y-chromosome (Kayser et al., 2001), a limited number of studies in other species have applied Y-chromosome microsatellite markers to population level questions, including canids (Olivier and Lust, 1998; Olivier et al., 1999; Sundqvist et al., 2001)

and homologous amplification in apes (Stone et al., 2002; Erler et al., 2004; Handley et al., 2006). The variation in the four canid microsatellite loci when applied to Scandinavian wolves showed differential patterns of colonization in Europe and reinforced results from mitochondrial and bi-parental markers, which indicate a recent population bottleneck (Sundqvist et al., 2001). Application of these same markers to domestic dogs demonstrated a restricted contribution of male founders and generally private haplotypes for different breeds (Sundqvist et al., 2006).

Homologous amplification of human microsatellite markers in the chimpanzee showed high levels of diversity from 56 polymorphic loci (Erler et al., 2004), whereas the number and extent of their utility was very restricted for bonobos, where only one microsatellite marker was found to be polymorphic in two populations of bonobos.

### 5.1.1 Y-chromosome markers in cetaceans

MtDNA demonstrates strong population structure within and among whale populations as a result of matrilineal social structure and high levels of female site fidelity in many whale species (Baker and Van Helden, 1990; Gladden et al., 1999; Lyrholm et al., 1999; Rosel et al., 1999; Escorza-Trevino and Dizon, 2000); Conversely, bi-parentally inherited microsatellites often exhibit low levels of differentiation (even after correction for differences in effective population sizes, see below), which suggests that males are more mobile and thereby maintain these low levels of population differentiation (Baker et al., 1993; Palumbi and Baker, 1994; Baker et al., 1998; Gladden et al., 1999; Lyrholm et al., 1999; Rosel et al., 1999; Escorza-Trevino and Dizon, 2000). By comparing mtDNA and autosomal microsatellite (aMS) variation it is possible to gain some inference about differential male and female gene flow, though this requires adjustment for the fourfold difference in effective population size, and even then the inference is limited by the fact that the two genomes evolve independently. Some further indication about which sex disperses more can be gained from comparing post-dispersal adults just at the nuclear markers, but this requires several assumptions, and has relatively low power (Goudet, 1995). Although comparison between female mtDNA and male mtDNA enables the

tracing of male patterns of dispersal, it does not provide evidence for contributions to subsequent generations. Using Y-specific markers provides information with specific regard to paternal lineages, and the interpretation is therefore much more straightforward.

In addition to the two genes commonly used for sexing in cetaceans, SRY, the 'sex-determining region', and ZFY, a 'zinc-finger protein' gene, (Brown et al., 1991; Palsboll et al., 1992; Richard, 1993; Berube and Palsboll, 1996), several additional Y-chromosome markers have been identified on the cetacean Y-chromosome.

Y-conserved anchor tagged sequences (Y-cats) are Y-chromosome sequences isolated and characterized by Hellborg and Ellegren (2003) in a number of species. The Y-cat markers are anchored in regions that are conserved across species with expected variation in the product region. Caballero et al. (2008) applied the Y-cats to dolphins for phylogenetic analysis but showed there to be very little variation and an absence of intra-specific variation across four loci in several cetacean species, despite higher levels of nucleotide diversity at these loci in other mammalian species (Hellborg and Ellegren, 2004; Luo et al., 2006). Hatch et al. (2004) developed five microsatellite and three (~1.5 kb) 'anonymous' markers for the fin whale (*Baleanoptera physalus*), and found that variation was sufficient for inter-specific phylogenetic analysis but showed limited population genetic differentiation. Although the y-markers were useful when combined with mtDNA and aMS loci, the information obtained from the Y-chromosome itself was limited. Similarly, Nishida et al. (2007) applied 1.7kb of non-recombining Y-chromosome taken from near the SRY gene to phylogenetic analysis across cetacean species and showed low inter-specific variation, particularly in the delphinidae where it was not possible to resolve relationships due to a lack of SNPs between them. Conversely, Mace and Crouau-Roy (2008), using 1kb of the amelogenin-y (AMELY) gene on the Y-chromosome found relatively high levels of inter-specific variation (7 haplotypes with 64 polymorphic sites).

Although in the majority of studies markers have been useful for investigating inter-specific phylogenetic questions, thus far markers from cetacean Y-chromosomes have shown little intra-specific population variation. Locating male-specific markers useful for resolving

population structure within cetacean species requires identification of additional genes or sequences on the non-recombining region of the Y chromosome. To this end, this chapter describes the isolation of male-specific markers from three cetacean species - killer whale (O*rcinus orca*), bottlenose dolphin (*Tursiops truncatus*) and sperm whale (*Physeter macrocephalus)*, and their application in the sperm whale.

Using Y-chromosome markers it should also be possible to gain insight into the degree of male reproductive skew in a population and the male-specific ecology. If there is sufficient difference between Y-specific markers it is possible to identify the precise paternity of individuals (Jobling and Tyler-Smith, 1995) and thereby ascertain the males that are contributing to the progeny. The Y-chromosome is haploid and therefore has an effective population size (Ne) ¼ that of autosomes. It follows therefore, that the breeding system, mating strategy and levels of assortative mating, (such as reproductive behaviour, polygyny and high reproductive skew) will have a significant impact on the genetic variation found on the Y-chromosome in mammals. A combination of genomic processes, including 'background selection', 'hitchhiking' and 'Müller's Ratchet' affect the level of variation among Y-chromosomes (see Chapter 4). Y-chromosome diversity may therefore result from an interaction between the effect of these genomic processes and demographic processes such as bottlenecks, dispersal and male reproductive skew, which may be magnified on the Y-chromosome as a result.

### 5.1.2 Introduction to species

Although there has been considerable assessment of population genetic structure from mitochondrial and autosomal microsatellites from the focal species, the addition of male-specific markers would help to elucidate a better understanding of the different sex-specific components of the overall population structure. Mitochondrial markers have been used to demonstrate patterns of maternal inheritance that affect the genetic structure of the population while autosomal markers have given an indication of the population structure as a whole (Hoelzel and Dover, 1991; Hoelzel et al., 1998; Hoelzel et al., 2002; Natoli et al., 2004; Gero et al., 2008;

Engelhaupt et al., 2009). However, the patterns demonstrated by the two groups of markers are not always in accord, not least because of differences in their effective population sizes, which makes direct comparison between them difficult, in spite of attempts to account for differences with modelling methods (see above).

According to theoretical models of mammalian reproductive strategy (Greenwood, 1980), greater female philopatry and male dispersal is expected. It is often suggested that male cetaceans demonstrate less site fidelity to natal breeding grounds than do females, which is expected to result in males contributing substantially to the maintenance of gene flow within the species and consequently they are expected to exhibit less differentiation between them, resulting in reduced male driven population genetic structure compared with females (Palumbi and Baker, 1994; Baker et al., 1998; Lyrholm et al., 1999; Rosel et al., 1999; Escorza-Trevino and Dizon, 2000). Furthermore, although many cetacean species are characterised by male dispersal, strategies are highly variable within the cetacea between species and even within species. Killer whale males generally mediate gene flow but some remain with their natal pod throughout their life, making temporary associations with other groups for mating whilst others permanently disperse to distant localities (Hoelzel and Dover, 1991; Baird and Dill, 1996; Hoelzel, 1998; Baird and Whitehead, 2000; Hoelzel et al., 2002; Hoelzel et al., 2007). By contrast, male sperm whales tend to live in 'bachelor' pods at a different latitude to the females, migrating to mate (Lyrholm et al., 1999; Pinela et al., 2009), and bottlenose dolphins live in fission-fusion societies (Connor et al., 2000; Natoli et al., 2005), changing their associations throughout their lives. Despite an abundance of studies comparing the relative contribution to population structure of maternal and bi-parental markers, there are relatively few studies that have been able to directly quantify the differential contribution of the sexes to the overall distribution of the species. The study of population genetics of cetaceans would therefore profit considerably from the development of male-specific markers from the non recombining Y-chromosome, which would further our understanding of the sex-specific contributions to the overall population genetic structure, in direct comparison to mtDNA.

### 5.1.3 Sperm Whales

Sperm whales are characterized by matrifocal societies with long-range movements (Whitehead and Weilgart, 2000) and a cosmopolitan distribution (Rice et al., 1989). Both male and female sperm whales live within mixed sex groups of varying stability until they are sexually mature at about 9 to 14 years, after which the females remain within similar groups, while males typically separate from the females to join 'bachelor' groups (Best, 1979; Arnbom and Whitehead, 1989; Lyrholm and Gyllensten, 1998; Lyrholm et al., 1999), usually of similar aged individuals, or become solitary; either way, ranging over large distances (Whitehead, 1993; Whitehead and Weilgart, 2000). Whilst the females live a nomadic existence centering around the tropical and temperate waters, where the sea surface temperature is generally above 15°C, the males generally reside most of the year near the ice edge in both polar waters and 'migrate' to the temperate and tropical waters to interact with the females (Rice et al., 1989; Whitehead, 2003). It has been well established, largely based on mitochondrial markers, that female sperm whales exhibit philopatry in the lower latitudes (see Engelhaupt et al., 2009 for review); however, evidence from microsatellite markers is inconsistent with philopatry for the species as a whole. Bi-parental microsatellite markers suggest male-specific dispersal may maintain continuity between distant philopatric female populations (Engelhaupt et al., 2009). There is generally very little data on the specific movement patterns of male sperm whales (Whitehead and Weilgart, 2000). Lyrholm et al. (1999) found that there was no significant difference in nuclear microsatellite markers between northern and southern groups of sperm whales, suggesting that at least some gene flow is maintained between the two hemispheres and that females do not show a preference for mating exclusively with particular males from specific 'populations'. There has therefore been consistent evidence for limited movement of females and bi-parental homogeneity mediated by widespread male movement. It is not known however, whether the males form a continuous breeding population readily moving between hemispheres and migrating to tropical and temperate waters for mating.

The higher levels of diversity at nuclear compared to mtDNA markers suggests that males are responsible for distributing genetic variation among populations (Lyrholm et al., 1999). The movement of males has important implications for the maintenance of gene flow in sperm whales and will determine how the population structure and mating strategies will affect the overall levels of diversity of the species, therefore the ability to specifically identify and trace patterns of male movement will provide valuable insight into the population dynamics and ecology of the species. Using male-specific markers it could be possible to establish whether males from different regions have restricted patterns of movement and mating behaviours resulting in a certain amount of assortative mating and may give insights into the behavioural patterns of males, in terms of their long-term dispersal patterns and any mating preferences for particular populations. There is great potential for male-mediated gene flow in large, highly mobile marine species such as the sperm whale to limit differentiation among populations. Using bi-parental and mitochondrial markers it appears that male sperm whales disperse more than females (Engelhaupt et al., 2009), therefore Y-chromosome markers will enable investigations that address the degree of male-mediated gene flow.

### 5.1.4 Introduction to methods

The methods explained in the chapters thus far, detail the methodological approaches adopted and optimized to overcome the technical difficulties associated with development of Y-chromosome markers, as reviewed in Petit et al. (2002), including the need for live cell cultures, high quality and abundant starting materials and challenging techniques. This study used primary cell culture followed by microdissection to develop Y-specific libraries for subsequent screening for male-specific markers (see Chapter 2 and 3).

Recent advances in cytogenetic technologies and comparative genomics have led to the development of techniques that can facilitate the discovery of Y-specific characters. Two main groups of strategy have been adopted in the endeavour to identify Y-specific polymorphisms. One strategy uses database mining and cross amplification of either Y-chromosome sequences

developed in a closely related species (e.g. Baboons-Human; Handley et al., 2006), or markers designed from Y-chromosome regions conserved across distantly related species (Y-CATS) (Hellborg and Ellegren, 2003; Erler et al., 2004). An alternative strategy involves the direct isolation of SNPs and/or Microsatellites from partial genomic libraries constructed from Y-chromosomes isolated directly from the focal species using BACs identified as originating from the Y-chromosome (Wallner et al., 2004); microdissection (Shibata et al., 1999); flow sorting (Bergstrom et al., 1998), or subtractive hybridization (Bergstrom et al., 1997). Incorporating these strategies together into a study, maximizes the potential for identification of both conserved regions, which permit investigations that can highlight patterns of inter-specific phylogenetics and the evolution of the Y-chromosome, as well as polymorphic regions that can further the understanding of intra-specific population level dynamics of the focal species. Specific application of these methods for developing sex-specific markers in non-model organisms, have been described in the literature (reviewed in Petit et al., 2002).

