Phylogenetic Relationships, Population Genetics and Hybridisation of Two Species of Southern Fur Seal (*Arctocephalus* spp.)

by

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"The seals of the world were divided into two major groups by Allen (1880); the 'walkers' and the 'wrigglers'."

C.A Repenning and R.H Tedford, 1977

Declaration of Originality

This thesis contains no material which has been accepted for a degree or diploma by the University or any other institution, and to the best of my knowledge and belief, contains no material previously published or written by another person, except when due acknowledgment is made in the text of the thesis.

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Declaration by candidate

I certify that this thesis represents an original and independent piece of research. All of the significant aspects of analysis and interpretation of the results were done by myself.

This thesis is being presented as a series of already published or submitted papers. The nature of the collaborations indicated by the co-authorship of these papers takes two forms:

1. Supervisors (White, Goldsworthy, Slade): as senior author I have exercised my prerogative to recognize the contribution made by my supervisors to my training by including them as co-authors.
2. Individuals that have supplied samples essential to the study. Several chapters of the thesis required tissue samples from all sub-Antarctic islands on which fur seals breed. Given the number and remote location of these islands it would have been impossible for me to collect these samples during the course of my study, so I organized for biologists from several countries to collect them for me. As these samples were expensive and difficult to collect, I have chosen to include those who contributed samples as co-authors. This in no way diminishes the originality or my overall contribution to the thesis.

Louise Wynen

Abstract

This research investigated the phylogenetic relationships and population genetics of two species of fur seal in order to provide a basis for the study of the hybridisation of these species at Macquarie Island. The Antarctic (*Arctocephalus gazella*) and subantarctic fur seals (*Arctocephalus tropicalis*) occur throughout the subantarctic region of the Southern Ocean, hauling out on remote islands throughout the region to breed. Both species were subject to intense sealing activities in the 18th and 19th centuries. The competitive and indiscriminate nature of the industry ensured that all populations were greatly reduced in size, with some becoming extinct. The cessation of sealing in the early 20th century has allowed both species to recover in number, and recolonise islands across their former range. The current range for the Antarctic fur seal overlaps with that of the subantarctic fur seal at Iles Crozet, Marion Island and Macquarie Island. Hybridisation has been reported at low levels at Marion Island, and at higher levels at Macquarie Island. The situation at Macquarie Island is further complicated by the presence of an additional species, the New Zealand fur seal (*Arctocephalus forsteri*) While this species is not breeding on the island, some males participate in the breeding process, with some hybrids being produced This research seeks to apply molecular methods to investigate the hybridisation that is occurring at Macquarie Island within the context of the evolutionary and recent history of the two breeding fur seal species, and to a lesser extent, the New Zealand fur seal.

The phylogenetic relationships of the Antarctic and subantarctic fur seals were investigated within the context of the family Otariidae. This family include the nine species of fur seal (Genera *Arctocephalus* and *Callorhinus*), and five species of sea lion (Genera *Neophoca*, *Phocarctos*, *Eumetopias*, *Zalophus*, and *Otaria*). A 360 base pair region of the cytochrome *b* gene in the maternally inherited mitochondrial genome was used for the primary phylogenetic analysis of the family, while a 356 base pair fragment of the mitochondrial control region was used to enhance resolution of the terminal nodes. The traditional classification of the family into the two subfamilies Arctocephalinae (fur seals) and Otariinae (sea lions) was not supported, as the fur seal *Callorhinus ursinus* was found to be basal to all other fur seal and sea lion taxa. While four sea lion clades and five fur seal clades were consistently observed through all analyses conducted, it was not possible to adequately resolve the relationships among these clades. This probably reflects the rapid radiation of these taxa that occurred about 3 million years ago. The subantarctic fur seal was found to be most closely related to the Australian and Cape fur seals (*A. pusillus*) while the closest species related to the Antarctic fur seal was not clearly resolved. However, there were discrete species specific differences observed between the Antarctic, subantarctic and New Zealand fur seals in both the cytochrome *b* gene and the control region, providing the basis for species identification within the hybridising population at Macquarie Island.

The investigation into the level and distribution of genetic variation in the Antarctic and subantarctic fur seals was conducted using two classes of molecular marker. The mitochondrial control region is a commonly used marker for investigation into population genetics issues, due

in part to the relatively high rate of mutation. Microsatellites are highly variable regions within the nuclear genome, and with a bi-parental mode of inheritance, provide a natural complement to the maternally inherited mitochondrial genes. Given that historic records indicate both fur seal species had passed through population bottlenecks as a result of sealing, it was suspected that the current level of genetic variation may be low. This is because reduced levels of variation have been observed in other species that have passed through extreme population bottlenecks at some stage in the recent past (eg. the northern elephant seal, *Mirounga angustirostris*). Surprisingly, the nucleotide diversities of the Antarctic and subantarctic fur seals, as determined by the mitochondrial control region, were found to be high (3.2% and 4.8% respectively). The level of genetic variation as exhibited by the 10 microsatellite loci was generally high (overall heterozygosity levels 0.54-0.62 for the three species), though variable between loci. Despite the overlapping allele size ranges for most of the loci, significant allelic and genotypic differentiation was observed between the three species ($P < 0.000$). Significant population structure was evident within the subantarctic fur seal with both the mitochondrial (Φ_{ST}=0.19) and microsatellite DNA (unbiased R_{ST}=0.122; P<<0.001). Population pairwise comparisons among subantarctic fur seals, suggest gene flow from Gough Island in the South Atlantic eastwards to Marion Island and Iles Amsterdam, and from Marion Island in the South Indian Ocean eastwards to the recolonised population at Iles Crozet. Less population structure was evident within the Antarctic fur seal based on mitochondrial DNA (Φ_{ST} =0.074), but two genetically differentiated regions were recognised. In contrast, no genetic heterogeneity was observed with microsatellite DNA (unbiased R_{ST}=0.003; P=0.501), suggesting panmixia despite the large geographic range of the species. Overall, less structure was evident with microsatellite DNA compared with mtDNA data for both species. This could be due to there being a greater effective population size for microsatellites compared with mitochondrial DNA , or the results may reflect the greater male mediated dispersal reported in fur seals. In any case, both markers indicate that the recolonisation of Macquarie Island is most likely to have originated from Iles Kerguelen for the Antarctic fur seals, and Marion Island and Ile Amsterdam for the subantarctic fur seals.

The presence of three fur seal species and their hybrids within the Macquarie Island population renders the identification of individuals to species problematic. The extent of hybridisation was initially investigated within a single cohort of pups (n=130), through the comparison of mitochondrial and microsatellite DNA methods developed in previous chapters, with a number of field-based methods The latter group include: the 'Overall Phenotype' method, where the investigator considers traits such as external appearance, behaviour, and vocalisations in providing an overall picture of the individual; and the 'Phenotype Score' method, where a prescribed set of phenotype traits are scored in an objective manner for each individual. This study also aimed to investigate the direction of hybridisation by comparing the incidence within the pup population with that observed in the breeding population of males and females. The two phenotype-based methods found that the incidence of hybridisation within the cohort ranged from 5.5% to 7.7%. The molecular methods relied on comparing the genotypes of all pups with a reference data set of the species, either with an assignment test or by eye. The

assignment test method was easy to use, but was unable to categorically assign individuals to a hybrid class in an objective manner. If a subjective variation of the test is used, then the estimated level of hybridisation was found to be 18.4%. This is lower than the estimate obtained when assessing the genotypes by eye, which was 30.4%. While the phenotype-based methods had a high success rate in classifying individuals to the correct species (75.8% to 70.9%), they severely underestimated the number of hybrids in the population. The incidence of hybridisation within the pup cohort was much greater than observed in the breeding female population (17.5%), but much less than was observed in the breeding male population (48.5%). If the males are grouped as territorial males and challenger males, it can be seen that the proportion of hybrids within each class also differs (58.8% and 37.5% respectively).

The study of the Antarctic and subantarctic fur seals in the light of their evolutionary relationships, combined with an investigation into the levels and distribution of genetic variation in the context of their recent history, has laid the ground work for a comprehensive examination of hybridisation at Macquarie Island. However, there are still many questions remaining, and a longitudinal study adopting a holistic approach (encompassing molecular data, field observations, morphological measurements, and the study of pre- and post-mating isolating systems) is required to understand the longer term implications of hybridisation in this population.

Acknowledgments

As this thesis presents a series of papers that have either been published, or are in the process of publication, the acknowledgments that are particular to each paper are presented at the end of each of the relevant chapters. However, not being one to be confined to the few words allowed by journals, I wish to include some further thoughts here.

My PhD adventure started at Macquarie Island.....three seasons of wandering over North Head in the wind and rain, with only vague thoughts of the times that lay ahead in the lab and in front of the computer. Working with the fur seals was a magical experience, and life on the station was pretty special too. I wish to thank all the expeditioners at Macquarie Island from 1994-1997 for much fun and parties, and for all their support in the field. A special thankyou to Kiwi who could make anything from the seal hide on Secluded Beach to Oscar the Grouch costumes, and to all the tradies who helped in some way make our life in the field easier. From all of those who passed on resights from around the island, to those who helped catch the odd seal, through to those who collected samples and extra observations while I wasn't about, I thank you. Sue Robinson (a vision in black plastic and chains), David van Smeerdijk and Eddie Firth were great mates to work with in the field, while the 'cutie patrol' - Elwood, Malbert, and Clive, was just something else!

Some sunshine and warmth were called for in the next phase of the adventure Craig Moritz of the Molecular Genetics Laboratory, University of Queensland was generous in allowing me loose in the lab, learning the ropes and harassing anyone within sight. All of the other students and post-docs were very welcoming and patient, and made my life there very happy. I thank Marcia, Chris, Katrina, Andrew, Veronica, Damien, Jo, Nancy, Conrad, Lisa, Jacob and the rest of the crew for all their help and guidance. I especially thank Kathryn and Claire for some great times together, especially at Wordsmiths and the staff club, and Petra and Dave for helping me cycle it all off. A special thanks to Reg and Liz, for dragging me off to Heron Island as a fieldie at a time when I most needed it, and Tweedie, who will always hold a special place for his "ccooffffeeeeeeeeeeeeeeee" phone calls, laughter and general insanity. To my fun-filled flatmates, Chloe and Fi for generally putting up with me, and making me laugh my way through the hard work.

A move to lovely Hobart was next on the agenda, to finish up at the Antarctic Wildlife Research Unit, University of Tasmania. All of the PhD and Honours students who passed through the AWRU at this time (especially Corey, Petra, Megan, Brad, Jane, Tony, Cath, Peter, Emma, Cait and Jacqui) made the indoors existence in front of a computer highly entertaining. Adam Smolenski was a legendary lab manager, helping and guiding all the way through the lab side of things, and Chris Burridge was also a source of great knowledge and advice. My supervisor Robert White was generous in support for a number of chapters, and of course, providing the opportunity to present my work at the Marine Mammal Society conference in Hawaii in 2000. Sherrin Bowden is the best departmental secretary a student could ask for. Sherrin was always friendly and willing to help, a wealth of knowledge, and totally reliable in making sure all the

paperwork got to the right person and that everything operated smoothly. The biggest hugs go to Kirsten, M-A, and Karen for all the laughter and distractions, and their seemingly endless patience and encouragement on the rocky road to the final hand in.

Just to make life really interesting, I started working full time with Forensic Science Service Tasmania in October 2000, ensuring that the hand-in was dragged out for another year All of my work mates at FSST have been great! Their tolerance at my grumpiness and their enthusiasm for life made the final write-up so much easier. I especially thank Stephen, Laszlo and Pam for their unquestionable support, especially during the last few weeks.

I also thank the whole tribe of co-authors who have been so generous with their samples, advice and their time in reading drafts. I thank them all for their enthusiasm for this work, and their timeliness in getting back to me with comments and all the paper work associated with publication.

Throughout the course of this large body of work, there were a number people who were instrumental in its completion. Rob Slade was the man who showed me the ropes in the lab at UQ, and was always willing to help with ideas and plans for the thesis. He put up with my arguments and my 'slowly slowly' approach to learning, and with his outrageous sense of humour and fun has helped me through the seemingly impenetrable world of genetics. Mark Hindell has been a mentor of mine for many years, and his open door and generosity despite not being a formal supervisor in this work has been an inspiration. Simon Goldsworthy is my principal supervisor for this work, and the person whose vision and foresight instigated the whole program. I thankyou Simon for all your support, undying enthusiasm and your hard working ethic that has helped me through the past 6 years.

And of course, to all those friends who have somehow felt the brunt of my 'PhDisms' throughout the past years...to name a few...M-A, Margie, Lou, Karen, Kirsten, Kiwi, Snake, Corey, Karah, Ray, Ros, Tim, Sarah, Mark, Paula, Elwood, Malbert, Lemonhead, Smudge, Rags and Sooz, Josh and Harry, Jack, Barbara, Maddie and Jonathan, Trevor, and my family. Mum and Dad's enthusiasm alone would have had me finished on the dot at the 3-year mark!! But notwithstanding the passing of this deadline without an end in sight, their support and their unquestioning belief in my ability to finish was a source of inspiration. Oma, Leonard, Karen, Jim, Mark, Kel, Sal, Krysty, Jess, Ellen and Josh all probably think I'm a bit odd for doing this in the first place, and they're probably right, but I thank them for their love.

And finally, there is no way that this work could have been finished in such a (relatively) stress-free manner without Clive. He kept my feet on the ground, and was a rock of support during the times when things got a bit tough. He cooked for me, cleaned the house, drove me about, read my drafts, laughed at my silliness and generally put up with my shit. A legend for sure.

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CHAPTER 1: Introduction

Background

The study of pinnipeds in the wild has long been hampered by their widespread distribution, the remote areas they inhabit, and their inaccessibility while at sea. While the southern fur seals (genus *Arctocephalus*) spend a proportionately larger amount of time on land relative to other marine mammals, thus providing the window of opportunity for research, their marine existence presents a barrier to obtaining an in-depth understanding of their ecology. There have been many novel approaches developed to circumvent this problem (eg. satellite telemetry, fatty acid analysis, etc, see Hindell and Kemper, 1997 for examples), and the development and refinement of molecular techniques has provided another dimension into understanding the ecology of these species. This has been achieved through the examination of evolutionary relationships, current and historical biogeography as well as inter- and intra-population level issues such as immigration, pedigree analysis, patterns of inheritance and reproductive success (Hillis and Moritz, 1990; Avise, 1994).

The Antarctic (*Arctocephalus gazella*) and subantarctic fur seals (*Arctocephalus tropicalis*) are currently some of the best studied of the fur seals, despite their large distribution throughout the Southern Ocean and the remoteness of the islands on which they breed. Research on these species has been ongoing since the 1930s, and has documented remarkable recoveries in population size and distribution in the wake of 19th century commercial sealing, as well as other information relating to taxonomy, demography, reproduction, and foraging ecology (eg. Olstad, 1929; Sivertsen, 1954; Payne, 1977; Jouventin *et al.*, 1982; Kerley, 1983; Boyd and Croxall, 1992; Goldsworthy, 1992; Guinet *et al.*, 1994; Gemmell *et al.*, 2001). Such research has also led to the discovery of hybridisation between these species at the three sites where they overlap in range. While such hybridisation is thought to occur only at low levels at Iles Crozet and Marion Island (Condy 1978; Jouventin *et al.*, 1982; Hofmeyr *et al.*, 1997), the incidence at Macquarie Island is suspected to be quite large (Goldsworthy *et al.* 1999).

The initial aim of this study seeks to investigate the incidence of hybridisation at Macquarie Island. Since this population is small, recovering from extinction, and has been monitored more or less continually since the birth of the first pup in the post sealing era, it provides a natural laboratory into intra-population studies. This population is also unique in that it is the only place where the Antarctic fur seal, subantarctic fur seal and the New Zealand fur seal (*Arctocephalus forsteri*) occur in sympatry. While the New Zealand fur seal is represented almost entirely by males who haul out on the island to moult, occasional individuals have participated in the breeding process.

Reliable species identification methods are pivotal for the recognition of hybrids as distinct from the parental species. While several studies have reported on a number of external traits that distinguish between fur seal species (eg. Condy, 1978; Goldsworthy *et al.*, 1999), their utility for the identification of hybrids may be equivocal due to their generally being

polygenic. It is assumed that hybrids would either exhibit phenotypes that are intermediate between parental species, or a mix of those expected of the parental species. Either way, difficulties arise when trying to distinguish between a naturally occurring variant of a parental species, and a hybrid. This is especially so for backcrossed individuals. An alternative approach is to use molecular methods as a basis for species identification. Such an approach is being widely used for a range of species, especially after the advent of PCR (polymerase chain reaction) technology. However, if hybridisation has been occurring for some time, and backcrossing is common, it is likely that the genes of one species have introgressed into one or both of the other species.

Therefore, this study aims to provide a comprehensive molecular assessment of the fur seal species that occur at Macquarie Island, their taxonomic relationships, and the level of genetic variation that occurs within and between each species is. The taxonomic relationships of all fur seal species have been contentious. Initially confusion arose primarily due to a paucity of samples from different taxa, a consequence of their wide distribution. However, even when reference material from all species was obtained, there were still problems in discerning and understanding the relationships (King, 1959; 1960; 1969; Repenning *et al.*, 1971). Even the more recent studies based on a wide range of different techniques (eg. skull morphology, fossil data, or a composite approach) have differed in their interpretations of these relationships (Berta and Wyss, 1994; Bininda-Emonds *et al.*, 1999). The one study where molecular evidence was provided (Lento *et al.*, 1997), did not have representative samples from all species, but hinted that the sharing of lineages detected for the Antarctic and subantarctic fur seal species indicated a very close relationship. Further, these fur seals were regarded at one time to be the same species (Sivertsen, 1954; Scheffer 1958). Such suggestions of a very close relationship between the Antarctic and subantarctic fur seal species necessitates a thorough investigation prior to the pursuance of intra-specific questions such as hybridisation.

Further, this study aims to assess the level and distribution of genetic variation of both the Antarctic and subantarctic fur seals through the sampling of all major populations throughout the range of both species. Commercial sealing in the 18th and 19th centuries had a major impact on the Antarctic and subantarctic fur seal populations in the Southern Ocean. The intensive and unrestricted nature of the industry ensured substantial reductions in population sizes and resulted in both species becoming locally extinct at some sites. However, both species are continuing to recover, through the recolonisation of islands across their former range and increasing population size. Such population bottlenecks may have a major effect on the level of genetic variation within a species, as exhibited by other marine mammals such as the northern elephant seal and Hawaiian monk seal (*Mirounga angustirostris* and *Monachus schauinslandi* respectively, Hoelzel *et al.* 1993; Kretzmann *et al.* 1997). Therefore, in order to adequately address intra-population questions such as those pertaining to the Macquarie Island population, a full assessment of the level and distribution of genetic variation is required for the Antarctic and subantarctic fur seals.

The attainment of these general aims will provide a solid basis upon which specific differences between species can be identified for the application of hybrid identification within the Macquarie Island population. These molecular methods will therefore form a natural complement to the field observations that have been recorded since the inception of breeding on Macquarie Island in the post-sealing era.

Objectives of the Study

This study aims to employ molecular techniques to provide a thorough investigation into the population genetics of Antarctic and subantarctic fur seals in the light of their evolutionary relationships. Such an investigation will provide the ground work to study the species composition of the Macquarie Island population, and to identify the extent of hybridisation. The specific aims of the study are to:

1. Investigate the phylogenetic relationships among taxa within the Family Otariidae.
2. Examine the post-sealing mitochondrial DNA variation in the Antarctic and subantarctic fur seals to determine whether the differences in their respective exploitation histories are reflected in the levels and distribution of observed genetic variation.
3. Examine the post-sealing genetic variation as exhibited by microsatellite DNA in the Antarctic and subantarctic fur seals, to determine whether the level and distribution of genetic variation observed with these bi-parentally inherited markers is different to that observed with the maternally inherited mitochondrial DNA.
4. Examine the extent of hybridisation in the Macquarie Island fur seal population as exhibited in a single pup cohort, through the application of both field and molecular methods of species identification
5. Determine the incidence of hybridisation within the breeding population of Macquarie Island through the examination of adult males and females, and to determine if possible, the direction of hybridisation within the population as a whole.
6. Assess the reliability of field based methods of species identification in comparison with molecular methods.

Life History and General Biology of the Antarctic and Subantarctic Fur Seals

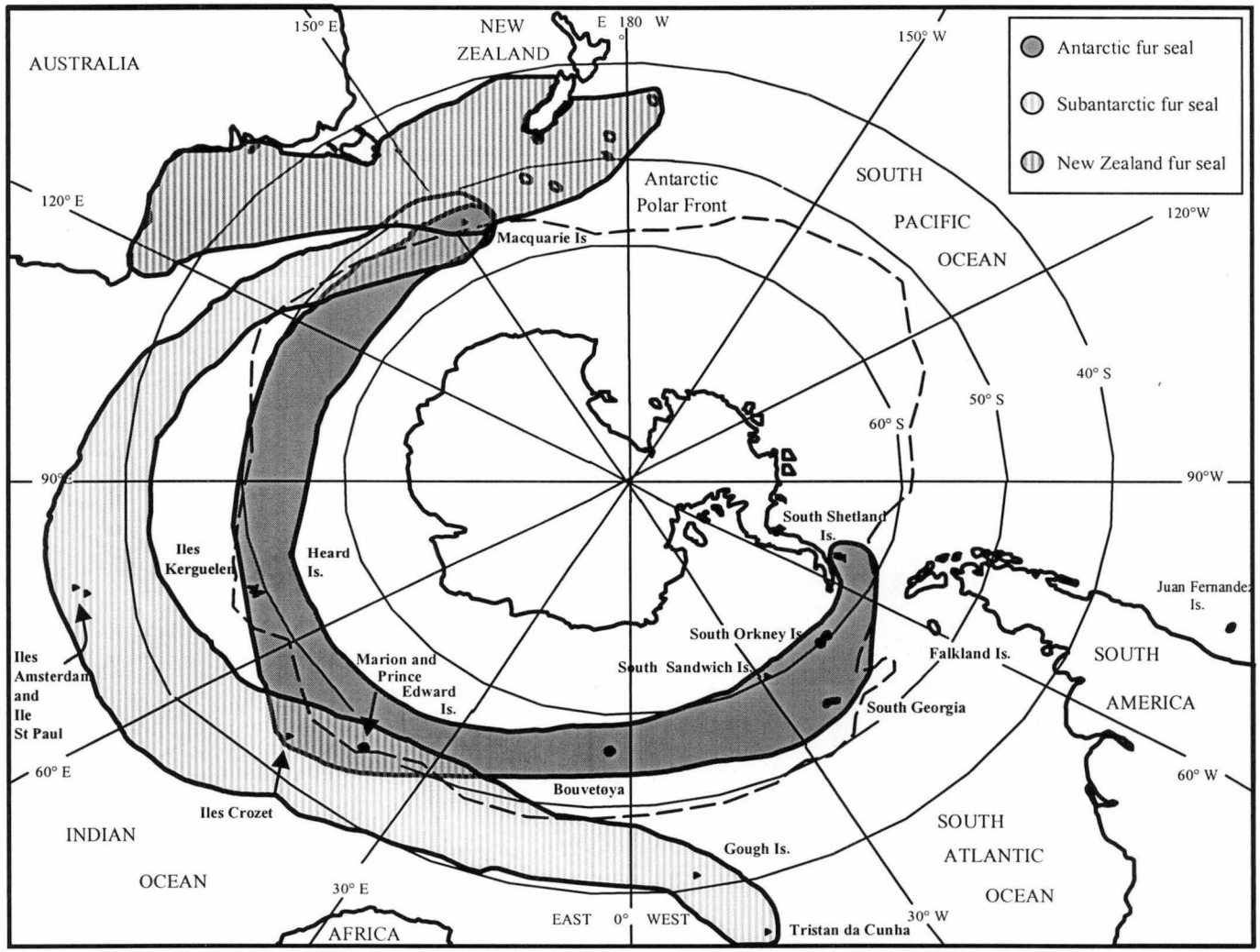
The distribution of the Antarctic fur seal has altered dramatically over the past few centuries as a result of the sealing activities that commenced in the late 18th century. This species was thought to have been driven to the brink of extinction, but recovery since the cessation of sealing has been dramatic and has seen a major range extension in recent years. Currently, the range of the Antarctic fur seal extends from the South Shetland Islands close to the Antarctic Peninsula, to islands in the south Atlantic and Indian oceans, through to Macquarie Island in the South Pacific Ocean. Despite such an extensive range, approximately 97% of the species occur at South Georgia (Hofmeyr *et al.*, 1997) (Figure 1.1).

While the subantarctic fur seal was also heavily exploited by sealing gangs, this species was not as adversely affected as the Antarctic fur seal. The subantarctic fur seal survived at three different locations, and records suggest that these sites were not primary targets for sealers due to the lower density of seals compared with populations elsewhere (eg. the South Shetland Islands). The subantarctic fur seal occur on islands to the north of the Antarctic Polar Front, ranging from Tristan da Cunha and Gough Island in the South Atlantic Ocean, through to Macquarie Island to the east (Figure 1.1). The major population centre for this species is at Gough Island, but large populations are also present at Ile Amsterdam and the Prince Edward Islands. The Antarctic and subantarctic fur seals occur in sympatry on three islands: Iles Crozet, Marion Island and Macquarie Island. While the New Zealand fur seal also occur on Macquarie Island, they generally do not participate in breeding. Their range extends from islands off the coast of Western Australia and South Australia, on mainland New Zealand and some of the subantarctic islands of New Zealand. There is also a small breeding population on Maatsuyker Island, Tasmania (Figure 1.1).

Much of the life history of fur seals conform to a generalised pattern, with some differences evident between the species, and among populations of the same species. All fur seals exhibit sexual dimorphism, with males reaching sizes up to four times that of the females. They have a polygynous mating system, where a single male will hold a territory where they can attract and control multiple females. Males arrive on the breeding beaches ahead of the females, and select sites which will attract females when they arrive to pup. The prime sites are hotly contested, and males need to defend their territory throughout the breeding season. As such, males fast during the breeding period, leaving only when driven out or when the breeding season ends. Females arrive a few weeks after the males, and generally produce a pup within two to three days of arrival. They remain to nurse their pups in the breeding territories for approximately six to eight days (Boyd *et al.*, 1991), when they are believed to come into oestrus. Mating is thought to occur with the territorial male prior to the departure of the females on the first of many foraging trips throughout the lactation period. However, recent studies have discovered that non-territorial males are responsible for a many of the conceptions, some of which may occur at sea (Goldsworthy *et al.* 1999; Gemmell *et al.*, 2001). The lactation period varies between species; about 120 days for the Antarctic fur seal, and ranging between 280 to 300 days for the subantarctic fur seal (Kerley, 1985; Goldsworthy, 1992). Female fur seals reach sexual maturity at a young age, and produce their first pup when three to fours years old. Males on the other hand, although reaching sexual maturity at a similar age to females, will not be big enough to successfully compete for a territory until reaching about eight years (King, 1983).

Fur seals are generally regarded as being highly philopatric, especially the females, although there have been no quantitative studies conducted on Antarctic or subantarctic fur seals to

Figure 1.1 (over page): Distribution of Antarctic, subantarctic and New Zealand fur seals throughout the Southern Ocean. Map modified from original by John Cox (Australian Antarctic Division).


Antarctic fur seal
Subantarctic fur seal
New Zealand fur seal
AUSTRALIA
NEW ZEALAND
150° E
E 180 W
150° W
120° E
120°W
Antarctic Polar Front
SOUTH PACIFIC OCEAN
Macquarie Is
40° S
50° S
60° S
90°E
90°W
South Shetland Is.
Iles Kerguelen
Heard Is.
Juan Fernandez Is.
South Orkney Is
Falkland Is.
SOUTH AMERICA
Iles Amsterdam and Ile St Paul
Marion and Prince Edward Is.
South Sandwich Is.
South Georgia
60° E
60° W
Bouvetøya
Iles Crozet
INDIAN OCEAN
SOUTH ATLANTIC OCEAN
Gough Is.
30° E
EAST 0° WEST
30° W
AFRICA
Tristan da Cunha

date. Nonetheless, fur seals marked as part of tagging studies have been sighted away from their natal island. These include; three subantarctic fur seal individuals tagged at Marion Island resighted at Heard Island and South Africa (Bester, 1989), another tagged at Ile Amsterdam resighted at Macquarie Island (S.D. Goldsworthy, personal communication), and a subantarctic fur seal and an Antarctic fur seal tagged at Macquarie Island, both resighted at Iles Kerguelen (C. Guinet and M.-A. Lea, personal communication). While these sightings are not indicative of migration *per se*, they indicate the enormous capability that these seals have for dispersal.

History of the Macquarie Island Fur Seal Population

The fur seal population at Macquarie Island is believed to have once numbered approximately 200,000, but was rendered extinct as a result of sealing activities by about 1840 (Ling, 1999). At this time, fur seal skins commanded a high price on the international market, ensuring a competitive and secretive sealing industry. Sealers were constantly on the look out for new sealing grounds, and when found, they were kept secret for as long as possible to enable exploitation without competition. Such was the situation at Macquarie Island, which was discovered in 1810 by Captain Frederick Hasselburgh (Cumpston, 1968). While Hasselburgh tried to keep the discovery of the island a secret, he failed, and by 1820, the population was declared commercially extinct (Cumpston, 1968), with very few seals found after this time (Shaughnessy and Fletcher, 1987). The sealing effort then shifted to the elephant seal (*Mirounga leonina*) and penguin populations (*Aptenodytes patagonicus* and *Eudyptes schlegeli*) (Cumpston, 1968), which resulted in successions of sealing gangs residing on the island almost continually until the 1900s. Fur seal skins were still valuable, so when fur seals were sighted on the island, they were killed (Shaughnessy and Fletcher, 1987). A small number of skins were harvested from 1874-1914 (n=180), and such sustained pressure ensured that the fur seal population was unable to re-establish for over 100 years. Macquarie Island was declared a sanctuary in 1933 (Cumpston, 1968), and commercial hunting of the wildlife was prohibited. The Australian National Antarctic Research Expeditions (ANARE) established a permanent station on the island in 1948, which has led to almost constant observations of the fur seal population since. New Zealand fur seals were reported on the island since these observations commenced, but breeding was not recorded until the 1954/1955 season, when a single pup was reported with its mother at Goat Bay (Csordas, 1958). Since then, the fur seal population has been in the process of recovery, with only one or two pups born in the early years, then increasing more rapidly (13.5% *per annum* between 1954-1996) with the total pup production for 2000 being 160 (S.D. Goldsworthy, unpublished data). The identity of the original species of fur seal on Macquarie Island prior to sealing is unknown, as there are no known specimens from this time, although many theories are proposed (Richards, 1994; Shaughnessy and Fletcher, 1987).

History of the Southern Ocean Fur Seal Populations

All fur seal populations in the Southern Ocean were subjected to similar levels of exploitation as observed at Macquarie Island. The secretive nature of the sealing industry has meant that few records of the era remain, and thus it is difficult to ascertain the true extent of sealing at each of the populations. Nevertheless, it is apparent that there were massive reductions in all of the populations throughout the region, with many believed to have been driven to extinction. A further complication is that few records exist that make any attempt to differentiate between the species of fur seal that were taken from each island. It is unclear whether the species that were affected by sealing were those that currently occur throughout the Southern Ocean region, and/or were species that have become extinct as a result of the these activities. Nevertheless, it is assumed that the current distribution of the Antarctic and subantarctic fur seal probably reflects their historical distribution, as it seems unlikely with the rapidity of colonisation that has occurred since the cessation of sealing that these species would not have recolonised islands that they inhabited previously. However, it is also possible that these species have colonised islands that they had not previously inhabited, especially if some historical constraints are no longer operating.

The Antarctic fur seal was thought to be extinct until small populations were discovered at Bouvetøya in 1928, and on Bird Island near South Georgia in the early 1930s (Olstad, 1929 as cited in Fevoden and Sømme, 1976; Bonner, 1968; Laws, 1973). While it is generally assumed from the records that populations at Iles Kerguelen, Heard Island, the South Sandwich Islands and Iles Crozet were rendered extinct from sealing, clear records are only present for Macquarie Island, and possibly the South Shetland Islands. Nonetheless, the recovery of this species from the low numbers that were reported from the 1930s has been spectacular, with the population growing from approximately 100 individuals detailed above to an annual pup production of 378,000 in 1990/1991 (Hofmeyr *et al.*, 1997). The Antarctic fur seal now has an almost circumpolar distribution as described above.

The subantarctic fur seal also suffered major population reductions, and a range contraction as a result of sealing, but probably to a lesser extent than that observed for the Antarctic fur seal. Populations at Ile Amsterdam, Gough Island and Marion Island are believed to have survived the era, while those at Iles Crozet and Macquarie Island did not.

Field and Laboratory Work

I collected skin biopsy samples and made field observations at Macquarie Island between 1994 and 1996, with the help of some field assistants. All of the samples that were collected as part of the phylogenetic chapter (Chapter 2) and the population genetics chapters (Chapters 3 and 4) were collected as part of collaboration agreements with a number of biologists around the world. Due to the wide and remote distribution of the otariid seals, the logistical and financial expense of contributing samples to this study is large. As such, contributors were offered co-authorship to the resulting paper, which accounts for the large number of co-authors listed for two of the papers in the thesis.

I conducted all of the laboratory work at the Molecular Genetics Laboratory, University of Queensland and the Molecular Zoology Laboratory, University of Tasmania.

Organisation of the Thesis

The thesis is presented as a series of papers that have either been published in scientific journals, or are in the process of publication. As such, each chapter is completely self-contained, which may lead to some repetition in content throughout the thesis. Chapter 2 contains the phylogenetic analysis of the family Otariidae, and was published in the journal Molecular Phylogenetics and Evolution in November 2001. Chapter 3 contains the population genetics analysis of the Antarctic and subantarctic fur seals as exhibited by mitochondrial DNA, and was published in Molecular Ecology in 2000. Chapter 4 contains population genetics analysis of these same two species but based on microsatellite DNA. This chapter has been submitted for publication to the Journal of Heredity, and is currently in review. Chapter 5 contains the investigation into the species composition and extent and direction of hybridisation within the Macquarie Island fur seal population. This chapter is currently being prepared for submission to the Journal of Zoology, London, Series B. Chapter 6 contains some concluding comments and suggestions for further research.

All citation and co-author details for published chapters are included at the start of each chapter. The content of each chapter is exactly as has been published/submitted for publication, except that the references have been removed and incorporated at the end of the thesis in one section. Appendices (where relevant) were also removed and incorporated separately at the end of the thesis.

In all cases, I was the senior author, and conducted all of the laboratory work, analysis of data and writing of the paper. My co-authors were generous in their contributions of samples for the project, advice on analysis, and/or contributed by reviewing the papers in preparation for publication.

CHAPTER 2: Phylogenetic relationships within the eared seals (Otariidae: Carnivora), with implications for the historical biogeography of the family

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Abstract

Phylogenetic relationships within the family Otariidae were investigated using two regions of the mitochondrial genome. A 360 base pair region of the cytochrome *b* gene was employed for the primary phylogenetic analysis, while a 356 base pair segment of the control region was used to enhance resolution of the terminal nodes. Traditional classification of the family into the subfamilies Arctocephalinae (fur seals) and Otariinae (sea lions) is not supported, with the fur seal *Callorhinus ursinus* having a basal relationship relative to the rest of the family. This is consistent with the fossil record which suggests that this genus diverged from the line leading to the remaining fur seals and sea lions about 6 million years ago (mya). There is also little evidence to support or refute the monophyly of sea lions. Four sea lion clades and five fur seal clades were observed, but relationships among these clades are unclear. Similar genetic divergences between the sea lion clades (D_a=0.054-0.078), as well as between the major *Arctocephalus* fur seal clades (D_a=0.040-0.069) suggest that these groups underwent periods of rapid radiation at about the time they diverged from each other. Rapid radiations of this type make the resolution of relationships between the resulting species difficult and indicate the requirement for additional molecular data from both nuclear and mitochondrial genes. The phylogenetic relationships within the family and the genetic distances among some taxa highlight inconsistencies in the current taxonomic classification of the family.

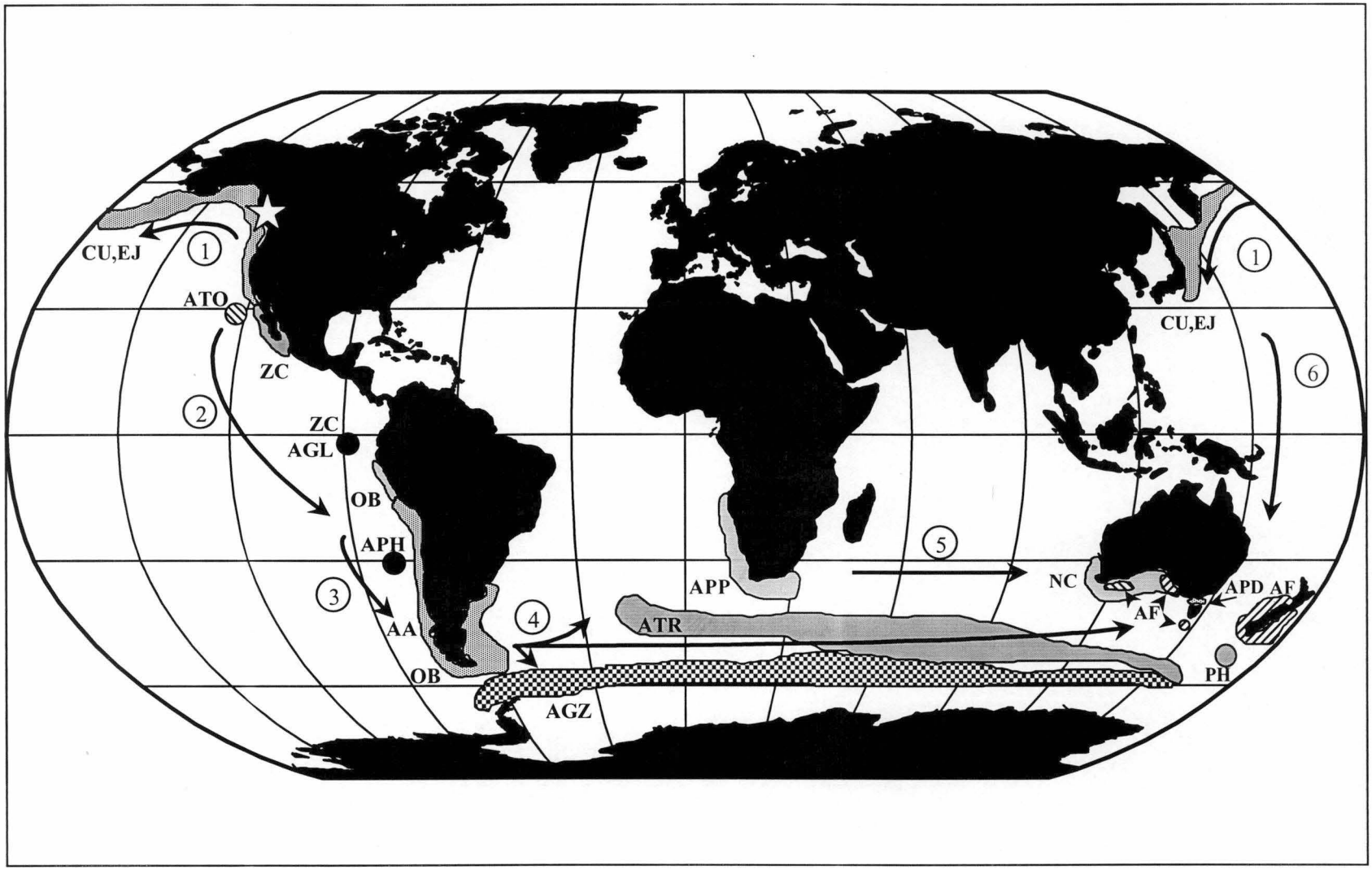
*** This chapter is presented as published in the journal Molecular Phylogenetics and Evolution but with a number of minor editorial changes as suggested by PhD Thesis Examiners. Where further comment was required, these have been included as footnotes under the heading "Post-Publication Note"**

Introduction

The family Otariidae (Order Carnivora) contains the 14 extant species of fur seals and sea lions. The most recent classification of the family (Rice, 1998) is based upon skull morphology, dentition and geographic distribution as proposed in the historic assessments of both fur seal and sea lion species (Scheffer, 1958; King, 1960; King, 1969; Repenning *et al.*, 1971). However in the past, there has been some confusion with otariid taxonomy, primarily because the broad geographic distribution of the family (Figure 2.1) made it difficult to obtain sufficient skeletal material from all species, and because there appears to be convergence of some morphological characters noted for some taxa (Berta and Demere, 1986). While a more comprehensive selection of material and a greater understanding of the distribution and ecology of species has served to reduce this confusion, there are still some elements of the current taxonomy that require further study.