**Hypotheses:**

1. There should be reduced Y-specific MS variation comparative to mtDNA and aMS
2. The Y-specific variation of the sperm whale should indicate homogeneity between putative mitochondrial-defined populations.

### 5.2.0 Methods

This study used the following methods in an attempt to maximize the isolation and development of markers for Y-chromosome population genetics in three odontocete species.

Method A – heterologous amplification from published sequences

1) Primers were designed from published Y-chromosome sequences from fin whale (Hatch, 2004), they were then amplified in sperm whales and then species-specific primers were re-designed from the sperm whale sequences and then tested in the population fro their efficacy, male-specificity and polymorphism in the focal species.

Method B – direct amplification of markers from focal species

1) Plasmid libraries enriched for 'anonymous' Y-chromosome sequences, were constructed from DOP-PCR DNA amplified from the microdissected Y-chromosomes from killer whale and bottlenose dolphin, without further enrichment (as detailed in Chapter 4).
2) Microsatellite-enriched libraries were created from the Y-chromosome specific DOP-PCR DNA, from killer whale and bottlenose dolphin enabling direct isolation of Y-specific microsatellite markers from the focal species (as described in Chapter 4).
3) Male-specific markers from killer whale and bottlenose dolphin libraries were tested for cross-specific amplification in the sperm whale.

The Y-specific libraries were created as described in Chapters 3 and 4. Briefly, individual microdissected Y-chromosomes were PCR amplified using a whole genome amplification method (WGA): degenerate oligonucleotide primer – polymerase chain reaction (DOP-PCR). The DOP-PCR product was used as the starting material for subsequent microsatellite enrichment and cloning.

10µl of DOP-PCR product was digested using *Sau 3AI* enzyme (Promega) and incubated at 37℃ for 4 hours in the following reaction: 16.3 µl sterile, deionized water; 2µl RE 10X Buffer; 0.2µl acetylated BSA, 10µg/µl; 1µl DNA, 1µg/µl; *Sau 3AI* restriction enzyme10µg/µl for a final volume of 20µl**.**

The entire digestion was loaded on to an agarose gel in 1xTBE buffer along with a 1kb ladder. Fractions between 400-1500bp were excised from the gel over a UV box using a razor blade. The DNA was purified from the agarose following the instructions of the 'QIAquick gel extraction kit', and resuspended in a final volume of 50µl $dH_2O$.

Various methods were used to optimize the amplification, enrichment and cloning processes. Initial libraries were digested with *Sau 3AI* to facilitate the addition of linkers for pre- and post-enrichment amplification. However, the additional fractionation of the product reduced the fragment size substantially and therefore later libraries were created without digestion and with the addition of a modified linker-$B^1$ that attached to the DOP sequence end directly (6MW: 5'-CCG ACT CGA $G(N)_{24}$AT GTG primer sequence). All libraries were used to obtain the fragments for sequence analysis and microsatellite screening; however, the latter methodology greatly improved the fragment length.

Using either 10µl of gel purified *Sau 3AI* digested product or 10µl gel purified DOP-PCR product, linkers were added to both ends of the fragments. Firstly, two separate linkers A and B ($B^1$) (LinkerA: 5'GCGGTACCCGGGAAGCTTGG3'; LinkerB: P-5'GATCCCAAGCTTCCCGGGTACCGC3'; Linker$B^2$: 5'CACATAAGCTTCCCGGGTACCGC3') were ligated together as follows: 50µM of each linker were combined together in an equal ratios (eg. 5µl each) to make a final concentration of 25µM each and annealed together at 68°C for 5min. The combined linker A-B was then ligated to the fractionated DOP-PCR DNA in the following reaction: 7.0µl sterile, deionized water; 1.5µl T4 DNA ligase buffer; 0.5µl annealed linkers; 10.0µl gel extracted DNA, 1.0µl T4 DNA Ligase enzyme for a final volume of 20µl.

Following ligation of the linkers the DNA product was PCR amplified prior to enriching the library for microsatellites. Using 1µl of linker-ligated purified product and linker-A as the primer, a PCR reaction with an annealing temperature of 68.6°C was used to amplify the fragments to create a chromosome-specific PCR-amplified library, with the following reaction conditions: 96°C for 2 min; 30 cycles of 94°C for 40 secs, 68.6°C for 40 secs and 72°C for 1 min; then elongation

at 72℃ for 10 min. The PCR product was purified following the Qiagen PCR purification kit protocol with a final resuspension in 50µl of $dH_2O$.

The purified PCR product was subsequently used in a hybridization reaction to isolate microsatellite-containing fragments. 1.5µl of probe (B-ATAGAATAT($CA_{16}$, $GCC_{16}$, $GATA_{16}$) or (CA3'dideoxy: 5'Biotin-ATA GAA TAT $(CA)_{16}$ -3'2',3'-dideoxyC) , 5µl of primer-A (5'GCGGTACCCGGGAAGCTTGG3'), 15µl of 20xSSC and 0.5µl 10% w/v SDS was heated to a hybridization temperature (68, 64, 68℃) for CA, GCC and GATA, respectively. Concurrently 100-350ng of the linker ligated PCR-product was denatured for 5-10 min at 95℃. The probe and denatured product were combined immediately and hybridized at 68℃ overnight on a shaker.

Whilst the hybridization was taking place, 100µl of 10mg/ml magnetic beads were prepared by washing four times with buffer-A* (10mM sodium phosphate, pH7, 0.1% w/v SDS, 0.1M NaCl), whilst the suspension was removed the beads were held against the side of the eppendorf using a magnetic stand. The beads were resuspended in a final volume of 50µl buffer-A, as above. Following hybridization and washing of the beads, they were combined and incubated at room temperature for 12-24 hours at room temperature with gentle shaking. This process attaches the biotin to the magnetic beads which will therefore label the biotin-CA hybridized PCR such that, when the magnetic beads are kept at the side of the tube and the liquid pipetted off, the biotinylated probe will also be held, as will the DNA fragments containing the microsatellite-rich sequences thus separating these fragments from the remainder of the DNA.

After incubation the reaction was placed in the magnetic stand and the suspension removed and fresh buffer-A added, six times to remove unbound DNA. The DNA was denatured from the beads by heating in 50µl of 0.1xTE at 95℃ for 5 minutes. Immediately, the suspension was placed in the magnet stand and the supernatant was drawn off to remove the enriched DNA from the beads.

1µl of the suspension was used in a post-hybridization PCR (protocol as for the pre-hybridization PCR, above) to amplify the microsatellite-containing fragments for subsequent isolation of individual fragments using cloning procedures as described in Chapter 4. Following PCR the reactions were run out on an agarose gel to verify the presence of a smear of fragments and then purified through Qiagen purification columns, resuspended in 30-50µl, for a final concentration of 50ng/µl.

Briefly, 1µl of purified microsatellite-enriched PCR product was ligated into a P-Gem T-Easy vector (Promega, A1360) overnight and then transformed into XL-1 Blue bacteria cells and plated out overnight. Individual positive colonies were picked and amplified overnight in LB-ampicillin broth for subsequent miniprep. Positive colonies were identified by blue-white screening followed by PCR screening as follows. Colonies containing an insert (white) were picked with a pipette tip and transferred to fresh plates for overnight re-growth. Each tip was also dipped into a PCR reaction containing vector and microsatellite-specific primers as a pre-screening method to verify the presence of microsatellite inserts following enrichment. Each 10µl reaction contained the following conditions: 1x PCR buffer containing 10 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 50 mM KCl, 2 mM $MgSO_4$, 0.1% w/v Triton X-100, pH 8.8, 250µM of each of the four dNTPs, 0.4U of Taq polymerase, 2pmole each primer (SP6: 5'-CAT TTA GGT GAC ACT ATA G-3'; T7: 5'-TAA TAC GAC TCA CTA TAG GG-3'); microsatellite-specific primers: 5' – TGT GGC GGC CGC ($TG^6$, $CGG^5$, $CTCT^4$)– 3') and $dH_2O$ to a final volume of 10µl. The amplification protocol had a denaturation time of 10 min at 95°C, 30 cycles of 95°C for 30 sec, 54°C for 1 min 30, 72°C for 50sec, followed by an elongation at 72°C for 10 min. Inserts containing a microsatellite produced two distinct bands when run out on an agarose gel and the corresponding re-streak colony was grown-up overnight for miniprep using a Fermentas Miniprep Kit (K0503). Following miniprep, clones were verified by restriction digest with *EcoR1*, as detailed in Chapter 4. Positive clones were sequenced with M13F/R vector primers on an ABI 3730 Capillary DNA Analyzer (Applied Biosystems, Warrington, UK). Primers were designed from microsatellite-containing sequences using 'Oligo 5.0' executable primer design

software (Medprobe). Primers were also designed from sequences obtained from non-enriched libraries that produced 'anonymous' sequences, with which samples were screened for Y-specific SNPs. Each marker was initially tested for amplification and specificity with eight male and four female samples from the focal species, to assess male-specificity of the markers. Following optimization, primer pairs were tested for cross-specific amplification in the killer whale, bottlenose dolphin and sperm whale. The amplification reaction and protocol, detailed above, were adapted following reagent, protocol and primer combination optimization. The primer-specific conditions are detailed in Table 1. Amplified DNA was analyzed for length variation on an ABI 3730 Capillary DNA Analyzer (Applied Biosystems, Warrington, UK), following incorporation of 1/10 fluorescent labelled primer for reactions that amplified successfully. An internal standard marker (Genescan-500 ROX, Applied Biosystems, Warrington, UK) was used to determine allele sizes. Polymorphism was assessed as the number of alleles per locus and subsequently analyzed using GENEPOP 3.1d (Raymond and Rousset, 1995), for haplotypic diversity.

**Table 1** shows the male-specific microsatellite loci with details of the amplification conditions for each locus. TD – Touchdown: the annealing temperature reduced from 60 to 50℃ in 0.5℃ increments per cycle

| Primer | temp | TD | MgCl | forward | reverse |
|---|---|---|---|---|---|
| VEY20 | 50.8 | ✔ | 1.5 | TAATGCAAGCCTATAACA | TGCGCCTATAGACAGTGC |
| VEY21 | 55.3 | | 1.5 | CCTGAAAGTTGGGGTTGT | TATTTGTGCATGCGTGCG |
| VEY22 | 57.7 | ✔ | 2 | AAGTTCCCAGACGTCACA | CTTTCAGGAGGAGCTGTA |
| VEY23 | 51.4 | ✔ | 1.5 | CGTGGACGGGTGTACTCAAT | GCGCATTAAGTCAANTCT |
| VEY24 | 56.8 | | 2 | CTCTAACTCAATGAACCACC | TCCGTACTACCAGACACA |
| VEY25 | 56.8 | | 2 | GAAACCATATGGACCCGT | CGAACACAATAGGCTGTT |
| VEY27 | 52 | | 1.5 | TGCAAGCCTATAACAGCA | GTGTTCGTGTGCTGCTAT |
| VEY28 | 57.4 | | 2 | ATGACTCAATGCAAGCCT | TGAGTCACAGTGAGCCGT |
| VEY29 | 50 | ✔ | 1.5 | TGCTGGTATAGAGAGTGC | GTGACTAAATGCTCCATC |

### 5.2.1 Population screening: DNA sequencing and genotyping

Primers were also designed for anonymous loci and the sequences screened for SNPs. Products from twelve of each of the killer whales and bottlenose dolphin were amplified for the non-enriched (anonymous) loci and those that amplified successfully were sequenced using an ABI 3730 DNA Sequencer to verify locus-specificity and in attempt to assess for variable sites. One microsatellite primer for each locus was fluorescently 5'-labelled with 6-FAM or HEX for detection on an ABI Automated 3730 DNA Analyzer (Applied Biosystems, Warrington, UK).

Between 20 and 85 sperm whales were genotyped at up to eight male-specific microsatellite loci for three previously identified populations from the Mediterranean (41), North Sea (20) and the Gulf of Mexico (27), using primer-specific protocols optimized for individual loci as detailed above.

Male sperm whale samples were taken from archived samples obtained from free-ranging whales by skin slough or biopsy sampling and from stranded whales. Samples are representative of three locations surveyed; the Gulf of Mexico (May-Sept, 2000-2005); the Mediterranean (Jun-Sept, 2000-2005) and the North Sea (May-Sept, 2003-2007) as part of a long term population genetic study (see Englehaupt, 2009, for details).

### 5.3.0 Results

#### 5.3.1 Novel Y-chromosome microsatellites

A total of 133 enriched and 162 non-enriched clones were sequenced from 10 (CA, GCC and GATA) enriched libraries, 8 (dideoxy-CA) enriched libraries and 20 non-enriched libraries. The majority (98%) of the enriched clones that contained a microsatellite contained the motif 'CA'. Although the addition of the 'dideoxy' group increased the efficiency of the probe, the libraries without the 'CA' modification produced clones containing the 'TG' motifs at a lesser efficiency. In contrast the 'GCC' and 'GATA' probes failed to produce clones containing the respective motifs.

In total 73 and 60 clones from the microsatellite-enriched libraries were sequenced for the killer whale and bottlenose dolphin, respectively. 39/73 killer whale and 29/70 bottlenose dolphin (68 total) sequences produced good quality sequences, containing a microsatellite motif with greater than six repeats. Among the 84 excluded sequences, 10 were either a re-sequence of the same locus or may have been part of a clone family on the Y-chromosome, as indicated by small differences in the flanking regions. 19 killer whale and 14 bottlenose dolphin sequences had sufficient suitable flanking sequences from which primers could be designed. In addition, 162 (79 killer whale / 83 BND) clones were sequenced from non-enriched libraries and yielded 11 microsatellite-containing sequences where the motif was greater than six repeats. Of the 11 sequences it was possible to design four primer sets of which two (both originating from killer whale sequences) were successfully amplified in one or more species tested. A total of 35 clones, including two anonymous killer whale microsatellites, were screened for male-specificity and variation. Ten out of the 35 primer sets from unique microsatellite-containing sequences failed to amplify a PCR product of the correct size in any of the three species, despite optimization attempts. Four additional primers failed to amplify consistently and did not produce a repeatable Genescan profile, therefore they were excluded from further efforts. Of the 21

remaining primer sets, four were not male-specific in any of the three species; two showed multi-locus amplification in all three species; and five were monomorphic in all three species. Four primer sets either failed, were not male-specific or produced multi-locus amplification in all three species. Six primers therefore remained and showed polymorphic variation in one or more of the three species and were screened for population variation.

Ten additional microsatellite markers and two additional anonymous primer sets (the latter discussed below) were designed from sperm whale sequences obtained using primers designed from the fin whale Y-chromosome sequences (Method A) (Hatch, 2004). Of the ten microsatellite primer sets, six successfully amplified in all species but three were not male-specific in any of the three species, leaving three that were polymorphic in one or more species. The three fin whale derived primer sets added to the six primer sets derived from original killer whale and bottlenose dolphin sequences resulted in nine primer sets, which were screened for variation across the three cetacean species, with varying success detailed in Table 2**.** In addition, Table 2 demonstrates the relative success of the various methods for obtaining Y-chromosome, male-specific microsatellite markers from the killer whale, bottlenose dolphin and sperm whale. Microsatellite markers obtained from species-specific sequences amplified using markers designed from Y-chromosome microsatellite-containing fin whale sequences showed phylogenetic patterns of cross-homology and amplification success. The sperm whale, which is taxonomically closer to the fin whale than are the other two species, showed more X-chromosome homology at more loci than did either the killer whale or bottlenose dolphin.

**Table 2:** 25 y-chromosome microsatellite markers designed from killer whale and bottlenose dolphin microdissected y-chromosomes and six microsatellite loci re-designed from sperm whale sequences amplified with markers designed from Fin whale sequences obtained from NCBI.
*Failed – Samples that failed across all species failed to reliably amplify a microsatellite profile following genotyping. Samples that failed exclusively in one species, generally failed to amplify a unique product at the PCR titration stage. All primers were initially tested with their species of origin, or with the sperm whale (in the case of the Fin whale derived sequence primers) which were re-designed from sperm whale sequences. Bold – BND derived, Plain text – Killer whale derived, Highlighted – anonymous sequence derived (i.e. not from enriched sequences. Multi = multicopy loci, mono = monomorphic, sgl = a single band was produced but the locus was not screened for variation in that species. No MS = there was not a microsatellite at the locus for the species. MS-Lib= primers designed from enriched libraries, anon Lib= enriched from non-enriched libraries.

| Primer Name | Size (bp) | Sperm Whale | Killler Whale | Bottlenose Dolphin | MS MOTIF | Origin Species | Method of Isolation |
|---|---|---|---|---|---|---|---|
| VEY1 | 380 | failed | failed | Failed | $(CA)_6$ | KW | MS-Lib |
| VEY2 | 181 | failed | failed | Failed | $(CA)_6$ | KW | MS-Lib |
| VEY3 | 386 | failed | failed | Failed | $(TC)_6$ | KW | MS-Lib |
| VEY4 | 272 | failed | mono | Sgl | $(CCTTTT)_7$ | KW | MS-Lib |
| VEY5 | 328 | failed | failed | Sgl | $(CA)_6$ | KW | MS-Lib |
| VEY6 | 224 | failed | mono | Sgl | $(CA)_6$ | KW | MS-Lib |
| **VEY7** | **171** | **mono** | **mono** | **mono** | **$(CA)_{7,15}$** | **BND** | MS-Lib |
| VEY8 | 271 | multi | mono | failed | $(TG)_{8,6}$ | KW | anon Lib |
| VEY9 | 200 | mono | failed | Sgl | $(GA)_5$ | KW | anon Lib |
| **VEY10** | **230** | **multi** | **multi** | **multi** | **$(TG)_{8,8,6,11}$** | **BND** | MS-Lib |
| **VEY11** | **122** | **Not Male Specific** | | | **$(CA)_8$** | **BND** | MS-Lib |
| **VEY12** | **250** | **mono** | **mono** | **mono** | **$(GT)_5(GA)_5(TG)_9$** | **BND** | MS-Lib |
| **VEY13** | **481** | **mono** | **mono** | **mono** | **$(TG)_{6,8}$** | **BND** | MS-Lib |
| **VEY14** | **250** | **mono** | **mono** | **mono** | **$(TG)_8$** | **BND** | MS-Lib |
| VEY15 | 207 | multi | multi | multi | $(GA)_{8,5}(TG)_6$ | KW | MS-Lib |
| VEY16 | 122 | Not Male Specific | | | $(CA)_{23}$ | KW | MS-Lib |
| VEY17 | 272 | Not Male Specific | | | $(CA)_{12}(CA)_6$ | KW | MS-Lib |
| VEY18 | 204 | Not Male Specific | | | $(CA)_9$ | KW | MS-Lib |
| VEY19 | 222 | mono | mono | mono | $(CA)_{13}(CA)_{10,5}$ | KW | MS-Lib |
| VEY20 | 348 | 2 all | mono | mono | $(TG)_{14}$ | KW | MS-Lib |
| VEY21 | 157 | 2all | mono | no ms | $(GA)_{16}$ | KW | MS-Lib |
| VEY22 | 285 | 3all | mono | mono | $(TG)_9$ | BND | MS-Lib |
| VEY23 | 189 | 4 all | 2all | mono | $(CA)^8$ | BND | MS-Lib |
| VEY24 | 280 | 3 all | failed | mono | $(TG)^9$ | BND | MS-Lib |
| VEY25 | 171 | 3 all | mono | failed | $(CA)_{5,7,12}$ | BND | MS-Lib |
| VEY26 | 600 | Not Male Specific | | | no MS | Fin | redesign |
| VEY27 | 287 | 4 all | 2all | mono | $(CA)_6$ | Fin | redesign |
| VEY28 | 189 | 3 all | mono | mono | $(CA)^)_8$ | Fin | redesign |
| VEY29 | 295 | 3 all | mono | mono | $(CA)_{9,4}$ | Fin | redesign |
| VEY30 | 388 | Not male specific | | | ca int rpt | Fin | redesign |
| VEY31 | 200 | Not Male Specific | | | ca int rpt | Fin | redesign |

**Table 3:** anonymous loci screened for SNPs in 12 orcas and 12 bottlenose dolphins. Markers highlighted in grey were derived from bottlenose dolphin libraries. NT= not tested, multi= multiple loci amplified, as determined by multiple bands on an agarose gel, 2 loci = two separate loci were amplifed and could not be isolated by gel extraction, sgl = a single product was produced and sequenced. Mono = one sequence represented all of the 12 individuals sequenced from each species .

| Primer | temp | forward | reverse | size | KW | BND | MOTIF | origin |
|---|---|---|---|---|---|---|---|---|
| L4F1N13 | 50 | ATA AAG AGG AAA CAG AGC | tCT CCA GtG AtG CTT TGT | 174 | failed | 2 loci | anon | bnd |
| L4F1N15 | 49 | GAG AcA AAT TAA ACA CCA | ACT CGT GTT ATG ACC TGT | 235 | mono | sgl | anon | bnd |
| L4F1N18 | 54 | ACT CGT GTT ATG ACC TGT | CcC GAG cAC AGT TGA TTA | 190 | multi | 2 loci | anon | bnd |
| L4F2N12 | 54 | GCA CGA TTT GAG TAG GGA | CGA AAA GCG TCA AAG CCA | 296 | mono | mono | anon | bnd |
| L4F2N2 | 52 | cgG GAA TAC TAC ACG ACA | GAT TTC GCT CCC ACT TAT | 152 | multi | mono | anon | bnd |
| L4I1N1 | 46 | TtT TCG ATT TAT CCT CAA | TCT GTT GAt tAC TAT GTC | 102 | mono | failed | anon | kw |
| L4I1N2 | 56 | TCT ATT ACG AAG CCA CGC | CGA CTG GCA ACG GGA TAC | 184 | multi | failed | anon | kw |
| L4I1N16 | 58 | GTATTGACCTAACACTCT | GTTTGCCCACTTGAGATG | NT | mono | mono | anon | kw |
| L4I2N14 | 46 | ATA TTG ATT TTT ATG GCG | CGA AAA TCC AGA ATA CAT | 298 | mono | failed | anon | kw |
| L5I34 | 42 | GGA TAT CGG TTT ATT AAA | CAC CAC CAC CAA CCT ACC | 108 | mono | 2 loci | anon | kw |
| L5I37 | 55 | GCT GCG AGA GTT GCT TGA | AGC ATC TTC AAC ACC ACC | 263 | multi | NT | anon | kw |
| L5I43 | 50 | TGG GGC TGT TTT GCT CTT | GCC AAA GAA AAA AAG AAA | 111 | multi | NT | anon | kw |
| L5I51 | 49 | GCT CGG GCT ATT ATT GTA | TAT CGT GGG CTT CGT TAT | 126 | mono | Multi | anon | kw |
| L5I66 | 43 | TGA AAT TTA ATG AGC TGT | ACA GGC GAG GAA TAC AGG | 123 | multi | NT | anon | kw |
| L5I80 | 50 | TCT AGG ATG CTT CTA TTA | AGC ACT GTA TTG TAG CGG | 141 | failed | sgl | anon | kw |
| L5I83 | 47 | AgA AAG ACA GTg TAA AGT | TGG GTT ACA TTG ATT TTC | 183 | sgl | sgl | anon | kw |
| L5I85 | 56 | GTG GAA GTG GGC GAA GAG | AAA GCG ACG AAG TTG GGT | 404 | sgl | sgl | anon | kw |
| L5I89 | 57 | AGA GAA AGG AGC AGG GGA | cCC AGT AGG CTT GAG ACC | 317 | sgl | sgl | anon | kw |

### 5.3.2 Anonymous sequence data

20 primers were designed from anonymous sequences, of which two contained microsatellites (as detailed in Table 2). The markers that amplified anonymous sequence (as detailed in Table 3) were applied to the killer whale and bottlenose dolphin to assess for variation at non-microsatellite loci. Up to 6 male and 3 female samples for each of the killer whale and bottlenose dolphin were assessed for single nucleotide polymorphisms (SNPs) by sequencing. Two loci failed to amplify at the PCR stage, two failed to produce a readable sequence from the original species of the marker and a further six produced multi-locus amplification. Preliminary alignments indicated that the markers that produced a single band showed neither intra- nor inter- specific variation at these loci. Only sequences that

successfully amplified from the species of origin for the clone were further assessed for homologous amplification.