The division of the Otariidae into two subfamilies, Arctocephalinae (fur seals) and Otariinae (sea lions), has been commonly recognised in the literature (e.g. Riedman, 1990; Reynolds *et al.*, 1999). The fur seals are represented by two genera (*Callorhinus* and *Arctocephalus*), while the sea lions are represented by five (*Phocarctos*, *Neophoca*, *Zalophus*, *Eumetopias* and *Otaria*). However, two lines of evidence suggest such a subfamilial classification is misleading. Only a few diagnostic characters separate the groups (e.g. the presence/absence of underfur, the presence of 5 or 6 upper canines), and these may not be not sufficient to warrant the division (Repenning *et al.*, 1971; King, 1983). Further, a number of recent studies have suggested that the subdivision into two subfamilies is ambivalent. This includes a taxonomic review based on anatomical descriptions (Brunner, 2000) and a comprehensive phylogenetic analysis based primarily on fossil and morphological evidence (Bininda-Emonds *et al.*, 1999). The latter suggests that the sea lions and the genera *Arctocephalus* and *Callorhinus* form a polytomy, indicating that the monophyly of the Arctocephalinae could not be assured.

Figure 2.1 (over page): Current distribution and dispersal patterns of the extant Otariidae, adapted from King (1983) and Repenning *et al* (1979): CU= *Callorhinus ursinus*; EJ= *Eumetopias jubatus*; ATO= *Arctocephalus townsendi*; ZC= *Zalophus californianus*; AGL= *A. galapagoensis*; OB= *Otaria byronia**; APH= *A. philippii*, AA= *A. australis*; AGZ= *A. gazella*; ATR= *A. tropicalis*; APP= *A. pusillus pusillus*; NC= *Neophoca cinerea*; AF= *A. forsteri*; APD= *A. pusillus doriferus*; PH= *Phocarctos hookeri.* Star indicates approximate centre of evolution of the Otariidae, and arrows indicate the proposed pattern of dispersal of both fur seals and sea lions determined from the literature and the presented phylogeny. (1) The Otariodea (which includes the Enaliarctidae) had an amphi-Pacific distribution from at least 18 mya. (2) After the divergence of *Callorhinus* (~6 mya), the fur seals disperse south, before 5 mya and after the closure of the Central American Seaway. Sea lions followed about 3 mya. (3) Fur seals disperse to both coasts of South America Sea lions follow later. (4) Fur seals disperse to Africa and the subantarctic with the assistance of the West Wind Drift. (5) *A. pusillus* colonises Australia from South Africa. (6) Sea lions move south from the north western Pacific into Australia and New Zealand about 3 mya. *We use *Otaria byronia* (de Blainville, 1820) rather than *Otaria flavescens* following the arguments of King (1978).


CU,EJ
1
ATO
ZC
2
ZC
AGL
OB
APH
3
AA
OB
4
AGZ
APP
ATR
5
NC
AF
1
CU,EJ
6
APD
AF
PH

Such a relationship between *Callorhinus*, *Arctocephalus* and the sea lions is also proposed in the fossil record (e.g. Repenning *et al.*, 1979; Miyazaki *et al.*, 1994). The modern fur seals and sea lions evolved from the ancestral family Enaliarctidae about 11 million years ago (mya) (Repenning, 1976; Repenning *et al.*, 1979; Miyazaki *et al.*, 1994). *Arctocephalus* is characterised by ancestral character states such as dense underfur and the presence of double rooted cheek teeth and is thus thought to represent the most 'primitive' line (Kim *et al.*, 1975). It was from this basal line that both the sea lions and the remaining fur seal genus, *Callorhinus*, are thought to have diverged. The fossil record from the western coast of North America presents evidence for the divergence of *Callorhinus* about 6 mya, whereas fossils in both California and Japan suggest that sea lions did not diverge until some 3 million years later (Kim *et al.*, 1975; Repenning *et al.*, 1979; Miyazaki *et al.*, 1994).

Both the sea lions and the genus *Arctocephalus* underwent a period of rapid radiation after the lines diverged. The exact process of these radiation events is unknown, aside from inferences made from the distribution and relationships of the resulting 13 extant species. However, the systematic relationships of some of these taxa are unclear, partly because of the high degree of convergence of several morphological characters in a number of taxa (Berta and Demere, 1986). One species that presents difficulties is *Arctocephalus pusillus*. This species has been regarded as having an "enigmatic taxonomic position" because of its similarity to sea lions in size, skull morphology and behaviour (Stirling and Warneke, 1971; Trillmich and Majluf, 1981; Goldsworthy *et al.*, 1997). C.A. Repenning even suggests that the subfamilial classification of the Otariidae could be better upheld if *A. pusillus* was removed from the equation, as these subfamilies could then be separated on the basis of skull morphology (as cited in Stirling and Warneke, 1971). Furthermore, the status of the two subspecies of *A. pusillus*, the Cape fur seal from South Africa (*A. p. pusillus*) and the Australian fur seal (*A. p. doriferus*) is also questionable. The skulls of both taxa are indistinguishable from each other, yet Repenning *et al.*, (1971) tentatively maintained the subspecific classification based on only one character. The reports of interspecific and intergeneric hybrids within the Otariidae (e.g. Goldsworthy *et al.*, 1999; Rice, 1998 and references therein) also motivates questions about the closeness of the putative species' and genera. Thus, further evidence is required to better investigate taxonomic relationships within the Otariidae.

Molecular genetic techniques are useful for providing evidence for taxonomic studies, as shown by those that have investigated the phylogenetic relationships of the Pinnipedia within the Carnivora (e.g. Sarich, 1969; Slade *et al.*, 1994; Arnason *et al.*, 1995). Only two studies have sought to address the question of intra-familial relationships within the Otariidae using molecular techniques (Lento *et al.* 1995; Lento *et al.*, 1997). However, these studies contained only representative taxa of the family. A more comprehensive approach was adopted by Bininda-Emonds *et al.*, (1999) who compiled data from a range of studies pertaining to all species within the Carnivora for phylogenetic analysis. These data included morphological, molecular and fossil data. While it included the work of Lento *et al.* (1995),

molecular information for all species in the family were not available. Here we present additional molecular evidence of the phylogenetic relationships of the Otariidae through the screening of all species within the family using two regions of mitochondrial DNA.

Materials and Methods

Sample Collection

All extant fur seal and sea lion species within the family Otariidae are represented in the phylogenetic analysis. Details of the sampling of each species are presented in Tables 2.1 and 2.2. Skin biopsies or blood were obtained from most species for extraction of genomic DNA and sequencing. For the remaining species, either genomic DNA was supplied, or the sequence data for the relevant genes were obtained from Genbank.

Laboratory Analysis

Total genomic DNA was extracted from blood and skin biopsies using the modified CTAB/proteinase K extraction protocol outlined in Wynen *et al.* (2000). The polymerase chain reaction (PCR) was used to amplify a region of the mitochondrial $tRNA^{thr}$ -control region using the primers Thr/Pro and Cent (Wynen *et al.*, 2000). PCR was also used to amplify the first 429 base pairs (bp) of the cytochrome *b* gene. A 25 µl reaction volume consisted of 17.775 µl milliQ water, 0.125 µl 10 mM dNTPs, 1.5 µl 25 mM $MgCl_2$, 2.5 µl 10xbuffer (500 mM KCl, 100 mM Tris pH=9.0, 1% Triton X), 1.0 µl each of 10 µM primers, 0.1 µl Taq polymerase (5-10 units), 1.0 µl extracted DNA and was overlaid with oil. Amplifications were conducted under the following conditions: 1 cycle of 94 °C for 2 min.; 8 cycles of 94 °C for 30 sec., 48 °C for 30 sec., 72 °C for 40 sec.; 25 cycles of 94 °C for 15 sec., 52 °C for 15 sec., 72 °C for 40 sec; and 1 cycle of 25 °C for 1 min. The primers used were adapted from Lento *et al.* (1994): Cyb2 and B-Glu-L (but without 5'-biotinylation).

All PCR products were purified using the Concert Rapid PCR Purification System (Life Technologies). Sequencing of the 5' end of the control region and 360bp of the cytochrome *b* gene was conducted using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer). The products were run through a 6M urea/5% acrylamide gel (FMC Long Ranger Singel) on an ABI Prism 377 DNA sequencer (PE Applied Biosystems). The primers employed for sequencing the regions were the same as used for amplification. Each fragment was sequenced initially from the 5' end and only sequenced from the 3' end if the first sequence was too short and/or contained too many ambiguous sites. The sequenced product of the $tRNA^{thr}$ -control region corresponds to sites 16342-16680 of the Gen Bank sequence for *Phoca vitulina* (accession number X63726, Arnason and Johnsson, 1992). The cytochrome *b* region employed corresponds to sites 15094-15454 of the same *P. vitulina* sequence.

Species	Label	Common Name	Populations Represented
Arctocephalus gazella	AGZ	Antarctic fur seal	Bouvetøya*
A. tropicalis	ATR	Subantarctic fur seal	Amsterdam Is., Iles Crozet*
A. forsteri	AF	New Zealand fur seal	Kaikoura, Taumaka Peninsula, Cape Saunders & Snares Is., N.Z., Maatsuyker Is., Tasmania*
A. philippii	APH	Juan Fernàndez fur seal	El Tongo & Los Harenes, Juan Fernàndez Archipelago*
A. galapagoensis	AGL	Galapagos fur seal	Galapagos Islands
A. australis	AA	South American fur seal	Punta San Juan, Peru
A. townsendi	ATO	Guadelupe fur seal	Guadelupe Islands
A. pusillus pusillus	APP	Cape fur seal	South Africa
A. p. doriferus	APD	Australian fur seal	Moriarty Rocks, Tenth Is., Reid Rocks & Judgement Rocks, Tasmania
Callorhinus ursinus	CU	Northern fur seal	St Paul Is., Alaska
Eumetopias jubatus	EJ	Steller's sea lion	Aleutian Islands & Kuril Islands
Zalophus californianus	ZC	Californian sea lion	San Nicolas, San Miguel, Punta Banda & Gulf of California, California
Otaria byronia	OB	Southern sea lion	Punta San Juan, Peru
Neophoca cinerea	NC	Australian sea lion	Western Australia
Phocarctos hookeri	PH	Hooker's sea lion	Figure of Eight Is., Enderby Is., & Macquarie Is.
Phoca vitulina	PV	Harbour seal	Iceland

Table 2.1: Details of fur seal and sea lion taxa used for phylogenetic analysis, including the abbreviated label used to identify individuals in analyses, the common name and the populations from which samples were obtained. *Haplotypes were selected from these species to exhibit the range of variation known to occur within that species based on previous studies (Lento, 1995; Lento *et al.*, 1994; Wynen *et al.*, 2000; Goldsworthy *et al.*, 2000).

2.2A. Cytochrome *b*

Taxa	ID	OTU	Hap.	π	Var.	Ts	1	2	3	Tv	1	2	3	Accession No.
AGZ	1-5 [1]	5	5	0.012	10	**10**	0	0	10	**0**	0	0	0	AF380878-882
ATR	1-5 [1]	5	3	0.007	4	**4**	0	1	3	**0**	0	0	0	AF380883-887
AF	1-5 [3]	5	5	0.023	15	**12**	0	2	10	**3**	1	0	2	U12837, U12839, U12841,U18537-38
APH	2-3, 5 [1]	3	3	0.009	5	**4**	1	0	3	**1**	1	0	0	AF380893-895
AGL	1-3 [6]	3	1	0.000	0	**0**	0	0	0	**0**	0	0	0	AF380898-900
AA	1-5 [1]	5	2	0.015	9	**9**	0	0	9	**0**	0	0	0	AF380901-905
ATO	5-6 [1]	2	1	0.000	0	**0**	0	0	0	**0**	0	0	0	AF380896-897
APP	1-5 [3]	5	5	0.006	5	**5**	0	1	4	**0**	0	0	0	U18448-52
APD	2-4 [1]	3	2	0.002	1	**1**	0	0	1	**0**	0	0	0	AF380916-918
CU	1-5 [1]	5	3	0.006	2	**1**	0	0	1	**1**	0	0	1	AF380888-892
EJ	1-5 [1]	5	2	0.001	1	**1**	0	0	1	**0**	0	0	0	AF380920-924
ZC	10-11 [8,9]	2	1	0.000	0	**0**	0	0	0	**0**	0	0	0	D26524, X82310
OB	1-5 [1]	5	2	0.002	2	**2**	0	0	2	**0**	0	0	0	AF380906-910
NC	1-5 [1]	5	1	0.000	0	**0**	0	0	0	**0**	0	0	0	AF380911-915
PH	6 [3], 7 [1]	2	2	0.003	1	**1**	0	0	1	**0**	0	0	0	U12851, AF380919
PV	PV [10]	1	1	-	-	**-**	-	-	-	**-**	-	-	-	X63726
OVERALL*		**60**	**38**		**93**	**76**	**8**	**8**	**60**	**17**	**5**	**0**	**12**	

2.2B. Control Region

Taxa	ID	OTU	Hap.	π	Var.	Ts	Tv	Seq. length	Accession No.
AGZ	1-5 [2]	5	5	0.050	32	31	1	289-97	AF384376-380
ATR	1-5 [2]	5	5	0.054	32	29	3	290-91	AF384381-385
AF	1-5 [4]	5	5	0.055	30	25	5	293-98	U12837, U12839, U12841,U18537-38
APH	1-5 [5]	5	5	0.038	22	22	0	285-91	AF384403-407
AGL	1 [6]	1	1	-	-	-	-	286	AF384386
AA	1-5 [1]	5	4	0.078	39	38	1	295-97	AF384398-402
ATO	4, 6 [1]	2	2	0.021	6	6	0	289	AF384396-397
APP	-	-	-	-	-	-	-	-	
APD	1-4 [1]	4	3	0.004	2	2	0	288	AF384392-395
CU	1-5 [1]	5	4	0.027	17	16	1	287-90	AF384387-391
EJ	1-5 [1]	5	4	0.004	3	3	0	291-92	AF384414-418
ZC	1-5 [7]	5	5	0.032	18	12	6	276	L37023-25,28,32
OB	1-5 [1]	5	4	0.008	5	5	0	288-89	AF384419-423
NC	1-5 [1]	5	1	0.000	0	0	0	289	AF384408-412
PH	1-5 [4], 7 [1]	6	4	0.004	3	1	2	287-89	AF384413 (PH7)
PV	PV [10]	1	1	-	-	-	-	339	X63726
OVERALL*		**63**	**52**		**113**	**69**	**44**		

Table 2.2 (previous page): Details of sequences used for phylogenetic analysis and intra-specific variation for **A.** partial cytochrome *b* (360bp) and **B.** control region (356bp) sequences. The identifying (ID) number of each individual sequence and its origin are presented, along with the total number of operational taxonomic units (OTUs), haplotypes (Hap.) and the nucleotide diversity (π) for each species and both regions. The number of variable sites (Var.) are also presented, including the number of transition (Ts) or transversion (Tv) mutations. For cytochrome *b*, the numbers of these mutations occurring at each codon position (1,2 or 3) are also given. For the control region, the range of sequence lengths prior to alignment are also presented. Taxa are abbreviated according to their scientific names and are as described in Table 2 1. [1]Sequenced as part of this study; [2]Sequenced as part of Wynen *et al.* (2000); [3]Genbank (Lento *et al.*, 1997); [4]Lento (1995); [5]Goldsworthy *et al* (2000); [6]Sequenced as part of this study by Rus Hoelzel; [7]Genbank (Maldonado *et al.*, 1995); [8]Genbank (Masuda and Yoshida, 1994); [9]Genbank (Arnason *et al.*, 1995); [10]Genbank (Arnason and Johnsson, 1992). Genbank accession numbers are listed where relevant; * Overall values presented for ingroup taxa only.

Data Analysis

Sequences were examined using Seqed (version 1.0.3; Applied Biosystems Inc.) to ascertain quality and to verify scoring. Sequences were aligned using CLUSTAL W (Thompson *et al.*, 1994) and the resulting alignments were evaluated by eye and corrected where required[1]. Intra- and inter-specific sequence statistics were calculated using DnaSP version 2.2 (Rozas and Rozas, 1997). These data included the number of polymorphic sites, nucleotide diversities and divergences. Nucleotide diversities (π) and nucleotide divergences (D_a) were calculated from Nei (1987; equations 10.5 and 10.21 respectively).

The phylogenetic relationships of the family were examined primarily using cytochrome *b* sequence data, as it has been shown to be useful for the inference of intra-specific to inter-generic relationships (Kocher *et al.*, 1989). Prior to analysis, the presence of a hierarchical structure in the data was determined using PAUP* version 4.0ba (Swofford, 1999). A frequency distribution of 500,000 randomly generated, equiprobable trees was plotted and the g_1 score determined (Hillis and Huelsenbeck, 1992). This sample statistic (an estimate of the population parameter, γ_1) was used to test for asymmetry by comparing it with the critical values in Table B.22 (Zar, 1996).

Subsequently, three methods of reconstructing phylogenetic relationships were employed: the Maximum Parsimony (MP), Maximum Likelihood (ML) and Neighbour Joining (NJ) methods. All tree reconstructions proceeded without a specified root, with *P. vitulina* nominated as the outgroup *post priori.* This species was chosen as it is from the Phocidae, a sister family to the Otariidae (Vrana *et al.*, 1994).

MP analysis was conducted in PAUP*. A full heuristic search was conducted with 100 random stepwise additions (5 trees held over at each replicate), accelerated character transformation (ACCTRAN), and employing the tree bisection-reconnection (TBR) branch swapping algorithm. Where a consensus tree needed to be generated, it was either by strict

[1] Post Publication Note - Evaluation by eye was conducted solely for the highly variable 'CT region' of the control region sequence which alignment program used could not cope with due to the dramatic length variations observed.

consensus or in accordance to the 50% majority rule criterion. Bootstrap analysis employing the full heuristic search conditions as described above was conducted with 2000 replications.

This analysis was repeated for a range of weighting schemes that were employed to investigate whether different mutational models might better reflect the relationships. These include (1) all mutations unweighted; (2) transversion (Tv) mutations weighted as the reciprocal of the transition (Ts)/transversion ratio (Ts/Tv)[2], which in this case equalled 7; (3) using the same weighting scheme as described in (2) but only at the 3rd codon position; (4) excluding all Ts mutations; or (5) excluding all 3rd codon positions.

NJ trees were reconstructed in MEGA version 1.01 (Kumar *et al.*, 1993) using the algorithm of Saitou and Nei (1987), and based on distances calculated using Kimura's 2 parameter model (Kimura, 1980). A bootstrap analysis was performed on each of the resulting trees, and values were obtained after 2000 replications. A standard error test was also performed (Rzhetsky and Nei, 1992; 1993) to examine the significance of the interior branch lengths. Sequences for reconstructing NJ trees include all nucleotides or Tv changes only.

The ML analyses were conducted in PHYLIP 3.57c (Felsenstein, 1993) using DNAML. All trees were reconstructed with global rearrangements, 10 randomised additions, and without a specified outgroup. The Ts/Tv ratio was varied until the maximum log likelihood value was obtained. The analysis was also conducted with three independent rates of mutation specified, 0, 1 and 2. Trees were also reconstructed using DNAMLK, where the ML method is employed as in DNAML, but under the constraint of a molecular clock. Evidence for a molecular clock is then examined by comparing trees from DNAML and DNAMLK using the likelihood ratio test as described in the DNAMLK notes (Felsenstein, 1993). A relative rate test was also employed to test for molecular clock-like sequence evolution (the two-cluster test as implemented in LINTRE, Takezaki *et al.*, 1995).

The control region data were also used to examine the phylogenetic relationships within the Otariidae. However, the rapid mutation rate of this region suggests that it is more suitable for the inference of relationships at the terminal nodes, rather than at the internal nodes. Therefore, these data were employed to examine those major clades observed in the phylogenies based on cytochrome *b* in greater detail. Phylogenetic analysis proceeded using the same methods as described above. Characters were treated the same as specified in (1) and (4) above, as well as in (2) but with Ts/Tv = 4. In all cases, alignment gaps were removed prior to analysis, except for one MP analysis where gaps were treated as a 5th character state.

Phylogenetic analysis using all three above-mentioned methods with unweighted characters was conducted with both the cytochrome *b* and control region combined. A partition

[2] Post Publication Note: Tv - pyrimidine base substituted for a purine base, or vice versa eg. A-C, T-G ; Ts - purine base is substituted for another purine, or a pyrimidine for another pyrimidine eg. A-G, T-C.

homogeneity test was conducted using PAUP* prior to further analysis to ascertain phylogenetic congruence. A heuristic search was employed, with 100 random stepwise additions, accelerated character transformation (ACCTRAN), and with the tree bisection-reconnection (TBR) branch swapping algorithm. Results were obtained after 1000 homogeneity replicates.

Results

A total of 61 cytochrome *b* sequences were obtained for analysis, and a further 64 from the control region. All taxa were represented in both data sets, with the exception of *A. p. pusillus* for which there were no control region sequences. There were large variations in sequence length observed in the control region (276bp to 298bp for the ingroup taxa; up to 339bp for the outgroup taxon; Table 2.2B) making sequence alignment problematic. The alignment between *A. forsteri* and *P. vitulina* in Slade *et al.*, (1994) was used as a guide[3]. The final aligned 'array' was 356bp. There were no alignment gaps required for the 360bp of the cytochrome *b* region used for analysis.

Details regarding intra-specific genetic variation for both cytochrome *b* and control region, and inter-specific nucleotide divergences for cytochrome *b* only, are presented in Tables 2.2 and 2.3 respectively. There is considerable variability in nucleotide diversities within species. *Neophoca cinerea* showed no variation in either cytochrome *b* or the control region, while the maximum nucleotide diversities for each region were 2.3% (*A. forsteri*, Table 2.2A) and 7.8% (*A. australis*, Table 2.2B) respectively. The two species that showed the highest nucleotide diversities for the more conserved cytochrome *b* region (*A. forsteri* 2.3%, *A. australis* 1.5%) each contained two highly divergent clades. The pairwise divergence between these intra-specific clades (0.031 and 0.025 respectively) were larger than those observed between *A. forsteri*, *A. australis* and *A. galapagoensis* (0.008-0.017, mean 0.011; Table 2.3). These intra-specific divergences are also much larger than observed between the subspecies *A. p. pusillus* and *A. p. doriferus* (0.002).

The above results and some preliminary phylogenetic analyses based on cytochrome *b* revealed a number of relationships requiring further attention. A two tiered approach to analysis was thus adopted to investigate these relationships. Firstly, the more conserved cytochrome *b* region was employed to investigate all relationships within the family. The control region data were then included to try and improve the resolution of these relationships. The inclusion of the two regions together necessitated a rationalisation of the dataset, as only individuals for which both regions had been sequenced could be included. Further, to adequately investigate the phylogenetic relationships of the family based on the cytochrome *b* data, such a rationalisation was required to ensure the successful completion of the more demanding analyses, such as the bootstrap analyses. Secondly, a more

[3] Post Publication Note: The alignment of Slade *et al.* was used as a guide as it was the only published alignment available at the time for this region.

	AGZ	AA	AF	AGL	APH	ATO	ATR	APD	APP	CU	EJ	ZC	OB	NC	PH
AGZ		11	9	16	17	18	15	20	18	35	23	23	17	21	17
AA	0 038		0	2	13	14	12	17	17	35	19	21	17	21	13
AF	0 034	0 008		4	13	14	13	18	17	35	20	20	16	20	13
AGL	0 049	0 009	0 017		16	18	19	25	24	42	22	24	23	25	18
APH	0 055	0 041	0 045	0 046		1	21	26	24	34	24	25	24	23	16
ATO	0 057	0 044	0 046	0 050	0 004		22	27	25	38	26	29	25	25	17
ATR	0 049	0 039	0 040	0 054	0 062	0 063		5	4	29	18	20	17	21	17
APD	0 063	0.052	0 056	0 070	0 074	0.076	0 017		0	33	23	26	19	25	20
APP	0 059	0 050	0 052	0 067	0 070	0 071	0 014	0 002		31	22	25	19	23	20
CU	0 105	0 107	0 105	0 121	0 100	0 110	0 089	0 094	0 092		34	33	36	33	37
EJ	0 066	0 058	0 062	0 061	0 070	0 072	0 052	0 065	0 061	0 099		19	27	27	24
ZC	0 069	0 063	0 061	0 067	0 071	0 081	0 058	0 073	0 070	0 096	0 054		22	28	23
OB	0.055	0 054	0 049	0 066	0 069	0.071	0 051	0 055	0 055	0 104	0 077	0 063		25	20
NC	0 065	0 063	0 064	0 069	0 066	0 060	0 061	0 069	0 066	0 096	0 075	0 078	0 073		20
PH	0 056	0.040	0 041	0 050	0.045	0 047	0.048	0 056	0 055	0 107	0 067	0 064	0 059	0 056	
PV	0 157	0 150	0 146	0 156	0 163	0 172	0 148	0 150	0 152	0 148	0 163	0 156	0 149	0 150	0 156

Table 2.3: Cytochrome *b* data - pairwise comparisons of the number of substitutions (above diagonal) and the nucleotide divergence (D_a) (below diagonal). Taxa are abbreviated according to their scientific names and are as described in Table 2.1.

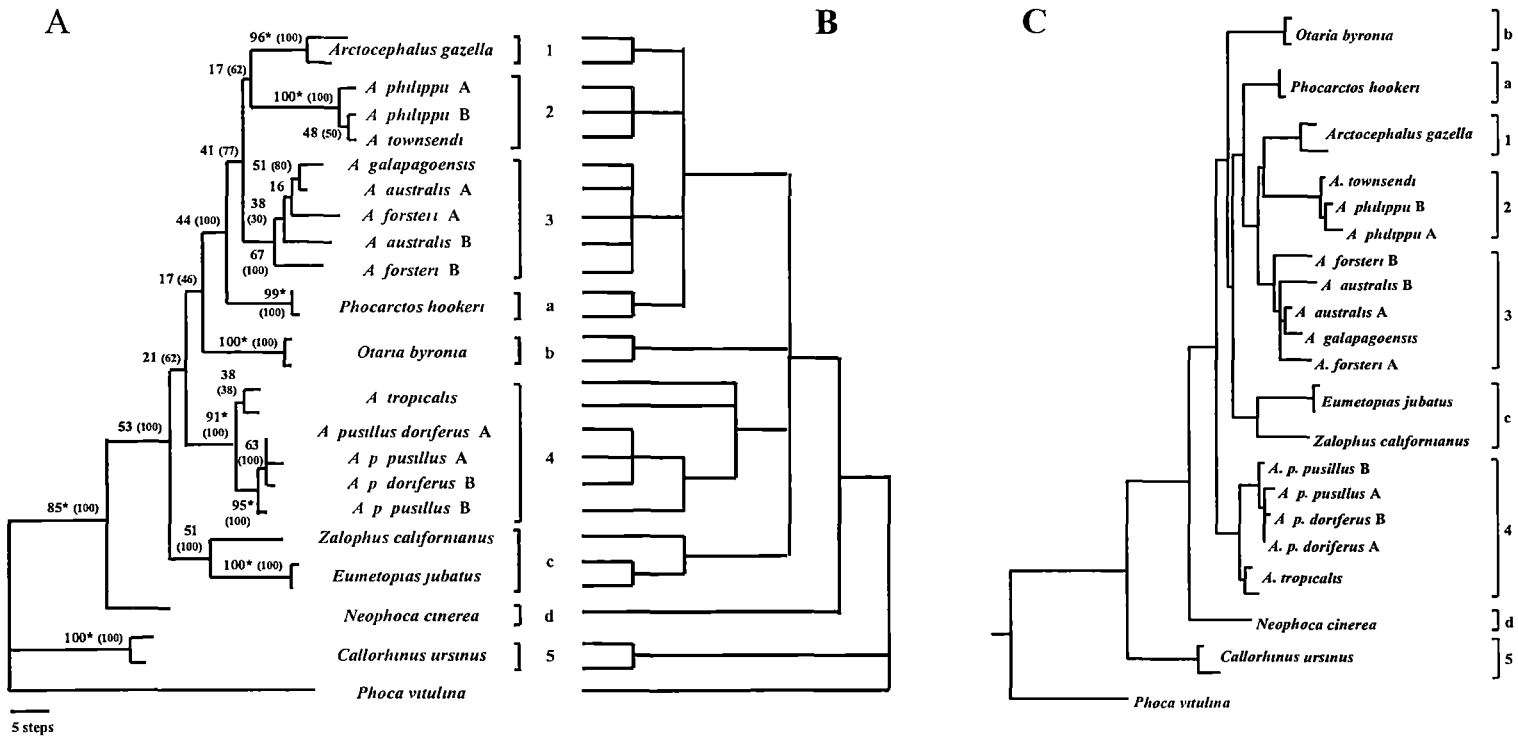
focussed approach was undertaken with a number of taxa where intraspecific relationships were obscuring the interspecific relationships. Therefore, further phylogenetic analyses were conducted on these and closely related taxa, encompassing as many individuals as possible

The following criteria were used when removing individuals prior to more in depth phylogenetic analysis. Where no intra-specific variation was observed, only one individual was included for the species for the phylogenetic analysis (e.g *Neophoca cinerea*). Where highly divergent lineages were evident within some species (e.g. *A. forsteri*, *A. australis*), the two most divergent individuals for these species was included for analysis. This ensured that the range of detected genetic variation for that species as sampled in this study was encompassed in the analyses. A total of 27 and 28 operational taxonomic units (OTUs) were used for analyses based on cytochrome *b* and control region respectively.

It has been demonstrated that the addition of taxa improves the accuracy of the phylogenetic tree (Wheeler, 1992; Graybeal, 1998). The removal of the above taxa in this case could be interpreted as a reduction in the accuracy of the resulting phylogenies. However, Graybeal (1998) also suggested that the addition of taxa is particularly beneficial if these taxa "break up long branches", and with the only taxa removed in this study being those that contain little or no additional phylogenetic information, the reduction in phylogenetic accuracy is likely to be minimal. Where the problems arise with the highly divergent taxa, the more focussed analysis serves to overcome these.

Only individuals for which both cytochrome *b* and control region sequences had been obtained were used for the combined region analyses. There are two exceptions: one OTU each for *Z. californianus* and *A. townsendi*. Since Maldonado *et al.* (1995) found no variation in 368bp of the same cytochrome *b* region of 40 *Z. californianus* individuals, the combination of the regions from individuals of unknown origin was not considered problematic in this case. The *A. townsendi* OTU (ATO45) resulted from the control region sequence of ATO4 and cytochrome *b* sequence of ATO5. While this is not ideal, this combined sequence was used only for subsequent phylogenetic analyses aimed at better resolving relationships of some fur seal clades and not for any intraspecific analysis. A total of 26 OTUs were used for these analyses where both control region and cytochrome *b* sequences were combined.

Figure 2.2 (over page): Results of phylogenetic analyses of 15 species of the family Otariidae, inferred from partial cytochrome *b* sequences: **A.** MP tree obtained through 50% majority rule consensus from 260 MP trees (TL=221; CI=0.593; RI=0.720). Figures at the nodes indicate bootstrap values obtained after 2000 replications, and those in brackets show the percent agreement for that node by the 260 MP trees. * indicates where branch lengths are significant based on the NJ tree; **B.** Strict consensus of the 260 MP trees; **C.** ML phenogram obtained where the Ts:Tv ratio was 6. Major fur seal and sea lion clades are respectively labelled 1-5 and a-d.


A
B
C
96* (100)
17 (62)
100* (100)
48 (50)
41 (77)
51 (80)
16
38
(30)
44 (100)
67
(100)
17 (46)
99*
(100)
100* (100)
21 (62)
38
(38)
91*
(100)
53 (100)
63
(100)
95*
(100)
85* (100)
51
(100)
100* (100)
100* (100)
Arctocephalus gazella
A philippii A
A philippii B
A townsendi
A galapagoensis
A australis A
A forsteri A
A australis B
A forsteri B
Phocarctos hookeri
Otaria byronia
A tropicalis
A pusillus doriferus A
A p pusillus A
A p doriferus B
A p pusillus B
Zalophus californianus
Eumetopias jubatus
Neophoca cinerea
Callorhinus ursinus
Phoca vitulina
1
2
3
a
b
4
c
d
5
5 steps
Otaria byronia
Phocarctos hookeri
Arctocephalus gazella
A. townsendi
A philippii B
A philippii A
A forsteri B
A australis B
A australis A
A galapagoensis
A. forsteri A
Eumetopias jubatus
Zalophus californianus
A. p. pusillus B
A p. pusillus A
A p. doriferus B
A. p. doriferus A
A. tropicalis
Neophoca cinerea
Callorhinus ursinus
Phoca vitulina
b
a
1
2
3
c
4
d
5

Phylogenetic analysis - cytochrome b

Of the 360bp of the cytochrome *b* gene available for analysis, 112 sites were variable, and 86 were parsimony informative where the outgroup taxon is included. Excluding this taxon, these figures change to 93 and 86 respectively (Table 2.2A). The frequency histogram of tree lengths from the 500,000 randomly generated trees was significantly skewed (g_1=-0.75634; 112 variable sites; P<0.01), indicating the presence of hierarchical structure within the data.

The phylogenetic relationships of the Otariidae as inferred by the cytochrome *b* region employing the MP and ML methods are displayed in Figure 2.2. The tree in Figure 2.2A was obtained using the MP method using unweighted characters and is the 50% majority rule consensus representation of the 260 most parsimonious trees. The tree length (TL) was 221 steps, with a consistency index (CI) of 0.593, and a retention index (RI) of 0.720. A series of major fur seal and sea lion clades with medium to high levels of bootstrap support were identified, and these are labelled 1-5 for fur seals and a-d for sea lions (Figure 2.2). A strict consensus representation is also presented (Figure 2.2B) and shows how these major clades were maintained in all of the 260 MP trees, as well as the maintenance of the basal positions of both *C. ursinus* and *N. cinerea*.

Further MP analyses proved less revealing with an overall reduction in resolution. When Tv and Ts were weighted as 7:1 (Ts/Tv=7), both overall and at 3rd codon positions only, 12 most parsimonious trees were obtained in both cases (TL=413; CI=0.680; RI=0.740 and TL=377; CI=0.650; RI=0.737). The two consensus topologies were identical to each other and were very similar to that shown in Figure 2.2A, with the same major clades (as described above) being recognised in all cases. The differences between these MP trees and that in Figure 2.2A lay in how the major clades were related. This reflects the poor resolution between these major clades that was consistently observed throughout our analyses. Even less support for these relationships was observed when the phylogeny was reconstructed based on the sequences excluding 3rd codon positions (no. sites = 240, no. parsimony sites/variable sites = 19/31, and 19/21 excluding the outgroup). Eighteen most parsimonious trees were obtained (TL=46, CI=0.696, RI=0.791), but with about 79% of Ts and 71% of Tv occurring at 3rd codon positions, there was insufficient variation remaining to resolve relationships (frequency histogram of 400,000 randomly generated trees was not significantly skewed, g_1=-0.453965, 31 variable sites, P>0.2). While our overall sequence was quite short, (Kallersjo *et al.*, 1999) also noted that despite popular belief, the 3rd codon positions contain most of the phylogenetic structure in the data set, and does not recommend their exclusion. Three most parsimonious trees were obtained using only Tv mutations (TL=30; CI=0.833; RI=0.839), but again, there was insufficient variation in this dataset to be of use in resolving relationships of the taxa (no. sites = 25 or 16 excluding the outgroup; frequency histogram of 400,000 randomly generated trees was not significantly skewed, g_1=-0.648180, 25 variable sites, P>0.1).

The reconstruction of phylogenetic relationships employing the ML method produced a range of topologies that were obtained using different weighting schemes. The tree that had the highest ML value (Ts/Tv ratio = 6, ln likelihood = -1633.10) is presented in Figure 2.2C. The major fur seal and sea lion clades identified in the MP analyses were also identified here. All topologies obtained through ML analysis contained all of these major clades, with the differences between topologies lying in the relationships of these clades to each other.

Similarly, NJ topologies showed all of the major fur seal and sea lion clades noted in Figure 2.2. Some differences were observed in the relationships between these clades, but these differences occurred where there was very poor bootstrap support (ranging from 20-41%). The standard error test showed that the interior branch lengths that were significant (ie. where the confidence probability, CP > 0.95 or 0.99) corresponded to those nodes where the bootstrap support was 92 or greater. These nodes are the same nodes in Figure 2.2A with bootstrap support greater than 91. As found for MP analysis, the NJ tree based on the exclusion of 3rd codon positions resulted in very poor resolution.