### 5.3.3 Amplification patterns of Y Microsatellite markers in three Odontocetes

A total of 31 microsatellite primer sets were designed from the various methods detailed above and were subsequently tested for amplification success, male-specificity and polymorphism from three focal species (killer whale, bottlenose dolphin and sperm whale), using 12 males and 6 females. The loci found to produce male-specific single copy products were subsequently used to amplify products from at least 24 individuals for each species. Five of the loci were found to be monomorphic across all species but there was no variation found at any locus of the loci in the bottlenose dolphin. There were nine and two polymorphic loci for the sperm whale and killer whale, respectively. There were two to four alleles at polymorphic loci in the sperm whale, and two alleles for the killer whale, (see table 4). All polymorphic loci had a $(TG/CA)_n$ simple repeat motif.

Generally, dinucleotide microsatellites were more common and had higher repeat numbers than trinucleotide or tetranucleotide microsatellites, and with one exception, it was only possible to design primers for dinucleotide microsatellite loci as, by chance the sequences containing the tri- and tetra-nucleotide repeats did not present a suitable priming site. Furthermore, coincidentally no suitable priming sites were identified from sequences with the highest repeat numbers.

| Polymorphic Microsatellites | | | | | |
|---|---|---|---|---|---|
| **Primer** | **Species** | **Size range** | **# of alleles** | **# of Individuals** | **Microsatellite motifs** |
| **VEY20** | **Pm** | 226, 228? | 2? | 28 | $(TG)_{14}$ |
| | **KW** | 348 | mono | 24 | $(TG)_{14}$ |
| | **BND** | 348 | mono | 24 | Unknown |
| **VEY21** | **Pm** | 157, 163? | 2? | 20 | $(GA)_{16}$ |
| | **KW** | 157 | mono | 18 | $(GA)_{16}$ |
| | **BND** | x | x | x | not MS |
| **VEY22** | **Pm** | 283,285,287, 289 | 3 | 76 | $(TG)_{5-9}$ |
| | **KW** | 426 | mono | 24 | $(TG)_{9}$ |
| | **BND** | 285 | mono | 24 | $(TG)_{17}$ |
| **VEY23** | **Pm** | 155, 157, 159, 161 | 4 | 82 | $(CA)_{6-10}$ |
| | **KW** | 187,189 | 2 | 24 | $(CA)_{6-10}$ |
| | **BND** | 189 | mono | 24 | $(CA)_{6}$ |
| **VEY24** | **Pm** | 280, 284, 286 | 3 | 81 | $(TG)_{9}$ |
| | **KW** | x | x | x | x |
| | **BND** | 280 | mono | 24 | $(TG)_{9}$ |
| **VEY25** | **Pm** | 452, 456,458 | 3 | 78 | $(CA)_{5, 7, 12,10}$ |
| | **KW** | x | x | x | X |
| | **BND** | 171 | mono | 24 | $(CA)_{5,7,12}$ |
| **VEY27** | **Pm** | 285, 287, 289, 291 | 4 | 85 | $(CA)_{17}$ |
| | **KW** | 218 | mono | 24 | $(CA)_{7}$ |
| | **BND** | 220 | mono | 12 | $(CA)_{5}$ |
| **VEY28** | **Pm** | 187,189,191 | 3 | 81 | $(CA)_{8-10}$ |
| | **KW** | x | x | x | no MS |
| | **BND** | x | x | x | no MS |
| **VEY29** | **Pm** | 293, 295, 297 | 3 | 61 | $(CA)_{13-15,4}$ |
| | **KW** | 418 | mono | 2 | $(TG)_{12}$ |
| | **BND** | 420 | mono | 3 | $(TG)_{13}$ |

**Table 4:** Shows the polymorphic microsatellite loci and the details of the number of loci, individuals, size range and motif for the three species. Loci highlighted in grey were designed from sperm whale cross-specific amplification of markers derived from fin whale sequences.
Mono = monomorphic, ? = failed to amplify consistently, x= not yet tested with the species

Although clones from non-enriched libraries, containing 'anonymous' sequences did contain di-, tri- and tetra-nucleotide repeat motifs, the number of clones containing Microsatellites from enriched libraries was much higher, as expected. Markers identified from non-selected sequences, however did not differ in their motif or repeat number to those from the enriched library (i.e. the same motifs, similar numbers of repeats and similar characteristics of flanking sequence were found in the anonymous sequences that contained microsatellites, as was found through the enrichment process).

The pre-screening of clones using the microsatellite-specific primers in combination with the vector primers (SP6/T7) and resultant identification of two bands on the agarose gel, corresponding to the insert and the microsatellite motif, proved to be a successful method for maximizing the number of sequenced clones that contained a microsatellite motif. Furthermore, the use of microdissected material isolated directly from the Y-chromosome appears to have maximized the male-specificity of the material obtained for screening, since there was a high incidence of male-specific loci. Despite the application of probes with several different microsatellite motifs, the only motif that resulted in enriched sequence was the 'CA/TG' motif, suggesting a higher presence of this motif on the odontocete Y-chromosome. (See Chapter 4, for discussion).

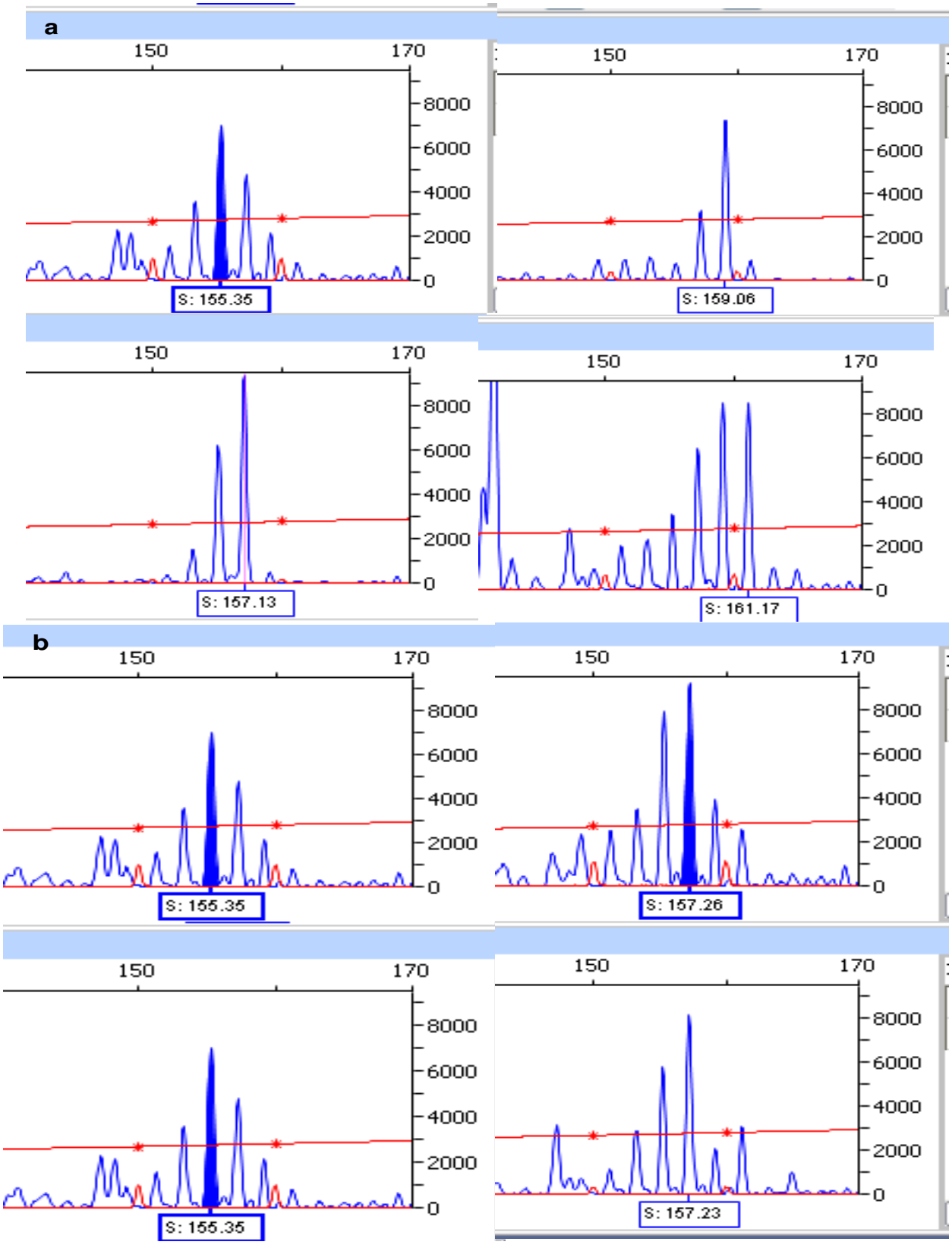
Surprisingly, primers designed from killer whale and bottlenose dolphin sequences were generally more successful and more variable when used for screening in the sperm whale, indicating retention of these loci over time, and that they were either never variable in the source species, or that variation in those species has been lost.

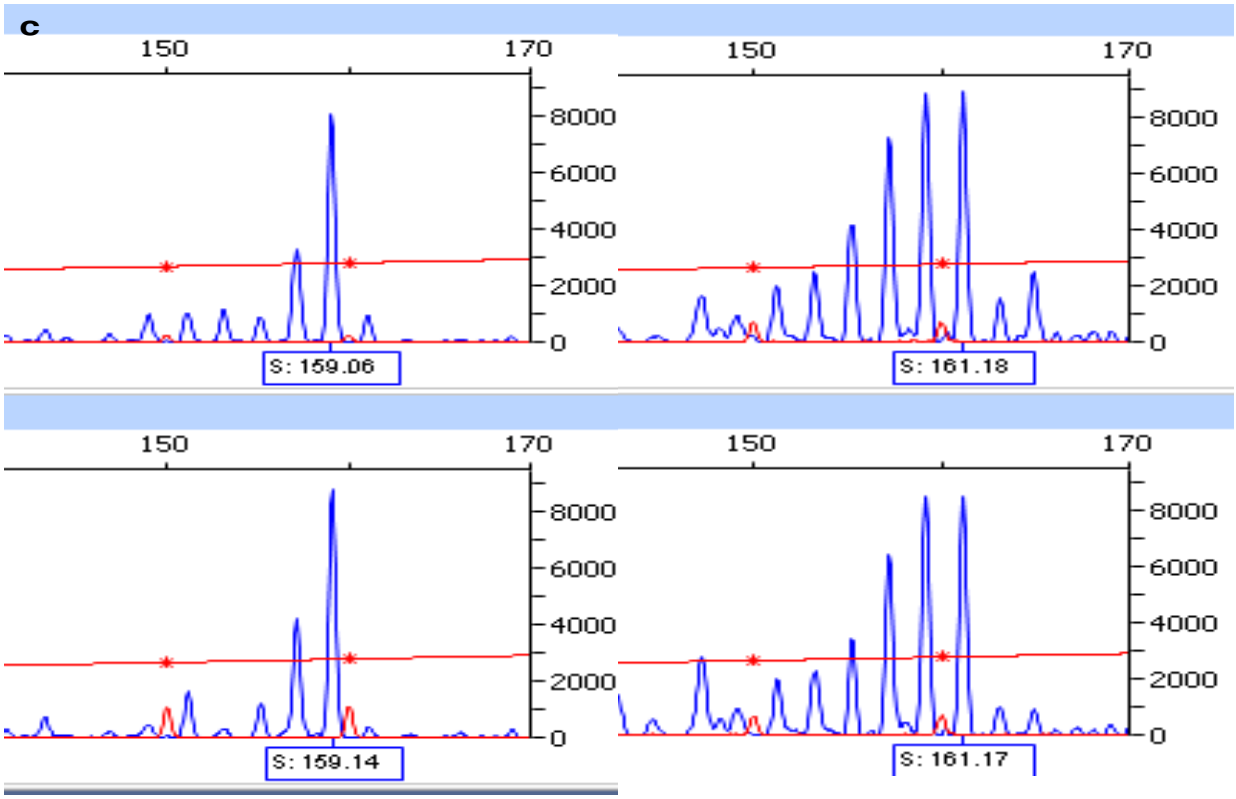
### 5.3.4 Population Screening with Microsatellite primers

Eight male-specific markers consistently amplified a PCR product from sperm whale samples. One however, failed to consistently produce a repeatable profile following genescan analysis and was therefore excluded from further analysis. Polymorphic loci ranged from two to four alleles, with the unequal distribution of alleles, one predominating across all populations

(Table 5). Stutter patterns were consistent with those seen in other studies using Y-chromosome dinucleotide loci (e.g. Butler and Schoske, 2004; Butler et al., 2005; Cryer et al., 2005; Luo et al., 2006; Thompson and Salipante, 2009). Where the stutter pattern of an allele could be construed as equivocal, samples were repeated three or more times and verified for a consistent profile between amplifications and individuals (Fig. 1). All samples that produced rare alleles were repeated to check the voracity of the allele. Figure 1 shows an example of the electropherogram amplification profile of a marker (VEY23) demonstrating how the amplification consistency of alleles was checked across replicates and individuals (See appendix 1 for profiles of other selected loci. Two loci produced a pre- and post-peak stutter pattern, which was superficially reminiscent of multi-locus alleles, however sequencing of the markers verified the single locus origin of the allele and consistent repeatable amplification of an identical profile was confirmed. It is not clear why it was nto possible to obtain clearer profiles. .

**Figure 1:** Y-chromosome microsatellite locus VEY23 has four alleles. The locus size is indicated below the main peak. Alleles 157 and 159 demonstrate a typical dinucleotide microsatellite stutter profile whereas allele 155 shows post-peak stutter and allele 161 shows an amplified pre-peak stutter band. a) shows the four alleles for the locus (155, 157, 159, 161); b) shows the consistency between individuals for the peak profile for alleles 155 and 157; and c) shows the consistency between repeats of the same individual between replicates for the peak profile, for alleles 159 and 161 (as an example).






The pre-peak amplification evident for allele 161 for VEY23, is consistent with other Y-chromosome microsatellite studies (Butler et al., 2005; Luo et al., 2007), as is the post-allelic stutter from complex microsatellite loci (Cryer et al., 2005; Thompson and Salipante, 2009). Despite the variability between the allelic patterns, the stutter pattern for each allele was very consistent and repeatable across individuals and repeats.

Between 20 and 85 sperm whales were genotyped (Table 5) at up to eight male-specific microsatellite loci for three previously identified populations from the Mediterranean (41), North Sea (20) and the Gulf of Mexico (27). Some samples failed to amplify across all loci and one locus (VEY20) was excluded from population genetic analysis due to failure to amplify consistently across samples. Sample identifications and population identifications are detailed in Engelhaupt (2004). Similar numbers of alleles were found across the three locations.

**Table 5:** frequency of each allele across sperm whales.

| Frequencies | VEY21 | VEY22 | VEY23 | VEY24 | VEY25 | VEY27 | VEY28 | VEY29 |
|---|---|---|---|---|---|---|---|---|
| Allele 1 | 11 | 14 | 5 | 74 | 2 | 14 | 6 | 10 |
| Allele 2 | 9 | 40 | 37 | 3 | 61 | 34 | 62 | 46 |
| Allele 3 | 0 | 19 | 31 | 4 | 15 | 33 | 13 | 5 |
| Allele 4 | 0 | 3 | 9 | 0 | 0 | 4 | 0 | 0 |
| Total | 20 | 76 | 82 | 81 | 78 | 85 | 81 | 61 |

There were no markers that were found to be private to any one population, although certain alleles were more common in some populations than others; all alleles for all loci were found in all populations. Two loci showed two common alleles with two rarer alleles; the remainder had one common allele with additional rarer alleles (Table 5).

The results of the Fisher's exact test and combined Pairwise $F_{ST}$ test performed with all loci combined into one haplotype, are shown in Table 6.

**Table 6:** Fst values and haplotypes from seven polymorphic y-chromosome microsatellite loci applied to the sperm whale. Lower diagonal: combined Y-haplotype Fst (above) and exact test p-values (below). Upper diagonal: Fst based on 16 autosomal microsatellite loci (above), and Fst based on mtDNA control region sequences (below) – from Engelhaupt et al. 2009. * = p<0.001.

| | GOM | NSea | Med |
|---|---|---|---|
| GOM | - | 0.000<br>0.425* | 0.037*<br>0.626* |
| NSea | 0.0194<br>(0.076) | - | 0.034*<br>0.560* |
| Med | 0.0241<br>(0.0003) | 0.0169<br>(0.0314) | - |

Assessment of population differentiation showed no significant difference between the population from the Gulf of Mexico (GoM) and the North Sea (NSea) (p=0.076), weak significant differentiation between the North Sea and Mediterranean (Med) (p=0.03) and a significant difference between the Gulf of Mexico and the Mediterranean (p=0.0003). It is of note that the $F_{ST}$ values are of similar magnitude to those previously reported for autosomal microsatellites (Fig. 6) consistent with expectations that males are more mobile than the females and maintain gene flow between the quite distinctive female populations (as indicated by high mtDNA $F_{ST}$

values; see Engelhaupt et al. 2009). Despite evidence for male movement, significant differentiation remains between the Gulf of Mexico and Mediterranean, which suggests that although males maintain continuity between the three populations, the Mediterranean is isolated from the other populations to a certain extent.

## 5.4.0 Discussion

### 5.4.1 Isolation of Y-specific markers

By using a combined approach to isolate and identify Y-specific microsatellites from killer whale, bottlenose dolphin and sperm whale, it has been possible to develop 18 male-specific microsatellite markers, of which 16 were single copy and 9 were polymorphic in the sperm whale. All 18 were also male-specific in the killer whale and bottlenose dolphin but all but two in the killer whale were monomorphic and all were monomorphic in the bottlenose dolphin.

Possible reasons for these species-specific differences are discussed below. Although not an exhaustive search, the low number of high copy number, polymorphic microsatellites isolated from these species is consistent with recent studies in other non-human mammals (Wallner et al., 2004; Handley et al., 2006; Handley et al., 2006a; Handley and Perrin, 2006; MacDonald et al., 2006; Luo et al., 2007). Indeed, prior to the genome sequencing effort there were also very few Y-chromosome microsatellite markers for human population studies and several of them were multi-locus markers (Butler et al., 2005).

### 5.4.2 Fewer Y-chromosome microsatellites

As discussed in Chapter 4, the number of microsatellites on the Y-chromosome is intuitively a function of its size. However, the level of polymorphism may also be related to Y-chromosome-specific characters, resulting in the degeneration of microsatellite loci and reduced slippage due to interrupting point mutations. Furthermore, the size of the Y-chromosome is not the only factor determining the number and character of microsatellites that are available for isolation. Most studies that aimed to isolate Y-chromosome microsatellites describe multi-copy loci and large quantities of interrupted-repeat loci. Microsatellites are interrupted stretches of tandemly-repetitive sequence both during the inception and disintegration phases of their life-cycle (Balaresque, 2007) and therefore, interrupted microsatellites would represent double the number of ‘simple’, uninterrupted microsatellite regions. Therefore (as detailed in Chapter 4), on a small chromosome that represents less than 1% of the genome as a whole, characterized by

high levels of repetitive DNA, the number of good quality microsatellites will be inherently lower. The overall lower representation of long-perfect microsatellites and consequent lack of slippage variation (Almeida and Penha-Goncalves, 2004), may simply be a function of the size of the Y-chromosome without needing to propose additional interpretation. Bachtrog and Charlesworth (2000), showed that microsatellite loci from the *Drosophila miranda* neo-y have significantly lower levels of variation than their X-chromosome counterparts from which they evolved, and proposed the higher levels of Y-chromosome degeneration as a causative element in this process. Many other Y-chromosome studies have also shown reduced levels of Y-chromosome microsatellite variation (Wallner et al., 2004; Handley et al., 2006), suggesting that it is possibly a Y-specific trend in microsatellite variability.

### 5.4.3 Lower levels of polymorphism on the Y

These results support early evidence from humans that levels of polymorphism are lower on the Y-chromosome than in the rest of the genome, as a result of reduced effective population (Ne); as discussed by the International SNP Map Working Group (2001) and Shen et al. (2000); however, additional support is needed to assess the extent and strength of this evidence across mammalian Y-chromosomes. Amplification of loci outside of conserved gene regions (such as SRY and ZFX/Y) may provide a better understanding of the evolutionary forces acting on the mammalian Y-chromosomes, and help identify species-specific ecology that impacts the evolution of the Y-chromosome and interactions between characters of the Y-chromosome and the male-specific history of species.

The amount of polymorphism at microsatellite markers on the Y-chromosome is a function of the purifying effect of a lack of recombination, the ecology of a species and the slippage patterns. The slippage pattern is directly affected by the birth pattern of microsatellites, and the length and motif of the microsatellite (Almeida and Penha-Goncalves, 2004). Therefore, if the number of long microsatellites relative to the number of short microsatellites throughout the genome is one in ten, the number will be proportionately fewer on the Y-chromosome because

of its reduced amount of sequence as a result of its size, independent of any other demographic processes specific to the Y-chromosome. In addition however, because there is a greater likelihood of interruption on the Y-chromosome due to increased mutation in the male germline (Bartosch-Harlid et al., 2003; Ellegren, 2009), which in turn increases the rate of microsatellite degeneration by the accumulation of point mutations (Kruglyak et al., 1998), the number of 'pure' microsatellites will potentially be further reduced. The ecology of the species will often act to further reduce the y-microsatellite variation. Demographic processes affecting the species as a whole will be amplified on the Y-chromosome (Kayser et al., 2000). For example, if the species has sex-specific patterns of dispersal and/or male reproductive skew, the already reduced male Ne of the Y-chromosome results in the amplification of the effects of these demographic processes resulting in a further loss of variation (Kayser et al., 2003). At least two of the odontocetes studied herein (sperm whales and killer whales) have potentially been affected by bottlenecks, have generally reduced Ne and exhibit some levels of skewed reproductive success which may reduce the Y-specific variation expected (Hoelzel et al., 2002; Hoelzel et al., 2007; Engelhaupt et al., 2009). Both a reduced survival to breeding and a reduced breeding population of males due to biased male mating success will substantially reduce the amount of variation found on the Y-chromosome.

### 5.4.4 Demography, ecology and evolutionary history

In the killer whale mtDNA haplotypes have been found to be unique and fixed at a regional level, which suggests very little movement of females and restricted breeding patterns, however a certain amount of male-mediated dispersal was detected throughout the North Pacific (Hoelzel et al., 2007). The reduced genetic variation at mitochondrial and autosomal markers in the killer whale is consistent with expectations of reduced effective population size and there is evidence that the killer whale suffered the effects of a bottleneck sometime during the late Pleistocene (Hoelzel et al., 2002). A study based on paternity and kinship assessment suggested that reproductive skew is low in this species for both sexes (Pilot et al., in press).