Overall, the best resolved topologies showed the highly supported basal position of *C. ursinus*, and the maintenance of the major fur seal and sea lion clades identified in Figure 2.2. The position of *N. cinerea* basal to the rest of the fur seal and sea lion taxa was also maintained, albeit with less bootstrap support. The very high bootstrap support for many of the major clades is significant, because it was noted in a study by Cummings *et al.* (1995), that while phylogenies obtained from data sets with small numbers of nucleotides (such as is the case in this study) infrequently reflect the true phylogeny of the genome, clades with bootstrap support > 95% in the former are usually present in the latter. The major differences observed between topologies came in the relationships between the remaining major clades. There were also discrepancies in the internal arrangements of both the *A. australis/forsteri/galapagoensis* clade (clade 3) and the *A. philippii/townsendi* clade (clade 2), which were investigated further as described below.

The topologies created by DNAML and DNAMLK for Ts/Tv=6 were not identical, and therefore the likelihood ratio test as described by Felsenstein (1993) was unable to be used. Instead, the relative rate test of Takezaki *et al.* (1995) was employed, and no significant differences in the relative mutation rates of all the sequences were observed (Q=22.23; $0.5<p<0.75$). A linear regression analysis was conducted of genetic divergence (calculated using the cytochrome *b* data) versus estimated time of divergence based on the fossil record. The dates used were of the proposed divergences: *Phoca vitulina* (representing Phocidae) from *Callorhinus* (23 mya); *Phoca vitulina* from the sea lion and *Arctocephalus* taxa (23 mya); *Callorhinus* from the sea lion and *Arctocephalus* taxa (6 mya); sea lion taxa from *Arctocephalus* taxa (3 mya) (Kim *et al.*, 1975; Repenning *et al.*, 1979; Miyazaki *et al.*, 1994; Slade *et al.*, 1994). While the analysis showed a significant relationship (R^2=0.868, $P<<0.001$), the 95% confidence limits were very large, and little faith could be placed in estimating the time of divergence using the line of regression, especially for those

divergences close to the time of interest (ie. 3 mya). Further, these data assume the monophyly of the sea lion and *Arctocephalus* groups, and our analysis has shown that this assumption is not valid. Therefore, estimating times of divergence from this regression would not be valid.

Phylogenetic analysis - control region

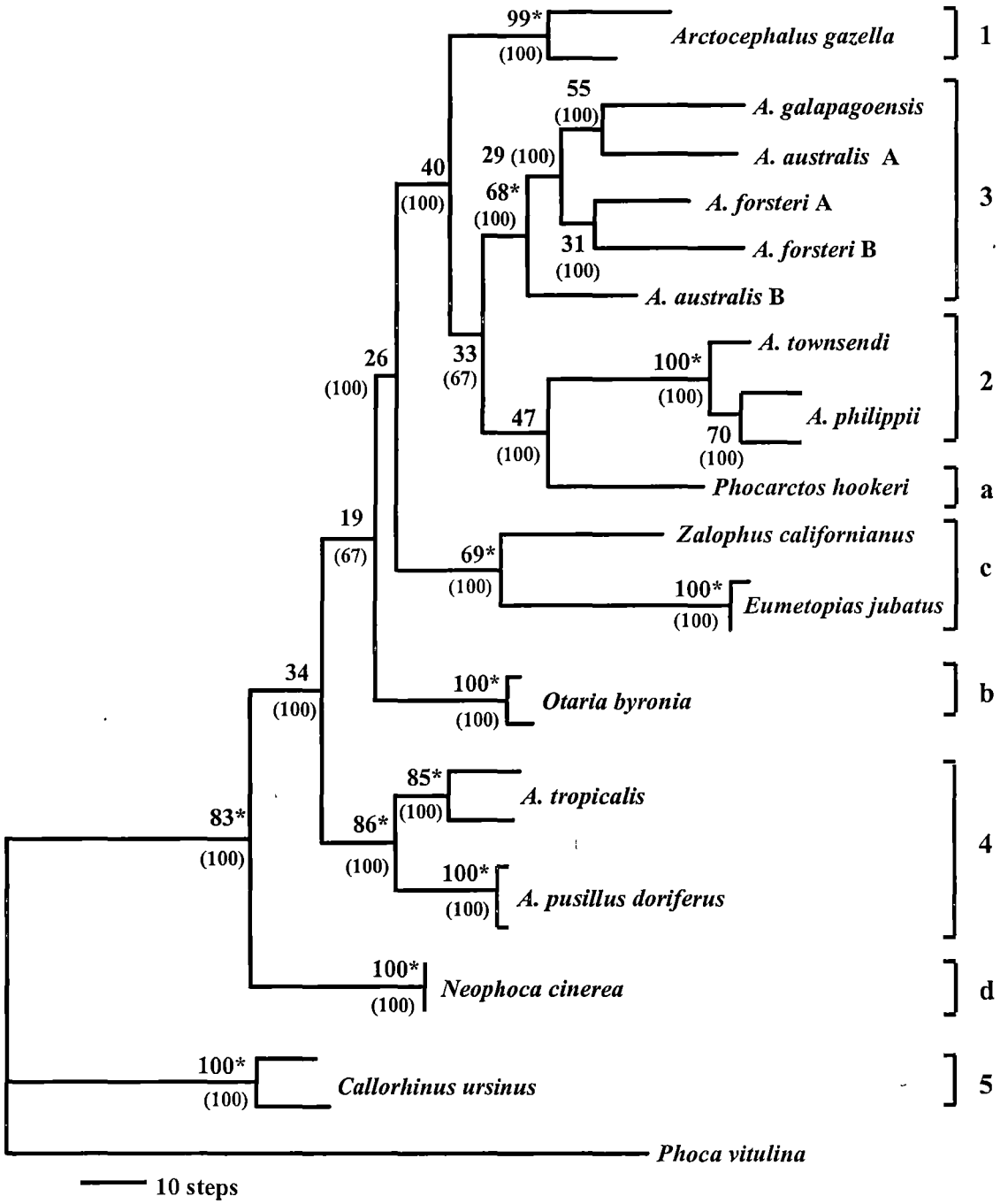
A 356bp 'array' of the 5' end of the control region was used for analysis, with 162 variable sites and 117 of these being informative (outgroup taxon included). The removal of alignment gaps and missing information left 253bp, with 118 variable sites and 89 parsimony informative sites (outgroup taxon included). These figures changed to 106 and 89 respectively when the outgroup was excluded (Table 2.2B). Evidence of hierarchical structure was observed as the histogram of the 500,000 random trees was skewed (g_1=-0.7937; 158 variable sites; P<0.001).

MP analysis of the control region data returned a single most parsimonious tree of 442 steps (CI=0.500; RI=0.589). A single most parsimonious tree was also found when alignment gaps were treated as a 5th character (TL=613; CI=0.520; RI=0.584;). The two topologies differed greatly from those obtained using cytochrome *b*, as the bootstrap support for internal nodes were extremely low (3-37), with most being below 10 (trees not shown). The two least supported of the major clades observed in Figure 2.2A (bootstrap values < 70%) were split: *E. jubatus* from *Z. californianus*; and *A. galapagoensis* from *A. forsteri/A. australis*. Similar results were observed in the topologies that were created using the NJ algorithm. The phylogeny with the highest ML value was obtained for a Ts/Tv ratio of 3 (ln likelihood = -2221.45). This topology retained all the major clades except that of *E. jubatus/Z. californianus*.

Phylogenetic analysis - cytochrome b and control region

A partition-homogeneity test was performed on the combined cytochrome *b*-control region data and found that the null hypothesis of homogeneity within the data set could not be rejected (P=0.482). As expected, there was evidence of structure within these data (g_1=-1.034; 265 variable sites; P<<0.001). Three most parsimonious trees were found (TL=654; CI=0.53; RI=0.602), and the consensus topology (obtained through 50% majority rule criteria) is presented in Figure 2.3. All of the major fur seal and sea lion clades are recognised with medium to high level of bootstrap support, and all of the branch lengths leading to these major clades in the NJ tree were significant when tested with the standard

Figure 2.3 (over page): Results of phylogenetic analysis of 15 species of the family Otariidae, inferred from combined cytochrome *b*/control region sequences and using the MP algorithm. The MP tree was obtained through 50% majority rule consensus from 3 MP trees (TL=654; CI=0.53, RI=0.602) Figures at the nodes indicate bootstrap values obtained after 2000 replications, and those in brackets show the percent agreement for that node by the 3 MP trees. * indicates where branch lengths are significant based on the NJ tree. As for Figure 2.2, major fur seal clades are numbered from 1-5, and major sea lion clades are marked a-d.

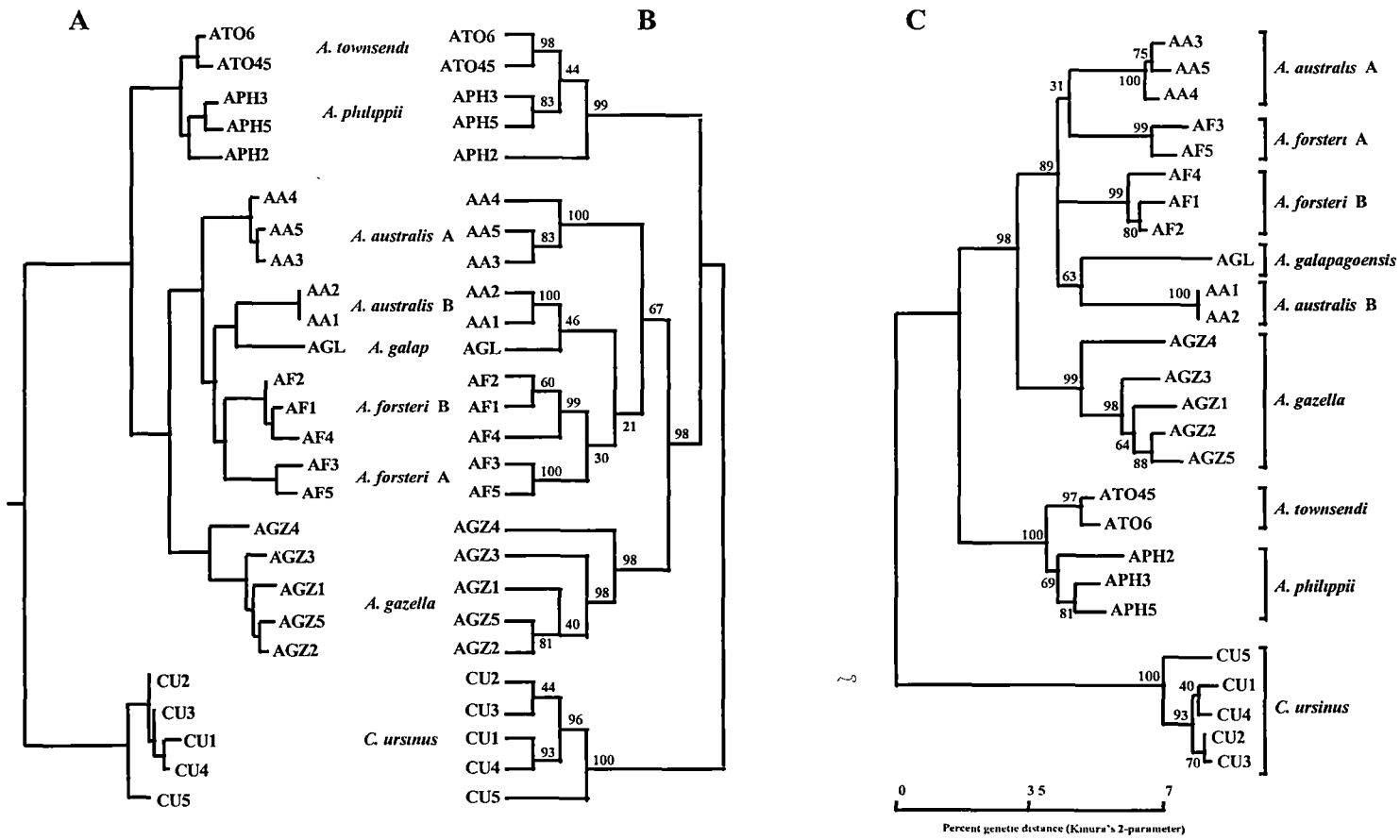

99*
(100)
Arctocephalus gazella
1
55
(100)
A. galapagoensis
A. australis A
29 (100)
40
(100)
68*
(100)
A. forsteri A
31
(100)
A. forsteri B
A. australis B
3
33
(67)
A. townsendi
100*
(100)
26
(100)
A. philippii
70
(100)
47
(100)
Phocarctos hookeri
2
a
19
(67)
Zalophus californianus
69*
(100)
100*
(100)
Eumetopias jubatus
c
34
(100)
100*
(100)
Otaria byronia
b
85*
(100)
A. tropicalis
83*
(100)
86*
(100)
100*
(100)
A. pusillus doriferus
4
100*
(100)
Neophoca cinerea
d
100*
(100)
Callorhinus ursinus
5
Phoca vitulina
10 steps

error test. However, while the combining of data sets has been reported to increase internal support in topologies (Soltis *et al.*, 1998) as well as increasing the probability of reflecting the true whole genome tree (Cummings *et al.*, 1995), there are still difficulties in resolving the internal relationships of these major clades for our data.

The results from Figures 2.2 and 2.3 reveal some interesting and inconclusive relationships between fur seal taxa, e.g. between *A. australis*, *A. forsteri* and *A. galapagoensis* or *A. philippii* and *A. townsendi* (clades 3 and 2 respectively). To better investigate these relationships, all of the individuals available for these taxa and those of *A. gazella* (clade 1) were incorporated into an analysis based on combined control region/cytochrome *b* sequence data. All three methods of tree reconstruction were used, and the results are presented in Figure 2.4. Greater resolution is observed for relationships between species with the inclusion of the control region data and additional individuals. However, the relationships of the divergent lineages within *A. forsteri* and *A. australis* to each other and to *A. galapagoensis* remain poorly resolved. In all cases, *A. gazella* is shown to be a sister taxon to these three species. The relationships of *A. philippii* and *A. townsendi* are better resolved apart from the position of one *A. philippii* lineage (APH2). Either the lineage is included with the remaining *A. philippii* lineages (as loosely supported in the ML and NJ trees: Figure 2.4A and C), or as a sister taxon to both species (see MP tree: Figure 2 4B). Those topologies reconstructed from the control region data alone (not presented here), give greater support to *A. philippii* being distinct from *A. townsendi* while still being retained in the same clade (bootstrap support to each monophyletic group of 60 and 96 respectively after 2000 replicates using MP).

Although further investigation into the close relationship of *A. p. doriferus* and *A. p. pusillus* is required, this was not pursued in this study due to the absence of control region sequences for the latter species. No further improvement in resolution of sea lion taxa (clades a-d, Figures 2.2 and 2.3) was possible with the data currently in hand. Additional, more conserved markers e.g. ND2, ND5 and/or a nuclear gene are required to adequately investigate these relationships.

Figure 2.4 (over page): Phylogenetic relationships of six species of the genus *Arctocephalus* from clades 1, 2 and 3 in Figures 2.2 and 2.3 with *Callorhinus ursinus* as outgroup. All trees reconstructed using combined cytochrome *b*/control region sequence data. Details for each are as follows: A. ML phenogram obtained where the Ts:Tv ratio was 8; B. MP tree obtained through 50% majority rule from 40 MP trees (TL=355; CI=0.586; RI=0.825); C. NJ tree; Figures at the nodes of trees B and C indicating bootstrap values obtained after 2000 replications. Labels as listed in Table 2.1.


A
B
C
ATO6
ATO45
APH3
APH5
APH2
AA4
AA5
AA3
AA2
AA1
AGL
AF2
AF1
AF4
AF3
AF5
AGZ4
AGZ3
AGZ1
AGZ5
AGZ2
CU2
CU3
CU1
CU4
CU5
A. townsendi
A. philippii
A. australis A
A. australis B
A. galap
A. forsteri B
A. forsteri A
A. gazella
C. ursinus
A. australis A
A. forsteri A
A. forsteri B
A. galapagoensis
A. australis B
A. gazella
A. townsendi
A. philippii
C. ursinus
0
3 5
7
Percent genetic distance (Kimura's 2-parameter)

Discussion

Phylogenetic Relationships

This study presents molecular data for investigation into the phylogenetic relationships within the family Otariidae. We found no support for the recognition of two subfamilies containing the fur seals (Arctocephalinae) and sea lions (Otariinae). The fur seal *Callorhinus ursinus* was found to be basal to the remaining fur seal and sea lion taxa in the family, a relationship that received high bootstrap support across all analytical methods employed. This supports evidence from the fossil record that suggests *Callorhinus* diverged from the line leading to extant sea lion and *Arctocephalus* fur seal species about 6 mya (Miyazaki *et al.*, 1994). While some studies argue that morphological data support our findings by suggesting that *Callorhinus* is distinct compared to the rest of the family (Brunner, 2000; Repenning *et al.*, 1971), others show findings to the contrary (Berta and Demere, 1986). A further study, based on a compilation of available morphological, fossil and molecular data indicated that neither the basal position of *Callorhinus* nor the monophyly of Arctocephalinae could be assured (Bininda-Emonds *et al.*, 1999).

This study presented some evidence to refute the monophyly of sea lions, a relationship that has been claimed elsewhere (Kim *et al.*, 1975; Morejohn, 1975; Berta and Demere, 1986; Bininda-Emonds *et al.*, 1999). We identified four major sea lion clades and four southern fur seal clades, all of which received high levels of bootstrap support. The relationships between these clades consistently infer polyphyly for sea lions and fur seals, although there was very little support for the deep internal nodes. We found that divergences between sea lion taxa are large (D_a=0.054-0.078, mean=0.067; Table 2.3), with the average being similar to that between the sea lion group and *Arctocephalus* (D_a=0.061). Therefore, if there was a single sea lion ancestor as proposed by other studies, then our data implies that at the time of divergence from the *Arctocephalus* line about 3 mya, there was a rapid radiation within the sea lion group that resulted in the five extant monotypic genera. Such a rapid radiation and/or population expansion has made the resolution of relationships at these deep nodes difficult using the markers employed in this study. Nonetheless, the phylogeny presented here (with respect to the sea lion taxa) is very similar to that presented by Barnes *et al.* (1985), with the exception of the relatively close relationship between the northern sea lions, *Eumetopias* and *Zalophus*, identified here. Neither was this relationship evident in other phylogenies (Morejohn, 1975; Bininda-Emonds *et al.*, 1999). Rather, it was between *Neophoca* and *Phocarctos* that the closest intergeneric relationship was observed (Morejohn, 1975; Barnes *et al.*, 1985; Bininda-Emonds *et al.*, 1999). Although the pairwise divergence between these species is similar to that between *Eumetopias* and *Zalophus* (0.056 and 0.054 respectively), this is not reflected in the phylogenies presented here (Figures 2.2 and 2.3).

A sister-taxon relationship for *Arctocephalus pusillus* and *A. tropicalis* was consistently observed in our analyses. This is interesting because *A. pusillus* has often been regarded as phenotypically intermediate between the southern fur seals and sea lions on the basis of

its behaviour, size, vocalisation and morphology (Repenning *et al.*, 1971; Stirling and Warneke, 1971; Trillmich and Majluf, 1981; Goldsworthy *et al.*, 1997). There is no evidence in the molecular data that the phenotypic intermediacy of *A. pusillus* reflects a close phylogenetic affinity with any sea lion lineage. Berta and Demere (1986) shows a similar close relationship, although *A. gazella* was also found to make up the third taxon of a trichotomy. In our study however, *A. gazella* appeared to be more closely related to the *A. australis*/*A. galapagoensis*/*A. forsteri* clade (Figure 2.4).

However, the relationship between *A. pusillus* and *A. tropicalis* is inconsistent with the study by Lento *et al.* (1997) which showed reciprocal paraphyly of haplotypes between *A. tropicalis* and *A. gazella*. The samples used in that study were obtained from the small population at Macquarie Island, where hybridisation has been reported between these species (Shaughnessy *et al.*, 1988; Goldsworthy *et al.*, 1999). In the current study, we found a large number of species-specific nucleotide differences in cytochrome *b* between *A. gazella* and *A. tropicalis* (n=15). When we compare the sequences from Lento *et al.* (1997) with those in this study, their *A. tropicalis* samples show haplotypes identical to our *A. gazella* samples. Further, these Macquarie Island samples were also used by Lento (1995) to obtain control region sequences. These sequences were subsequently compared with a larger study of 248 individuals of both species (Wynen *et al.*, 2000). Species-specific haplotypes were observed in the control region, and again, the *A. tropicalis* samples of Lento (1995) were found to have haplotypes identical to those of *A. gazella*. It is therefore probable that these *A. tropicalis* samples from Macquarie Island were from hybrid individuals. As such, this interspecific hybridisation might have affected the composite phylogeny produced by Bininda-Emonds *et al.* (1999) whose study incorporated the phylogeny of Lento *et al.* (1995).

The phylogenetic relationships as inferred in Figures 2.2, 2.3 and 2.4 suggest a close relationship between *A. philippii* and *A. townsendi* (bootstrap values of 100). The interspecific divergence between these species was extremely low (D_a=0.004, Table 2.3), and is similar to that observed between the subspecies *A. pusillus pusillus* and *A. p. doriferus* (D_a=0.002, Table 2.3). However, these results differ from those of some other studies which propose that *A. philippii* is more closely related to the *A. australis*/*A. forsteri*/*A. galapagoensis* group than to *A. townsendi* (Berta and Demere, 1986; Bininda-Emonds *et al.*, 1999). Prior to these studies, the close relationship between *A. townsendi* and *A. philippii* had been recognised to the extent where it had been proposed that they be classed as subspecies (Scheffer, 1958) or placed together into the separate genus *Arctophoca* (Sivertsen, 1954). This view is supported by a recent taxonomic review of the family by Brunner (2000). However, Repenning *et al* (1971) retained them as separate species based on skull morphology but conceded that this conclusion was based on a small sample size. Our results are also from a small sample size, and thus a reassessment of the taxonomic position of *A. philippii* and *A. townsendi* would require a greater sampling effort encompassing all populations of both species.

Highly divergent lineages were observed within both *A. forsteri* (D_a=0.031; 11 fixed differences) and *A. australis* (D_a=0.025; 9 fixed differences). The relationships of these lineages are poorly resolved, and while the inclusion of control region data and additional individuals provided greater support for the intra-clade relationships (Figure 2.4), there is still no evidence of monophyly of both *A. australis* and *A. forsteri*. Regional differences in *A. forsteri* had been previously reported by Shaughnessy (1970) based on transferrin types, and the divergent lineages within this species had also been reported by Lento *et al.*, (1997), based on cytochrome *b*. The latter study suggested these lineages might be a result of two pre-sealing populations: one extending from southern and western Australia to the east coast of New Zealand (which might be called *A. forsteri forsteri)*, and the other primarily occurring in the subantarctic islands of New Zealand (*A. f. snaresensis*). Lento *et al.* (1997) proposed that the current distribution and geographic overlap of haplotypes from each clade may be explained by secondary contact as the latter moved north and west, colonising empty rookeries that were a result of sealing. A similar scenario might explain the two divergent lineages observed in *A. australis*. King (1983) notes that there is some evidence for the presence of two subspecies occurring within *A. australis*, with those occurring on mainland South America (*A. australis gracilis*) being smaller than those occurring at the Falkland Islands (*A. a. australis*). It is possible that the two subspecies are represented by the two divergent lineages found in this study. However, all of the *A. australis* skin samples obtained for this study came from Punta San Juan in Peru. So, if the lineages are representative of the subspecies, then like *A. forsteri*, it is possible that there is some secondary contact between these two populations. However, more extensive sampling is required for both species to better investigate these intra-specific questions. This is achieved not only through increasing the number of individuals sampled, but also by ensuring all populations throughout the entire geographic range are represented.

Repenning *et al.* (1971) had noted that the skull characters of many of the *Arctocephalus* species' suggest a relationship to, and possible descent from *A. australis* or an *A. australis*-like ancestor. The authors also suggest that there is a gradational series from *A. australis* to *A. forsteri* to *A. gazella*, although it was noted that it was unknown how this applied to phylogeny. Our phylogenetic analysis found no evidence that *A. pusillus*, *A. tropicalis*, *A. philippii* or *A. townsendi* were direct descendants or close relatives of *A. australis*, but rather evolved from lineages that diverged at more or less the same time from the ancestral *Arctocephalus* line. However, Figure 2.4 shows a relatively close relationship between *A. australis*, *A. forsteri* and *A. galapagoensis* despite the poor resolution of these relationships. Further, it was also shown that *A. gazella* shared a common ancestor with this clade, suggesting that the gradational series above may be in some way indicative of the phylogenetic relationships of these taxa. The relatively close relationship of *A. australis*, *A. forsteri* and *A. galapagoensis* was also noted through anatomical similarities (Brunner, 2000).

Biogeography - Current and Historical

While many relationships in the presented phylogenies remain unresolved, those that are supported are consistent with the proposed dispersal patterns of the Otariidae (Repenning *et al.*, 1979) (Figure 2.1). The north-east Pacific region is considered the centre of origin of the family, which evolved under temperate climatic conditions (Repenning *et al.*, 1979; Miyazaki *et al.*, 1994). However two equatorial crossings and subsequent dispersal and radiation has led to a much broader geographical distribution of this family in recent times (Figure 2.1). *Callorhinus* stayed in the northern hemisphere, but both the sea lion and *Arctocephalus* fur seal groups dispersed further afield.

Fossils found in Peru of seals ancestral to *Arctocephalus* suggest that this line had dispersed south along the coast of North and South America some time prior to 5 mya (Repenning, 1976). The average genetic divergence between the major fur seal clades (excluding *Callorhinus*) tends to be lower than observed between the sea lions (D_a=0.040-0.069, mean =0.047, cf. 0.054-0.078, mean=0.067). Assuming a clock-like rate of mutation, as demonstrated with the cytochrome *b* data in this study, these results suggest that a similar radiation occurred in the fur seals as the sea lions, but at a slightly later time. Such a radiation probably accompanied the far-reaching dispersals of fur seals throughout the Southern Ocean. The resulting broad distribution of the *Arctocephalus* fur seals (Figure 2.1) is a reflection of their remarkable dispersal abilities as has been reported in the literature (e.g. Torres and Aguayo, 1984; Shaughnessy and Burton, 1986).

The common ancestor to *A. tropicalis* and *A. pusillus* probably moved around Cape Horn, and dispersed to the east facilitated by the West Wind Drift. *A. tropicalis* colonised islands in the north subantarctic, while *A. pusillus* colonised south west Africa. Recent migration events from Africa to Australia led to the subspecies, *A. pusillus doriferus*. The large geographical separation, yet the close genetic relationship of *A. townsendi* and *A. philippii* makes it difficult to speculate on the dispersal of their ancestral line. This is especially so given that the range of *A. galapagoensis* lies in between the ranges of these two species. While it is likely that the Peru Current sweeping up the west coast of South America, bypassing the Juan Fernàndez Islands, would facilitate immigration to the Galapagos Islands from the south, it is difficult to hypothesize on the mechanisms leading to current distribution of *A. philippii* and *A. townsendi*, straddling that of a less related species. The remaining fur seal species, *A. gazella*, *A. australis*, *A. galapagoensis* and *A. forsteri* appear to share a common ancestor (Figure 2.4). The line leading to *A. gazella* dispersed into the subantarctic, probably colonising islands to the south of South America and eventually throughout the Southern Ocean. *A. forsteri* and *A. galapagoensis* diverged later, as the former probably dispersed east or west to Australia and New Zealand.

Fossil evidence also suggests a major dispersal south by the sea lions about 3 mya, but with separate dispersals occurring on both the east and west sides of the Pacific (Repenning *et al.*, 1979) (Figure 2.1). Aside from suggesting that all sea lion genera diverged at

approximately the same time, there is little our data can add with regards to dispersal patterns. Although the geographic proximity of *Neophoca cinerea* and *Phocarctos hookeri* would suggest a close relationship, there is no evidence of this in the data presented. The similar distributions of *Eumetopias jubatus* and *Zalophus californianus* might be a reflection of a more recent common ancestry, but this is speculative at best. While our molecular data allows some speculation as to the dispersal patterns of both the fur seals and sea lions, additional evidence from fossil deposits, for example, are required to substantiate these speculations. However, there are only a few such deposits from the appropriate time period documented at this stage.

Taxonomic Considerations

Some of the relationships inferred in this study through the use of molecular markers, are inconsistent with current classification. This study raises concern as to how great the genetic distance should be between species, and between subspecies. For example, the divergence between *A. pusillus pusillus* and *A. p. doriferus* (D_a=0.002) is very low, and yet subspecific classification for these taxa was only tentatively maintained by Repenning *et al.* (1971) based on one slight difference in skull morphology, and geographic separation. The divergence between *A. townsendi* and *A. philippii* is comparable (D_a=0.004), yet the retention of these groups as separate species on the basis of skull morphology is supported (Repenning *et al.*, 1971). A contrasting scenario is evident within *A. forsteri* and *A. australis*, where genetic divergences between clades within each species are much larger (D_a=0.031 and 0.025 respectively), even larger than observed between some species.

Clearly such discussion regarding taxonomic classification and phylogenetic relationships can only proceed in the light of all evidence presented. Such an attempt was made by Bininda-Emonds *et al.* (1999), but there was an absence of comprehensive molecular data for the family Otariidae for inclusion in the study. While the current study has presented the first comprehensive molecular study based primarily on one mitochondrial gene, the inference of phylogenetic relationships on the basis of a single locus is to be regarded with caution (Harpending *et al.*, 1998). Further, the number of nucleotides used in the study were very low when compared to more extensive phylogenetic analyses in the literature. It has been demonstrated that increasing the number of characters improves phylogenetic accuracy and resolution (Wheeler, 1992; Graybeal, 1998; Soltis *et al.* 1998). This is especially so when many different loci are included from the genome (in this case, the mitochondrial genome) instead of using large blocks of sequence from the same gene (Cummings *et al.*, 1995). Additional loci incorporated from the nuclear genome would also provide further resolution.

The addition of sequence data from multiple loci is especially required for interpreting the phylogenetic relationships of the Otariidae given the rapid radiation events that appear to have occurred about 3 mya. While it has been suggested that the addition of taxa leads to greater improvements in phylogenetic accuracy relative to the addition of characters

(Graybeal, 1998), this could be arguable in the case of the otariid data set because all extant species within the family are currently represented. What is likely to be more important for future analyses, is the incorporation of all extant genetic variation for all of these species. While only a small sample size is sufficient to investigate interspecific relationships (Tajima, 1983), this is only on the proviso that the samples are randomly collected. With the broad geographic distribution of this family, and of some species in particular, a random sample is difficult to collect. It is imperative, however, that future studies make efforts to sample all populations within a species to cover all the intraspecific genetic variation, a necessary step for the evaluation of phylogenetic relationships within the family Otariidae.

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CHAPTER 3: Post-sealing genetic variation and population structure of two species of fur seal (*Arctocephalus gazella* and *A. tropicalis*)

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Abstract

Commercial sealing in the 18th and 19th centuries had a major impact on the Antarctic and subantarctic fur seal populations (*Arctocephalus gazella* and *A. tropicalis*) in the Southern Ocean. The intensive and unrestricted nature of the industry ensured substantial reductions in population sizes and resulted in both species becoming locally extinct at some sites. However, both species are continuing to recover, through the recolonisation of islands across their former range and increasing population size. This study investigated the extent and pattern of genetic variation in each species to examine the hypothesis that higher levels of historic sealing in *A. gazella* have resulted in a greater loss of genetic variability and population structure compared with *A. tropicalis*. A 316 base pair section of the mitochondrial control region was sequenced and revealed nucleotide diversities of 3.2% and 4.8% for *A. gazella* and *A. tropicalis* respectively. There was no phylogeographic distribution of lineages observed within either species, although the respective Φ_{ST} values of 0.074 and 0.19 were significantly greater than zero. These data indicate low levels of population structure in *A. gazella* and relatively high levels in *A. tropicalis.* Additional samples screened with restriction endonucleases were incorporated, and the distribution of restriction fragment length polymorphism (RFLP) and sequence haplotypes were examined to identify the main source populations of newly recolonised islands. For *A. tropicalis*, the data suggest that Macquarie Island and Iles Crozet were probably recolonised by females from Marion Island, and to a lesser extent Ile Amsterdam. Although there was less population structure within *A. gazella*, there were two phylogeographic regions identified: a western region containing the populations of South Georgia and Bouvetøya, which were the probable sources for populations at Marion, the South Shetland and Heard Islands; and an eastern region containing the panmictic populations of Iles Kerguelen and Macquarie Island. The latter region may be a result of a pronounced founder effect, or represent a remnant population that survived sealing at Iles Kerguelen.

*** This chapter is presented as published in the journal Molecular Ecology but with a number of minor editorial changes as suggested by PhD Thesis Examiners. Where further comment was required, these have been included as footnotes under the heading "Post-Publication Comment"**

Introduction

Commercial sealing during the 18th and 19th centuries resulted in substantial declines in the number and size of fur seal populations throughout the Southern Ocean (Bonner and Laws 1964). The Antarctic and subantarctic fur seal (*Arctocephalus gazella* and *A. tropicalis*) suffered differing degrees of exploitation during this time, with the former reportedly brought to the brink of extinction (Bonner and Laws 1964). However, since the cessation of sealing, these species have recolonised islands throughout their former range, with most populations experiencing rapid increases in size (Hofmeyr *et al.* 1997; Wickens and York 1997). The Antarctic fur seal currently breeds on islands predominantly to the south of the Antarctic Polar Front (APF) (Figure 3.1). Approximately 97% of the species occur in populations at South Georgia (Hofmeyr *et al.* 1997), but its range has recently extended as far west as Macquarie Island. Subantarctic fur seals breed on islands to the north of the APF with the major concentrations occurring at Gough, Amsterdam and the Prince Edward Islands (Hofmeyr *et al.* 1997). Antarctic and subantarctic fur seals occur sympatrically at Iles Crozet, the Prince Edward and Macquarie Islands (Figure 3.1). Hybridisation has been reported at two of these sites (Condy 1978; Kerley 1983a, 1983b; Shaughnessy and Fletcher 1987; Goldsworthy *et al.* 1999).

The discovery of South Georgia in 1775 by James Cook led to the commencement of sealing in this region (Bonner 1958). The vast numbers of seals reported here and on islands further south were harvested in such an intense and indiscriminate fashion, that stocks were rapidly exhausted (Headland 1984). Such a pattern of discovery and subsequent depletion was paralleled throughout the subantarctic. As fur seal stocks declined, the focus shifted to the exploitation of southern elephant seals (*Mirounga leonina*) and southern right whales (*Eubalaena australis*), which were harvested for their blubber (Roberts 1950; Bonner and Laws 1964). Fur sealing was most intense at islands that contained, or were close to large populations of these other species, because fur seals continued to be killed whenever they hauled out (Rand 1956; Bonner and Laws 1964). Many local extinctions resulted, and since the largest populations of elephant seals were on islands south or just north of the APF, *A. gazella* was the most severely exploited. This species suffered a major range contraction and was considered virtually extinct early this century (Bonner and Laws 1964). However, records from early this century indicate that it is likely that *A. gazella* survived in remnant populations at Bouvetøya (numbering approximately 1000-1200 in 1928; Olstad 1929 as cited in Fevoden and Sømme 1976), and on islands off the north-west coast of South Georgia (Bird Island and the Willis group -

Figure 3.1: Map of the islands in the subantarctic region upon which *Arctocephalus gazella* and *A. tropicalis* breed. Reported remnant populations and indications of current population size are shown through estimates of annual pup production figures (Hofmeyr *et al.* 1997; Isaksen *et al.* 1997; Shaughnessy *et al.* 1998, S.D. Goldsworthy, unpublished data). Map modified from original by John Cox (Australian Antarctic Division).

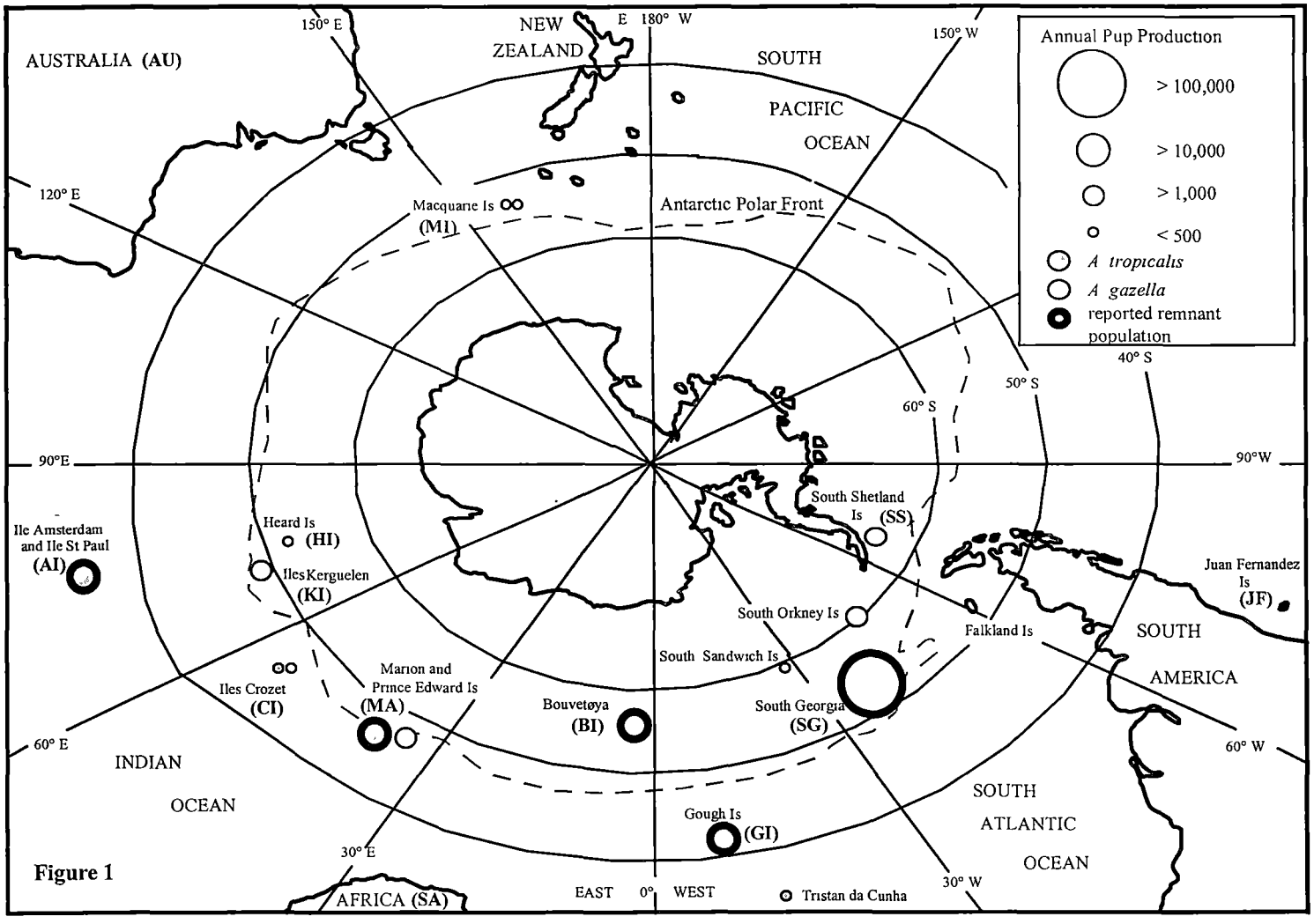

AUSTRALIA (AU)
NEW ZEALAND
SOUTH PACIFIC OCEAN
150° E
E 180° W
150° W
120° E
90°E
90°W
60° E
30° E
EAST 0° WEST
30° W
60° W
40° S
50° S
60° S
Antarctic Polar Front
Macquarie Is (MI)
Heard Is (HI)
Ile Amsterdam and Ile St Paul (AI)
Iles Kerguelen (KI)
Iles Crozet (CI)
Marion and Prince Edward Is (MA)
Bouvetøya (BI)
South Georgia (SG)
South Shetland Is (SS)
South Orkney Is
South Sandwich Is
Falkland Is
Juan Fernandez Is (JF)
Gough Is (GI)
Tristan da Cunha
INDIAN OCEAN
SOUTH AMERICA
SOUTH ATLANTIC OCEAN
AFRICA (SA)
Annual Pup Production
> 100,000
> 10,000
> 1,000
< 500
A tropicalis
A gazella
reported remnant population

Figure 1

estimated at less than 100 in the 1930s: Laws 1973; Bonner 1968). This species has since recovered with a world-wide annual pup production of approximately 400,000 (Hofmeyr 1997; Isaksen 1997).

Records indicate that prior to sealing, *A. tropicalis* were abundant on the Tristan da Cunha group, Ile Amsterdam and Ile St Paul (Clark 1875; Wace and Holdgate 1976). As major populations of *A. tropicalis* occur on islands north of the APF which did not support large populations of elephant seals, they were probably visited by sealers only when fur seal numbers were large enough to ensure an economic return. As such, local extinctions in this species are known to have occurred only at Tristan da Cunha and Ile St Paul (Shaughnessy 1982; Roux 1987). The post-sealing status of *A. tropicalis* is questionable at a number of sites, such as on Prince Edward Island (De Villiers and Ross 1976) and Iles Crozet. There is no evidence that this species occurred at the latter site prior to sealing, (Jouventin *et al.* 1982; Roux 1987), while the identity of the species occurring at Macquarie Island prior to sealing is unknown (Shaughnessy and Fletcher 1987; Richards 1994). There were however, three remnant populations of *A. tropicalis* documented: at Gough, Amsterdam and Marion Islands (Roux 1987; Bester 1987; Kerley 1987). It is at these three islands that the bulk of this species currently resides, containing approximately 99% of the annual pup production for *A. tropicalis* occurring here (73,000 recorded between 1988-94: Hofmeyr *et al.* 1997).

The aim of this study was to examine post-sealing mitochondrial DNA variation in *A. gazella* and *A. tropicalis* to determine whether differences in their respective exploitation histories are reflected in the levels and distribution of observed genetic variation. We use reports of historic sealing records and contemporary data on population recovery to examine two hypotheses. Firstly, if *A. gazella* has passed through a more intense population bottleneck, then it is expected that this species will exhibit lower levels of genetic variation compared with *A. tropicalis*. Secondly, given that *A. gazella* suffered a greater reduction in range due to commercial exploitation, this species will exhibit reduced population structure relative to *A. tropicalis*.

Materials and Methods

Sample collection

Skin biopsies were collected from both *A. tropicalis* and *A. gazella* from all of the major populations across each species' range. These include South Georgia (SG), South Shetland Islands (Seal Island and Cape Shireff) (SS), Bouvetøya (BI), Marion Island (MA), Iles Crozet (CI), Iles Kerguelen (KI), Heard Island (HI) and Macquarie Island (MI) for *A. gazella*; and Iles Crozet (CI), Ile Amsterdam (AI), Gough Island (GI), Marion Island (MA) and Macquarie Island (MI) for *A. tropicalis*. A number of samples from this species were also available from vagrant seals found in the Juan Fernandez Islands (JF), South Africa (SA) and Australia (Melbourne Zoo) (AU) (Table 3.1).

For all populations, pups were targeted to ensure that members of the breeding population, and not vagrants were being sampled. Such a protocol also ensured that the sampled individuals were not full-siblings. At populations where *A. gazella* and *A. tropicalis* occur sympatrically, efforts were made to ensure that individuals sampled were not phenotypic hybrids. At MI, where a large proportion of the pups born annually are hybrid, and the *A. tropicalis* population is small (pup production for 1997/1998 = 27; Goldsworthy *et al.* unpublished data) it was possible that some samples were from hybrid pups. The *A. tropicalis* samples incorporated into the sequence analysis from MI were consistent with the above sampling regime, but the small population of this species meant that the extra samples included for the restriction fragment length polymorphism (RFLP) analyses (described below) may have been full siblings and/or hybrids.

Population	Acronym	Sequenced	RFLP only	Comments
A. gazella				
Sth Shetland Is.	SS	5[1]	26[2]	[1]Seal Is., [2]Cape Shireff
Sth Georgia	SG	20	20	
Bouvetøya	BI	20	20	
Marion Is.	MA	20	34	
Iles Crozet	CI	20	20	
Iles Kerguelen	KI	20	20	
Heard Is.	HI	20[3]	20	[3]only 7 are pups
Macquarie Is.	MI	20	30	
		145	**190**	
A. tropicalis				
Gough Is.	GI	20	19	
Marion Is.	MA	20	19	
Iles Crozet	CI	20	19	
Ile Amsterdam	AI	20	20	
Macquarie Is.	MI	17	12	
South Africa	SA	1		vagrant
Australia	AU	2		vagrants
Juan Fernandez	JF	3		vagrants
		103	**89**	
A. forsteri				
Australia/NZ		17	0	

Table 3.1: Populations of *A. gazella* and *A tropicalis* sampled. Individuals screened for RFLPs were additional to those sequenced. All samples collected from pups unless otherwise stated.

Biopsies were obtained using a 6mm biopsy punch and stored in salt saturated 20% dimethylsulphoxide (DMSO). Attempts were made to obtain samples from at least 20 individuals from each population for DNA sequencing, and an additional number (~20 per population) for RFLP analysis (Table 3.1). This was not possible for all sites. For HI, only 7 of the 40 biopsies collected from *A. gazella* were from pups and only 5 of the *A. gazella* samples obtained from SS (Seal Island) were sequenced. As a result of the problem of hybridisation outlined above, there were only 17 sequences obtained for *A. tropicalis* at MI, and a further 12 individuals screened for RFLPs.

Additional control region sequences were obtained from the New Zealand fur seal, *Arctocephalus forsteri* to assist in examining phylogenetic relationships. Individuals from across the species' geographical distribution were included. Fifteen of these sequences were from Lento (1995), one from Slade *et al.* (1994) (Genbank accession number UO3576) and another from an individual at Taronga Zoo, Australia (sequenced as part of this study). A harbour seal sequence (*Phoca vitulina*) (Arnason and Johnsson 1992; Genbank accession no. X63726 S37044) was used as an outgroup.

Laboratory Analysis

Total genomic DNA was extracted from each skin biopsy using a CTAB (Hexadecyltrimethylammonium bromide)/proteinase K incubation procedure and phenol/chloroform extraction adapted from Grewe *et al.* (1993). DNA was precipitated using a standard ethanol precipitation protocol (Grewe *et al* 1993) and resuspended in sterile distilled water. A 457 base pair (bp) fragment of the maternally inherited mitochondrial $tRNA^{thr}$ -control region was amplified using polymerase chain reaction (PCR). In a 25µl reaction volume: 17.8µl milliQ water, 0.1µl 10mM dNTPs (combined), 1.5µl 25mM $MgCl_2$, 2.5µl 10xbuffer (500mM KCl, 100mM Tris pH=9.0, 1% Triton X), 1.0µl each of 10µM primers: TDKD (Slade *et al.* 1994) and 10µM L15926 (Kocher *et al.* 1989), 0.1µl Taq polymerase[4] (5-10 units), 1.0µl extracted DNA and overlaid with oil. The amplification parameters are as outlined in Slade *et al.* 1994. The product was purified by gel purification using 1.5% agarose in TAE.

A 316bp fragment of the PCR product was sequenced with the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) using internal primers: Thr/Pro (5'-TCCCTAAGACTCAAGGAAGAG-3') and Cent (5'-GAGCGAGAAGAGGTACACTTT-3'). Both internal primers were designed for this study using an *A. forsteri* sequence (Slade *et al.* 1994). The fragment was sequenced initially from the 5' end and only sequenced from the 3'

[4] Post Publication Note - The brand of *Taq* polymerase used varied, but was mainly home made at the University of Queensland.

end if the first sequence was too short and/or there were too many ambiguous sites. The sequenced product corresponds to sites 68-373 of the Gen Bank sequence for *A. forsteri* (UO3576).

Additional samples from each population were screened with a series of restriction endonucleases in order to enable a frequency based analysis for the examination of population structure. The sequences obtained above were used as a template to identify a series of restriction sites that could recognise species haplotypes as well as group individuals into the clades identified from sequence analysis. DNA was extracted and amplified as above using internal primers before digestion with each enzyme. *Nde* I and *Tsp*509 I were the enzymes employed to identify species haplotypes by targeting specific differences in the sequences. Additional enzymes, namely *Bcl* I, *Ssp* I and *Hin*f I were employed to further classify each individual into one of the major clades identified by the neighbour-joining tree (for *A. tropicalis* only). All enzymes were obtained from New England Biolabs Inc. Digestion of the PCR product proceeded as directed by the manufacturer, but using 4 units of enzyme per reaction instead of 5 for all enzymes except *Nde* I, which required 6 units. Digests were run out on 2% NuSieve 3:1 agarose (FMC BioProducts) stained with ethidium bromide and scored over a UV illuminator.

Data Analysis

Sequences were examined using Seqed (version 1.0.3; Applied Biosystems Inc.) to ascertain quality and to verify the scoring. Sequences were aligned using CLUSTAL W (Thompson *et al.* 1994) and resulting alignments were evaluated by eye and corrected where required. All sites containing insertion/deletions and/or missing information were removed prior to further analysis. Data from individuals that were not sampled as part of a breeding population (i.e., the vagrants) were not included in any population analyses.

The program MEGA (Kumar *et al.* 1993) was used for creating neighbour-joining (NJ) trees which were based on distances calculated using Kimura's 2-parameter model (Kimura 1980). The method of tree construction was based on the algorithm of Saitou and Nei (1987). A bootstrap test was performed on each tree and values were obtained after 1000 replications. Data on polymorphic sites, nucleotide diversities and divergences within populations and species were obtained using the program DnaSP (Rozas and Rozas 1997). Uncorrected nucleotide diversities (π) were calculated from Nei (1987; equations 10.5 or 10.6). Uncorrected nucleotide divergence data (D_{xy} and D_a) were obtained from respective equations 10.20 and 10.21 (Nei 1987).

To examine within species population structure based on sequence data, the Analysis of Molecular Variance (AMOVA; Excoffier *et al.* 1992) was employed using the program Arlequin (Schneider *et al.* 1997). Population pairwise Φ_{ST} were calculated in Arlequin based on both sequence data and sequence haplotype frequencies (Weir and Cockerham 1984; Weir 1990). Testing for differences between populations was performed by permuting

haplotypes between populations and presenting a P-value that is the proportion of permutations showing an Φ_{ST} greater than or equal to the observed one. All significance levels were adjusted for multiple comparisons using the Bonferroni test (Rice 1989).

Analysis of heterogeneity was conducted using the Monte program within REAP (McElroy *et al.* 1992) where significance testing of the estimate of χ^2 (X^2) was calculated after 1000 replications (Roff and Bentzen 1989) and adjusted for multiple comparisons (Rice 1989). Analysis of isolation-by-distance was performed by regressing $\Phi_{ST}/1-\Phi_{ST}$ with the natural logarithm of geographical distance in Genepop (version 3.1; see Raymond and Rousset 1995; Rousset 1997).

Results

A total of 248 sequences was obtained for analysis, which comprised of 103 from *A. tropicalis* and 145 from *A. gazella* (Table 3.1). A further 17 sequences from *A. forsteri* were used for comparison. Significant length variation was observed in all sequences, primarily due to a highly variable TC region from site 91 to 122 (Figure 3.2). This "TC landmark" (as recognised by Lento *et al.* 1995) caused problems with alignment despite highly conserved flanking regions, due to the length variations and polymorphisms. Sequences varied in length from 294bp to 309bp, but were all aligned to form a 316bp data matrix (Figure 3.2). The TC landmark was removed from all individuals after alignment and prior to analysis.

Interspecific Analysis

Species specific sequence differences were observed through fixed polymorphisms as well as length variation in the TC landmark (Figure 3.2). Overall there were 8 fixed differences between the three species, all within the first 80bp. These largely confirm those found by Goldsworthy *et al.* (1999) who identified a total of 11 differences in the same region. One was altered due to alignment differences and the other two were found not to be fixed across species when a larger sample size was examined. There were 5 fixed differences observed between *A. gazella* and *A. forsteri*, 13 between the latter and *A. tropicalis*, and 9 between *A. tropicalis* and *A. gazella*. In *A. gazella* and *A. forsteri* sequences, greater length variation was observed in the TC landmark relative to *A. tropicalis* sequences, which were much more conserved. Summary sequence details of each species are presented in Tables 3.2.1 and 3.2.2, and the phylogenetic relationships[5] among the species are shown in Figure 3.3. This phylogeny employed the range of lineages for both *A. gazella* and *A. tropicalis* that were obtained in this study. While *A. tropicalis* forms a well supported monophyletic group, the relationship between *A. gazella* and *A. forsteri* is paraphyletic, with the latter species characterised by two highly divergent clades.

[5] Post Publication Note: The phylogenetic reconstruction conducted here was solely to ascertain that *A. gazella* and *A. tropicalis* had sufficient genetic differences to be considered separate species. As this chapter was published ahead of Chapter 2, a simple phylogenetic reconstruction was required here to address this point. The authors acknowledge that such a reconstruction may not represent the true species phylogeny of these fur seals.

Intraspecific Analysis

A. tropicalis

DNA from 103 individuals were sequenced for 316bp of the $tRNA^{thr}$-control region. Included in these samples were seals from 5 major breeding populations (Figure 3.1, Table 3.1) plus vagrants from the Juan Fernandez archipelago (n=3), Australia (n=2) and South Africa (n=1). Three individuals from MI were found to have a control region sequence haplotype of *A. gazella*, despite having phenotypic characteristics of *A. tropicalis*. These putative hybrids were not included in any further analysis in this chapter.

There were 33 haplotypes, 13 of which were represented in more than one individual. The relationships of these haplotypes to each other, the variable sites that characterise each haplotype and their geographic distribution is displayed in Figure 3.4. There is a high degree of lineage structure within the species, with three divergent clades apparent, but no obvious geographic structure in the distribution of lineages. The nucleotide diversities within clades are low (2.1%, 0.5% and 1.1% for I, II and III respectively) relative to the overall value of 4.8% for the species. Sequence statistics for the species and each population are presented in Tables 3.2.1 and 3.2.2. Each population has a high level of diversity relative to the low within clade diversities reflecting the presence in each population of representatives from more than one clade. This is further reflected in the AMOVA results which indicate that 81% of the variation was distributed within, rather than among populations. An overall Φ_{ST} value of 0.19 was calculated for *A. tropicalis*. To examine the level of population structure within the species, pairwise Φ_{ST}'s were calculated and are presented in Table 3.3. Those calculated based solely on haplotype distribution showed significant structure for 8 of the 10 pairwise comparisons. However, when molecular information was also considered, the number dropped to only 4. Overall, the latter Φ_{ST} values were higher than those calculated on haplotype frequency alone, which is not unexpected. However, the CI-MI and AI-GI pairwise Φ_{ST} values from haplotype frequency alone are not only greater, but they are also significantly different from zero ($P<0.01$). The isolation-by-distance analysis based on sequence haplotype distribution among populations revealed no significant linear relationship between geographic distance and Φ_{ST} ($R^2=0.024$, Appendix 1). A hierarchical AMOVA was then performed grouping populations by geographical region (Indian, Atlantic and Pacific Ocean groups) and found that little of the variation could be explained by geography (1.35% between groups). However, when the two recolonised populations were grouped together, 21.9% of the variation was among groups and negligible variation within (-1.4%). The between group variation dropped to 18.1% when MA was included in the group, and to 16.2 when AI replaced MA.

To further examine levels of population structure, 83 additional samples from all populations were screened with a series of restriction enzymes (Table 3.1) which targeted sites that distinguished between the major clades. The individuals whose DNA had been sequenced were also included as the RFLP haplotype could be determined from each sequence. A total of 7 haplotypes were obtained (Table 3.4.1), and the distribution of these haplotypes showed

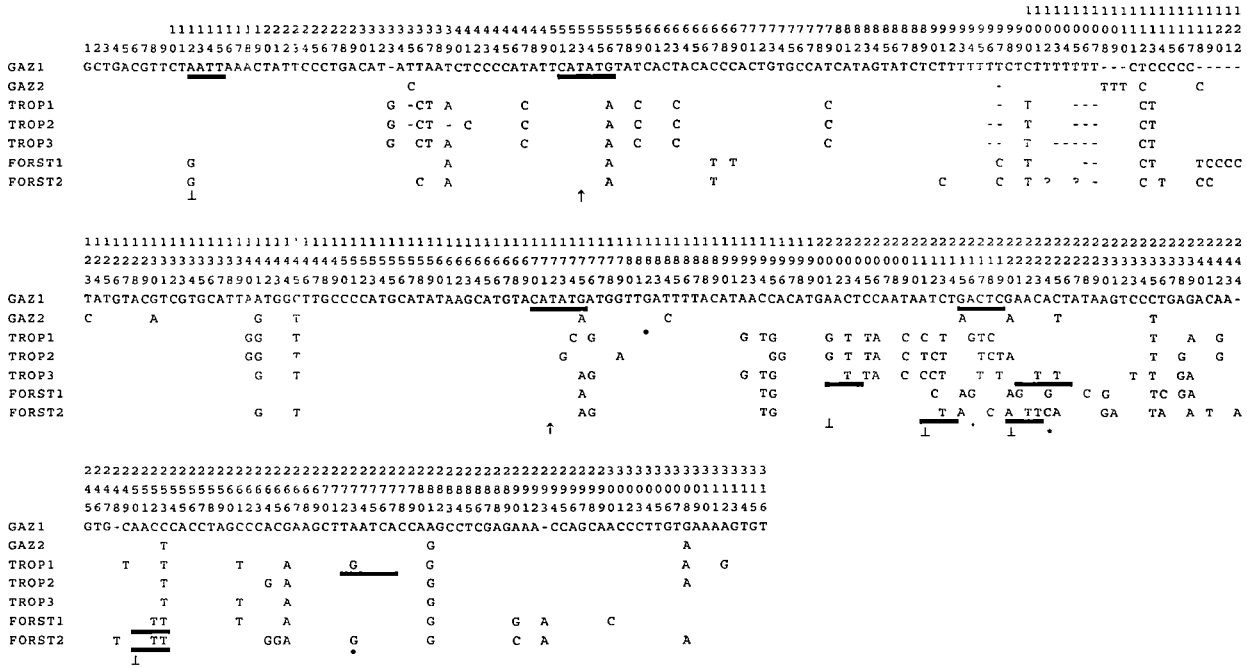


Figure 3.2: The 316bp sequence of the mitochondrial tRNA^{thr}-control region from three species of *Arctocephalus*. Identity of sequences as follows: Gaz1 and Gaz2 from *A gazella*, haplotype numbers 01 and 03; Trop1, Trop2 and Trop3 are haplotypes 01, 18 and 22 respectively from *A. tropicalis,* and Forst1 and Forst2 from *A. forsteri* are haplotypes A and E as determined by Lento (1995) Shaded regions indicate species specific nucleotide differences Underlined sequence indicate recognition sequences of restriction endonucleases, and markings below each block of sequence indicate the restriction site as follows: ↑=*Nde*I which distinguishes *A. gazella* from the other species; ⊥=*Tsp*509I, distinguishes *A. tropicalis* from *A forsteri*; +=*Hin*fI, distinguishes between two groups of *A gazella*; *=*Ssp*I, distinguishes clade 3 of *A .tropicalis* from clades 1 and 2; •=*Bcl*I, distinguishes clade 1 of *A. tropicalis* from clade 2.

	Number of individuals	Total number of haplotypes	Haplotypes n>1	Haplotypes unique to a population	Variable sites	Nucleotide diversity
A . gazella	**145**	**26**	**16**	**10**	**45**	**0.032**
BI	20	9	4	3	37	0.042
CI	20	8	4	1	28	0.029
HI	20	11	4	1	35	0.031
HI-P	7	4	2	0	14	0 025
KI	20	7	5	1	21	0.023
MI	20	9	5	3	23	0.021
MA	20	8	4	0	34	0.034
SG	20	8	4	0	28	0.032
SS	5	4	1	1	11	0.022
A. tropicalis	**103**	**33**	**13**	**28**	**46**	**0.048**
AI	20	9	4	7	28	0.041
CI	20	8	4	5	35	0.044
GI	20	8	5	6	33	0.030
MA	20	9	3	6	31	0.045
MI	17	9	4	4	32	0.046
A. forsteri	**17**	**16**	**1**	**15**	**40**	**0.051**

Table 3.2.1: MtDNA control region sequence and haplotype statistics for three species of *Arctocephalus* and their populations. Sequences had all insertion/deletions, sites with missing information and the TC landmark removed. HI-P =Heard Island pups only

that the populations are heterogeneous (X^2[24]=99.96; P<0.001). In contrast to Φ_{ST} results, all population pairwise comparisons from RFLP data were significant (Table 3.5).

	A. gazella	*A. tropicalis*	*A. forsteri*
A. gazella	-	0.122	0.091
A. tropicalis	0.080	-	0.123
A. forsteri	0.050	0.075	-

Table 3.2.2: Nucleotide diversity (Dxy) and divergence (Da) for mtDNA control region between populations above and below the diagonal respectively.

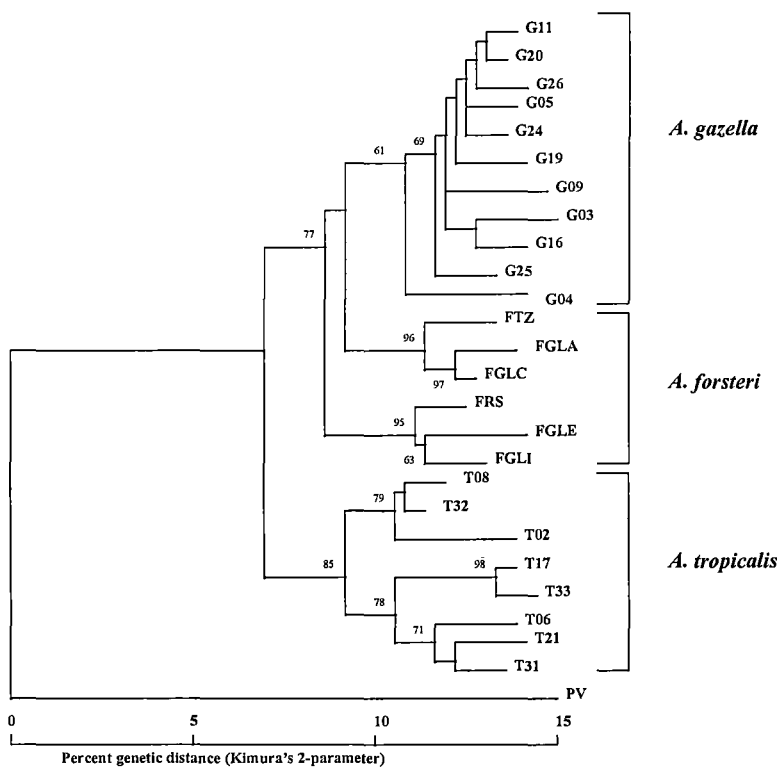


Figure 3.3: NJ tree of representative haplotypes of mtDNA control region from three species of fur seal with *Phoca vitulina* outgroup The letter of each of the labels denotes species (G=*A gazella*, T=*A tropicalis*, F=*A forsteri*), while the number corresponds to the haplotype For *A forsteri*, FRS from Slade *et al.* (1994), FGL are from Lento (1995: where the last letter corresponds to haplotypes identified therein) and FTZ from Taronga Zoo, Australia. Bootstrap values are shown only at nodes which were supported in over 60% of the 1000 replications.

A. tropicalis - Vagrants

Sequence data from six vagrant individuals were compared with that from the breeding populations. The individual from South Africa was found to have a haplotype unique to the GI population. The haplotype from one of the vagrants from Australia was unique to the AI population. The other was shared with AI, MI and GI, indicating a probable origin from one of these sites. However, this un-tagged juvenile is less likely to have come from MI as all pups from this population have been tagged since the mid 1980s. Two of the Juan Fernandez vagrants had haplotypes shared by all populations except GI, making it difficult to assess from where they originated. The haplotype of the third however, was unique to AI.

A. gazella

DNA sequences were obtained for a total of 145 individuals from 8 populations (Table 3.1). There were 26 haplotypes found, 16 of which were represented by more than one individual (Table 3.2.1). The relationship of these haplotypes (Figure 3.5) shows little clade structure in the tree, relative to that observed in *A. tropicalis*, and the nucleotide diversity of 3.2% for this species is also lower. The sequence statistics for *A. gazella* and each of its populations are displayed in Table 3.2.1. AMOVA results reveal that 92.6% of the observed genetic variation occurred within the populations. The overall Φ_{ST} for *A. gazella* is 0.074. Pairwise Φ_{ST} calculated for populations using sequence and haplotype frequency data are presented in Table 3.6. Of the 28 pairwise comparisons, 6 or 7 were significant, depending on the data used for the analysis. The KI and MI populations were found to be significantly different to all both BI and SG, which are the supposed source populations for the recolonised populations.

These data, along with haplotype distributions, suggest that *A. gazella* consist of 2 broad regional groups: Region 1 containing SG, SS, BI and MA populations; and Region 2 containing KI and MI. The two populations of CI and HI are intermediate, with the former containing haplotypes otherwise found exclusively in each of Regions 1 and 2. While HI is not significantly different to the KI/MI region, all the sequence haplotypes found within the known breeding population (n=7) are shared primarily with Region 1. However, pairwise Φ_{ST}'s calculated between the groups of known and unknown breeding status within the HI population were found to be not significantly different from zero.

Isolation-by-distance analysis, with the assumption that all islands were re-colonised from the SG or BI populations, showed that some variation could be explained by geographic distance (R^2=0.228, Appendix 1). However, if there was an additional population within the KI/MI region that survived sealing, then the distribution of genetic variation within *A. gazella* would not be expected to conform to an isolation-by-distance model alone. If the data from Region 2 are excluded from the analysis (ie. that from the KI and MI populations), a greater correlation resulted with an R^2 value of 0.600 for Region 1. Furthermore, a hierarchical AMOVA was also used to investigate potential geographic structure. Southern elephant

	Al (n=20)	Cl (n=20)	Gl (n=20)	Ml (n=17)	MA (n=20)
Al	-	0.098	0.123	0.121	0.289**
Cl	0.066	-	0.268**	-0.021	0.097
Gl	0.148**	0.197**	-	0.303**	0.390**
Ml	0.097**	0.083**	0.133**	-	0.062
MA	0.120**	0.085*	0.179**	0.043	-

Table 3.3: Population pairwise Φ_{ST} within *A. tropicalis* based on mtDNA control region sequence and frequency data (above diagonal) and on haplotype frequency data only (below diagonal). Significance testing of Φ_{ST} performed through 992 permutations and adjusted for multiple comparisons: $P \leq 0.05$ * and $P \leq 0.01$ **.

3.4.1: *A. tropicalis*

No.	Haplotype	Clade	Al	Cl	Gl	Ml	MA	Total
1	0101	I	0	1	1	0	0	**2**
2	0001	I	25	10	28	10	3	**76**
3	0000	II	0	6	5	4	15	**30**
4	0011	III	0	0	0	1	0	**1**
5	0010	III	12	20	0	7	15	**54**
6	0110	III	0	0	5	0	1	**6**
7	1110	III	3	2	0	7	5	**17**
Total			**40**	**39**	**39**	**29**	**39**	**186**

3.4.2: *A. gazella*

No.	Haplotype	SS	SG	Bl	MA	Cl	Kl	Hl	Ml	Total
1	00	13	7	15	8	11	19	12	19	**104**
2	01	17	24	13	13	17	12	16	21	**133**
3	11	1	9	12	13	12	9	12	10	**78**
Total		**31**	**40**	**40**	**34**	**40**	**40**	**40**	**50**	**315**

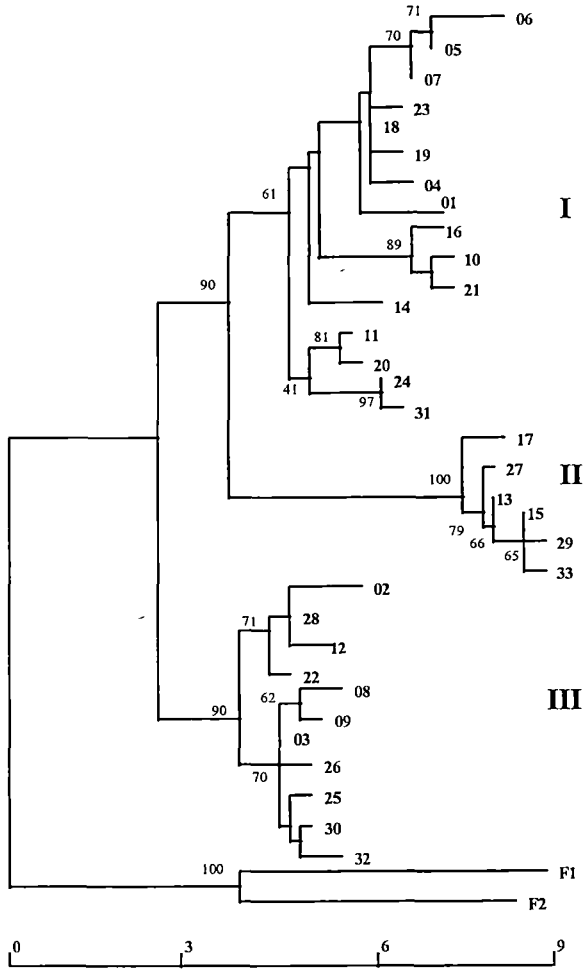
Tables 3.4.1 and 3.4.2: RFLP haplotypes of mtDNA control region for *A. tropicalis* and *A. gazella*, where 1=restriction site and 0=no restriction site.

	CI (n=39)	GI (n=39)	MI (n=29)	MA (n=39)
AI	15.6**	26.2**	12.9*	34.1**
CI	-	35.6**	10.2*	11.6*
GI		-	28.8**	48.8*
MI			-	14.2*

Table 3.5: Estimated pairwise population chi-squared values from the mtDNA control region RFLP data for *A tropicalis* adjusted for multiple comparisons Significance levels shown are P≤0.05 * and P≤0.01 **

Figure 3.4 (over page): NJ tree of 33 mtDNA control region sequence haplotypes observed in 5 populations of *A. tropicalis* and vagrants, the variable sites for each haplotype and its geographical distribution. Labels are arbitrarily assigned to haplotypes from 1 to 33. F1 and F2 are *A. forsteri* outgroups corresponding to FORST1 and FORST2 in Figure 4.2. Bootstrap values are shown only at nodes which were supported in over 60% of the 1000 replications. Three major clades labelled as I, II and III. Variable sites are numbered according to their position within the 316bp aligned sequence. Geographic labels are described in Table 4.1.

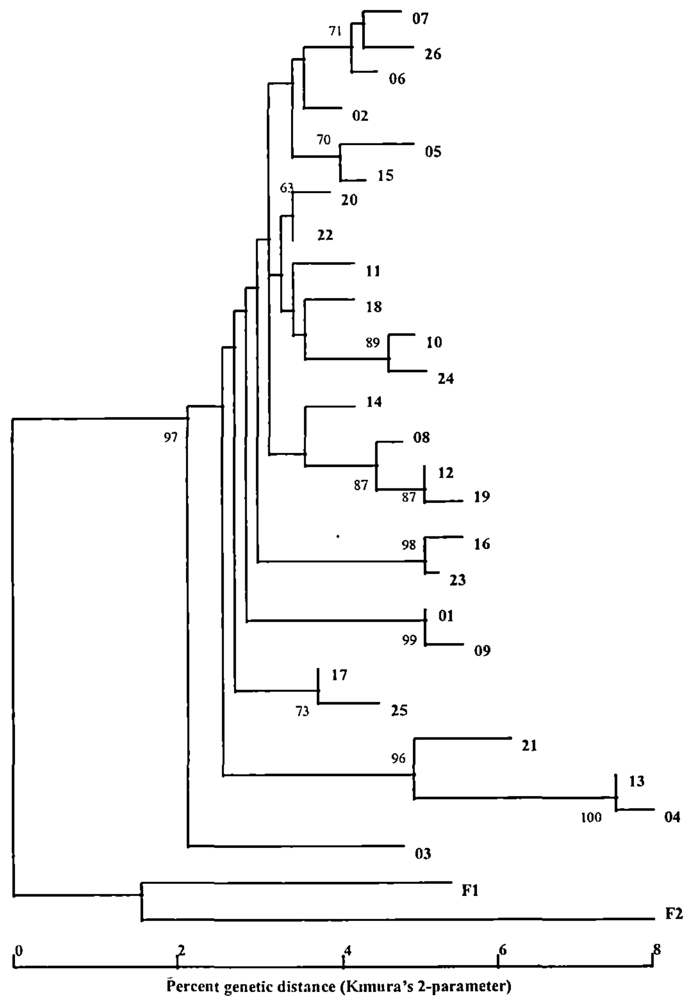
Figure 3.5 (over two pages): NJ tree of 26 sequence haplotypes observed in 8 populations of *A. gazella,* the variable sites for each haplotype with its geographical distribution. Labels are arbitrarily assigned to haplotypes from 1 to 26 F1 and F2 are *A. forsteri* outgroups corresponding to FORST1 and FORST2 in Figure 4.2. Bootstrap values are shown only at nodes which were supported in over 60% of the 1000 replications. Variable sites are numbered according to their position within the 316bp aligned sequence. Geographic labels are described in Table 4 1. HI haplotypes that are emboldened and italicised represent those which are represented in pups



Percent genetic distance (Kimura's 2-parameter)

```
  11111111112222222222222222222222222222333333
14477777899900001111112223333445666778890000001
1103456952460136124689035358929101434147456782   GI SA MA CI AI MI AU JF  Total
ATAATAGGTATGGGTACTTGCTGCCCTAAGTGATAGAGCGTTGTAA                      1        1
T G..  ..  .              .     .G   . . . . .G                     3        1  4
T G .    .  .              .  AG.  .... ... G                      1           1
T G C.     A.                ..AG . ........GG   1                           1
T G.C   . .A ..            .   .AG  . . ......G   8                 1         9
T G.C  .   A .                 .AG  ...T .  ..G   2                           2
T G.C A    A.         A ..     AG   ..... ...G   1                 2  1  1    5
T G CG.  G A    ..   C    .   .AG   .... . ..G                      5     1    6
T G.CG   . .AAA TTC        C  A.AG   .. ..... G                 1              1
T G CG   G AA .TTC.       ..C. . AG  . . ......G                 1              1
T G CG  .. AA  TTC        .C    AG G.....  . .G   2                           2
T G ...    A    TC    A  .C  .  G   .... ...GG                 1              1
T G. .A.C  A    .  A  A   C  ..AG . ..... ..G                 3              3
T G. .A C .A       A  A . C. ..AG. . ........GG   3                           3
T GG. A    A .  T  A  A ..C.   AG.G.... . .. G                      2        2
T GG..A  . A.   T  A  A . C  . AG G....T.....G                 1              1
TCAG.GAA .C     TC A  A   .GG.CAGCGA.........   2  1                        3
TCGGCGAA  C    .TC A  A.  CGG.CAGCGA...... .G                      1        1
TCGGCGAA  C.    TC A  A . CGG CAGCGA . .....              8  2     2           12
TCGGCGAA  C   . TC A  A ..CGG CAGCGA.. T....              1                   1
TCGGCGAA .C     TCCA  A ..CGG CAGCGA. .T. ....           1                   1
TCGGCGAA  C     TC A  A.  CGG.CAGCGA.A.T.   ..           1                   1
T G..GA..G.A A   C A   TTT.G ACAG .A.. ..GTGG.                      1        1
T G .GA. G A A   C A  .TTT.G ACAG  A . .....G.           1                   1
T G .GA  G A A   C ATC.TTT G ACAG..A. ... . G.           3  2     6           11
T G.     G A A.  C AT  TTT G ACAG .A. ... . G   1                           1
  G.  A. ..A AC  C A C TTT G ACA  .A.... . .G.                      1        1
T G . A    A AC  C A  .TTT.G ACA   A    C...G.                      1        1
T G   A..  A AC  C A   TTT G ACAG .A   .....G           3  9  5  2     2     21
T G. .A    A.AC TC.A   TTT G.ACAG .AG.. . ..G                      1        1
T G.. A..  A AC..C A  .TTT.G.ACAG..A...A.. .G                      1        1
T G . A    A AC. C A  .TTT.G ACAG. A . T . .G.           1                   1
T G   A  . A AC TC A   TTT G.ACAG..A A T.. .G.           1                   1

                                          TOTAL    20 1  20 20 20 17 2  3   103
```



Position	MI	HI	KI	CI	MA	BI	SG	SS	TOTAL
`  11111111111112222222222222222222222222222333`									
`342344778888999011111112222333344555666788000`									
`573005452457234523567890358568959237146315189`									
`TTTGGTTAACTTAGTCTCGACTCACCCCTAGGCTTCCAGAATCGA`					1	2	1		4
`   A..    .       .   .          .. A  ...`								1	1
` .  A    .                          .    . ..`						1			1
`..  A   G   ..   T      .  .     C  .    .. .`				2	1	4	4	1	12
`..  A ..G .. .C.          C  . C.T.G     ...`		*2*		4	2	1	1		10
`    A...G   .C           TC     C ..       ..`	5	1	2						8
`..  A ..G   A     .    ..   .  TC ..  .. ...`	1	*1*					2		4
`..  A...G   .A  . . . . . .   .  C. .   .. ..`			1					1	2
`.      .G   A .   .          .  . C.      ... .`	2	3	3	1					9
` .. .   G  . A.  . . .     .C   .C ..  G....`		*1*			1		1		3
`..  . . GT    .   A  .      C    C  .    . .A`	2	1	2	2					7
`..   ...GT        A .      .C .  C     ... .G`	1								1
`..  A. .GTC .A .           C    C .     .. .`				1	1				2
` .. A ..GT  GA .   G       .C  . C..T    ....`						1			1
`... A . GT  GA.  . G        C..  .C .T  .. .A.`				1				2	3
`.. .A ..GT  GA  .  G .    . C..  C  T   . TA.`		1					1		2
`.. AA  .G C..AC .      T    G   C.      C A`		1							1
`  .AA  .G.C  AC. . .    T  . G . C .     C. .`	1								1
`... AC.GGT   AC        G  T.   . .CC .  .. ...`	2	*6*	4	8	10	4	5		39
`..  AC GGT.C.AC . .    .G  T    . .CC . .....`						1			1
`..   ...GT. .AC .   .C      TC .. C...   .....`	5	2	7						14
` . . . .GT   AC    .  C .   TC.A .C . .A   ... .`	1								1
`.C    . GTC. ACT T  T  C. TT .    C  T    .C ..`			1						1
`.C  A C.GTC .A. CT GTCTC  TT      C. . .G.C .`				1					1
`.C  A.C.GTC..A. CT GTCTC  TT.. A C     G C...`		1			2	5			8
`C.CAA...G    AC    .. .  .TTT .. .C   . . G. A.`					2	1	5		8
TOTAL	20	20	20	20	20	20	20	5	145

	BI (n=20)	CI (n=20)	HI (n=20)	KI (n=20)	MI (n=20)	MA (n=20)	SG (n=20)	SS (n=7)
BI	-	0 050	0.057	0.140*	0.163**	0.032	0.069	0.070
CI	0.051	-	-0.018	0.081	0.111	-0 011	0.045	0.119
HI	0.039	-0.006	-	0.003	0 034	0.012	0.042	0.106
KI	0.109**	0.090	0.011	-	-0.017	0.113	0.133*	0.232*
MI	0.103**	0.106**	0.025	-0.015	-	0.165*	0.138**	0.206
MA	0.043	-0.008	0.014	0.123*	0.144**	-	0.036	0.181
SG	0.024	0 041	0 037	0.111*	0 105**	0.033	-	0.087
SS	0.076	0 119	0.103	0.133	0.113	0.187	0.089	-

Table 3.6: Population pairwise Φ_{ST} within *A. gazella* based on mtDNA control region sequence and frequency data (above diagonal) and on haplotype frequency data only (below diagonal) Significance testing of Φ_{ST} performed through 992 permutations and adjusted for multiple comparisons: $P \leq 0.05$ * and $P \leq 0.01$ **.