Therefore it is possible that demographic history has contributed to low levels of variation on the Y-chromosome in this species, but reproductive behaviour is less likely to have been a significant factor.

Older lineages tend to obtain more variation than do more recently derived species, due to the founder effect of divergence. However, the TMRCA of segments of the genome can be much younger than the species as a whole, especially for the Y-chromosome and mtDNA, because of their effective population sizes. The more time that has passed since the 'founder event' the greater the number of mutations that will have occurred (Irvin et al., 1998; Goldstein and Schlotterer, 1999; Bachtrog and Charlesworth, 2000). The sperm whale, which diverged before the delphinidae, shows polymorphism consistent with an older lineage, though this also gives greater time for the accumulation of disruptive point mutations. It is also true that low mtDNA variation suggests the possibility of a historical bottleneck (Lyrholm et al. 1999) in this species, and that behavioural observations suggest male reproductive skew (e.g. Whitehead and Weilgart, 2000). Although levels of variation found at Y-chromosomes are consistent with the demography in killer whales, demography and ecology cannot explain the exceptional lack of variation found in bottlenose dolphins, suggesting some alternative casual explanation for the pattern observed.

The smaller size of the delphinid Y-chromosome compared to that of the sperm whale may explain the reduced polymorphism of the former. It is possible that the larger sperm whale Y-chromosome is the result of a more recent translocation event (Waters et al., 2001), which will have had less time to degenerate, consistent with the amount of variation observed. Whatever the cause of the larger chromosome, the increased content will in itself provide increased potential for polymorphic loci simply as a result of the greater volume increasing the opportunity. The complete and near-complete lack of variation for the bottlenose dolphin and killer whale are potentially a result of the smaller size of the chromosome but the small difference between them (i.e. two marginally polymorphic loci from the killer whale compared with zero in the bottlenose dolphin) may have simply been identified by chance, as only a small proportion was assessed.

However, the minimal Y-chromosome microsatellite shows inter-and intra-specific microsatellite variability (MacDonald et al., 2006). Nevertheless, a complete lack of variation from Y-chromosome microsatellites has been observed in other species, including the tiger (Luo et al., 2007) and lynx (Hellborg et al., 2002), both of which have suffered extreme bottlenecks. Low levels of variation are characteristic of all species; however, a trend consistent with a history of bottlenecks or founder events, as seen in domestic species (Edwards et al., 2000; Wallner et al., 2004; Meadows et al., 2006; Luo et al., 2007), appears to play a substantial role in the degree of the deficiency in variation across taxa.

Most other studies of Y-chromosome microsatellites have found similarly low Y-chromosome variation indicating that Y-specific patterns of degeneration, reduced Ne and lack of recombination of the mammalian Y-chromosome may be the main causative feature of reduced variability (Bachtrog and Charlesworth, 2000; Wallner et al., 2004; Handley et al., 2006; Luo et al., 2007). Although most species are characterized by low-Y-specific variation, there are some distinct, apparently species- and/or population- specific trends that have been noted (Hellborg and Ellegren, 2003; Kayser et al., 2003). Following the complete sequencing of the human-y, numerous polymorphic and variable markers were discovered, suggesting that the discovery of variability is also a function of the search effort and most recent investigations have not attempted to interpret a lack of variation but rather highlighted the necessity for more markers to provide a greater understanding (Handley et al., 2006; Luo et al., 2007; Kariya et al., 2009). Handley et al. (2006) using a comprehensive modelling approach attempted to isolate the various factors (Ne, demography, etc) on the Y-chromosome and identify their relative contribution to the mutation expectations in shrews. However, the model failed to pinpoint an explanation for the observed patterns, having accounted for the aforementioned Y and species-specific processes. Handley et al. (2006) cited ecological distinction between humans and baboons for the lack of variation found in the Y-chromosome of the latter species, also suggesting selection may be playing a more significant role in the baboon, which is subject to greater pressures than are found in human populations. Results from studies in non-human

mammals remain equivocal and an explanation for the Y-chromosome and its general lack of variation remains elusive.

In humans where reported levels of variation are generally higher than that of other species studied, there is a divergent pattern of diversity that correlates with population-specific demographic processes (Malaspina et al., 1997). These findings in the extensively studied human populations may shed some light on patterns and processes observed in other species, which tend to have less population-specific patterns of demography and ecology and conform more closely to the evolutionary histories of the less divergent human populations (e.g. showing reproductive skew, isolation and population bottlenecks or having a limited effective population size).

### 5.4.5 Population implications of Y-chromosome variation in sperm whales

The results of the population genetic analysis of the Y-chromosome showed variation consistent with a pattern of male-mediated gene flow in the species. Evidence from mitochondrial and microsatellite markers suggest female philopatry among three putative populations, but relatively high male mediated gene flow between the North Sea and Gulf of Mexico compared to restricted movement between either population and the Mediterranean Sea (Engelhaupt et al. 2009). Y-chromosome variation described here was broadly consistent with the autosomal microsatellite DNA pattern, showing some restricted movement with respect to the Mediterranean, and less so between the North Sea and Gulf of Mexico. However, the magnitudes of the $F_{ST}$ values were all low compared to expectations for a haploid marker, and consistent with relatively high levels of male-mediated gene flow, as suggested previously (Engelhaupt et al. 2009). Further, the exact tests, which have comparatively high power, showed a highly significant alpha value only for the comparison between the Gulf of Mexico and the Mediterranean Sea. The implications are that males disperse over a very broad geographic range in this species, in contrast to the philopatric behaviour of females, and that the Mediterranean is isolated to some extent with respect to the dispersal of both sexes.

The evidence of correspondence of the new Y-chromosome markers with results from previous studies validates the utility of Y-chromosome markers for informing an understanding of demographic processes in the sperm whale and although, the number and variability of the markers mean that inferences are limited, there is obvious potential.

### 5.4.6 Conclusions

The aims of this study were to isolate and apply male-specific Y-chromosome microsatellite markers to three odontocete species (sperm whale, killer whale and bottlenose dolphin). Although the success reported herein was limited, particularly in the latter two species, the lack of variability still has the potential to inform an understanding of the evolution of the Y-chromosome. Furthermore, the application of the seven polymorphic microsatellite loci in the sperm whale facilitated the investigation of male-mediated gene flow in this species. Although the extent of interpretation is limited it represents a substantial step towards isolating and studying the male-specific element of population ecology.

# 6.0 General Discussion

## 6.1 Trends in Y-chromosome evolution

The Y-chromosome is characterized by degeneration, previously described as 'a wasteland of junk DNA' (Graves, 2006; Noordam et al., 2006). It is not just because it has generally been regarded as uninformative that the Y-chromosome remains largely unstudied throughout the animal kingdom; the Y-chromosome is also recalcitrant, reluctant to give up its secrets, thus requiring extensive research effort to elucidate its unique character and composition (Skaletsky et al., 2003; Jobling and Tyler, 2003; Rozen et al., 2003; Steinemann and Steinemann, 2005; Charlesworth, 2003). Even with substantial efforts, the human Y-chromosome sequence is not definitive. The 'complete sequencing' of the human Y-chromosome, more accurately described as the complete euchromatic sequence; does not include the extensive ~30-60Mb heterochromatic region, which accounts for the majority (Skaletsky et al., 2003). Recent work in *D. melanogaster* has suggested that heterochromatin is not simply 'junk' and may itself perform important functions in gene regulation. Furthermore, although the majority of actively transcribed genes are found within the euchromatic region there are some genes that are transcribed at a lower rate in the heterochromatin of some species. Therefore, to not sequence the heterochromatin is potentially to miss some important information both about the regulation of genes and genomic processes and potentially also the identification of individual genes present on the Y-chromosome (Hughes et al., 2009). Two further indications of the importance of this so-called 'junk' are that the *D. melanogaster* Y-chromosome consists entirely of heterochromatin and yet still performs its essential male-determining role (Pisano et al., 1993); and the heterochromatic region has been implicated in correct segregation of chromosomes during meiosis, particularly in the absence of recombination, which may be important in the dunnart, which lacks a PAR and as such does not undergo recombination (Toder et al., 2000; de la Fuente et al., 2007). Recent years have seen a change in attitude to this essential male element, as genes and functional significance, aside from male-determination, have been

discovered (Delbridge and Graves, 1999; Murphy et al., 2006; Goodstadt et al., 2007; Li et al., 2008).

It is obviously not possible to dedicate the same resources to Y-chromosome studies of non-humans, and although the rat Y is nearing completion (Alfoldi, 2008), the myriad partial or complete genome sequencing projects have concentrated their endeavours on the female genome (NCBI). Whilst it appears these projects are lacking an essential element, (which most certainly they are), the impediment the inclusion of the Y-chromosome would create for the project as a whole, would exceed the benefits of elucidating the Y-chromosome sequence. As a result, the character of the Y-chromosome is largely being discovered piecemeal through periphrastic approaches, rather than through attempts to directly sequence large portions (Donnison et al., 1996; Navin et al., 1996; Bergstrom et al., 1998; Bello and Sanchez, 1999; Hellborg and Ellegren, 2003; Wallner et al., 2004; Hatch et al., 2006; Luo et al., 2007). These studies necessarily rely heavily on the information gained from the human Y-chromosome for expectations and comparison (eg Murphy 2006; Raudsepp, 2004; Natanaelsson, 2006). As well as discovering common mechanisms of Y-chromosome evolution (see Steinemann and Steinemann, 2000, for review), studies are also showing some distinct patterns across taxa. This reality is not entirely unexpected, as there is a general understanding of the divergent evolutionary mechanics of Y-chromosome evolution (Bachtrog, 2003).

Although nothing on the scale of human research is possible in other species, some inferences can be made by comparative investigation of gene regions (Delbridge and Graves, 1999; Murphy et al., 2006), investigating the extent of cross-homology (Hellborg and Ellegren, 2003) and comparative analysis of general sequence characteristics (Olivier and Lust, 1997; Olivier et al., 1999; Toder et al., 2000; Yi and Charlesworth, 2000; Yi et al., 2003; Wallner et al., 2004; Takehana et al., 2007). Furthermore, by comparative assessment of sequence characters it is possible to distinguish some common features and differences of mammalian Y-chromosomes. Recently several studies looking at gene regions of the cetacean Y identified

some common characters, however interpretation was mostly limited to phylogenetic inferences (Hatch et al., 2006; Nishida et al., 2007; Caballero et al., 2008; Mace and Crouau-Roy, 2008).

### 6.2 Common features of mammalian Y-chromosomes – evidence on the delphinid Y?

The Y-chromosome is a paradigm of degeneration throughout the animal kingdom. The human Y is a degenerate version of the X, with very few functional genes and clear evidence of x-degenerate genes throughout the non-recombining region (Skaletsky et al., 2003). Studies of the process of this degeneration have been facilitated by the existence of the neo-Y-chromosome, which presents a window to the ongoing process. *Drosophila miranda* carries a neo-Y allowing comparative assessment between the content and composition of this new chromosome in comparison to the advanced degenerative stage of the true Drosophila Y, which only carries two genes (Bachtrog et al., 2008). Similar comparative studies in the medaka, which has a young Y-chromosome and the muntjac, which also carries a neo-Y, have distinguished similar characters associated with degeneration in mammals (Zhou et al., 2008). Common evidence of degeneration is an increasing lack of cross-homology to the X-chromosome and a scarcity of functional genes (Steinemann and Steinemann, 2000) – and whilst this study did not specifically target the gene regions, the characters of the fragments analyzed are consistent with this expectation. The size and the Y-chromosome FISH paint not hybridizing to any other region of the genome is consistent with a reduction in cross-homology (Toder et al., 2000; Alkalaeva et al., 2002). Furthermore, the isolated fragments did not indicate sequence similarity to others found within the NCBI database, suggesting a lack of functional genes – and the low GC-content and high simplicity of the fragments further suggests sequence degeneration (Tautz and Schloetterer, 1994; Galtier et al., 2001; Galtier, 2004). There is therefore clearly evidence for degeneration of the odontocete Y-chromosome.