	CI (n=40)	HI (n=40)	KI (n=40)	MI (n=50)	MA (n=34)	SG (n=40)	SS (n=31)
BI	1.15	0.64	0.94	1.44	1.70	6.61	8.99
CI	-	0.07	3.42	1.65	0.56	2.51	8.47
HI		-	2 58	1.34	0.67	3.34	8.37
KI			-	1.41	4.79	9.54	7.36
MI				-	3.85	4.74	4.71
MA					-	3.60	11.9*
SG						-	8.39

Table 3.7: Estimated pairwise chi-squared values within *A. gazella* for mtDNA control region, with adjustment for multiple comparisons. Significance levels shown are $P \leq 0.05$ *.

seals (*Mirounga leonina*) show strong phylogeographic structure forming three major oceanic populations (Slade 1997). *A. gazella* were also grouped in this fashion: Pacific (MI), Atlantic (SS, SG, BI) and Indian (MA, CI, KI, HI) oceanic populations. This only accounted for 3.8% of the overall variation. Examining populations on a finer scale (SS,SG versus BI, versus MA,CI versus
KI,HI versus MI) resulted in 7.1% among groups. However, when HI was removed from the analysis due to the uncertainly of the sampled individuals' origin and KI included with MI, the amount of variation among groups was 10.2% with only 0.16% of the variation occurring within groups.

To further investigate structure within *A. gazella*, 315 individuals were screened with two restriction enzymes to produce three mtDNA control region RFLP haplotypes (Table 3.4.2). An analysis of heterogeneity on the frequency distribution of these haplotypes shows that the species is heterogeneous, but only at the 5% level ($X^2[14]=25.13$; $P<0.05$). The population pairwise estimated chi-squared values (Table 3.7) show little difference between all populations with respect to the distribution of haplotypes, except for the comparison between SS and MA. This would be due only one individual in SS with haplotype 3, whereas MA has the highest proportion of this haplotype within the population (Table 3.4.2). A greater number of enzymes could be used to increase the number of RFLP haplotypes observed within *A. gazella*, thereby increasing the resolution of this analysis.

A. forsteri

A. forsteri has high levels of diversity, with 16 haplotypes found from the 17 sequences examined (Table 3.2.1). There are two highly divergent clades (Figure 3.3) which are also apparent in cytochrome *b* (Lento 1995). The additional samples from Taronga Zoo and Slade *et al.* (1994) showed haplotypes that differed from those found by Lento (1995).

Discussion

The phylogenetic relationship between *A. tropicalis* and *A. gazella* is characterised by the absence of shared haplotypes, a divergence between the species of 8.0% and the presence of discrete clades in a NJ tree. These points indicate that the reported hybridisation between *A. gazella* and *A. tropicalis* is recent, not extensive, and is probably confined to the very small areas of range overlap. The most extensive current hybridisation is probably occurring at Macquarie Island, which has a very small population where both species breed on the same beach. This would explain why 3 individuals sampled from the island had a phenotype characteristic of *A. tropicalis* while having a mtDNA haplotype characteristic of *A. gazella*. The findings of this study and that of Goldsworthy *et al.* (1999) suggest that the results of reciprocal paraphyly of these two species reported by Lento *et al.* (1997) using samples from Macquarie Island, were probably based on hybrid individuals.

Levels of Genetic Variation

The levels of genetic variation that were detected in *A. tropicalis* and *A. gazella* are very high for control region I, especially when compared with other vertebrate species (see Table 2; Slade 1997). The respective nucleotide diversities of 4.8% and 3.2% for these species are among the highest reported for the listed mammalian species. These results might be considered surprising after both species are thought to have experienced recent population bottlenecks. However, the current levels of genetic variation within a species are the result of many factors. These include the amount of pre-existing genetic variation, pre- and post-bottleneck population sizes, as well as duration and extent of the bottleneck itself. Nei (1975) proposed that if a species with high pre-existing genetic variation was able to recover rapidly from a severe bottleneck, it would be expected to exhibit reduced haplotype variation while retaining pre-bottleneck nucleotide diversity.

Although the pre-bottleneck variation for both *A. tropicalis* and *A. gazella* are unknown, they are expected to be high given the large population sizes reported at that time. Despite the lack of specific data from the era, it appears that the population sizes of *A. gazella* were greater than those for *A. tropicalis*. The reported numbers of seal skins removed from *A. gazella* population centres is greater (1.2 million from South Georgia by 1822; approx. 250,000 from the South Shetlands 1820-1821: Bonner 1958, Bonner and Laws 1964) and it was noted that the South Shetland Islands "revealed what were probably the richest sealing grounds of the nineteenth century in the southern hemisphere" (Bonner and Laws 1964). Specific details pertaining to the duration and extent of the population bottlenecks for both *A. gazella* and *A. tropicalis* are incomplete. Although rapid population increases have been documented in both species (eg. McCann and Doidge 1987; Shaughnessy 1982), the estimation of pre-sealing population sizes is difficult, partly due to the secrecy shrouding a highly competitive sealing industry. Also, many islands in the subantarctic are difficult to search thoroughly due to the length and/or inaccessibility of sections of coast. Thus reports of population extinctions at islands infrequently visited and/or with inaccessible coastlines may be viewed with scepticism. The more credible reports are from islands with long periods of human habitation, such as Macquarie Island. Therefore, it is possible that the recent population reductions experienced by both species, particularly *A. gazella*, were not as severe as implied in historic reports. This and the known rapid post-bottleneck recoveries, suggest that neither species have suffered major reductions in their levels of genetic variation. Nonetheless, the lower level observed in *A. gazella* relative to *A. tropicalis* may be a result of the more intensive sealing efforts waged against this species.

High post-bottleneck levels of genetic variation are also found in two other species of fur seal. The Juan Fernandez and Guadelupe fur seals (*A. philippii* and *A. townsendi* respectively) were both subjected to major reductions in numbers through sealing (Hubbs 1956 as cited in Fleischer 1987; Hubbs and Norris 1971), but have retained high nucleotide diversities in control region I (π=3.0% and 2.0% respectively; Goldsworthy *et al.* 2000; G. Bernardi personal communication). These scenarios are in stark contrast to those seen

within the northern elephant and Hawaiian monk seals (*Mirounga angustirostris* and *Monachus schauinslandi* respectively). Both exhibit extremely low levels of variation in the mitochondrial control region, thought to result from severe bottlenecks (π=0.43% and 0.7% respectively) (Hoelzel *et al.* 1993; derived from Kretzmann *et al.* 1997). The differences seen between these two phocid species and the four fur seal species may be explained by the latter surviving sealing in greater numbers. Furthermore, the biology of the phocid species suggest that they were more vulnerable to exploitation. The northern elephant seals haul out to breed and/or moult on open beaches (Bonner 1994) relatively close to human civilisation, and thus would have been highly accessible to sealers. This may have ensured that low numbers were maintained for prolonged periods. The Hawaiian monk seal population was probably not large to start with. Although this species is solitary, wary and easily disturbed, sealing and other post-sealing human activities not only reduced the population markedly, but maintained a sustained pressure on the species (Busch 1987). In contrast, the closely related southern elephant seal breeds on remote subantarctic islands, probably allowing greater numbers to survive sealing (control region I π=1.95%; Slade 1997). This may also be the case for the Antarctic fur seal. On the other hand, the Juan Fernandez, Guadelupe and subantarctic fur seals all prefer a more rugged substrate for breeding, such as rocky sections of coast, often at the base of high cliffs and in caves (King 1983). The nature of this substrate decreases the visibility of the seals, thereby increasing the chance of survival.

Population Structure

The observed patterns of genetic variation within *A. tropicalis* and *A. gazella* are a result of a number of factors and processes: mutation, drift, migration, effective population size and selection. The time frame within which the latest recolonisation events have taken place (ie. within the last 100 years), suggests that effects due to mutation and selection would be negligible. Also, genetic drift is likely to be a consideration only in very small populations, such as at Macquarie Island and Iles Crozet. Therefore, the major contributors to the observed distribution of genetic variation are likely to stem from the associated effects of migration, such as founder effects. Furthermore, as there are large differences in the current sizes of many populations of both species, it would be expected that the strategy employed to sample current genetic variation would also influence results. All populations are represented by about 40 individuals, regardless of their size, which may lead to under-representation of the number and frequency of haplotypes sampled in the larger populations.

The higher level of population structure and genetic diversity observed within *A. tropicalis* supports historic records that suggest it was subjected to less intensive sealing than *A. gazella*. The sequence data revealed a high proportion of haplotypes within *A. tropicalis* to be unique to certain populations. Each population had between 4 to 7 unique haplotypes, representing 41% of all samples sequenced. Given the expectation that haplotypes in the recolonised populations would also occur in the source population/s, these data suggest that the sampling regime employed may have been insufficient. Although sampling of some

populations was conducted across many colonies (eg. Gough and Marion Islands), others may be represented only by a single colony (eg. Ile Amsterdam) or a very small colony (Macquarie Island). The high incidence of singleton sequence haplotypes observed in this species may also be an artefact of sampling, and/or be indicative of a species that has undergone recent rapid population expansion.

There are three highly divergent evolutionary lineages within the *A. tropicalis* tree. These indicate that at some time in the past this species showed phylogeographical structuring. It is impossible to suggest from the data presented here where the three ancient population centres were, although the patterns of distribution may provide some clues. There were no lineages from clade II found at Iles Amsterdam, while these are well represented in the Marion Island population. The Gough Island population is dominated by lineages from clade I, as is that at Iles Amsterdam, but to a lesser degree. It is possible that the three populations that survived the sealing era were the centres for each of these divergent clades, and that the current distribution of these lineages within the populations may be a reflection of the rapid post-sealing recovery of the species.

Despite the high incidence of singleton sequence haplotypes within *A. tropicalis*, the distribution of shared haplotypes allow some speculation on the pattern of female recolonisation. The three surviving populations at Gough, Amsterdam and Marion Islands differ in the distribution and proportion of sequence haplotypes. The population at Gough Island shares only one haplotype with Ile Amsterdam, and another with Macquarie Island. This suggests that Macquarie Island and Iles Crozet were most likely recolonised from either Ile Amsterdam, Marion Island, or both. This is further supported by the hierarchical AMOVA results as well as both pairwise Φ_{ST} (calculated either way) and chi-squared values suggest that Gough Island is highly significantly different to both Iles Crozet and Macquarie Island. Marion Island is the most likely major source population for Iles Crozet, as all of the shared sequence haplotypes found in the latter are shared with the former. One haplotype is also shared with Ile Amsterdam, indicating possible input from this population. The pairwise Φ_{ST} of Amsterdam-Crozet and Marion-Crozet are similar, 0.066/0.098 and 0.085/0.097 respectively. However, the geographic proximity of Marion Island to the west of Iles Crozet suggests that this population may have a greater influence. The Marion Island population appears also to be a source for Macquarie Island, with the pairwise Φ_{ST} suggesting panmixia. With the first breeding of *A. tropicalis* on Iles Crozet recorded in 1976 (Jouventin *et al.* 1982), about 5 years before this species was confirmed on Macquarie Island (Goldsworthy *et al.* unpublished data), it is possible that some immigrants came from here. But as these females would previously have come from Marion Island anyway, it is impossible to distinguish the exact source. Migration from Ile Amsterdam to Macquarie Island is also likely given that the populations share several lineages. The shared haplotype between the latter and Gough Island populations, suggest that at least one female made the 12,000 km journey from the south Atlantic ocean. Alternatively, this haplotype may be shared with either Ile Amsterdam or Marion Island, but was not sampled. Sampling may also

explain the lack of shared haplotypes between Gough Island and either Marion Island or Iles Crozet. The overall pattern described from the sequence data is supported by the RFLP data, although all populations were found to differ significantly from each other, possibly a function of the increased sample sizes.

In contrast to *A. tropicalis*, there is little structure seen within the *A. gazella* tree, although there were two genetically differentiated regions identified within the species. This was unexpected, as due to its rapid post-sealing recovery and current population size, it was thought that South Georgia would be the main source for recolonisation across the species' former range (Laws 1973; McCann and Doidge 1987). Although a wide range of the existing lineages are present in this population, 69% of the haplotypes found in *A. gazella* are not represented here. Furthermore, 10 of the sequence haplotypes were found to be unique to some populations, but none of these occurred at South Georgia. This observed haplotype distribution may be a result of drift within the small, recolonised populations and/or the sampling of only a small proportion of the South Georgia population (20 sequences from an estimated 1.5 million individuals; Boyd 1993). Alternately, it is possible there were other populations that survived sealing that contained additional unique lineages to those found at South Georgia.

Few records exist on the pre-sealing fur seal populations at Bouvetøya, McDonald Island and Iles Kerguelen. Although the two former populations were not considered large (Bonner and Laws 1964; Budd 1972), and the latter was harvested heavily (see Budd and Downes 1969), there is nothing to suggest that these populations actually became extinct. In fact, there were 1000-1200 fur seals reported on Bouvetøya in 1928 (Olstad 1929 as cited in Fevoden and Sømme 1976), after 800 had been collected by the crew of the "Norvegia" in 1927 (Holdgate *et al.* 1968). This is several years prior to the rediscovery of the small population at South Georgia in 1933, and therefore seems likely that the Bouvetøya population survived the sealing era. The presence of 3 unique haplotypes in this population supports this, although there is still evidence of extensive gene flow between here and South Georgia.

The discovery of two genetically differentiated regions within *A. gazella* may give evidence that other populations survived sealing. The first of these regions is represented by populations at South Georgia, Bouvetøya, Marion Island and the South Shetland Islands. The second region is located to the east and is represented by the panmictic populations of Iles Kerguelen and Macquarie Island. Although the two groups are not genetically isolated, there is a greater sharing of haplotypes within rather than between regions. The Iles Crozet population appears to be an intermediate, sharing haplotypes with both groups, while the Heard Island breeding population shares all haplotypes with South Georgia. This is unexpected given the close proximity of Heard Island to Iles Kerguelen, whose populations only share one haplotype.

Two hypotheses can be proposed to account for the observed regional differences. Firstly, the haplotype distribution within the eastern sector may be a result of a marked founder effect, with very few individuals colonising Iles Kerguelen from the western sector. Subsequent colonisation of Macquarie Island predominantly from Iles Kerguelen would account for the similarity of these populations. Alternately, such a distribution may be due to an additional post-sealing remnant population at Iles Kerguelen. The Kerguelen archipelago is a remote, extensive island group, and it is conceivable that small numbers of fur seals survived here. Each of these hypotheses is equally valid. The former is based on the premise that the current sampling regime was insufficient to detect representative haplotypes within the South Georgia/ Bouvetøya populations. But based on the data currently at hand, it is the latter that is the more plausible.

Because of the geographic proximity of the South Shetland Islands to South Georgia, it was expected that these populations would be more similar. Although there is no significant difference in their pairwise Φ_{ST}, the South Shetland Island population shares only one of its four haplotypes with South Georgia. The other two are shared with Iles Crozet and Kerguelen respectively. The small sample from the South Shetland population is likely to be a factor in its difference to South Georgia, but this was not borne out entirely with the RFLP data where the sample size was larger. Although both populations shared similar proportions of haplotype 2 (Table 3.4.2), the South Shetland population was distinguished by an almost complete absence of haplotype 3. It is possible that this distribution is a result of genetic drift and/or a founder effect, but would suggest limited migration of females between the two populations. This is supported by tag resight information, where only one case is documented of a female moving between South Georgia and the South Shetland Islands (Bengtson *et al.* 1990). The remaining sightings and additional satellite tracking data show that it is predominantly the males that are dispersing (Boyd *et al.* 1998; Bengtson *et al.* 1990; Laws 1973), a factor that would not be reflected in the mtDNA data.

Although fur seals are capable of dispersing huge distances providing the mechanism of rapid recolonisation, they are generally regarded as philopatric (Riedman 1990). However, there is little quantitative information pertaining to philopatry (especially in females) in *A. gazella* and *A. tropicalis*, and it is unknown whether any differences between the species may provide some explanation for the differences seen in genetic population structure. Nevertheless, resight information for *A. tropicalis* include: 3 individuals tagged at Marion Island sighted at Heard Island and South Africa (Bester 1989); one juvenile tagged as a pup at Ile Amsterdam and seen at Macquarie Island in 1998 (S.D. Goldsworthy, unpublished data); two Ile Amsterdam individuals sighted at Iles Kerguelen in early 1999 (C. Guinet, personal communication); and a 1-2 year old *A. tropicalis* tagged at Macquarie Island in 1997 sighted at Iles Kerguelen in early 1999 (M.-A. Lea, personal communication). This species has also been seen at Heard Island where a pup has been reported (Goldsworthy and Shaughnessy 1989). A juvenile *A. gazella* tagged at Macquarie Island was later sighted at Iles Kerguelen (M.-A. Lea personal communication). Additional sightings of this species

have been made on mainland Antarctica (Shaughnessy and Burton 1986) and South America (Payne 1977; 1979).

However, such a dispersal capacity does not automatically presuppose lower levels of population structure, as observed within *A. tropicalis* and *A. gazella*. The southern elephant seal (*M. leonina*) is a species that also breeds on subantarctic islands and has been reported to have a large dispersal capacity (eg. Hindell and McMahon 2000; Slip, 1997). Very high levels of population structure were found to occur in both mtDNA and nuclear DNA, with three genetically distinct populations being identified (Slade 1997). Gene flow between the most recently diverged populations is estimated to be only 3 to 4 females per generation, or if no gene flow, then having a divergence time of about 20,000 years. Such low levels of gene flow between populations is indicative of a species with high female philopatry. Although reported in fur seals, such philopatry was not as strongly evident in *A. tropicalis* and *A. gazella* in this study. Further research and analysis will be conducted to assist in evaluating the patterns of recolonisation more fully, employing these data as well as that obtained by screening with bi-parentally inherited microsatellite DNA.

Conclusion

Results from this study indicate high levels of genetic variation and significant population structure within *A. tropicalis*, and low but significant structure within *A. gazella*. Overall, *A. tropicalis* exhibited the higher levels of variation and structure, with a high proportion of unique haplotypes and haplotype singletons. The distribution of lineages within *A. tropicalis* suggests that the population at Marion Island as the major source for immigrants to Macquarie Island and Iles Crozet, although there is evidence for some input from Ile Amsterdam. There were two genetically differentiated regions observed within *A. gazella*. One centring on Iles Kerguelen and Macquarie Island, while the second is in the west, with South Georgia and Bouvetøya, probably the source of immigrants to Marion and the South Shetland Islands. The populations at Iles Crozet and Heard Island appear to be intermediates.

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CHAPTER 4: Genetic variation and population structure in two fur seal species, *Arctocephalus* spp.

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Abstract

Antarctic and subantarctic fur seals (*Arctocephalus gazella* and *A. tropicalis*) have endured major population fluctuations over the past 300 years due to human exploitation. We use 10 microsatellite loci in conjunction with mitochondrial DNA (mtDNA) sequence data to examine the levels and distribution of genetic variation within both species to ascertain whether there is any discernible effect from these fluctuations. Despite overlapping allele size ranges at most loci, significant allelic and genotypic differentiation was observed between these two species, and the New Zealand fur seal (*A. forsteri*) which occurs in sympatry at Macquarie Island ($P << 0.001$). Significant population structure was evident within the subantarctic fur seal (unbiased R_{ST}=0.122, F_{ST}=0.056; P<<0.001), with pairwise comparisons suggesting gene flow from Gough Island in the South Atlantic eastwards to Marion Island and Iles Amsterdam, and from Marion Island in the South Indian Ocean eastwards to the recolonised population at Iles Crozet. No genetic heterogeneity was observed within the Antarctic fur seal based on unbiased R_{ST} (0.003; P=0.501), suggesting panmixia despite the large geographic range, but significant structure was indicated with F_{ST} (0.021; P<<0.001). Generally, less structure was evident with microsatellites compared with mitochondrial data, a probable reflection of the smaller effective population size of the latter.

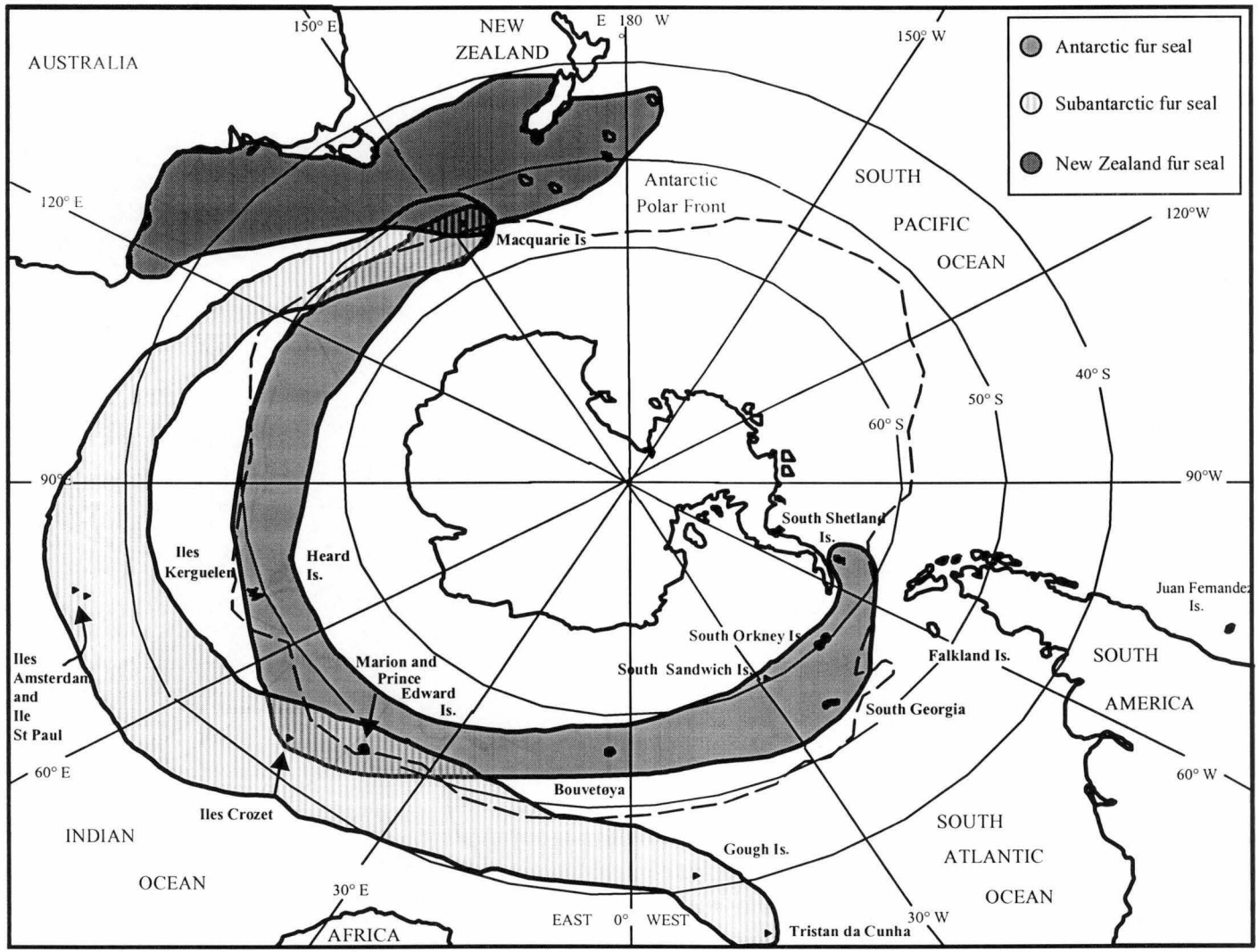
Introduction

Stock and population identification is essential for the conservation and sustainable management of marine mammals (Dizon *et al.* 1997). Such identification is difficult in the subantarctic region because many species have wide-ranging distributions throughout a region that is very remote. Many major predator populations are also recovering from human exploitation throughout the 18^{th}-20^{th} centuries, where populations were either greatly reduced in number or became extinct (Laws 1977). Effective management strategies for these species, and for the region as a whole requires an understanding of current population structure and dynamics to enable predictions of the effect of these recoveries. Traditional means of estimating and monitoring population abundances and structure are difficult to apply in the subantarctic because of its remoteness and because many species are at sea for a major proportion of their lives. Molecular techniques provide an alternate and relatively easy means of identifying populations and intra-specific gene flow required for effective and sustainable management.

One study aimed at increasing such an understanding was conducted on the fur seal species that occur throughout the subantarctic region (Wynen *et al.* 2000). Molecular techniques were employed to estimate the levels of mitochondrial DNA (mtDNA) diversity and population structure within the Antarctic fur seal (*Arctocephalus gazella*) (AFS) and subantarctic fur seal (*A. tropicalis*) (SAFS), and how these data might reflect the possible impact of major population reductions due to sealing. Here, we continue the study of molecular variation in these fur seal species, but using hypervariable microsatellite DNA.

The current distribution and recovery from past exploitation of AFS and SAFS are dealt with in some detail in Wynen *et al.* (2000). In summary, the AFS has a more southerly and extensive distribution relative to that of the SAFS (Figure 4.1), with the former being brought to the brink of extinction due to sealing activities from the late 1700s to the early 1900s (Bonner and Laws 1964). After the cessation of sealing, small relic populations at South Georgia (estimated at <100 individuals in the 1930s) and Bouvetøya (about 1000-1200 individuals in 1928) were reported (Bonner 1968; Fevoden and Sømme 1976; Laws 1973).

Figure 4.1 (over page): Distribution of subantarctic fur seals (SAFS) and Antarctic fur seals (AFS) throughout the Southern Ocean. All populations marked with a circle are current breeding populations of SAFS, while all those marked with a triangle are those for AFS. The open symbols ○△ represent populations known to have survived sealing for SAFS and AFS respectively. For AFS, all populations are representative of the genetically differentiated 'western sector' as described by Wynen *et al.* (2000), except those marked ▼ which are populations in the 'eastern sector', and ◭ representing populations containing representative mtDNA haplotypes from both western and eastern sectors. Populations sampled for this study are indicated by their abbreviations as described in the Methods. Map modified from original by John Cox (Australian Antarctic Division).


Antarctic fur seal
Subantarctic fur seal
New Zealand fur seal
AUSTRALIA
NEW ZEALAND
150° E
E 180 W
150° W
120° E
120°W
Antarctic Polar Front
SOUTH PACIFIC OCEAN
Macquarie Is
40° S
50° S
60° S
90°E
90°W
South Shetland Is.
Iles Kerguelen
Heard Is.
Juan Fernandez Is.
South Orkney Is.
Falkland Is.
SOUTH AMERICA
Iles Amsterdam and Ile St Paul
Marion and Prince Edward Is.
South Sandwich Is.
South Georgia
60° E
60° W
Bouvetøya
Iles Crozet
INDIAN OCEAN
SOUTH ATLANTIC OCEAN
Gough Is.
30° E
EAST 0° WEST
30° W
AFRICA
Tristan da Cunha

AFS numbers have increased rapidly in subsequent years, particularly at South Georgia (up to 16.8% per annum, 1957-72; Payne 1977) with a current annual pup production of 378,000 (estimated for 1990, Boyd 1993). Range expansion has occurred concurrently with increasing abundances, and while populations at South Georgia currently comprise 97% of the species, all other populations throughout the range are increasing (Hofmeyr *et al.* 1997).

The recent history of the SAFS is similar to that of the AFS, although the range contraction, the reduction in numbers, and the subsequent population recovery are thought to have occurred to a lesser degree (see Wynen *et al.* 2000). Three remnant populations are reported to have survived the sealing era, at Gough Island, Marion Island and Ile Amsterdam, with the post-sealing status of Iles Crozet and Prince Edward Island being uncertain (Bester 1987; Kerley 1987; Roux 1987a). No population estimates are available for post-sealing remnant populations, although anecdotal evidence suggests that the seals were scarce (see Bester 1987; Kerley 1987; Roux 1987a and 1987b). Approximately 99% of the current pup production is reported at Gough Island, the Prince Edward Islands and Ile Amsterdam (73,000 recorded between 1988-1994, Hofmeyr *et al.* 1997), but there are also small, increasing populations at Iles Crozet, Tristan da Cunha and Macquarie Island. There are sympatric populations of AFS and SAFS at Iles Crozet, Marion Island and Macquarie Island.

The study by Wynen *et al.* (2000) suggested that the extent and/or the duration of the population bottleneck experienced by AFS and SAFS was not as severe as historical records would suggest. High nucleotide diversities within the partial control region of mtDNA (3.2% for AFS and 4.8% for SAFS), and significant population structure were reported in both species (Φ_{ST} =0.074 and Φ_{ST} =0.19, respectively). All population pairwise comparisons of F_{ST} for SAFS (except for Iles Crozet/Ile Amsterdam, and Marion Island/Macquarie Island) and estimated chi-squared values of haplotype frequency showed statistically significant differences. Despite the lower overall structure evident within AFS, two genetically differentiated regions were identified (Figure 4.1). The presence of these differentiated regions suggest that an additional population may have survived the sealing era than reported, possibly at Iles Kerguelen.

The regional differences in the distribution of genetic variation identified by Wynen *et al.* (2000) may form the basis of a number of distinct Management Units (MU) as defined by Moritz (1994a,b). But as this study was based on a molecular marker that has a maternal mode of inheritance, the distribution of genetic variation shown for both species reflects only female dispersal patterns. Microsatellites have a bi-parental mode of inheritance, are highly variable and have previously been shown to be appropriate for addressing population based questions in pinnipeds (e.g. Allen *et al.* 1995; Goodman 1998). Here we use microsatellite loci identified in other pinniped species (Burg *et al.* 1999; Coltman *et al.* 1996; Gemmell *et al.* 1997) to address further questions regarding population structure of the AFS and SAFS. In particular, we predict: (1) the levels of microsatellite variation observed within SAFS and AFS

are high, consistent with the mtDNA results; (2) the levels of population structure based on microsatellites within both species are lower than observed in mtDNA; and (3) higher levels of microsatellite variation and structure observed in SAFS relative to AFS, consistent with mtDNA results.

Methods and Materials

Sample Collection

Skin biopsies were collected from individuals representing most of the major population centres for both SAFS and AFS (Figure 4.1) and were stored as outlined in Wynen *et al.* (2000). A total of 213 individuals (primarily pups) were sampled from the following areas: Iles Crozet (CI, n=20), Ile Amsterdam (AI, n=20), Gough Island (GI, n=20) and Marion Island (MA, n=15) for SAFS; and South Georgia (SG, n=20), South Shetland Islands (Seal Island and Cape Shireff) (SS, n=18), Bouvetøya (BI, n=20), Marion Island (MA, n=20), Iles Crozet (CI, n=20), Iles Kerguelen (KI, n=20) and Heard Island (HI, n=20) for AFS. A number of New Zealand fur seal (NZFS) samples from non-breeding individuals at Macquarie Island (MI) were also included in the study for comparative purposes (n=16) due to the range overlap with AFS and SAFS. All samples used in the present study are the same as those detailed in Wynen *et al.* (2000). Species specific differences in the mitochondrial control region had been identified in this study between AFS, SAFS and NZFS. Therefore, species identification of all individuals is based not only on external phenotypic characters and natal colony, but also from the mtDNA haplotype, as determined from sequencing or through RFLP analysis.

Skin biopsies for AFS and SAFS were also collected from MI, but only data from mother-pup pairs were used for verification of Mendelian inheritance of microsatellite alleles. The data were not otherwise included in further analysis due to high reported levels of hybridisation (estimated at 25% by Goldsworthy *et al.* 1999), and the uncertainty of species identification using a marker with a bi-parental mode of inheritance. The data for MI require a more detailed treatment, and are the subject of another study (Wynen *et al.* in prep.).

Laboratory Analysis

Total genomic DNA was extracted from skin biopsies as described in Wynen *et al.* (2000). Microsatellite analysis was conducted by two methods: polymerase chain reaction (PCR) using γ^{33}P ATP end-labelling of primers; or PCR using the fluorescence technology of Applied Biosystems The primers used in this study had been previously isolated from three other pinniped species (Table 4.1).

The first method was employed only for the loci Hg6.3 and Hg4.2, and was used prior to the availability of ABI technology. Typically 1.05 Ci of radiation was added to a 3.5 µl reaction mix, with ~60 ng genomic DNA, 2-3 mM PCR Buffer (Promega), 1.5 mM dNTPs, 0.4 µM each of the labelled forward and unlabelled reverse primers, 1.5-3.0 mM $MgCl_2$ (see Table 4.1 for details), 0.05 units Taq polymerase and distilled water to volume. PCR conditions

Locus	T_1 / T_2 (°C)	$MgCl_2$ (mM)	Label	Species of Origin	Source	Accession No.	Primer Sequence (5' to 3')
Aa4	55	2.5	HEX	*Arctocephalus australis*	A		
Hg1.4	47 / 52	2	FAM	*Halichoerus grypus*	A	AF055862	
Hg6.3	48 / 55	1.5	FAM*	*H. grypus*	B	G02092	
Hg4.2	48 / 55	1.5	HEX*	*H. grypus*	B	G02090	
Pvc19	46	1.5	HEX	*Phoca vitulina*	C	L40989	
Pvc78	53	1.75	FAM	*P. vitulina*	C	L40983	
3E3	56	2.5	HEX	*P. vitulina*	D		TTGACATTGATACAATCCACCC
							TGAATAAAGTGGGTGGAGGG
4A3	58	2.2	TET	*P. vitulina*	D		ATCAGTATGGAAAAAAAATACACAC
							TGATTGGGACTGGAATGTCA
10E4	47 / 52	3	FAM	*P. vitulina*	D		CCACCCAGTCTATGGCACTT
							CAGTTCAAAACAAGTCTCAATATCA
Pv9	54	1.5	HEX	*P. vitulina*	E	G02096	
Pv11	47 / 52	2.25	FAM	*P. vitulina*	E	U65444	

Table 4.1: Microsatellite loci, anneling temperatures for PCRs conducted with fluorescently labelled primers (T_1 and T_2) and $MgCl_2$ concentrations as used in this study. The fluorescent label of the forward primer is also given. Primers had been isolated from other species as described in published sources: [A]Gemmell *et al* (1997); [B]Allen *et al.* (1995); [C]Coltman *et al.* (1996); [D]Kappe (1998); [E]Goodman (1997a). Genbank accession numbers are presented where available *screening of these loci were primarily conducted using $\gamma^{33}P$ dATP (see text).

were typically [1 cycle of 94° C 1', 35 cycles of (94° C 1', 59°/60° 1', 72° C 1'), 10° C to soak]. The microsatellites were run through a 6% denaturing sequencing polyacrylamide gel and visualised using autoradiography. Scoring of allele sizes was conducted by comparison to a size ladder run in each gel (as described by Fitzsimmons *et al.* 1995). Some genotypes for these loci were obtained using the fluorescence technology. However, to ensure consistency in scoring across the two methods, individuals of known genotype were incorporated in both analyses as controls.

For all other loci, the forward primers were labelled with a fluorescent tag at the 5' end to enable visualisation of the microsatellites using ABI technology (Table 4.1). PCR reaction volumes were 20μl containing ~60 ng genomic DNA, 1x PCR buffer (Promega), 0.8 mM dNTPs, 0.3 μM each of the labelled forward and unlabelled reverse primers, 1.5-2.5 mM $MgCl_2$, 0.1 μl Taq polymerase and distilled water to volume. Typical PCR conditions were either: [1 cycle of 94° C 1', 35 cycles of (94° C 1', T_1 1', 72° C 1'), 10° C to soak]; or [94° C 2', 8 cycles of (94° C 30s, T_1 30s, 72° C 40s), 25 cycles of (94° C 15s, T_2 15s, 72° C 40s), 10° C to soak]. Details for each locus, including the annealing temperature/s (T_1 and T_2) and $MgCl_2$ concentration are presented in Table 4.1. All PCRs were conducted separately, but the resulting PCR products were combined, requiring only two gel lanes per sample for characterisation. The combined products were mixed with a formamide loading dye containing GS350 Tamara internal lane size standards (ABI Prism, Genescan 350 PE Applied Biosystems, England). The samples were run through a 4.8% 6M urea denaturing polyacrylamide gel on the ABI Prism 377 DNA sequencer (PE Applied Biosystems), and analysed using GENESCAN 3.1 collection software (PE Applied Biosystems) and local southern size calling method. Scoring of alleles was conducted using Genotyper (version 1.1.1, PE Applied Biosystems).

Data Analysis

All loci were tested for conformity to the Hardy-Weinberg equilibrium (HWE) and for genetic disequilibrium using GENEPOP 3.1 (Raymond and Rousset 1995). Significance testing was estimated by the Markov chain method with 150-batches, 1500 iterations per batch and the dememorization number of 1500. Loci were compared across all species, as well as across all populations within AFS and SAFS. Levels of observed heterozygosity per locus for each population and species were calculated using the program TFPGA (Tools for Population Genetic Analyses, version 1.3; Miller 1997).

GENEPOP was also employed to calculate allele frequencies for each locus within populations and species, as well as to conduct tests for allelic and genotypic differentiation. The degree of genetic heterogeneity within each species was further investigated through the calculation of biased and unbiased estimates of R_{ST} (ARLEQUIN version 1.1 Schneider *et al.* 1997; R_{ST}CALC Goodman 1997b), F_{ST} (ARLEQUIN) and through the use of the assignment test described by Paetkau *et al.* (1995). Both biased and unbiased estimates of R_{ST} were calculated according to Slatkin (1995), but the latter is standardised for allele and

population sizes (labelled as UR_{ST} for convenience). Significance of F_{ST} estimates were obtained after 1000 permutations. The assignment test assumes random mating and independence of loci within the populations (Paetkau *et al.* 1995), but makes no assumptions of population equilibrium, which cannot be assured in newly colonised populations (Davies *et al.* 1999). The probability of identity (see Paetkau *et al.* 1995 for description) was also determined for each species (Paetkau and Strobeck 1994).

Results

General

A total of 11 microsatellite loci were amplified in the three species of fur seals screened in this study. The number and sizes of alleles amplified for all microsatellite loci in each species are presented in Table 4.2. Overall, the loci were highly polymorphic, with an average of 12.8 alleles per locus for all three species combined (range = 6-34, Table 4.2). Allele frequencies for each locus in all populations and species are presented in the Appendix.

For most of the loci, allele distributions for the three species were overlapping. However, species specific alleles were observed at some loci, most notably 3E3 and Pv11. The locus 3E3 was found to be monomorphic for both AFS and SAFS, with the diagnostic alleles of 155 and 153 occurring respectively. This locus was more polymorphic in NZFS which shared the 155 allele with AFS. However, NZFS and AFS showed disjunct allele distributions at the Pv11 locus (see Table 4.2 and Appendix). While these loci are useful for inter-specific analyses, they were excluded from intra-specific analyses due to 3E3 being monomorphic within species, and an insufficient sample size for Pv11 across populations.

One AFS individual screened in this study had a genotype contrary to expectation (3E3 153,153; Pv11 148,158 - see Table 4.2 for expected ranges). This adult female of unknown age is from the South Shetland Islands population. Either the alleles occur rarely in AFS and thus not detected in this study, or this individual is of another species or a hybrid. That inconsistencies occur at more than one locus suggests that the latter interpretation is more likely. The genotype is not consistent with any of the species screened in this study, nor of the South American fur seal (*A. australis*) which occur along the coast of South America and the Falkland Islands (allele range for Pv11 164-180; Gemmell *et al.* 1997). Nevertheless, because of the uncertain species identity of this individual, it was excluded from further analysis.

Table 4.2 (over page): The number of alleles and size range for microsatellites employed in this study for 'n' individuals of Antarctic fur seal (AFS), subantarctic fur seal (SAFS) and New Zealand fur seal (NZFS) *allele size ranges found here are different to those observed in harbour seals (3E3. 336-348 bp; 10E4: 121-123 bp, Kappe 1998).

	AFS			SAFS			NZFS			Total		
Name	n	No. alleles	Size range	n	No. alleles	Size range	n	No. alleles	Size range	n	No. alleles	Size range
Aa4	138	6	204-218	76	3	214-218	16	2	216-218	**229**	**6**	**204-218**
Hg1.4	137	9	189-209	76	10	191-211	16	7	193-205	**227**	**12**	**189-211**
Hg6.3	137	16	216-252	75	11	222-250	16	6	236-246	**227**	**17**	**216-252**
Hg4.2	134	25	126-230	76	22	144-224	16	14	136-222	**227**	**34**	**126-230**
Pvc19	138	5	110-118	76	6	112-122	16	3	112-116	**229**	**7**	**110-122**
Pvc78	138	10	138-162	76	13	132-158	16	11	136-164	**229**	**16**	**132-164**
3E3*	138	1	155	76	1	153	16	6	155-165	**228**	**7**	**153-165**
4A3	138	8	142-156	76	10	146-164	16	5	142-150	**229**	**12**	**142-164**
Pv9	138	11	168-188	76	8	168-182	16	7	168-182	**229**	**11**	**168-188**
Pv11	38	2	146-148	13	6	154-164	10	5	156-168	**62**	**10**	**146-168**
10E4*	12	5	132-144	2	3	134-150	6	5	136-148	20	**9**	**132-150**

Eight of the 11 microsatellite loci in the present study were previously amplified in AFS and NZFS (Pvc19, Pvc78, Aa4, Pv9, Pv11, Hg1.4, Hg4.2 and Hg6.3; Coltman *et al.* 1996; Gemmell *et al.* 1997), with only two for SAFS (Pvc19, Pvc78; Coltman *et al.* 1996). The allele size ranges observed in the different studies are comparable, with greater average numbers of alleles per locus observed in the present study (10.5 for AFS, 6.9 for NZFS and 9.5 for SAFS compared with 7.0, 5.3 and 4.0 in previous studies). This probably reflects the larger sample size across a greater geographic range for SAFS and AFS in the present study. Whilst the sample sizes of NZFS in all studies were small, the number used in this study was greater (16 versus 7).

The three remaining microsatellite loci, 3E3, 10E4 and 4A3, have not been previously screened in otariid species to date, preventing any comparisons. However, some significant differences in allele size was observed between the source species, the harbour seal (*Phoca vitulina*) and the fur seal species studied here. While the allelic size range of 142-164 base pairs (bp) found in this study for 4A3 did not greatly differ from that found in the harbour seal (148-150bp, Kappe 1998), this was not the case for the remaining two loci. For 3E3, the expected allele size was about 336-348 bp (Kappe 1998) compared with 153-165 bp in the fur seals, and for 10E4 the expected range was 121-123 bp, which is smaller than found in the fur seals (132-150 bp) (see Kappe 1998 and Table 4.2). The larger allele sizes of 10E4 for the fur seals conflicted with the co-loading scheme of the fluorescent labelled loci, and was therefore no longer employed in this study.

Such size discrepancies in the amplified products of homologous microsatellite regions between source and non-source species have been reported in a range of taxa including birds (see Primmer *et al.* 1996 and references therein) and marine turtles (Fitzsimmons *et al.* 1995). Sequence conservation at the primer binding sites is not unusual in related species, and differences in allele sizes are probably a reflection of the phylogenetic distance between the fur seals (family Otariidae) and true seals (family Phocidae).

Independence of Microsatellite Loci

The independence of all pairs of microsatellite loci was tested across all three species as well as across all populations within AFS and SAFS. No significant linkage was found in the 45 pairwise comparisons made (sequential Bonferroni correction applied, Rice 1989).

Hardy Weinberg Equilibrium (HWE)

Observed levels of heterozygosity for all loci in each species and in populations within species are presented in Table 4.3. Heterozygosity levels are highly variable, ranging from 0.00 to 1.00 at both the species and population level. For each locus/species combination,

Table 4.3 (over page): Observed heterozygosity and departure from Hardy-Weinberg expectations in all three species, and populations of AFS and SAFS for 10 microsatellite loci (10E4 excluded). Departure from HWE (heterozygote deficiency) denoted as **P<0.01 and *P<0.05 (sequential Bonferroni correction applied).

Locus	Hg1.4	4A3	Pvc19	3E3	Pv9	Aa4	Pvc78	Hg6.3	Hg4.2	Pv11	Overall
BI	0.25**	0.60	0.65	0.00	0.80	0.80	0.90	0.60	0.60**	0.00	**0.52**
CI	0.35**	0.65	0.80	0.00	0.80	0 55	0.85	0.70	0.60**	0.00	**0.53**
HI	0.26**	0.45	0.65	0.00	0.95	0 55	0.90	0.68	0.65**	0.38	**0.55**
KI	0.55*	0.40	0.60	0.00	0.85	0.80	0.85	0.85	0.75*	0.00	**0.57**
MA	0.30*	0.50	0.70	0.00	0.85	0.60	0.70	1.00	0.56**	0.00	**0.52**
SG	0.50**	0.30**	0.65	0.00	0.60	0.90	0.70	0.85	0.53**	0.00	**0.50**
SS	0.83	0.61	0.78	0.00	0.94	0.67	0.89	0.67	0.71**	0.00	**0.61**
AFS	**0.43****	**0.50****	**0.69**	**0.00**	**0.83**	**0.70**	**0.83**	**0.77***	**0.63****	**0.08**	**0.54**
AI	0.50**	0.60	0.45*	0.00	0.75	0.25	0.30	0.74	0.75	0.80	**0.51****
CI	0.42**	0.89	0.11	0.00	0 79	0.16	0.63	0.79	0.79	1.00	**0.56****
GI	0.65**	0.90	0.40	0.00	0.85	0.20	0.70	0.90	0.85	-	**0.55**
MA	0.06**	0.65	0.76	0.00	0.82	0.12	0 71	0.76	0.88	0.71	**0.55***
SAFS	**0.42****	**0.76****	**0.42****	**0.00**	**0.80**	**0.18****	**0.58**	**0.80**	**0.82**	**0.77**	**0.56****
NZFS	**0.06****	**0.56***	**0.38**	**0.63**	**1.00**	**0.13**	**1.00**	**0.75**	**0.88**	**0.80**	**0.62**

10 of 30 showed significant deviation from HWE, while at the population level, this was reduced to 20 out of 110 (Table 4.3). Significant deviations in the former are highly likely due to population structure, as is evident for the loci 4A3 and Aa4 in SAFS and Hg6.3 in AFS, where distributions were found to deviate from HWE at the species level, yet not at the population level (Table 4.3). The observed levels of heterozygosity in NZFS should therefore be interpreted with caution as no population data were collected for this species. The significant deviations observed for Hg4.2 within AFS excluded this locus from further analysis concerning this species.

The deviations from HWE detected were a result of homozygote excess, and could be a reflection of the Wahlund principle, indicate the presence of null alleles or be attributed to the locus having a mode of inheritance in violation of HWE (Callen *et al.* 1993; Pemberton *et al.* 1995). The latter is unlikely as all of the microsatellite loci employed in this study (except 3E3, 4A3 and 10E4) have previously shown predictable Mendelian inheritance in mother-pup pairs of a range of pinniped species (Coltman *et al.* 1996; Gemmell *et al.* 1997). Further, seven of the 11 microsatellite loci used in the present study had been screened in 117 known mother-pup pairs from Macquarie Island as part of another study (data not shown). Five of these loci showed patterns of inheritance consistent with Mendelian principles for all mother-pup pairs (Pvc19, 3E3, Aa4, Pvc78 and Pv11), while two loci showed occasional non-inheritance (in one pair for Pv9, and in four pairs for Hg1.4) (L. Wynen, unpublished data).

The significant deviations from HWE in all species and populations (except SS) for Hg1.4 is possibly due to the locus being sex-linked. Where the sex of sampled individuals was known (for all three species combined), only 7 of 53 (13.2%) males were heterozygous, compared with 41 of 61 females (67 2%). In the Macquarie Island samples containing AFS and SAFS, no males were observed to be heterozygous (n=88) compared with 135 of 175 females (77.1%). While this locus has been shown previously not to be sex-linked for a number of seal species including the South American fur seal (Gemmell *et al.* 1997), the vastly differing ratios in males and females found in this study is worth noting. Further, the 7 males found to be heterozygous were sampled at GI, HI and MA, and thus the author cannot assure that these individuals were accurately sexed (especially as juvenile males can easily be mistaken for adult females in the field). In any case, the major violation of Hg1.4 from HWE necessitates its removal from all subsequent comparative analyses of species and populations.

Inter-specific Comparisons

Although there was considerable overlap in allele size ranges of AFS, SAFS and NZFS (see Appendix), comparisons between the species showed highly significant differences in allele and genotype distributions ($P<<0.001$). Pairwise comparisons showed significant differences in all cases (most $P<0.005$), except for between NZFS and SAFS for the loci Hg1.4 and Aa4, and for AFS and NZFS for the locus 4A3 (genotypes only)

(a)

	AFS (n=138)	SAFS (n=76)	NZFS (n=16)
AFS	-	0.530**	0.410**
SAFS	0.339**	-	0.644**
NZFS	0.195**	0.259**	-

(b)

	Assigned		
Nominal	**AFS**	**SAFS**	**NZFS**
AFS	**138**	0	0
SAFS	0	**76**	0
NZFS	0	0	**16**

(c)

Locus	SAFS	AFS	NZFS	Over All Species
4A3	0.051	0.119	0.090	0.040
Pvc19	0.254	0.130	0.332	0.142
3E3	1.000	1.000	0.141	0.357
Pv9	0.065	0.046	0.043	0.040
Aa4	0.534	0.135	0.770	0.187
Pvc78	0.200	0.051	0.025	0.027
Hg6.3	0.034	0.033	0.092	0.018
Hg4.2	0.010	-	0.008	-
8 loci	**3.06×10^{-8}**	-	**2.57×10^{-9}**	
7 loci	-	**1.62×10^{-7}**		**7.37×10^{-9}**

Table 4.4: Examination of population heterogeneity in three species of fur seal. (a) Pairwise comparisons of UR_{ST} (above diagonal) and F_{ST} (below diagonal) for SAFS, AFS and NZFS. Significance levels **P<0.01 determined after 1000 permutations and adjusted for multiple comparisons. All estimates are with the exclusion of Hg1.4 and Hg4.2. (b) Species-level assignment matrix where individuals sampled from each species (nominal) were assigned to one of the three species (assigned) based on their genotype. (c) Probability of identity for all species.

Significant genetic differentiation between species was also indicated by the pairwise comparisons of UR_{ST} and F_{ST} (P<0.001) (Table 4.4a) as well as by biased R_{ST} measures (results not shown, P<0.05). The probabilities of identity for each locus and species overall are presented in Table 4.4c. The low overall value (1 in $1.4x10^8$) relative to those for AFS and SAFS (1 in $6.2x10^6$ and $3.3x10^7$ respectively) indicate that the loci employed in this study have a great capacity for identification for these species. This is less so for NZFS and is probably a reflection of the small sample size.

Intra-specific Comparisons

The examination of population structure within the SAFS and AFS showed consistently greater levels within the former, than the latter. Allele and genotype distributions within SAFS showed significant differentiation at all loci (P<0.005) except Aa4 and Pvc78 (the latter with a significant genotype distribution P<0.05). For AFS, there were more loci that displayed no population structure (Pvc19 and Pvc78), while only a few showed significant structure (Aa4 and Pv9: P<0.02). This same pattern was observed in population pairwise comparisons of allele distributions, where 42.9% of SAFS population comparisons were significant compared with only 11.6% of those in AFS.

These results are further supported by both R_{ST} measures, which for SAFS differed significantly from zero (R_{ST}=0.125, UR_{ST}=0.122; P<<0.001), in contrast to AFS where they did not (R_{ST}=0.011, UR_{ST}=0.003; P<0.288). Interestingly, the F_{ST} estimates for both SAFS and AFS indicated significant structure (F_{ST}=0.056 and F_{ST}=0.021 respectively; P<<0.001). Population pairwise comparisons of UR_{ST} and F_{ST} are presented in Table 4.5 for both SAFS and AFS. While the biased R_{ST} estimates showed slight differences in value to their corresponding UR_{ST} estimate, the significance level of all pairwise comparisons was the same. For this reason, these data are not presented here.

The assignment test results present an additional means of visualising the degree of population structure within SAFS and AFS (Table 4.6). For SAFS 18 individuals (23.7%) were assigned to populations other than the one from which they came. This is in contrast to the AFS, where 80 individuals (57.6%) were assigned to non-natal populations. These results clearly show that populations of SAFS are more differentiated from each other, with a greater probability of recognising an individual's natal population than for AFS.

Table 5 (over page): Population pairwise comparisons of UR_{ST} (above diagonal) and F_{ST} (below) for populations of (a) SAFS and (b) AFS. Population labels as described in Figure 4.1 and Methods. All measures calculated excluding 3E3, Pv11, Hg1.4 and Hg4.2. Significance levels** P<0.01 and * P<0.05 after 1000 permutations and corrected for multiple comparisons.

(a)

	AI (n=20)	CI (n=19)	GI (n=20)	MA (n=17)
AI	-	0.130**	0.021	0.084**
CI	0.078**	-	0.045**	0 044*
GI	0.055**	0.041**	-	0.003
MA	0.062**	0.058**	0.020*	-

(b)

	BI (n=20) (n=18)	CI (n=20)	HI (n=20)	KI (n=20)	MA (n=20)	SG (n=20)	SS
BI	-	-0.005	-0.012	-0.007	-0.010	0.002	-0.001
CI	-0.001	-	0.007	0.005	0.009	0.002	0.011
HI	0.015	0.002	-	0.002	0.010	0.002	0.005
KI	0.025*	0.014	-0.002	-	-0 015	0.013	-0.003
MA	-0.004	-0.004	0.021	0.028*	-	0.037	0.010
SG	0 015	0.014	0.010	0.020	0.031*	-	0.004
SS	0.013	0.017	0.028*	0.039**	0.020	0.032*	-

(a)

Nominal	Assigned Al[1]	Cl	Gl	MA
Al	**18**	0	1	1
Cl	2	**16**	0	1
Gl	0	3	**13**	4
MA	0	2	4	**11**

(b)

Nominal	Assigned Bl[1]	Cl	Hl	Kl	MA	SG	SS
Bl	**11**	2	0	0	4	2	1
Cl	4	**6**	2	1	3	2	2
Hl	2	1	**11**	6	0	0	0
Kl	0	3	4	**7**	3	2	1
MA	5	4	1	3	**5**	0	2
SG	3	3	1	1	2	**8**	2
SS	1	1	0	1	3	3	**9**

Table 4.6: Population-level assignment matrices showing the populations into which individuals from each population (nominal) were assigned (assigned) for (a) SAFS and (b) AFS Population labels as described in Figure 4.1 and Methods.

The probabilities of identity reveal the same pattern. For all populations of SAFS, these values are at least an order of magnitude larger than observed for the species as a whole (ranging from ~10^{-8} for Al and Gl to 10^{-7} for Cl and MA, data not shown). The results indicate that individuals within a population are slightly more related to each other than to those from other populations. The probability of identity for all populations of AFS are of the same order of magnitude as overall (data not shown) except for SS (1.4×10^{-10}) and SG (1.7×10^{-8}). The great potential of these microsatellites for individual identification (as required for paternity analysis etc) is further demonstrated by the fact that all of the 214 individuals for which complete genotypes were obtained (excluding Pv11), displayed genotypes that were unique. Those genotypes that were very similar, were generally found between individuals sampled from the same population.

Discussion

General

This study has employed microsatellites to study the distribution of genetic variation within the Antarctic and subantarctic fur seals, to gain some insight into the population dynamics of species that are otherwise very difficult to study. It was predicted that there would be high levels of genetic variation within each species, with the greater levels being exhibited by SAFS. This species was also expected to show more pronounced population structure relative to the AFS, a pattern that was observed with mtDNA. It was further predicted that there would be greater levels of variation and less population structure observed in the microsatellite data relative to that observed in the mtDNA. In general, many of these predictions were upheld.

An interesting finding was the high degree of variability observed in the levels of diversity and differentiation both between microsatellite loci and between species. The numbers of alleles per locus at the species level ranged from 1 to 25, while heterozygosity levels ranged from 0.00 to 1.00, with overall species averages between 0.54 and 0.62. Such high variability in genetic variation at different loci highlights the wide-ranging applications to which these loci can be applied. Those with large numbers of alleles and high heterozygosity levels are useful for the examination of intra-specific issues such as those pertaining to population structure and paternity. However, loci that exhibit few alleles and low levels of heterozygosity, most notably 3E3 and Pv11, show great utility for species identification.

Population structure was investigated using a range of methods in an attempt to present the most complete representation for both AFS and SAFS. Estimates of R_{ST} are thought by some to be more appropriate for microsatellite data as they are calculated under a step-wise mutation model, rather than F_{ST} estimates which assumes mutation under an infinite alleles model (Jarne and Lagoda 1996). However, there is no consensus as to which model best reflects the microsatellite mutational process, and the application of these models to data from natural populations presents problems as these data may violate assumptions under these models (Jarne and Lagoda 1996; Goodman 1998). However, Gaggiotti *et al.* (1999) suggest that where sample sizes are small or moderate ($n<10$) and only a small number of loci are used ($n<20$), the most conservative approach is to use F_{ST}. While this scenario best fits our data, both estimates are presented for completeness. To assist in better visualisation of any population structure inherent within each of the species without assuming that the populations are in equilibrium, the assignment test was also employed.

Inter-specific Comparisons

Despite overlapping allele ranges, high levels of genetic differentiation were observed between all three species examined here. Differences in allele distribution and low probabilities of identity indicate that these microsatellites provide an excellent basis for species identification, as shown by assignment test results (Table 4.4b). This is not

surprising as previous comparative work including these species show pairwise sequence divergences of the partial cytochrome *b* gene ranging from 0.034-0.049 (Wynen *et al.* 2001).

The more pronounced population structure within SAFS relative to AFS as shown by microsatellites was also observed in mtDNA (Wynen *et al.* 2000) This is evident when all measures of population heterogeneity are examined, including the assignment test. However, it is difficult to categorically compare population structure as exhibited by mtDNA directly with that exhibited by microsatellites. The $F_{ST(msat)}$ for SAFS and AFS (0.056 and 0.021 respectively) are lower than the corresponding values derived from mtDNA data (0.19 and 0.074 respectively) suggesting less structure is detected with the nuclear markers. However, there are more pairwise significant differences of $F_{ST(msat)}$ between populations within each species than for $F_{ST(mtDNA)}$. For SAFS mtDNA data (excluding Macquarie Island comparisons), 83.3% of all $F_{ST(mtDNA)}$ comparisons differed significantly from zero (where $F_{ST(mtDNA)}$ = $_{ST}$ based on mtDNA 'haplotypes only' as described in Wynen *et al.* 2000), compared with 100% of all those for $F_{ST(msat)}$. The respective values for AFS are 14.3% and 28.6%. Far less structure is shown if UR_{ST} values are used, with 66.7% and 0.0% of pairwise comparisons significant in SAFS and AFS respectively. The difference between the F_{ST} and R_{ST} values and their patterns of significance, is probably due to the models upon which these measures are derived and their underlying assumptions, which makes the results difficult to interpret.

Intra-Specific Comparisons

At the intra-specific level, microsatellite variation within AFS and SAFS was similar, though variable. Generally, there was greater population structure within SAFS relative to AFS, with the assignment test results showing a higher proportion of SAFS individuals being correctly assigned to their natal population (Table 4.6). The two estimates of R_{ST} obtained for SAFS indicated significant structure whereas the corresponding estimates for AFS did not. The F_{ST} estimates for both species indicated significant structure, though at a greater level for SAFS.

Within the SAFS, the estimates of F_{ST} were greater than UR_{ST}, with all pairwise comparisons being significant for the former ($P<0.025$), compared with only three for the latter. While F_{ST} suggests restricted gene flow between all populations, UR_{ST} only reveal differences between the population at CI to those at both GI and AI, as well as between MA and AI. The lack of structure between the remaining pairwise comparisons suggest a pattern of historic and/or contemporary gene flow from GI in the west, to populations at AI and MA, as well as from MA to CI. CI is the only population screened in this study that became extinct as a result of sealing. The primary source of recolonisation as inferred from mtDNA data was from the population at MA, with possible contributions from AI. However, while our data support gene flow between MA and CI, there is no evidence from UR_{ST} and F_{ST} estimates of gene flow from AI to CI.

The lack of significant population pairwise comparisons of UR_{ST} within AFS suggest that despite the extraordinarily large geographic range of this species, all populations are panmictic. In contrast, the F_{ST} estimates suggest that there is some population structure evident within the species, with six pairwise comparisons ($P<0.05$), and the overall estimate for the species ($F_{ST}=0.021$; $P<<0.001$) found to differ from zero. Interestingly, three of the six significant comparisons were between KI and three populations from the western region as identified by mtDNA data (SS, MA and BI, Wynen *et al.* 2000). The presence of low levels of structure within the species is also evident in the assignment test with 42.4% of individuals being correctly assigned to their natal population.

Ecological and Management Applications

The distribution of genetic variation within each species as revealed by both molecular markers reflect a balance of the processes that both facilitate and degrade population structure. One of the most influential factors in the recent history of the AFS and SAFS populations is human exploitation (or sealing). While high pre-sealing population sizes for both species infers high levels of genetic variation, the population bottleneck that resulted from sealing, though not extreme, could have still altered these levels to varying degrees (Wynen *et al.* 2000). Therefore, the observed levels and distribution of genetic variation in AFS and SAFS is a result of the amount of historic variation and structure remaining after sealing, and how it has been altered through contemporary population dynamics.

The historical records suggest that AFS suffered greater levels of exploitation compared to SAFS, both in terms of larger reductions in overall abundance as well as a higher number of population extinctions. That AFS was more intensely hunted compared to SAFS suggests that the former would have lower levels of contemporary genetic variation. While this pattern is evident in mtDNA, it is not the case for the nuclear DNA. In fact, both species exhibit very high levels of genetic variation in mtDNA and variable levels for nuclear DNA, suggesting that the extent and/or duration of the population bottleneck was not as extreme as indicated in historic documentation (see Wynen *et al.* 2000 for discussion).

However, the reductions in the size of individual populations and population extinctions cannot be ignored entirely. Reduced population sizes at the height of sealing, and founder effects in newly recolonised populations would facilitate increased effects due to genetic drift, possibly leading to greater population differentiation. Mutation and selection are additional factors to consider, although these are unlikely to be major contributors to the distribution of genetic variation over the last 100 - 150 years of post-sealing recovery that is the primary focus.

Migration is the major factor acting against increased population structure. The capacity for far-ranging dispersal in both AFS and SAFS has long been recognised (e.g. Carr and Carr 1985; Castello and Pinedo 1977; Wynen *et al.* 2000 and references therein), and is clearly illustrated by the remarkable recolonisation efforts observed in the post-sealing era. If this

dispersal capacity translates into gene flow, then one might expect to observe intra-specific genetic homogeneity. Such was the expectation in two other marine mammals, the southern elephant seal (*Mirounga leonina*) and the polar bear (*Ursus maritimus*). These species are both known to range widely from their natal population, yet strong population structuring within these species at the molecular level suggest that individuals show high levels of philopatry and site fidelity (Paetkau *et al.* 1995; Slade 1997). Clearly, in these cases apparent migration does not equal actual migration, and the processes acting for population structure outweigh the effects due to migration.

However, in fur seals, the actual migration is known to have occurred at a relatively high rate as shown by the recolonisation of many islands over the past 100 years of the post-sealing era. Therefore, the factors acting for population structure are outweighing the effects of migration, as is evident in SAFS for both mtDNA and microsatellite DNA. Three populations survived sealing, and these were widely separated, ranging from the South Atlantic Ocean through to the South Indian Ocean (Figure 4.1). Evidence of high levels of pre-sealing population structure would be 'carried over' into the post-sealing populations, and possibly enhanced due to drift when the populations were small. While migration led to the recolonisation of populations at Iles Crozet, Macquarie Island and Tristan da Cunha, founder effects and genetic drift would also be contributing factors determining contemporary variation at these sites. AFS survived at South Georgia and Bouvetøya, as revealed from historic records, and possibly at Iles Kerguelen also (Wynen *et al.* 2000). Nevertheless the rapidity of recovery has seen a major recolonisation effort by AFS, resulting in levels of gene flow that would override any founder effects or those of genetic drift.

Our results generally suggest that the level of population structure as indicated by mtDNA is greater than that observed with the nuclear markers. The F_{ST} values based on microsatellite data are lower, as are the UR_{ST} values. While the significance of population pairwise UR_{ST} estimates also suggest less structure, those of F_{ST} suggest the reverse. While difficult to interpret data with conflicting results, a pattern of less structure evident with nuclear markers relative to mitochondrial markers is the expectation. Being maternally inherited, the effective population size of mtDNA is much smaller than for nuclear markers, and is thus more susceptible to genetic drift.

Fur seals are thought to conform to the theoretical pattern of mammalian dispersal (female philopatry, male dispersal), and is an alternate consideration when examining the difference in population structure as exhibited by mitochondrial and nuclear DNA. While female philopatry in AFS and SAFS is assumed, no studies have quantified it, although some have shown that females of both species have a high degree of breeding site fidelity (Lunn and Boyd 1991; C. Guinet personal communication). Anecdotal evidence also suggests that males of both species show high breeding site fidelity at reproductive age (at Macquarie Island, Iles Amsterdam; unpublished data S. Goldsworthy and C. Guinet), yet appear to disperse more readily than females (Boyd *et al.* 1998). However, without any quantitative

data, discussion of sex-biased dispersal remains somewhat speculative. This is especially so with the fur seals where the remarkable capacity of both sexes to disperse has been illustrated in the recolonisation efforts throughout the subantarctic. While the non-dispersing sex in mammals does occasionally disperse (Smale *et al.* 1997) as a balance between factors such as philopatry and breeding site density (Bradshaw *et al.* 2000), the rapid rate of recolonisation across such broad geographic distances suggest the presence of other contributing factors affecting fur seal females.

Fur seal populations have been in a state of flux over the past few centuries, with the effects of the sealing era still manifesting themselves today. This study and that of Wynen *et al.* (2000) have identified some genetically differentiated populations and regions within both AFS and SAFS, which might form the basis of a series of Management Units (MUs). However, the current ecological context of the definition of these units needs to be considered. The fur seal populations and those of all other species with which the fur seals interact are likely to continue to change throughout the early stages of this century. As such, the boundaries of these MUs may change over time, and constant reassessment is therefore required. Furthermore, the large geographic distances between populations within the fur seal species suggests that resources and environmental conditions may be quite different between sites, regardless of any perceived genetic differences or similarities. The significant dietary differences between AFS in the South Georgia/South Shetland Island populations compared with those in the South Indian and Pacific Oceans (e.g. Bonner 1968; Croll and Tershy 1998; Green *et al.* 1989, 1990) is a prime example, and is likely to lead to other flow-on effects such as differential interactions with fishing activities. There are also large differences in population density at different islands, and the corresponding affects that high levels have on other species. Such issues show that while molecular genetic techniques clearly provide a useful tool for the identification of potential management units, it should not be used in isolation of other ecological factors.

In summary, variable levels of genetic variation were observed in the range of microsatellite loci employed in this study. Many of the loci studied showed application for addressing a range of different questions. While SAFS exhibited comparable levels of genetic variation to AFS, the former species did show greater levels of population structure. The distribution of genetic variation in both mitochondrial and nuclear markers was not unexpected given the smaller effective population size of mitochondrial markers.

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CHAPTER 5: Hybridisation between fur seal species (*Arctocephalus* spp.) at Macquarie Island: a comparative analysis of molecular and field based techniques for species identification

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Abstract

The fur seal population at Macquarie Island is unique in that it is the only population where Antarctic (*Arctocephalus gazella*) (AFS), subantarctic (*A. tropicalis*) (SAFS) and New Zealand fur seals (*A. forsteri*) (NZFS) occur in sympatry. Although hybridisation among these species has been previously reported, the extent and direction of hybridisation has not been well documented. The aim of the study was to determine the incidence of hybridisation within the Macquarie Island population by undertaking genetic analysis of a single cohort of pups (1996/1997), their mothers and the adult males from the previous breeding season. This study compared analyses of both mitochondrial DNA (mtDNA) and six microsatellite loci with field identification methods based on external phenotypic traits. mtDNA haplotypes of 125 pups, 113 adult females and 33 territorial and challenger males were from either AFS (83%, 88% and 70% respectively) or SAFS (17%, 12% and 30% respectively). No NZFS mtDNA haplotypes were detected. Results of microsatellite allele frequencies using an assignment test also showed a predominance of AFS genotypes in pups, adult females and adult males (71%, 76% and 39% respectively), with lower levels of SAFS (24%, 30% and 39% respectively). Interestingly, some individuals were assigned to the NZFS class (5%, 4% and 21%). However, the assignment test does not allow for the identification of hybrids, without resorting to a subjective assessment. Therefore, individuals were identified to species or as a hybrid based on the more objective combined mtDNA/microsatellite genotype approach, which relies on the presence of species-specific alleles at some loci. AFS then accounted for 54%, 72% and 27% of all pups, adult females and adult males respectively, with the proportions for SAFS being 15%, 11% and 24%. No individuals were found to be NZFS. Hybridisation within the pup cohort (30.4%) was much greater than in the breeding females (17.5%), but much less than in the breeding males (48.5%). When the males are grouped as territorial and challenger males, the proportion of hybrids of each class differs (58 8% and 37.5% respectively) NZFS alleles were identified in 29.7% of all hybrid individuals, suggesting the contribution of this species is greater than expected. Further, 18.9% of hybrids are backcrossed, indicating that these fur seal hybrids are not sterile. The two field based methods of species identification of the pup cohort found the proportion of hybrids ranged from 5.5% to 7.7%. While these methods had a high success rate in classifying individuals to the correct species relative to the overall genotype approach (75.8% to 70.9%), they severely underestimated the number of hybrids in the population.

Introduction

Hybridisation between two or more species under natural conditions has been reported widely in a range of plant and animal taxa, including vertebrates (eg. deer, fish, wolves and frogs, Abernethy, 1994; Roy *et al.*, 1994; Mukai *et al.*, 1997; Fischer *et al.*, 2000; Lu *et al.*, 2001). In marine mammals, while there are a number of records of hybrids in captivity and of different species attempting to mate, there are few reports indicating that hybridisation occurs naturally (eg. Stewart *et al.*, 1987; Kovacs *et al.*, Baird *et al.*, 1998; Berube and Aguilar, 1998; 1997; Goldsworthy *et al.*, 1999). While this might be so for marine mammals, the interbreeding of individuals of different species or genetically differentiated populations is far from a novel process, and is considered an important mechanism of evolution, potentially contributing to the process of speciation (Barton, 2001). Some hybrid zones in the natural environment have been reported to be large and stable (see Hewitt, 2001), while others may arise as a consequence of human disturbance (Rhymer and Simberloff, 1996).

At Macquarie Island (54° 30'S, 59°57' E), a small population of fur seals (*Arctocephalus* spp.) is recovering from extinction brought about by sealing activities in the early 19th century (Csordas, 1963; Shaughnessy and Fletcher, 1987). Observations of the recovering population have reported the presence of mixed-species breeding territories, inter-specific matings, and the appearance of individuals with intermediate phenotypes (Shaughnessy and Fletcher, 1987; Shaughnessy *et al.*, 1988; Goldsworthy *et al.*, 1999). The presence of hybrids was confirmed by Goldsworthy *et al.* (1999), through comparison of species-specific mitochondrial DNA (mtDNA) haplotypes of pups with their parents as identified by DNA fingerprinting. Aside from this study, species identification to date has relied upon a series of external characters that discriminate among species. However, identification of hybrids based on morphological characters alone can be problematic (Bester and Wilkinson, 1989), especially after several generations of backcrossing (Rhymer and Simberloff, 1996), which is thought to occur at Macquarie Island.

This study proposes to address four main aims: to determine the species composition and extent of hybridisation among fur seals at Macquarie Island based on a single cohort of pups, and breeding females and males from the same breeding season; to determine if the species composition and extent of hybridisation differs between pups, adult females and adult males; to investigate the direction of hybridisation and its potential impact on species/population recovery; and to assess the accuracy of field based methods of species identification.

Materials and Methods

The fur seal population at Macquarie Island is small (pup production in 2000/2001 of 160), but increasing (13.5% *per annum* between 1954-1996, S.D. Goldsworthy, unpublished data) and is more or less confined to the northern tip of the island.

The three species of fur seal that occur at Macquarie Island are the Antarctic fur seal (*Arctocephalus gazella*, AFS), the subantarctic fur seal (*A. tropicalis*, SAFS) and the New Zealand fur seal (*A. forsteri*, NZFS). However, only the AFS and the SAFS comprise the breeding population in general, with approximately 80% of the breeding females being identified as AFS (L.P. Wynen, personal observation). The NZFS population is composed almost entirely of males, which primarily haul out on the island to moult. A small number of NZFS are present on the island at any time of the year, but the majority arrive in late summer/early autumn, approximately one month following the breeding season of the other species. While NZFS have occasionally bred at Macquarie Island, the incidence is very low, with some of these seals subsequently being identified as hybrids (Goldsworthy *et al.*, 1999; L.P. Wynen, unpublished data).

Field observations were conducted throughout the 1996 breeding season to record the arrival and pupping date of each adult female. As a large proportion of the adult population is marked with external tags in the front fore flippers (Dalton Tags, Woolgoolga NSW), the identity of these individuals was also recorded. Pups were captured and marked for individual recognition once the mother had left on the first foraging trip. Marking was conducted initially with paint or bleach and internal tags (TIRIS, Texas Instruments), and then with external flipper tags when they were older. At the time of marking, each pup was sexed, described in terms of physical characteristics, measured (see below), and had a 6 mm diameter skin biopsy collected for genetic analysis. Observations of the colony were conducted throughout the season to establish the identity of mother-pup pairs, and the birth date of the pup. Skin biopsies were also collected from most of the adult females from 1996 and adult males from 1995. Field observations conducted in the 1995 breeding season established the territory structure of the colony, and recorded the identity of territorial and challenger males.

Species Identification

Species identification of all age classes at Macquarie Island is currently based on a series of phenotypic characters that are readily recognisable in the field (Condy 1978; Goldsworthy *et al.*, 1999). However, definitive species identification is difficult because hybrid individuals may display phenotypes that are intermediate to the parental species. Therefore, a molecular based method is sought, and compared with the field based methods to assess their reliability. The primary emphasis is on the identification the AFS and SAFS, these being the two main breeding species. While the NZFS is regarded as rarely contributing to the breeding process, it cannot be ignored, and is thus included in the molecular analysis to determine its relative contribution. The specific methods used to assess the species composition of the Macquarie Island population are detailed as follows.