Along with the degeneration of the functional sequence, Y-chromosomes are characterized by the accumulation of repetitive sequences and translocations (Steinemann and Steinemann, 2000). In addition to the palindromes that have been identified on the human

(Skaletsky et al., 2003), chimpanzee (Hughes et al., 2005) and rat (Alfondi, 2008) there is evidence for palindromes and/or other repetitive sequence classes on most Y-chromosomes studied thus far. For example, population genetic studies have been hampered by the presence of multi-copy loci in a number of species including: canids (Sundqvist et al., 2001); horse (Wallner et al., 2004); rat (Nishioka et al., 1987); and felids (Luo et al., 2007) and direct sequencing of gene regions has revealed the presence of multiple gene copies (Chowdhary et al., 1998; Raudsepp et al., 2004; Murphy et al., 2006; Pearks Wilkerson et al., 2008).

Evidence for the presence of multiple copies in cetaceans was discovered for the fin whale in the form of putative gene families drawn out by representational difference analysis (Hatch, 2004). Here the repetitive nature of the odontocete Y-chromosome was not obvious, with fewer than expected multi-copy loci and insufficient data for identification of palindromes. Whilst it is likely that the lack of evidence was a function of the study itself, there is also evidence for a relative lack of repetitive DNA on the minimal Y-chromosomes of marsupials (Toder et al. 2000) and drosophila (Bachtrog et al., 2003), suggesting that it may also be a function of its size.

The 'complete' sequence of the human Y revealed the mosaic of sequence classes that characterize the mammalian Y-chromosome (Skaletsky et al., 2003). As a result of differential selective and stochastic evolutionary pressures on different portions of the Y-chromosome, sequence characters are highly divergent from one region to the next (Otto and Barton, 1997; Orr and Kim, 1998; Wiehe, 1998; Jobling and Tyler-Smith, 2000; Slattery et al., 2000; Yi and Charlesworth, 2000; Williamson and Orive, 2002; Steinemann and Steinemann, 2005; Pozzoli et al., 2008; Whitlock and Agrawal, 2009; Wilson and Makova, 2009).

The Y-chromosome either recombines extensively (in the PAR); is subject to high levels of homogenizing gene conversion (MSY-palindromes) or does not recombine at all (heterochromatic region) (Skaletsky et al., 2003). Furthermore, the sequence character changes according to its functional significance including: essential male-specific genes under high selective pressure (e.g. Waters et al., 2007), functional x-homologous genes (Slattery et al.,

2000), non-functional x-degenerate genes (e.g. Pearks Wilkerson et al., 2008), and non-functional repetitive heterochromatic regions (Skaletsky et al., 2003). Both evolutionary pressures and the functional significance will inevitably have profound effects on the character of the sequence. Limited evidence for a mosaic of sequence classes on the odontocete Y comes from the differential character of the sequences analyzed in this study. Whilst the majority of sequences were 'simple' and AT-rich as expected, there was also evidence for extremes of sequence characters (high GC and high simplicity), consistent with differential origin of the sequence, although no gene regions appear to have been represented in the dataset, thus limiting any interpretation.

A character of particular interest to this study is the apparent lack of Y-chromosome marker variability (Hellborg and Ellegren, 2003) and evidence of microsatellite degeneration (Buschiazzo and Gemmell, 2006). Most population genetic studies exploiting the Y-chromosome report high numbers of imperfect repeat loci and low variability (e.g. Bachtrog et al., 2000; Liu et al., 2000; Wallner et al., 2004; Sundqvist et al., 2001). Within the mammalia, these characters appear to be consistent across the majority of taxa with the only real exception being the human (Hurles and Jobling, 2003). Whether this disparity in the human is a function of the research effort or whether it is related to species ecology is not well understood or documented. The closely related chimpanzee (Erler et al., 2004) and bonobo (Eriksson et al., 2004), both show a relative lack of diversity at Y-chromosome microsatellite markers isolated from humans (see Kayser et al., 2006; Vowles and Amos, 2006; and Jorde et al., 2000, for example). There is clearly evidence from the cetacean Y-chromosome for reduced variation and abundance of complex microsatellites, both from Hatch (2003) and from this study. Consistent with Greminger et al. (in Press), the killer whale and bottlenose dolphin in this study were both lacking in polymorphic microsatellite loci whilst the sperm whale (this study) exhibited similar microsatellite characteristics to those of the fin whale (Hatch, 2003), the only other cetacean species from which polymorphic microsatellites have been identified.

## 6.3 Summary and Future Directions

The availability of a growing cell line is very useful for many of the latest techniques and so the development of a relatively straightforward method that others can replicate with a clear indication of the limits of storage will be beneficial to future research in this and other fields. The methodology described and applied herein to several marine mammal species represents a good starting point for effective exploitation of the lymphocyte assay without the need for minute optimization. The assay also has the potential to be made even more efficient with minor adaptations suggested in Chapter 2.

The study of most species will certainly be limited by the budget, and the magnitude of effort that was required to obtain the human Y-chromosome sequence is just not practicable in other species. Even the human Y sequence, with all the resources available, took a long time to resolve and is still not entirely complete (Skaletsky et al. 2003). Interpretations from this study are therefore inevitably limited by the amount of data available but also, because the sequences were taken from anonymous regions, it is not possible to make as much comparative interpretation as desired.

Other Y-chromosome studies have mainly focused on the homologous genes and intronic regions for which cross-species comparisons are possible (e.g. Raudsepp et al., 2004; Murphy et al., 2006); however, there is also limited inference to be obtained from focusing exclusively on genes, particularly if there is only partial cross-homology and/or variation restricted to the phylogenetic level (Caballero et al., 2008; Mace et al., 2008; Nishida et al., 2008).

This study has demonstrated the relevance of flow sorting and microdissection to the study of marine mammals and has indicated the potential future application of these methods for understanding their genomic organization (through ZOO-FISH, for example) and to the exploitation of their products (e.g. Fronicke et al., 1996; Raudsepp et al., 1996; Bielec et al., 1998; Glas et al., 1999; O'Brien et al., 1999; Raudsepp and Chowdhary, 1999; Coppola et al., 2007; Lear and Bailey, 2008).

Cytogenetics became a lost art in the late 1990s as the trend moved towards exploiting the benefits from the plethora of advancements in the molecular field and the new generation of researchers turned their focus to the fountain of knowledge that was available as a result of PCR (e.g. Gingeras et al., 1990; Hoelzel, 1992; Hoelzel and Green, 1992) and more recently from sequencing technologies, such as pyrosequencing (Ronaghi et al., 1999; Ahmadian et al., 2000; Nordstrom et al., 2000; Shi, 2000). However, specialist advances in the field of cytogenetics revealed new potential from the technique and ecology has profited from the use of ZOO-FISH and flow sorting technologies (e.g. Telenius et al., 1993; Scherthan et al., 1994; Solinastoldo et al., 1995; Froenicke et al., 1996).

A limited number of specialist groups turned their attentions back to the valuable information that can now be obtained from advances in these methodologies (Ferguson-Smith, 1996; O'Brien et al., 1999; Ferguson-Smith, 2001; Ferguson-Smith et al., 2001; Ferguson-Smith and Trifonov, 2007); indeed the human genome project relied heavily on them (Cram, 1990; Vandilla and Deaven, 1990; Roslaniec et al., 1997; Cram et al., 2002). Flow karyotyping can inform future research in other fields, whilst the paints themselves and the demonstrated potential of the technique could facilitate further investigation of the cetacean Y-chromosome and enable the transfer of information from the data-rich human genome project to other budget-poor species.

This study suffered from the separation efficiency and minute amounts of starting material obtained from microdissected chromosomes and the lack of identity of the large number of small chromosome fragments. The methodology presented here does, however, represent a cost-effective, efficient way of obtaining the entire Y-chromosome DNA sequence which could also be used to screen BAC libraries from the focal species, in the future.

Although limited, the sequence data were able to suggest characteristics of the Y-chromosome and in particular indicate that there is a combination of similarities and differences between species and ultimately that there is much more to learn about this elusive chromosome.

This minute genomic element has the potential not only to reveal its own peculiarities, but to inform ongoing research into evolutionary patterns of genomics.

Highly variable microsatellite-loci appear to be limited on the Y-chromosome; nevertheless there is an equal requirement to that of autosomal microsatellites for sufficient variable markers for interpretation. Therefore, although there are seven polymorphic Y-chromosome microsatellite markers reported herein for the first time for an odontocete, the limited variation does impact the amount of inference and interpretation that is possible. Nevertheless, this study has shown their utility for investigating the male movement of North Atlantic sperm whales and results herein are consistent with expectations based on the species ecology (Whitehead et al., 1998; Baumgartner et al., 2001; Gannier et al., 2002; Lettevall et al., 2002; Mullin and Fulling, 2003; Marcoux et al., 2007) and results of population genetic analysis for the sperm whale (Lyrholm and Gyllensten, 1998; Whitehead et al., 1998; Lyrholm et al., 1999; Vitalis, 2002; Engelhaupt et al., 2009; Pinela et al., 2009). These limitations were not entirely unexpected when considering the general lack of population level interpretation possible from non-human Y-chromosome microsatellite markers (Edwards et al., 2000; Liu et al., 2003; Hatch, 2004; Meadows et al., 2006; Luo et al., 2007; Greminger et al., In Press).

## 6.4 Conclusions

The differential characteristics of Y-chromosomes across the mammalia, suggests that it remains an enigma of genome evolution. Despite the opportunities provided by the complete sequencing of the human Y-chromosome for investigations of male-specific history and evolutionary mechanisms, the Y-chromosomes of other species remain recalcitrant to the probing of science. As whole genome projects continue to use female genomes for their studies the finer details of the Y-chromosome remain concealed within a repetitive and degenerative environment. Amid the doom and gloom of prophesied male extinction (Graves et al., 2006), however, comparative Y-chromosome studies have revealed many of the processes of genome evolution suggesting the vast potential that the Y-chromosome has to offer. Almost a decade

ago Petit et al. (2002) heralded the potential of the Y-chromosome for population genetic studies, since which faltering steps have been taken towards realizing this potential and yet so much more remains to be discovered. This first assessment of cetacean Y-chromosome sequence characters hints at the extensive promise it has to offer investigation both within the cetaceans and in comparison to other mammals. Having facilitated the process, it will be possible to extend its utility to a broader diversity of cetacean species. The markers and information generated by this study will be useful for gaining an understanding of the generalities associated with Y-chromosome evolution and the relevant context of differences. Finally, inferences gained from population analysis have the potential to inform our understanding of male movement of species for which it holds important conservation implications.