Molecular Analysis

Species-specific haplotypes have been recognised in the mitochondrial control region for the AFS, SAFS and NZFS (Goldsworthy *et al.* 1999; Wynen *et al.*, 2000). DNA was extracted from skin biopsies from the pups, adult males and females, and a section of the mitochondrial control region amplified and screened with a range of restriction enzymes that target the species-specific nucleotide changes previously recognised. Specific details pertaining to the collection of skin biopsies, their storage, DNA extraction, polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) procedures have been outlined previously (Wynen *et al.*, 2000).

Hypervariable microsatellites have also shown utility for the identification of AFS, SAFS and NZFS (Wynen *et al.*, in review). A total of seven loci were employed in this study, Pvc19, 3E3, Pv9, Aa4, Pvc78, Pv11 and Hg1.4. PCR conditions and characterisation conducted using ABI technology (PE Applied Biosystems, England) is detailed in Wynen *et al.* (in review).

Given the records of hybridisation and backcrossing within the Macquarie Island population in the post-sealing era (Goldsworthy *et al.*, 1999), reference DNA material from allopatric populations were used to assess species differences at the molecular level for AFS, SAFS and NZFS. Populations sampled for AFS include Bouvetøya, Marion Island, South Georgia, the South Shetland Islands, Iles Crozet, Iles Kerguelen and Heard Island. Those for SAFS include Ile Amsterdam, Gough Island, Iles Crozet and Marion Island. The NZFS samples were collected from itinerant non-breeding males from Macquarie Island (see Wynen *et al.*, 2000; in review).

The assignment test of Paetkau *et al.* (1995) was used to discriminate between the AFS, SAFS and NZFS using samples from all populations (excluding Macquarie Island) as the reference data set (n=230). Wynen *et al.* (in review) showed that the assignment test was accurate in re-assigning individuals to the correct species 99.6% of the time. We used these data as the basis for the identification of the Macquarie Island fur seals. The microsatellite data for the individuals were incorporated into the reference data set as of 'unknown' origin, and the test run, with the individual's scores for each species noted. The species class that yielded the highest assignment score was the class into which the pup, adult male or adult female was assigned.

An alternative approach to determine the species identity of the Macquarie Island fur seals was a direct comparison of their genotypes with the expected allele ranges for each species as revealed by the reference data set. Many of the loci employed in this study show species-specific alleles, and such a direct comparison of an individual's genotype will allow the identification of hybrids. Species-specific alleles are defined as those that appear in one species, and not in either of the other two species.

Phenotype Methods

The field based methods that rely on external traits were compared with the molecular data for pups only, because it is for this class that the data are most reliable. Two methods are addressed, and these are the 'Overall Phenotype' (OP) method and the 'Phenotype Score' (PS) method.

The OP method is where an observer makes an overall assessment of the individual, identifying the species to which it belongs based on a range of phenotypic traits including pelage, behaviour and vocalisations. Species identification by this method is easy, yet highly subjective, with results possibly varying from year to year, as well as with observer. The OP method has been the primary basis for species identification of fur seals, especially adults, at Macquarie Island over the past decade.

The PS method aims to adopt a more systematic means of the species identification of pups. Based on phenotype criteria developed by Condy (1978), Bester and Wilkinson (1989), and Goldsworthy *et al.* (1999), this method assesses four readily identifiable natal pelage characteristics in a systematic way. These characteristics include the colour of the pelage over the body, muzzle, and belly, as well as the presence/absence of grizzling (where grizzling refers to pale-tipped guard hairs). Scores are allocated as described in Table 5.1. An average of the four scores is obtained and the pup allocated to one of the following classes based on this final score: AFS 0 -0 25; SAFS 0.75 - 1; and hybrid 0.25 <x>0.75.

	Species			
Character	**AFS**	**Score**	**SAFS**	**Score**
Pelage colour	grey	0	glossy black	1
Muzzle colour	pale	0	dark	1
Belly colour	pale	0	dark	1
Grizzling	present	0	absent	1
Average Score		**0**		**1**

Table 5.1: Phenotype characters and corresponding scores employed for species identification of fur seal pups, where AFS = Antarctic fur seal and SAFS = subantarctic fur seal. Scores of 0.5 are assigned when the characters appear to be intermediate, and 0.75 or 0.25 assigned if the character is indicated to be only slightly different to the ideal.

Other Potential Field Methods

Studies into the AFS and SAFS over the years have recognised a range of differences in the species and their ecology that may provide a basis for a species identification method. Here we briefly examine a number of those differences, specifically hind flipper shape, the timing of birth and lactation length to ascertain their potential.

The shape of AFS and SAFS hind flippers are markedly different, with those of AFS being generally long and thin, while those of the latter being short and broad (Bester and Wilkinson, 1989). Hind flipper measurements were collected from the 1996 cohort of pups as follows. The distance from the base of the nail to the tip of the flipper was measured for the first, third and fifth digits (D1, D3 and D5 respectively, where D1 corresponds to HFT, Bester and Wilkinson, 1989). Measurements were also taken of the hind-flipper length (L) and width (W), where L is from the base of the heel to the tip of the first lateral digit (corresponds to HFS, Bester and Wilkinson, 1989), and W measured along the base of the nails with the flipper stretched out. The index, W/L (width/length), was created to provide a relative index of shape while removing the possibly confounding factor of pup size. To reduce any effect due to age, the data were separated into three age classes; Age Class 1 (0-20 days), Age Class 2 (21-39 days) and Age Class 3 (40+ days old). The data for each flipper measurement were checked for conformance to a normal distribution by visual inspection of probability plots. Differences between species and/or sex were tested for significance by Analysis of Variance and a post-hoc Tukey test. In a preliminary assessment of whether hind flipper measurements are likely to provide a suitable means of species identification, we also conducted a discriminant function analysis.

An offset in breeding period of AFS and SAFS and differences in the length of the lactation period have been previously recognised (Condy 1978; Kerley, 1983a; Goldsworthy, 1992). The field observations conducted throughout the 1996 breeding period enabled the recording of the birth date of the pups (described above) and some of their weaning dates. These data are evaluated in the light of the genetics results to determine their utility for species identification.

Additional differences between the species that may be useful but are not addressed in this study include vocalisations, and the timing of the natal coat moult (Condy 1978; Page *et al.*, 2001).

Results

One hundred and thirty pups were born at Macquarie Island during 1996. Most individuals were described, measured and had skin biopsies collected. However, a number of pups disappeared in the immediate weeks after birth, and some data could not be collected. Most of the mothers that pupped in 1996 were sampled (n=115, 88.5%), as were all territorial and most challenger males of the 1995 breeding season (n=33, with 51.5% being territorial males).

Molecular Analysis

The molecular markers employed in this study were chosen to investigate hybridisation because of the differences in the mode of inheritance of the mitochondrial markers relative to

the microsatellite markers (mtDNA having a maternal mode of inheritance and microsatellites, bi-parental). The AFS mtDNA haplotypes dominated the 1996 cohort of pups (83%), breeding females (88%) and adult males (70%) (Table 5.2). SAFS mtDNA haplotypes accounted for 17% of pups, 12% of adult females and 30% of adult males (Table 5.2). The proportion of AFS and SAFS mtDNA haplotypes was significantly different between adult females and adult males ($G=5.164$, $p<0.05$), but not between pups and adult males ($G=2.712$ $p>0.05$) or pups and adult females ($G=1.241$ $p>0.05$). No NZFS mtDNA

Method	AFS	SAFS	NZFS	HYB	No. (%) individuals sampled
Pups					
'Overall Phenotype'	98 (77.8)	21 (16.7)	0 (0.0)	7 (5.5)	126 (96.9)
Phenotype Score	82 (70.1)	26 (22.2)	-	9 (7.7)	117 (90.0)
mtDNA	104 (83.2)	21 (16.8)	0 (0.0)	-	125 (96.2)
Assign Test 1	89 (71.2)	30 (24.0)	6 (4.8)	-	125 (96.2)
Assign Test 2	80 (64.0)	22 (17.6)	0 (0.0)	23 (18.4)	125 (96.2)
Genotype	68 (54.4)	19 (15.2)	0 (0.0)	38 (30.4)	125 (96.2)
Adult Females					
mtDNA	99 (87.6)	14 (12.4)	0 (0.0)	-	113 (86.9)
Assign Test 1	87 (76.3)	23 (20.2)	4 (3.5)	-	114 (87.7)
Assign Test 2	86 (75.5)	16 (14.0)	0 (0.0)	12 (10.5)	114 (87.7)
Genotype	82 (72.0)	12 (10.5)	0 (0.0)	20 (17.5)	114 (87.7)
Adult Males					
mtDNA	23 (69.7)	10 (30.3)	0 (0.0)	-	33 (?)
Assign Test 1	13 (39.4)	13 (39.4)	7 (21.2)	-	33 (?)
Assign Test 2	10 (30.3)	8 (24.2)	0 (0.0)	15 (45.5)	33 (?)
Genotype (overall)	9 (27.3)	8 (24.2)	0 (0.0)	16 (48.5)	33 (?)
Genotype (TM)	2 (11.8)	5 (29.4)	0 (0.0)	10 (58.8)	17 (100)
Genotype (CM)	7 (43.7)	3 (18.8)	0 (0.0)	6 (37.5)	16 (?)

Table 5.2: Species composition of the 1996 cohort of pups at Macquarie Island (n=130) as determined by the different field and molecular methods as described in the text. Assign Test 2 differs from Assign Test 1 in that hybrids were identified subjectively by proximity to the line of equality (see text) Figures in brackets are percentages. Species labels as defined in text, with HYB = hybrid. The proportion of the cohort sampled for each method is also presented. Species composition of adult females that pupped in 1996 (n=115), and adult males in the colony in 1995 (n=33) determined by molecular methods only. Species composition of territorial males (TM) and challenger males (CM) also included for genotype method only.

haplotypes were detected in the population, which is not surprising given that NZFS females are rarely sighted on the island. The absence of NZFS mtDNA haplotypes was also reported by Goldsworthy *et al.* (1999).

Of the seven microsatellite loci used to screen the Macquarie Island fur seals, only six were included in the final analysis. Hg1.4 was found to deviate significantly from Hardy Weinberg Equilibrium in the AFS, SAFS and NZFS, and thus had to be excluded from the present analysis (see Wynen *et al.*, in review). The allele sizes and ranges for the six loci in the reference data set are presented in Table 5.3 Of the loci employed, 3E3 and Pv11 are particularly useful for species identification due to the presence of species-specific alleles as shown in the reference data set (eg. 148 is a species-specific allele for AFS at PV11, while 153 is one for SAFS at 3E3). There are some species-specific alleles in other loci, but differences between species are primarily a result of different allele frequency distributions.

Two different approaches were taken to analyse the genetic data. Firstly, an assignment test was used to assign pups to one of the three species (AFS, SAFS and NZFS, assign test 1). Further interpretation was conducted on the assignment test results to ascertain whether this test was able to identify hybrids (described in detail below, assign test 2). Secondly, the microsatellite genotype of each pup was compared with the expected allele size ranges from the reference data set, and the pup assigned to the species whose allele sizes were comparable. Hybrids were identified as sharing species specific alleles from more than one species, and/or having a mtDNA haplotype contrary to its microsatellite genotype.

The results of the assignment test are presented in Table 5.2 (assign test 1). Interestingly, a total of 17 individuals were classed as NZFS, suggesting that this species does, or has participated in breeding at Macquarie Island. The allele size ranges for the pup cohort are presented in Table 5.3, and when compared with the reference data set for each species, it is apparent that there is a broadening of the ranges in the Macquarie Island population. This is particularly noticeable in 3E3 and Pv11, and is probably a result of hybridisation.

The respective proportions of individual pups and breeding females assigned to AFS (71.2%, 76.3%), SAFS (24.0%, 20.2%) and NZFS groups (4.4%, 3.5%) using assign test 1 were not significantly different ($G=0.821$, $p>0.05$, Table 5.2). However, these proportions were different for both pups and breeding females when compared with adult males ($G=13.87$ $p<0.001$ and $G=16.958$ $p<0.001$ respectively). The differences lay in the much lower proportion of adult males that were assigned as AFS (39.4%) with a relatively large proportion as SAFS (39.4%) and NZFS (21.2%).

	Aa4	3E3	Pv9	Pvc19	Pvc78	Pv11
Reference Data Set						
AFS	204-218 (6)	155 (1)	168-188 (11)	110-118 (5)	138-162 (10)	146-148 (2)
SAFS	214-218 (3)	153 (1)	168-182 (8)	112-122 (6)	132-158 (13)	154-164 (6)
NZFS	216-218 (2)	155-165 (6)	168-182 (7)	112-116 (3)	136-164 (11)	156-168 (5)
Species Specific Alleles						
AFS	204-208 (3)		184-188 (3)	110 (1)		146 (1)
SAFS		153 (1)		120-122 (2)	132-4,140-2 (4)	154,8,162 (3)
NZFS		157-165 (5)			164 (1)	166-168 (2)
Macquarie Island Data - assign test 1						
AFS	206-218 (5)	153-155 (2)	168-188 (10)	110-120 (6)	136-162 (10)	148-168 (7)
SAFS	206-218 (4)	153-155 (2)	168-184 (9)	114-120 (4)	136-158 (9)	148-168 (9)
NZFS	206-218 (4)	155 (1)	168-182 (6)	112-116 (3)	136-160 (7)	148-166 (5)
Macquarie Island Data - assign test 2						
AFS	206-218 (5)	155 (1)	168-188 (9)	110-120 (6)	138-162 (8)	148-168 (5)
SAFS	206-218 (4)	153 (1)	168-182 (8)	114-120 (3)	136-158 (6)	148-164 (7)
NZFS	No individuals classed as this species					
HYB	206-218 (4)	153-155 (2)	168-184 (8)	110-120 (6)	136-162 (10)	148-168 (9)
Macquarie Island Data - Genotype						
AFS	206-218 (4)	155 (1)	168-188 (10)	110-118 (5)	138-162 (8)	148 (1)
SAFS	214-218 (3)	153 (1)	168-182 (8)	114-120 (3)	136-158 (6)	154-164 (6)
NZFS	No individuals classed as this species					
HYB	206-218 (5)	153-155 (2)	168-184 (8)	110-120 (6)	136-162 (12)	148-168 (9)

Table 5.3: The size ranges and number of alleles (in brackets) observed for the six microsatellite loci in the reference data set (n=230, Wynen *et al* , in review), and the Macquarie Island 1996 cohort of pups Species specific alleles are listed where observed in one species only for the reference data set Species identification of Macquarie Island pups was determined by the assignment test of Paetkau *et al.* (1995) (assign test 1), the subjective assessment of those assignment test results (assign test 2, see text) and by comparison of genotypes with the reference data set and mtDNA haplotype (Genotype).

However, this approach does not allow for the objective assignment of individuals to a hybrid class, as the results are 'unambiguous'. Each individual is assigned categorically into one of the three species classes without an estimate of confidence for each assignment, nor does it provide for an objective means of assigning individuals to the hybrid class in the absence of a reference hybrid data set To better examine the assignment test results, the three assignment scores (for AFS, SAFS and NZFS) obtained for each individual are plotted against each other. A confident assignment to a species class would be observed when the assignment score for this species is much greater than that obtained for the other species. Where the scores are similar and therefore causing the sample point to lie close to the line of equality, less confidence could be had in that assignment, and as such, these individuals may represent a hybrid class. The subjective assessment of whether a sample point is 'close' to the line of equality and thereby a hybrid is called 'assign test 2'. The results from both assignment tests are presented in Figures 5.1-5.5, where Figure 5.1 depicts the reference data set, Figures 5.2 and 5.3 the 1996 pup cohort, Figure 5.4 the breeding females and Figure 5.5 the adult males.

The assignment test results for the reference data show discrete clustering of individuals of each species (Figure 5.1). Generally, the AFS and NZFS individuals fall closer to the line of equality when compared with each other, relative to the comparisons with SAFS. This might indicate that the differences between these species are less pronounced than those observed between both species and SAFS, a finding that is consistent with previous studies (Wynen *et al.*, 2000; 2001). The equivalent plot for the 1996 data set shows that the differences between the species within the Macquarie Island population are less discrete (Figure 5.2). While there are individuals that are confidently assigned to a particular species, there are a number that are not (circled in Figure 5.2). These individuals lie close to the line of equality relative to the bulk of individuals, and it might be supposed that they are hybrids. Assuming this is true, the relative species composition of the cohort using this method is presented in Table 5.2 (assign test 2). The incidence of hybridisation within the pup cohort is now estimated at 23%, far greater than observed within the field-based methods. Also, those individuals that were previously identified as NZFS have now been classed as hybrids. Similar results were observed for the adult females and adult males (Figures 5.4 and 5.5 respectively).

The relative proportions of pups and adult females assigned to AFS (64.0%, 75.5%), SAFS (17.6%, 14.0%) and hybrid groups (18.4%, 10.5%) remained non-significant ($G=4.120$ $p>0.05$), while again, both differed significantly from the adult males ($G=13.012$ $p<0.005$ and $G=23.98$, $p<0.001$ respectively). Such differences are no doubt due to the very high proportion of hybrid (45.5%) and SAFS (24.2%) adult males identified with relatively low levels of AFS (30.3%). The allele size ranges for the pups are presented in Table 5.3 and while these ranges have narrowed in comparison with assign test 1 (especially in 3E3), they are still broader than what is expected, indicating that some hybrids are still not being

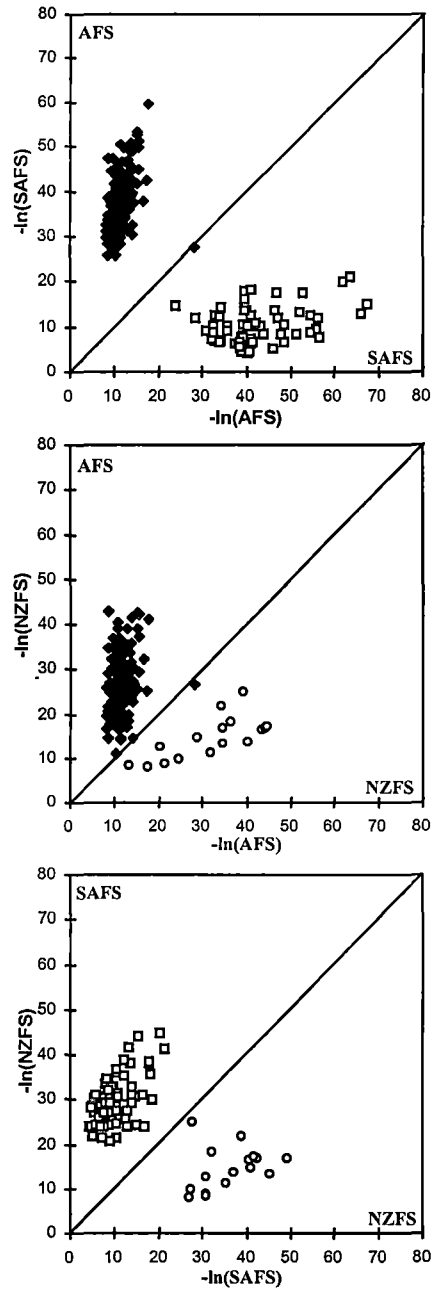

Figure 5.1: Assignment test results of the reference data set of AFS (◆), SAFS (□) and NZFS (O) (n=230) All individuals reassigned into the correct species class, except one AFS individual assigned to SAFS (see Wynen, Goldsworthy et al. in review for further details).

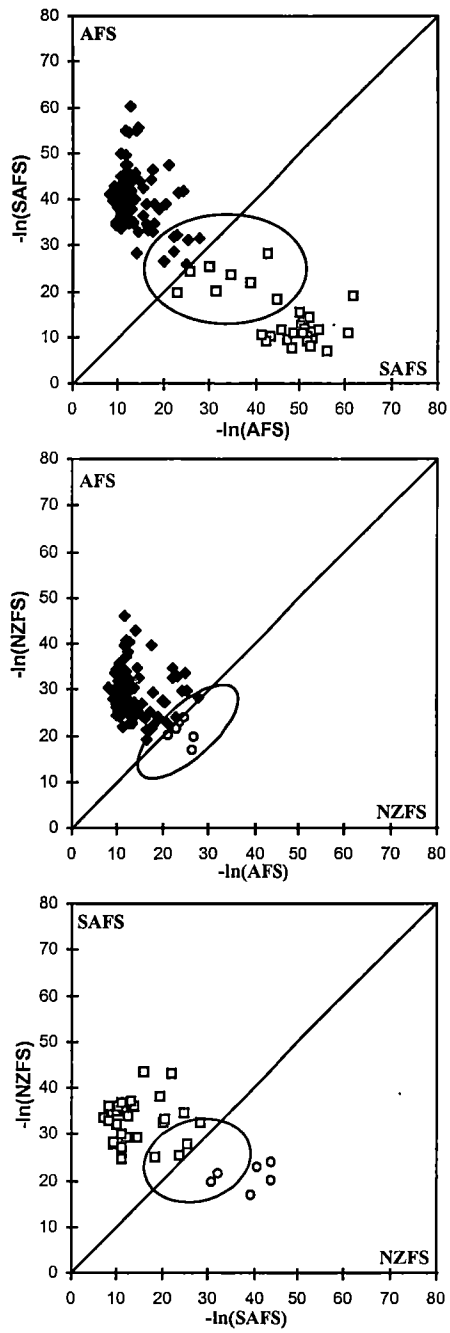

Figure 5.2: Assignment test results of the 1996 Macquarie Island pup cohort. Species labels are those assigned to the individuals as part of the test (AFS ◆, SAFS □, NZFS ○). Circles identify individuals close to the line of equality and therefore may be considered hybrids (see text).

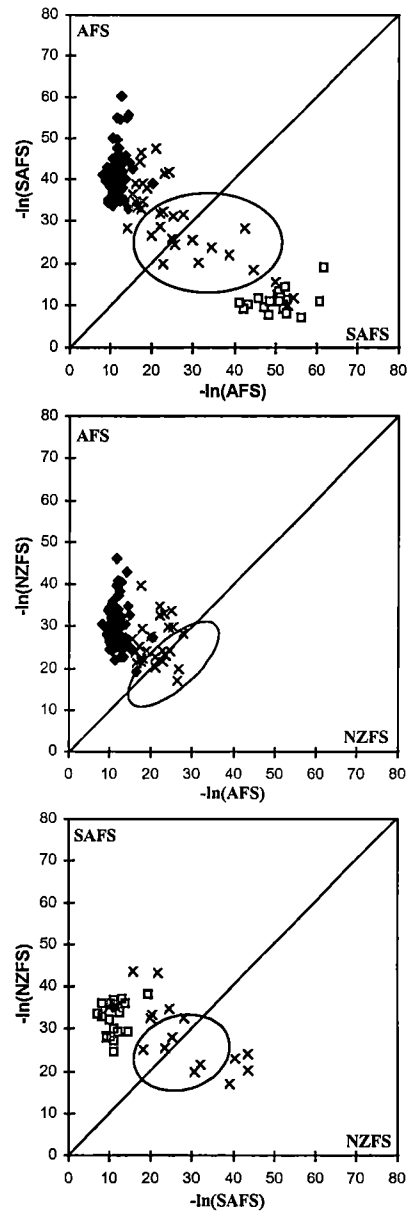

Figure 5.3: Assignment test results of the 1996 Macquarie Island pup cohort as shown in Figure 2, but where species labels of individuals as determined by the 'Genotype' method (AFS ◆, SAFS □ and HYB **x**)

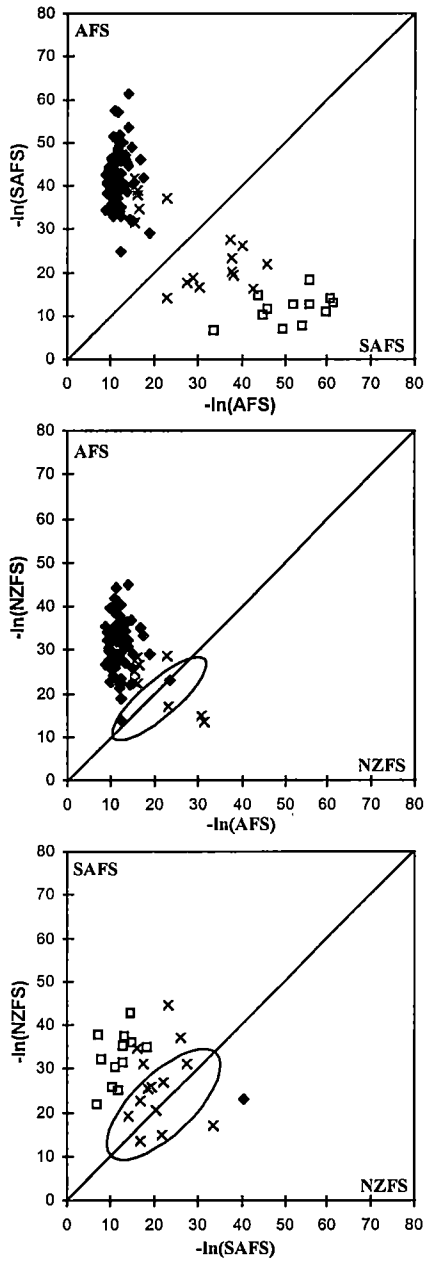

Figure 5.4: Assignment test results of adult females from the 1996/1997 breeding season. Species labels are those assigned to the individuals as determined by the 'Genotype' method (AFS ◆, SAFS □, HYB **x**).

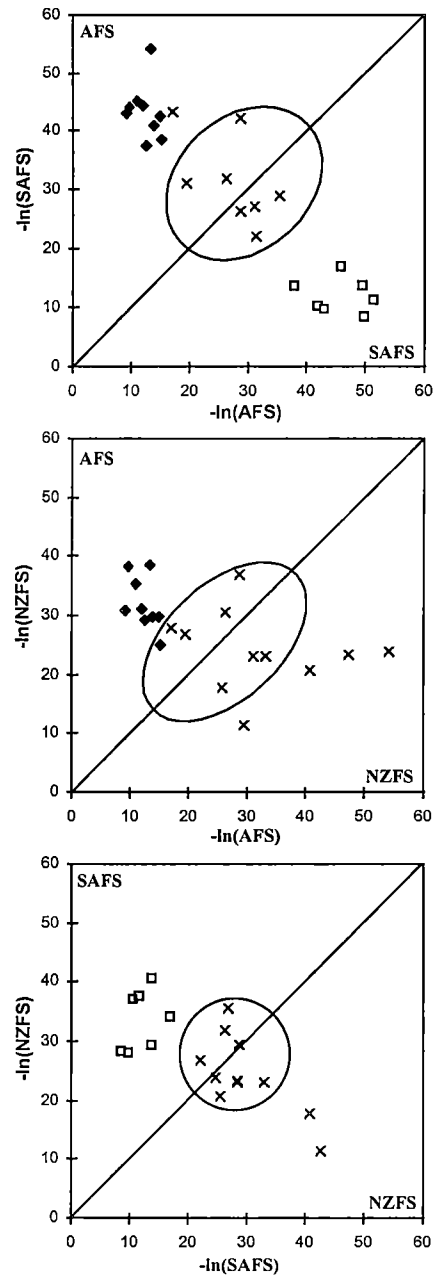

Figure 5.5: Assignment test results of the adult males the 1995 breeding season. Species labels are those assigned to the individuals as determined by the 'Genotype' method (AFS ◆, SAFS □, HYB x).

detected. Furthermore, the delineation of a hybrid from the parental species using this method is purely subjective, and these are results regarded with some caution.

An alternative means of examining the molecular data is by direct comparison of an individual's genotype with the allele size ranges observed for each species within the reference data set (Table 5.3). If there is a mixture of species-specific alleles observed, then it is clear that this individual is a hybrid. If the mtDNA RFLP haplotype of the individual is of a different species to what is indicated by the microsatellite results, then that individual is also classed as a hybrid. Due to the known existence of backcrossing, it would not be possible to categorically class an individual as one of the parental species, but it is possible to categorically class an individual as a hybrid. As such, a minimum level of hybridisation for the pup population can be determined, and this has been estimated as 30.4% (Table 5.2, 'Genotype' method). Once again, it is interesting to note that the individuals assigned to the NZFS class (see above) are recognised as hybrids. The allele size ranges for each species are presented in Table 5.3, and are very similar to those in the reference data set. The ranges for the hybrid class are generally broader than species classes, and contain more alleles per locus.

The use of this 'Genotype' method for species identification appears to be the most objective of the methods examined in this study. The species composition of the pups, adult males and breeding females as determined by this method all differ from each other. The greatest difference is observed between the adult males and breeding females ($G=20.785$ $p<0.001$), where 72 0% of females were classed as AFS compared with 27.3% of males. Similarly, differences were observed in proportions of SAFS (10.5% versus 24.2%) and hybrids (17.5% versus 48.5%). The pups were found to be intermediate between the adult male and female classes in the proportion of AFS (54.4%), SAFS (15.2%) and hybrids (30.4%), but were still significantly different ($G=7.740$ $p<0.025$ and $G=7.972$ $p<0.025$ respectively).

Interestingly, there were considerable differences within the adult male class, when separating out the territorial males from the challenger males. The species composition is very different, with the proportions of 11.8% AFS and 29.4% SAFS for the territorial males compared with the respective values of 43.7% and 18.8%% for the challengers. Similarly, the extent of hybridisation within the two male classes was significantly different, with 58.8% for territorial males versus 37.5% for challengers.

Just as the species composition of the pups, adult males and breeding females were found to differ based on the genotype data, so does the incidence of hybridisation. The proportion of hybrids to non-hybrids in all three classes showed that the differences between them were significant ($G=11.932$ $p<0.005$). Of the hybrids that were identified using the 'Genotype' method, the vast majority of them had mtDNA haplotype of AFS (94.7%, 87.5% and 85.0% for pups, adult males and breeding females respectively).

To visually compare the 'Genotype' method with the assignment test results, Figure 5.2 was re-drawn with the species labels of individuals being derived from the 'Genotype' method (Figure 5.3). It is interesting to note that those individuals that were thought to be hybrids due to their proximity to the line of equality (assign test 2) were also found to be so by the 'Genotype' method. However, it is also apparent by Figure 5.3 that the assignment test underestimates the detectable level of hybridisation by about 10% (Table 5.2). So although the assignment test allows some useful insights into an individual's genetic heritage, it does not allow for an objective identification of hybrids without *a priori* sampling of a known hybrid population. And even if such a sample was obtained, the likely overlap with both/all parental species may erode the utility of the test. Further, where backcrossing occurs continually over time, the ability to detect hybrids would decrease.

Hybrid Identification

Using the overall genotypes of all individuals (see Appendix 3), it was possible to examine the types of hybrids that occur within the Macquarie Island population (Table 5.4). Interestingly, 14 of the total number of hybrids (18.9%) were definitely backcrossed individuals, indicating that these fur seal hybrids at Macquarie Island are not sterile. Also, it is interesting to note that 22 of the hybrids had NZFS alleles present in the genotype (29.7%), and this could possibly be as high as 38 (51.4%). The presence of NZFS alleles in such a large proportion of the hybrids suggest that this species has either had, or is currently having a far greater impact on the breeding population than was first acknowledged.

Phenotype Methods and Comparison with Molecular Data

The data for all pups that were described using the 'Overall Phenotype' (OP) and 'Phenotype Score' (PS) methods are presented in Table 5.2. Most of the pups were described as AFS (77 8% and 70.1% respectively) and SAFS (16.7% and 22.2%), with hybrids estimated to make up 5.5% and 7.7% of the population. There was no significant difference between the proportion of AFS, SAFS and hybrid pups as estimated by the two phenotype methods ($G=1.835$ $p>0.05$). Neither of these methods indicated that any individual was a 'pure' NZFS, although some individuals had phenotypes that suggested that at least one parent/grandparent was NZFS.

There was no significant difference observed between the proportion of pups nominally assigned to AFS, SAFS or hybrid groups using either of the phenotype methods ($G=1.835$ $p>0.05$). However, the OP and PS methods provided significantly different proportions to those derived from the assignment test 2 ($G=10.680$ $p<0.01$ and $G=6.345$ $p<0.05$, respectively), and the 'Genotype' method ($G=28.677$ $p<0.001$, $G=21.142$ $p<0.001$ respectively). When comparing methods within species, the phenotypic assessment was most accurate among SAFS pups with their being no difference to proportion assigned to this species using both phenotype methods and mtDNA haplotypes (OP v mtDNA, $G=0.0008$ $p>0.05$; PS v mtDNA, $G=1$ 123 $p>0.05$) and genotype methods ($G=0.100$ $p>0.05$ and $G=1.949$ $p>0.05$ respectively). However, with the exception of assignment on the basis of

Adult Females

genotype	mtDNA haplotype possibly F_1		not F_1
	AFS	**SAFS**	
SAFS	11	-	0
NZFS	4	1	0
SAFS or NZFS	2	0	0
AFS or NZFS	0	2	0
Total	**17**	**3**	**0**

Adult Males

genotype	mtDNA haplotype possibly F_1		not F_1
	AFS	**SAFS**	**AFS**
AFS	-	1	-
SAFS	6	-	0
NZFS	3	0	1
SAFS or NZFS	1	0	1
SAFS and NZFS	-	-	3
Total	**10**	**1**	**5**

Pups

genotype	mtDNA haplotype possibly F_1		not F_1
	AFS	**SAFS**	**AFS**
AFS	-	2	-
SAFS	9	-	4
NZFS	6	0	1
SAFS or NZFS	12	0	0
SAFS and NZFS	-	-	4
Total	**27**	**2**	**9**

Table 5.4: Hybrid classes of the different age and sex classes of fur seals in the 1996 breeding season at Macquarie Island Hybrids grouped by the species identity of their mtDNA haplotype and microsatellite genotype. Hybrids that were definitely backcrossed were identified as 'not F_1', and all other hybrids are 'possibly F_1' .

OP and mtDNA (G=1 165 p>0.05), the proportion of pups assigned to AFS on the basis of either of the phenotype methods were significantly different to proportions assigned on the basis of mtDNA (PS v mtDNA, G=5.825 p<0.005) or genotype (OP v genotype, G=15 437 p<0.001; PG v genotype, G=6.314 p<0.025). Similarly, there were significant differences in proportions in assigning hybrids (OP v genotype, G=28.145, p<0.001; PS v genotype, G=21.016 p<0.001).

Species Identification - Other Potential Methods

One potential method of species identification is the use of hind flipper shape. The measurements of the 1996 cohort of pups are presented in Table 5.5 for three age classes,

and where the species identity of each pup was determined by the 'Genotype' method. Due to the small sample size in age class 3 these data were not analysed further. Each of the six flipper measurements conformed to a normal distribution as observed through a series of probability plots. There were only two significant differences observed between males and females for all 30 possible species/measurement combination (SAFS, age class 1, W/L, $p<0$ 002 and AFS age class 2, W, $p<0$ 001) As such, the data for males and females within each age class were pooled for the purposes of further analysis. All length measurements were larger in AFS relative to SAFS for age classes 1 and 2 ($p \leq 0.038$), while the W/L index was greater in SAFS for all age classes ($P<<0.001$) (Table 5.5). There was no significant difference in the width of the hind flipper between the species. Generally, these data conform to the expected pattern of AFS having longer and more slender hind flippers relative to SAFS as reported by (Bester and Wilkinson 1989). Hybrids differed significantly from SAFS for all measures in both age classes ($p \leq 0.031$), except for D5 and length (age class 2), and width (both age classes) where there were no differences detected. Hybrid flippers were more similar in size to AFS, with no significant differences observed in both age classes. For age class 2, hybrids showed average measurements that were intermediate between the parental species. This pattern of intermediate morphological measurements has been demonstrated previously by Kerley and Robinson (1987) for skull measurements in AFS, SAFS and their hybrids. However, this pattern was not evident in age class 1, and may be a reflection of small sample sizes and/or because there may be some influence from an additional parental species, namely NZFS.

To address the utility of hind flipper measurements as a tool for species identification of individuals, the data were analysed using a discriminant function analysis. When hybrids were excluded from the analysis, the success rate of reassigning individuals back into the correct species class was 100% and 89% for age classes 1 and 2 respectively. However, when the hybrid class was included, the success rates for the same age classes dropped 39% and 44%. Such a level of success is too low, and shows that in its present form, the analysis is having difficulty identifying hybrids from the parental species, particularly between AFS and hybrids. However, this might be a reflection on the small sample size, and improved representation of the different species and age classes by sampling across additional breeding seasons and/or including measurements from allopatric populations may enhance the utility of the test. Further, Bester and Wilkinson (1989) had suggested that the most enhanced differences between AFS and SAFS occurred when pups were 40 days or older, an age class that is under represented here.

An additional approach to species identification is to explore the differences in birth dates and lactation length. The offset in the breeding period of AFS and SAFS was investigated by comparing the birth dates of pups whose species identity was determined on the basis of the 'Genotype' method. While SAFS were generally born later compared with AFS (median dates of 21 December and 4 December respectively), there was considerable overlap in the respective ranges (5 December - 4 January versus 16 November - 4 January for AFS).

Class	Age	Number	D1	D3	D5	Length	Width	W/L
AFS	0-20	17	**58.00**	**46.00**	**45.53**	**163.71**	**109.76**	**0.67**
			(49-65)	(33-58)	(37-53)	(139-179)	(100-120)	(0 58-0 76)
HYB		6	**58.33**	**48.67**	**46.17**	**161.67**	**107.50**	**0.67**
			(55-62)	(46-51)	(42-48)	(155-170)	(101-115)	(0 63-0 72)
SAFS		4	**47.50**	**40.25**	**40.25**	**149.75**	**111.00**	**0.75**
			(40-56)	(33-47)	(35-46)	(130-172)	(102-116)	(0 59-0 87)
AFS	21-40	47	**57.79**	**48.45**	**45.47**	**166.85**	**109.21**	**0.65**
			(49-68)	(38-58)	(36-56)	(146-194)	(84-132)	(0.54-0.77)
HYB		19		**45.95**	**43.58**	**162.11**	**107.63**	**0.67**
				(37-52)	(35-51)	(137-192)	(93-125)	(0 58-0 77)
SAFS		9	**48.11**	**40.89**	**39.44**	**148.44**	**121.56**	**0.82**
			(44-54)	(37-46)	(35-45)	(136-169)	(113-138)	(0 77-0 89)
AFS	40+	2	**56.50**	**49.00**	**44.50**	**162.50**	**114.00**	**0.70**
			(55-58)	(45-53)	(39-50)	(158-167)	(111-117)	(0 70-0 70)
HYB		0						
SAFS		2	**43.50**	**38.00**	**35.00**	**138.50**	**117.00**	**0.81**
			(42-45)	(36-40)	(34-36)	(136-151)	(116-118)	(0 78-0 85)

Table 5.5: Mean (range) hind flipper measurements of the 1996 cohort of pups in three age classes. Flipper measurements are as described in the Materials and Methods. Species identities, Antarctic fur seal (AFS), subantarctic fur seal (SAFS) and putative hybrids (HYB) were determined using the 'Genotype' method

Such an overlap in conjunction with the overlapping range of the hybrids (17 November to 12 January) suggest insufficient resolution for species identification. Similarly, the hybrid class showed considerable overlap between the parental species in lactation length. Although the sample size was small (n=41) due to predation by a Hooker's sea lion (*Phocarctos hookeri*) (see Robinson *et al.*, 1999), the average weaning age of hybrids was not only intermediate between the parental species, but also more variable (157.42 days, s.d.=50.62, compared with 118.63 days, s.d.=9.15 for AFS and 283.30 days, s.d.=27.22 for SAFS). While each method alone may not prove adequate as a means of species identification, a multivariate approach similar to that used for the hind flipper measurements may be suitable, especially if access to genetic analysis is difficult or unlikely.