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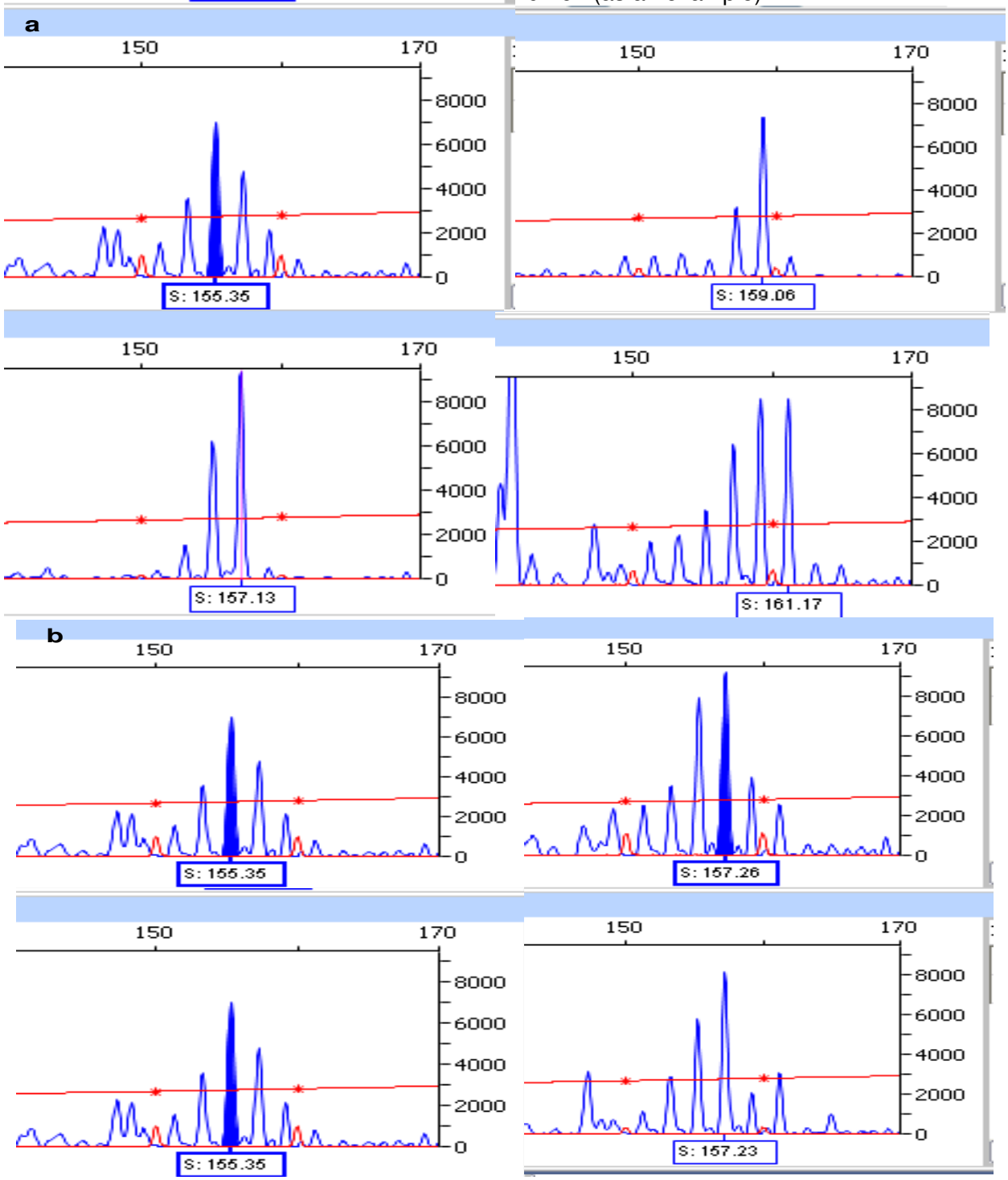
**Appendix 1**

**Figure 1:** Y-chromosome microsatellite locus VEY23 has four alleles. The locus size is indicated below the main peak. Alleles 157 and 159 demonstrate a typical dinucleotide microsatellite stutter profile whereas allele 155 shows post-peak stutter and allele 161 shows an amplified pre-peak stutter band. a) shows the four alleles for the locus (155, 157, 159, 161); b) shows the consistency between individuals for the peak profile for alleles 155 and 157; and c) shows the consistency between repeats of the same individual between replicates for the peak profile. for alleles 159 and 161 (as an example).



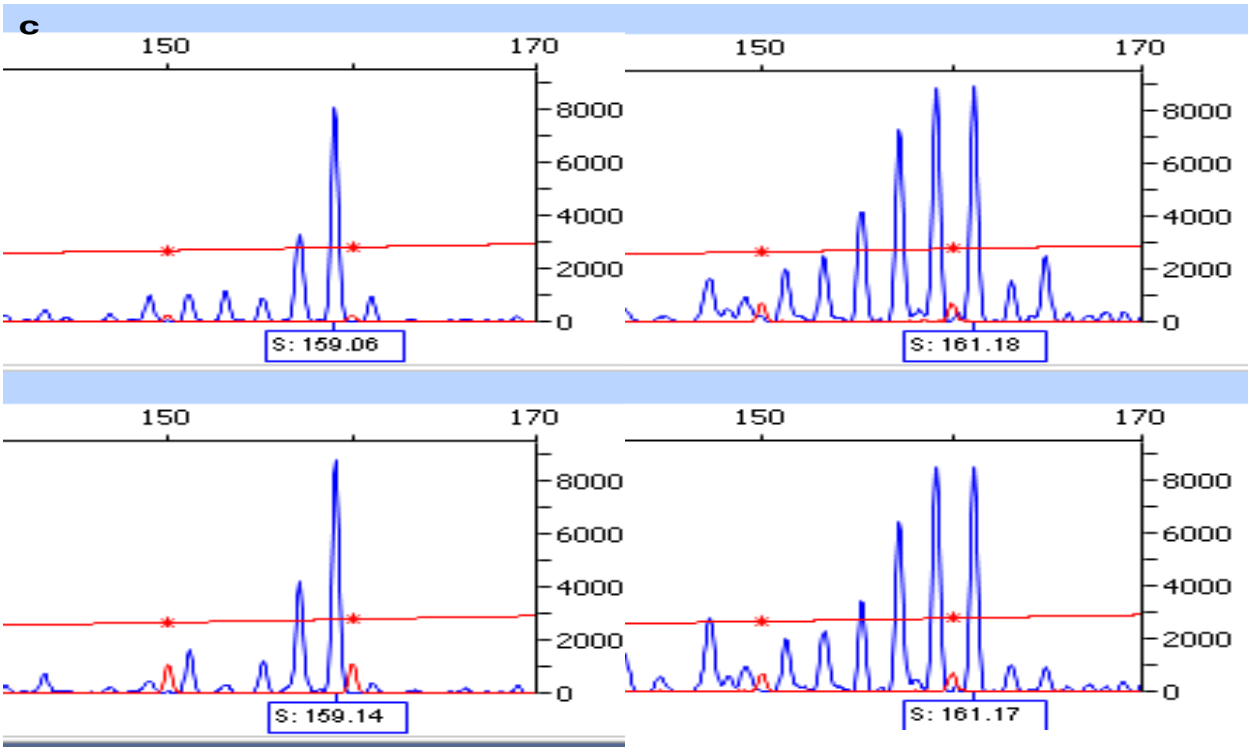
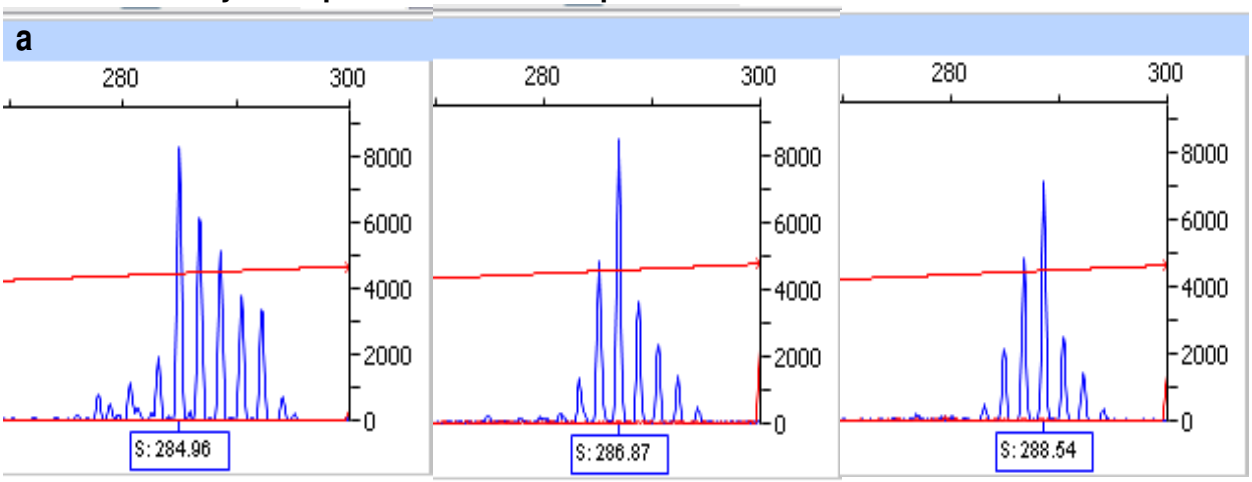



**Figure 2 shows the genotype profiles of locus VEY27 genotyping profiles showing a) the three alleles for the locus (285, 287, 289) and b) the consistency of amplification across replicates and individuals.**



b) Locus 287 (top) and 289 (bottom) showing consistent amplification profile across amplifications for the same sample i and ii; and different samples iii and iv

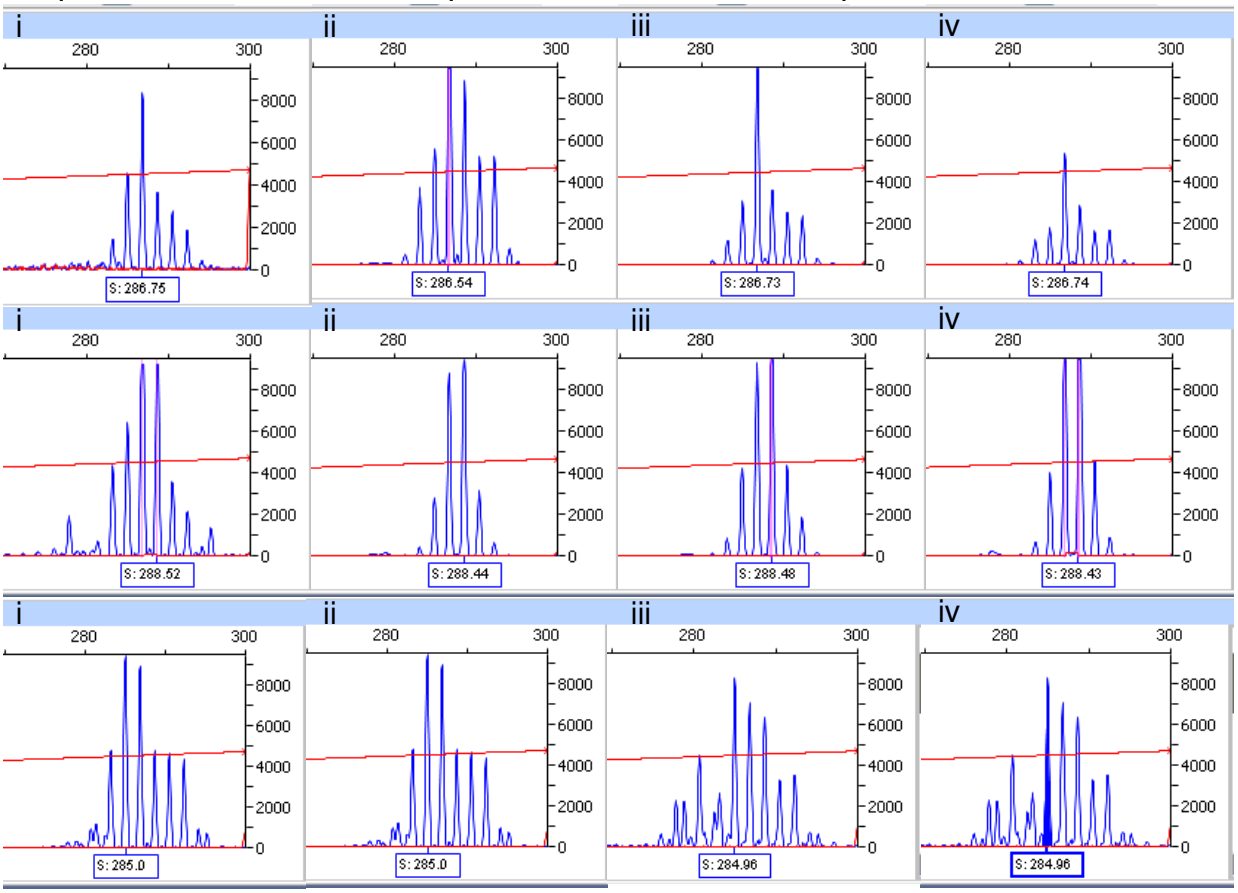


**Figure 3 Profiles of the Y-microsatellite locus VEY28 Primer showing the two alleles 187 and 191 that amplify consistently between individuals**