Such a 'total evidence' approach could also include data on the timing of the natal coat moult, which has been shown to occur a few weeks earlier in AFS than in SAFS (Condy, 1978). While these data are collected routinely at Macquarie Island, the data for the 1996 cohort are sparse due to a large proportion of the cohort being lost to the Hooker's sea lion detailed above. As such, these data were not analysed, but it is surmised that the level of overlap in hybrid pups as is evident with the weaning data would also be evident here. Another difference reported between the species are their vocalisations (Page *et al.*, 2001), and might also be useful as a means of species identification. As such, while there are a range of different factors pertaining to the different fur seal species that may assist an observer in the identification of species and their hybrids, few would be useful in isolation. A total evidence approach using multivariate techniques to isolate the different species could possibly allow an alternative means of identification that does not rely on genetic analysis, and may provide greater success in the identification of hybrids than the current field based methods.

Discussion

Species Identification - Choice of Method

Of the different field and genetics based methods of species identification examined in this study, the one that most reflects the species composition at Macquarie Island appears to be the 'Genotype' method. The other methods underestimate the incidence of hybridisation to a lesser or greater degree. While it is suspected that the 'Genotype' method also underestimates hybridisation in that it cannot detect all backcrossed individuals, the estimate provided is likely to be closer to the true level than provided by the other methods. While a laboratory based approach means a delay in results relative to a field based approach, the 'Genotype' method is preferred because it is objective, reproducible and categorical. Individuals can be assigned to each of the parental species or the hybrid class with some confidence. This confidence, however, is dependent on a number of assumptions.

The first is that the reference data set provides an accurate representation of each of the parental species. In this study, the reference samples for AFS and SAFS have been collected from all of the major breeding populations across the entire range for each species. While increasing the sample size would no doubt improve representation, the current data set is likely to contain most of the genetic variation contained within these species. The reference data set for NZFS, however, could be greatly improved with increased sampling across the range, to boost the sample size and improve the chance of sampling most of the genetic variation within that species.

The second assumption is that the actions of genetic drift and mutation have not had a marked effect on the allele distributions of the different species at Macquarie Island. While mutation is unlikely to have a major effect due to the relatively short time since breeding re-commenced on Macquarie Island, the same cannot be said for genetic drift. The size of the population over the past 50 years has been very small, and thus the action of genetic drift might be large. If there was one or more rare alleles present in the founding population (eg. an allele that has not been detected in the reference data set), the action of drift may result in that allele becoming common in this population. However, there is no way to detect such an effect as a result of drift, because differences in the presence of alleles and their frequencies may also be a result of hybridisation. Nonetheless, there is some evidence that immigration may be acting against drift at Macquarie Island. Each summer, a large number of fur seals that have not been tagged previously are seen on the island. Since the current tagging program at Macquarie Island has ensured that all pups born on the island since the 1980s have been tagged, these untagged seals are likely to have originated from another population. Further, some females that have never been tagged have also been reported as breeding in some years (L.P. Wynen, personal observation), and these are also possible immigrants. While such evidence is circumstantial, the number of immigrants per generation required to offset the effects of genetic drift is small, and it would only take one or two to have bred in the past decade to have a marked effect.

Notwithstanding the assumptions required in the application of the 'Genotype' method, this method appears to provide the most accurate assessment of the level of hybridisation within the Macquarie Island population. According to this method, we found 54.4% of the 1996 pup cohort for which data are available (n=125) are AFS, 15.2% are SAFS, and 30.4% are hybrid (Table 5.2). There was 86.4% congruency between the assignment test and the 'Genotype' method. One AFS was mis-assigned as a hybrid by the assignment test as it was deemed too close to the line of equality, whereas 16 hybrids were mis-assigned as AFS (n=13) and SAFS (n=3). As such, the assignment test was only able to identify 61.1% of the known hybrids, which would result in an underestimate of the detectable hybridisation in the population if this method were used.

The comparison between the field based and molecular methods showed that both the 'Overall Phenotype' and 'Phenotype Score' methods were also quite successful in identifying

pups to the correct species class (respective success rates of 75.8% and 70.9%). The majority of incorrect classifications were primarily due to the failure to recognise hybrids. For the 'Overall Phenotype' method, 90% of incorrectly classified individuals were classed as AFS when they were hybrids, and 6.7% were incorrectly classed as SAFS. Similarly for the 'Phenotype Score' method, the respective figures are 61.8% and 20.6%. Interestingly, there were also AFS individuals that were mis-classed as hybrids by the 'Phenotype Score' method (14.7%).

Despite a general congruence between field and molecular methods, those based on phenotype underestimate the incidence of hybridisation within the population. This is possibly because the phenotypic traits measured are not a reflection of the underlying genotype as determined in this study. Many phenotypic traits are polygenic, and thus their expression in hybrid and backcrossed individuals are not likely to be predictable in the absence of a clear understanding of the nature of their inheritance. As the level of backcrossing within a population increases, the reliability of these phenotypic traits to detect hybrids would decrease. Backcrossing is known to occur with the Macquarie Island population, as shown in this study, that of Goldsworthy *et al.* (1999), and through field observations. For example, one female in Goldsworthy *et al.* (1999) tagged "602", produced a hybrid pup in 1992/1993, which was tagged prior to weaning as "870/871". This female was observed to pup in 1996 as well as in subsequent years. Further, 17.5% of the females in the present study are identified as hybrids, and these all produced pups. These observations indicate that backcrossing is not uncommon within this population.

While data on weaning, flipper measurements, species identity of mother, vocalisations, etc may provide a basis for a species identification method, these data are likely to provide a poor estimate of species composition in such a population where hybridisation is so high. Given the extent of backcrossing within the Macquarie Island population, the genetic methods are the most appropriate. But, in the absence of molecular data, a multivariate approach based on as much of these phenotypic data as can be compiled might assist in providing a reasonable assessment of species composition.

Species Composition of Macquarie Island Population

This study presents the first and most detailed assessment of the species composition and extent of hybridisation in the Macquarie Island population using genetic techniques. The data presented here suggest that a determination of the species composition of the population is not straight forward, with differences observed within the different classes of individuals examined. The breeding female population is dominated by AFS, which comprises about three quarters of this group. However, only about half of the pups and a third of the adult males are AFS. The pattern of SAFS distribution within these classes is the reverse, with the greatest proportion observed in the adult males (about 25%) compared with about 15% and 11% for the pups and females respectively. The high level of NZFS

contribution to the hybrid class indicates that this species has or continues to play a greater role than expected in the breeding process.

The incidence of hybridisation as determined by this study is high, with about a third of the pup population, a fifth of the breeding female population and nearly half of the breeding male population found to be hybrids. Furthermore, of the adult males sampled, nearly 60% of the territorial males were hybrid, compared with about 38% of challenger males. The only other molecular data for the population at Macquarie Island comes from a study into mate choice by Goldsworthy *et al.* (1999) over the 1992 and 1993 breeding seasons. Here, the authors used species-specific mtDNA haplotypes in conjunction with phenotype for species identification of, identifying hybrid pups when either their phenotype and mtDNA haplotype and/or when their parent's mtDNA haplotype were incongruent. Based on this approach, 41.2% of the 1992 cohort that was sampled (n=17, 20% of the cohort) were found to be hybrids compared with 19.4% of the 1993 cohort (n=36, 38.3% of the cohort). Goldsworthy *et al.* (1999) also estimated the incidence of hybridisation within the breeding population, with the figure of 12.5% for males being markedly different to that observed in 1996. However, the estimate obtained for breeding females (15.79%) was not dissimilar to that obtained in 1996. The difference in male estimates may be a reflection of the change in breeding males present in the colony at the time, or may reflect the limitations of the method of hybrid identification used in 1992-1993. The only way to adequately test this is to screen the 1992-1993 populations with the same microsatellite loci as applied here.

Using the species-specific alleles identified in this study to try and assess the direction of hybridisation is difficult, because these alleles within a locus are often specific to one of the species, with the other two having overlapping ranges. The dominance of AFS mtDNA throughout the hybrid population suggests that this is the major maternal line. This is supported by field observations that suggest that AFS comprise the bulk of the breeding female population, and the lack of NZFS females observed on the island at any time over the past few decades. While it might be supposed that the main hybrid crosses are AFS female/SAFS male, the presence of NZFS alleles within the population (eg. 166 and 168 at Pv11), indicate that this species has or is participating in the breeding process. Backcrossing is also clearly occurring (eg. pup1039 and pup1045, Appendix 3) and the ability of hybrid females to produce pups suggest that the fur seal hybrids on Macquarie Island are clearly not sterile.

Hybridisation at Macquarie Island - Historical Context

The interpretation of the high levels of hybridisation detected in our study is difficult due to the lack of comparable data from other years. However, field observations conducted over the past 50 years of the breeding population at Macquarie Island may provide a useful starting point.

The first pup born on Macquarie Island since the cessation of sealing was reported in March 1955 (Csordas, 1958). Initially this pup, and the few that were born in subsequent years were thought to be NZFS (Csordas and Ingham, 1965). However, a detailed examination of photographs of these early pups led to the conclusion that they were more likely to be AFS (Shaughnessy and Fletcher, 1987). Further analyses of these photographs and records made of these pups has led to the conclusion that they were probably hybrids (S.D. Goldsworthy, unpublished data). These conclusions and records of subsequent field observations suggest that hybridisation has been prevalent at Macquarie Island since 1955.

Additional observations made throughout the 1980s revealed that while the fur seal population was increasing, there was a predominance of mixed species territories, and the appearance of fur seals showing an intermediate phenotype (Shaughnessy and Fletcher, 1987; Shaughnessy *et al.*, 1988). Most of the territorial males were SAFS, while almost all of the breeding females were AFS. Shaughnessy *et al.* (1988) notes that while there were a few AFS males about, they were smaller than the SAFS, and had difficulty challenging them for access to the females. If they were able to hold a breeding territory, it was usually only for a few days, and when there were only a few females present. So in summary, the SAFS males generally controlled access to the breeding females, most of which were AFS. NZFS were not reported in the breeding colony, and if they were (Shaughnessy and Fletcher, 1987), it was conceded that they were probably AFS that had been mis-identified (Shaughnessy *et al.*, 1988). From these field observations it might be inferred that the majority of pups born during this era may be hybrids, and it is these pups that probably form the basis of the current breeding population (taking immigration into account). As such, it is apparent why there is a predominance of AFS mtDNA haplotypes observed within the extant hybrid population, and only small number from SAFS mtDNA (5.3% of hybrid pups).

Further observations made during the 1990s suggest that there has been a trend away from the mixed species territories seen in the 1980s. While the major breeding colonies are still confined to the same beaches, the different species have started to segregate based on their different preferences for breeding substrate. For instance, SAFS prefer rugged sections of coast with a rocky substrate, and tend to breed at these sites rather than along the flat, pebbly beaches that are the preference of AFS (Condy, 1978). Most of the territories that are formed during the breeding season are single species territories, aside from the presence of hybrids. While hybridisation is undoubtedly still occurring, there appears to be some selection by individuals for mates that at least appear to be from the same species (Goldsworthy *et al.*, 1999).

Such a trend towards ecological segregation of the species within the breeding population at Macquarie Island suggests that the population is moving towards a *status quo* that is evident at the other two locations where AFS and SAFS occur in sympatry. At Iles Crozet and Marion Island, the colonies of AFS and SAFS tend to be geographically separated, with only low levels of hybridisation being reported/suspected (Condy 1978; Jouventin *et al.*, 1982).

Ecological segregation of the species is enhanced by such factors such as the difference in preference of breeding substrate, an offset in the pupping season by a few weeks, and positive assortative mating based on physical and vocal cues (Condy 1978; Kerley, 1983b; Goldsworthy *et al.*, 1999; Page *et al.*, 2001).

However, the small size of the population at Macquarie Island since recolonisation has meant that in these early years, such segregative factors might have been overridden by the basic urge to breed. In the early decades since 1954, the availability of mates *per se* was likely to be of primary concern, let alone any attempt to select mates of the same species. As such, the breeding strategy of these early colonisers was likely to be opportunistic, supporting the suggestion that hybridisation may have been present in the population from the start. Now with the increase in population size, there are signs that the factors that ultimately lead to ecological segregation are becoming more pronounced, suggesting that these may operate more effectively at higher population densities. As such, it is expected that there will be a reduction in the level of hybridisation in the years to come. This concurs with observations at Marion Island, where the level of apparent hybridisation has been decreasing as the population sizes of AFS and SAFS increase (Hofmeyr *et al.*, 1997).

In addition to the high levels of hybridisation observed within the Macquarie Island population, there also appears to be large differences in the incidence of hybridisation within different age and sex classes. The proportion of breeding males that are hybrid is far greater than that observed within the breeding females, with the proportion of the pup population being intermediate. If the assumption is that each male within the breeding population equally contributes to the subsequent pup cohort, then the expectation is that the incidence of hybridisation within this cohort would at least equal that observed within the male population. However, the levels presented here are not comparable, suggesting that this assumption is not valid. One possible reason is that a certain percentage of backcrossed pups are not detected by the molecular techniques used in this study. However, this may not account for all of the large differences observed in the extent of hybridisation of different age/sex classes.

The general expectation in the past has been that because territorial males are thought to have the primary access to females at the time of oestrus, they are assumed to account for most of the successful copulations (where success is defined as resulting in conception). However, at Macquarie Island, nearly 60% of these males are hybrids, and thus the level of hybridisation in the pup cohort would be expected to much higher than the observed 30.4%. Why is such a disparity observed? In a study at South Georgia, Gemmell *et al.* (2001) estimated that only 28% of the pups sampled were fathered by territorial males, and at Macquarie Island, Goldsworthy *et al.* (1999) showed that the paternity of 22.6% of the pups screened (n=53) could not be assigned to the territorial males. These data reveal that non-territorial males, where the incidence of hybridisation is much less than the territorial males (about 38%), have a substantial contribution to the pup gene pool.

Another possible reason for a disparity in hybridisation levels between pups and adults is positive assortative mating. Goldsworthy *et al.* (1999) have showed that breeding females at Macquarie Island can actively select their mates, and often do so on the basis of phenotype, selecting males that exhibit phenotype traits of the same species as themselves. Therefore, the combination of positive assortative mating, where females select both territorial and challenger males partly on the basis of phenotypic traits, and the lower level of hybridisation within the non-territorial male population, may thus assist in the reduction of hybridisation in the subsequent pup cohort. However, while plausible, this theory can only be confirmed by additional paternity analysis, where the identity of the fathers of the 1996 cohort can be established.

An alternative interpretation of the disparate levels of hybridisation within the different age and sex classes within the population is that we are simply not detecting all of the hybrids present within the pup cohort as a result of backcrossing. The breeding males present in the colony in 1995 were probably born in the 1980s when there was a predominance of SAFS males and AFS females. The continuing dominance of AFS females in the population suggests that backcrossing is likely to occur, and our method is not capable of identifying all backcrossed individuals. So it is possible that the number of hybrid individuals within the pup cohort is much higher than the 30.4% reported, but there is a proportion that we are not able to detect.

A further interpretation of the results found in this study focuses on the possibility of hybrid sterility, or hybrid breakdown. There has been no long term study into reproductive success nor into the possibility of differential survival of these species and their hybrids. If hybrids have a lower fitness, even to the point where hybrid males are sterile (as per Haldane's Rule), the collective reproductive output of the large number of hybrid males in the breeding population may be quite low relative to males of the parental species. Interestingly, in the study by Goldsworthy *et al.* (1999), the one territorial male that was identified as a hybrid was found not to father the three pups born the following year to females that were in his territory, despite observed copulations occurring. So despite the ability of hybrid males to successfully compete for and hold breeding territories, there may be some pre-zygotic isolating mechanisms, such as mechanical isolation or gametic mortality/incompatibility that prevent successful copulations (Avise, 1994). This may result in a female not producing a pup the following year, or more likely, being successfully mated by another male. While observations confirming this are scarce, in general it is known that the error associated with field observations in relation to paternity can be quite large (Boness *et al.*, 1993; Amos *et al.*, 1995; Goldsworthy *et al.*, 1999; Gemmell *et al.* 2001). Further data are required regarding paternity of pup cohorts over a number of seasons to adequately address these issues.

Yet another consideration to be made in addressing the differential level of hybridisation in the male and female breeding populations is the possibility of differential survival rates.

Perhaps female hybrids are less likely to survive to maturity compared with hybrid males. There are no data to support or confirm this, and once more, additional long term studies on the Macquarie Island population are required before this issue can be adequately addressed. Solid demographic information, molecular screening of all individuals and continual monitoring over many years are required to ascertain the relative contributions of the above factors to the results observed in this study.

While there is still much work to be done, there are signals within our data in relation to the field observations made over the past 50 years that suggest that the relative levels of hybridisation within the Macquarie Island will decline over time. One such signal is the relatively small numbers of hybrids within the challenger male population. Given that these males are yet to be competitive enough to hold breeding territories, it might be supposed that they are younger than the territorial males. Perhaps the disparity observed between the groups (37.5% versus 58.8%, Table 5.2) is a reflection of an overall trend towards lower levels of hybridisation. This is further supported by the female population which has fewer hybrids again (17.5%, Table 5.2). Females pup for the first time at around three to four years old, and thus the females as a group might be younger than the challenger male group. However, the pups do not follow this trend, but this might be because most of these individuals will not survive to breeding. And if there is a differential survival rate for the parental species and the hybrids, this might lead to much lower numbers than are currently observed.

Taking a global perspective, the distribution of the AFS and SAFS are generally discrete, with the latter occurring on islands to the north of the Antarctic Polar Front while the AFS primarily inhabit islands to the south of this front. At Iles Crozet and Marion Island where the distributions overlap, the species appear to breed in colonies that are geographically segregated, and reports suggest that the incidence of hybridisation is low. Analysis of the available mtDNA data across the ranges of both species (excluding Macquarie Island), it is apparent that there are no shared lineages detected (Wynen *et al.*, 2000). If historical hybridisation had occurred between these species, then it was either at very low levels and/or all shared lineages were eliminated as a result of sealing. While there is no evidence from the mtDNA of introgression outside Macquarie Island, it is more difficult to assess the microsatellite data due to the overlapping allele size ranges. Where species-specific alleles have been detected at certain loci, there certainly have not been any individuals with shared alleles outside of Macquarie Island, but this argument is circular since these alleles (if detected) would not have been classed as species-specific. However, the absence of apparent introgression outside of Macquarie Island may indicate that hybridisation between the species is not normally of a high level. This further suggests that the high level of hybridisation at Macquarie Island is highly unusual for these species, and probably an artefact of a disturbed system. Field observations over the past 50 years, and to a lesser extent the limited molecular data presented here, indicate that the relative incidence of

hybridisation within the population will decrease with time and an increase in population density.

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CHAPTER 6: Concluding Comments and Further Research

Summary of Findings

This study employed a range of molecular methods to investigate different aspects of the ecology of the Antarctic and subantarctic fur seals. These molecular data were analysed in the light of other types of data available (eg. historical records, fossil data, field observations, etc) and thereby presenting a total evidence approach where possible. Historical records, field observations and the results of research programs into various facets of fur seal ecology can all be used in conjunction with information obtained from molecular methods to present a fuller assessment of the ecology of the Antarctic and subantarctic fur seals.

The phylogenetic relationships of the Antarctic fur seal and the subantarctic fur seal were investigated within the context of the family Otariidae, which comprises all of the fur seal and sea lion taxa. This was done to study the level of genetic differentiation between these two species, and to establish that such a differentiation is sufficient to warrant the classification of the Antarctic and subantarctic fur seal as distinct species.

The traditional classification of the Otariidae into the subfamilies Arctocephalinae (fur seals) and Otariinae (sea lions) was not supported in this study, as the northern fur seal (*Callorhinus ursinus*) was found to be basal to all other taxa within the family. While four sea lion clades and five fur seal clades were recognised, the molecular information obtained in this study was unable to resolve the specific relationships among these clades. Such poor resolution is probably a reflection of the rapid radiation that is thought to have occurred within the family about 3 million years ago. Some interesting findings include the genetic similarity of the Guadelupe (*A. townsendi*) and Juan Fernandez fur seals (*A. philippii*) which are regarded as separate species, relative to the similar level of divergence observed between the two subspecies of *A. pusillus*, the Australian and Cape fur seals (*A. p. doriferus* and *A p. pusillus*). This is contrasted by the highly divergent lineages evident within both the New Zealand and South American fur seal species, where the divergences between intra-specific lineages were much greater that observed between some species. These two species formed a clade with the Galapagos fur seal (*A. galapagoensis*), yet the relationships between the divergent lineages within the New Zealand and South American species and that of the Galapagos fur seal remained poorly resolved. The genetic distance between Antarctic and subantarctic fur seals and the absence of shared lineages indicate that they are discrete species, a conclusion that differed from the study by Lento *et al.* (1997), where there was some uncertainty in the origin of the some samples (Wynen *et al.* 2000).

The intra-specific genetic variation was investigated for both the Antarctic and subantarctic fur seals with a view to establishing whether there are any apparent effects of the intensive sealing activities of the 19th century on the levels and distribution of this variation. The levels that were observed within both the species of fur seal was far greater than initially anticipated. This was observed for all of the molecular markers used. Given the recent exploitation histories of the two species and the severity of the resulting population

bottlenecks, it was thought that reduced levels of intra-specific genetic variation would be apparent, such as that observed in the Hawaiian monk seal and the northern elephant seal (Hoelzel *et al.*, 1993; Kretzmann *et al.*, 1997). However, it is clear that either the levels of genetic variation within both species was very high prior to the sealing era, and/or that the extent and duration of the population bottleneck was not as extreme as suggested in the literature. The genetic variation in the microsatellite DNA was highly variable and was dependent on the locus employed. While some loci were found to be decidedly useful for species identification because there were discrete differences between species, and because they had low levels of variation, other loci were informative for addressing intra-specific questions, such as those pertaining to paternity and reproductive success.

The population genetics data also allowed some speculation as to the population dynamics of both the Antarctic and subantarctic fur seals, and therefore provides a background picture within which the Macquarie Island population can be studied. There was significant population structure evident within the subantarctic fur seal as exhibited by mtDNA. While significant structure was also evident within the Antarctic fur seal, the level was less than that observed within the subantarctic fur seal. It is possible that this may be a result of the greater sealing effort against the former, forcing the species to be reduced to very small numbers. Further, it might also reflect the subantarctic fur seal surviving the sealing era at more isolated populations, thereby preserving a higher proportion of the pre-sealing genetic variation. The subantarctic fur seal is thought to have survived at Ile Amsterdam, Marion Island and Gough Island, and immigration from these sites has resulted in the recolonisation of this species at Iles Crozet and Macquarie Island. The distribution of mtDNA haplotypes within the recolonised populations relative to those in the colonising populations enables some speculation into the origin of the source populations. The Antarctic fur seal was thought to have survived the sealing era at South Georgia and Bouvetøya, and subsequent recolonisation across the current range having stemmed from here. When the molecular data are examined, there is a suggestion of two genetically differentiated regions, suggesting that an additional population may have survived at Iles Kerguelen. Alternatively, this differentiation may be an indication of a founder effect. Greater levels of variation and population subdivision in the subantarctic fur seal relative to the Antarctic fur seal was also observed with the microsatellite data (nuclear DNA that is bi-parentally inherited).

The application of the molecular methods developed in the phylogenetic and population genetics aspects of this study to the Macquarie Island population, enables an investigation into the species composition of the population, and an estimation of the extent of hybridisation that is occurring there. The species composition was found to highly variable depending on the age and sex class examined. Within the pup population, 54.4% were found to be Antarctic fur seals, compared with 72.0% and 27.3% of breeding females and males respectively. Similarly, disparate estimates of the subantarctic fur seal population were evident, with 15.2%, 10.5% and 24.2% of pups, breeding females and breeding males belonging to this species. Such variable estimates also translate to the level of hybridisation

detected, with the corresponding values being 30.4%, 17.5% and 48.5%. These estimates of hybridisation are greater than previously published, and represent a minimum level due to the constraints of the method.

The species composition of the Macquarie Island population as determined by a number of field based approaches showed consistent under-estimates of the level of hybridisation. Such results are expected in a population where hybridisation has been occurring for some time, and backcrossing is likely to be common. With phenotypic traits being polygenic, the identification of hybrid individuals based on such traits becomes increasingly difficult with increasing levels of backcrossing. Nonetheless, the field based methods had success rates of 70.9-75.8%, and thus will be useful to provide an overview of the population prior to laboratory analysis can be conducted.

The high incidence of hybridisation at Macquarie Island is likely to have been promoted by the sealing era, and the high levels of sympatry of closely related species. While hybridisation between these species has been reported/suspected at Iles Crozet and Marion Island, the incidence is thought to be low, and the ecological segregation of the species more pronounced then currently observed at Macquarie Island. The lack of shared mtDNA lineages between the species outside of Macquarie Island is further evidence that hybridisation is a recent occurrence, or otherwise had occurred at such low levels that it has yet to be detected. Field observations of the breeding colonies at Macquarie Island over the past few decades, and the disparate levels evident within the different age and sex classes suggest that hybridisation will decline in the coming years in concert with an increase in the species populations

Further Research

This study commenced with an investigation into the phylogenetic relationships within the family Otariidae. Insufficient resolution was obtained with the molecular markers employed to present a definitive picture of these relationships. Increased resolution might be obtained by increasing the number of genes sampled, and by sampling both the mitochondrial and nuclear genomes. Furthermore, these data should be combined with, or analysed in the light of data obtained from other sources, as the genes sequenced may not provide an accurate reflection of phylogeny on their own. These issues are not unique to molecular data, and apply equally to other data types such as those derived from the fossil record, morphological studies etc. However, by considering all of the data types together in a 'total evidence' approach, not only might there be an improvement in the level of resolution obtained, but a more accurate reflection of the phylogenetic relationships can be achieved.

This study has also found that there can be considerable genetic variation observed within some otariid species, and that this variation may not be uniformly distributed across the range of the species. Clearly, an accurate phylogenetic study would require a more strategic approach to sampling of the species. Many have broad distributions encompassing many

populations, and a concerted effort to sample across the range and all populations is required if a representative sample of the genetic variation for that species is to be obtained. While this is a large undertaking, requiring a large amount of collaboration and logistical expense, this study has presented only a first step to the identification of priority areas for sampling. Clearly, the divergent lineages observed within the New Zealand fur seal and the South American fur seal suggest that these species need to be sampled more thoroughly. While this has happened to a degree with the New Zealand fur seal (Lento, 1995), little has been published to date regarding the South American fur seal. Also, the population structure within the Australian sea lion has recently been shown to be strong, with different colonies exhibiting different mtDNA lineages, suggesting that all colonies would require sampling (Campbell *et al.*, 2001). These present examples of how a less than comprehensive sampling can affect the outcomes of the study.

The reliance of the species identity methods based on microsatellite and mitochondrial DNA on the reference data sets for each species, necessitates that these data sets be continually improved to ensure that they representative. This would include the sampling of populations of Antarctic and subantarctic fur seals not included in this study, such as Tristan da Cunha, the South Orkney Islands, the South Sandwich Islands, Prince Edward Island and Saint Paul Island. Given the recent history of exploitation and recovery of both species, and the nature of the distribution of genetic variation as shown in this study, it is unlikely that the sampling of these populations will alter the overall picture appreciably. However, the level of confidence that can be had with species identification will increase dramatically with every population that is included.

Furthermore, the sampling of the New Zealand fur seal species for the study into hybridisation was minimal. In the light of the highly divergent lineages observed in the mitochondrial DNA, it is clear that the individuals sampled as part of this study are not likely to be representative of the species as a whole. While the New Zealand fur seal appears not to participate to any large extent in the breeding at Macquarie Island, there needs to be a greater understanding of the level of genetic variation within the species to ensure greater confidence in the conclusions drawn from the molecular data. This would involve sampling individuals throughout the entire range of the species, from Western Australia through to the populations in New Zealand and on the offshore islands.

Further investigation is also required into the use of a larger range and variety of microsatellite loci that can be readily amplified in the fur seal species. This study provides the results of a handful of loci that each can prove useful for addressing a range of questions. However, different qualities are required from microsatellite loci depending on the questions asked. For instance, when aiming to develop a species identification method, then loci that have little intra-specific variation, yet pronounced inter-specific differentiation are required. This is in contrast to questions pertaining to paternity, for instance, where high levels of intra-specific variation are required. Improved confidence can be had when

investigating such issues as hybridisation and reproductive success within the Macquarie Island population if a larger number of loci are employed, that have the specific qualities required.

This study has presented a comprehensive overview of the evolutionary relationships and population genetics of two species of fur seal, which provides a solid grounding for further in depth research into the unique fur seal population at Macquarie Island, and among other populations and species. Detailed field observations at Macquarie Island have been conducted since the 1980s, and sporadic data are available prior to this time. Further, skin biopsies have been collected from all pup cohorts since the early 1990s, as well as from most of the breeding population since this time. The combination of these data, with the extensive genetic analysis presented here, along with the preliminary work on hybridisation provide the backbone for a comprehensive assessment of hybridisation at Macquarie Island. Such a study would address the reproductive success of males and females of different species, survival rates of the different species and their hybrids, questions relating to mating systems, and so on. Furthermore, comparative studies on foraging ecology, reproductive strategies, mating systems, etc can be conducted on the basis of a solid species identification method, and thereby allowing comparisons to be made without the complication of inter-island, inter-population and inter-colony effects.

Such a comprehensive molecular study into single species is not common. However, with the increasing emphasis on an ecosystem based approach to the development of conservation strategies and their management, molecular methods provide an essential tool to assist in addressing a wide range of questions. These methods should not be used in isolation, but rather in concert with all other data available, to provide the 'total evidence' approach required to obtain an understanding of the organisms and the systems within which they occur.

References

Abernethy, K. (1994) The establishment of a hybrid zone between red and sika deer (genus *Cervus*). *Molecular Ecology* **3:** 551-562.

Allen, P J , W Amos, P P Pomeroy and D. Twiss (1995) Microsatellite variation in grey seals (*Halichoerus grypus*) shows evidence of genetic differentiation between two British breeding colonies. *Molecular Ecology* **4:** 653-662.

Amos, B., S. Twiss, P. Pomeroy and S. Anderson (1995) Evidence for mate fidelity in the gray seal. *Science* **268:** 1897-1898.

Arnason, U., K. Bodin, A. Gullberg, C. Ledje and S. Mouchaty (1995) A molecular view of pinniped relationships with particular emphasis on the true seals. *Journal of Molecular Evolution* **40:** 78-85.

Arnason, U., and E. Johnsson (1992) The complete mitochondrial sequence of the harbor seal, *Phoca vitulina*. *Journal of Molecular Evolution* **34:** 493-505.

Avise, J.C. (1994) *Molecular Markers, Natural History and Evolution*. New York, Chapman and Hall Inc.

Baird, R.W., P.M. Willis, T.J. Guenther, P.J. Wilson and B.N. White (1998) An intergeneric hybrid in the family Phocoenidae. *Canadian Journal of Zoology* **76:** 198-204.

Barnes, L.G , D.P. Domning and C.E. Ray (1985) Status of studies on fossil marine mammals. *Marine Mammal Science* **1:** 15-53.

Barton, N.H. (2001) The role of hybridization in evolution. *Molecular Ecology* **10:** 551-568.

Bengtson, J.L., L M. Ferm, T.J. Harkonen and B.S. Stewart (1990) Abundance of Antarctic fur seals in the South Shetland Islands, Antarctica, during the 1986/87 austral summer. In: *Antarctic Ecosystems. Ecological Change and Conservation* (ed. K.R. Kerry and G. Hempel), pp. 265-270. Springer-Verlag, Berlin.

Berta, A., and T.A. Demere (1986) *Callorhinus gilmorei* n. sp., (Carnivora: Otariidae) from the San Diego formation (Blancan) and its implications for otariid phylogeny. *Transactions of the San Diego Society of Natural History* **21:** 111-126.

Berta, A. and A.R. Wyss (1994) Pinniped phylogeny. *Contributions in Marine Mammal Paleontology Honouring Frank C. Whitmore Jr.* (ed. A. Berta and T.A. Demere). San Diego Society of Natural History. **29:** 33-56.

Berube, M. and A. Aguilar (1998) A new hybrid between a blue whale, *Balaenoptera musculus*, and a fin whale, *B. physalus*: frequency and implications of hybridization. *Marine Mammal Science* **14:** 82-98.

Bester, M N (1989) Movements of southern elephant seals and subantarctic fur seals in relation to Marion Island *Marine Mammal Science* **5:** 257-265.

Bester, M.N. (1987) Subantarctic fur seal, *Arctocephalus tropicalis*, at Gough Island (Tristan da Cunha group). In: *Status, Biology and Ecology of Fur Seals, Proceedings of an International Symposium and Workshop* (ed. J.P. Croxall and R.L. Gentry), pp. 57-64. Cambridge, England.

Bester, M.N. and I.S. Wilkinson (1989) Field identification of Antarctic and subantarctic fur seal pups. *South African Journal of Wildlife Research* **19:** 140-144.

Bininda-Emonds, O.R.P., J.L. Gittleman and A. Purvis (1999) Building large trees by combining phylogenetic information: a complete phylogeny of the extant Carnivora (Mammalia) *Biological Review of the Cambridge Philosophical Society* **74:** 143-175.

Boness, D.J., W.D. Bowen and J.M. Francis (1993) Implications of DNA fingerprinting for mating systems and reproductive strategies of pinnipeds. *Symposium of the Zoological Society of London* **66:** 61-93.

Bonner, W.N. (1994) *Seals and Sea Lions of the World.* Blandford, London.

Bonner, W.N. (1968) The fur seal of South Georgia. *British Antarctic Survey Scientific Reports* **56:** 1-82.

Bonner, W.N. (1958) Notes on the southern fur seal in South Georgia. *Proceedings of the Zoological Society of London* **130:** 241-252.

Bonner, W.N. and R.M. Laws (1964) Seals and sealing. In: *Antarctic Research* (ed. R. Priestley, R.J. Adie and G.D.Q. Robin), pp. 163-190. Butterworths, London.

Boyd, I.L. (1993) Pup production and distribution of breeding Antarctic fur seals (*Arctocephalus gazella*) at South Georgia. *Antarctic Science* **5:** 17-24.

Boyd, I.L., D.J. McCafferty, K. Reid, R. Taylor and T.R. Walker (1998) Dispersal of male and female Antarctic fur seals (*Arctocephalus gazella). Canadian Journal of Fisheries and Aquatic Science* **55:** 845-852.

Boyd, I.L. and J.P. Croxall (1992) Diving behaviour of lactating Antarctic fur seals. *Canadian Journal of Zoology* **70:** 919-928.

Boyd, I.L., N.J. Lunn and T. Barton (1991) Time budgets and foraging characteristics of lactating Antarctic fur seals. *Journal of Animal Ecology* **60:** 577-592.

Bradshaw, C.J A, C Lalas, and C.M. Thompson (2000) Clustering of colonies in an expanding population of New Zealand fur seals (*Arctocephalus forsteri*). *Journal of Zoology, London* **250:** 105-112.

Brunner, S. (2000) *Cranial Morphometrics of the Fur Seals and Sea Lions (Family: Otariidae) – Systematics, Geographic Variation and Growth.* PhD Thesis, Department of Veterinary Anatomy and Pathology, University of Sydney.

Budd, G.M. (1972) Breeding of the fur seal at McDonald Islands and further population growth at Heard Island. *Mammalia* **36:** 423-427.

Budd, G.M. and M.C. Downes (1969) Population increase and breeding in the Kerguelen fur seal, *Arctocephalus tropicalis gazella*, at Heard Island. *Mammalia* **33:** 58-67.

Burg, T.M., A.W. Trites and M.J. Smith (1999) Mitochondrial and microsatellite DNA analyses of harbour seal population structure in the northeast Pacific Ocean. *Canadian Journal of Zoology* **77:**930-943.

Busch, B.C. (1987) *The War Against the Seals.* McGill-Queen's University Press, Kingston.

Callen, D.F., A.D. Thompson, Y. Shen, H.A. Phillips, R.I. Richards, J.C. Mulley and G.R. Sutherland (1993) Incidence and origin of "null" alleles in the $(AC)_n$ microsatellite markers. *American Journal of Human Genetics* **52:** 922-927.

Campbell, R., N. Gales, G Lento and S Baker (2001) Population genetics and its importance in effective conservation management: a case study involving the Australian

sea lion, *Neophoca cinerea*. As presented at the Southern Hemisphere Marine Mammal Conference 2001. Phillip Island.

Carr T. and N. Carr (1985) A record of the subantarctic fur seal *Arctocephalus tropicalis* in Angola. *South African Journal of Zoology* **20:** 77.

Castello, H.P. and M.C. Pinedo (1977) *Arctocephalus tropicalis*, first record for Rio Grande do Sul coast (Pinnipedia Otariidae). *Atlantica Rio Grande* **2:** 111-119.

Clark, J.W. (1875) On the eared seals of the islands of St. Paul and Amsterdam, with a description of the fur-seal of New Zealand, and an attempt to distinguish and rearrange the New Zealand Otariidae. *Proceedings of the Zoological Society* **1875:** 650-677.

Coltman, D.W., W.D. Bowen and J.M. Wright (1996) PCR primers for harbour seal (*Phoca vitulina concolour*) microsatellites amplify polymorphic loci in other pinniped species. *Molecular Ecology* **5:** 161-163.

Condy, P.R. (1978) Distribution, abundance and annual cycle of fur seals (*Arctocephalus* spp.) on the Prince Edward Islands. *South African Journal of Wildlife Research* **8:** 159-168.

Croll, D A and B R. Tershy (1998) Penguins, fur seals, and fishing: prey requirements and potential competition in the South Shetland Islands, Antarctica. *Polar Biology* **19:** 365-374.

Csordas, S.E. (1963) The history of fur seals on Macquarie Island. *Victorian Naturalist* **80:** 255-258.

Csordas, S.E. (1958) Breeding of the fur seal, *Arctocephalus forsteri* Lesson, at Macquarie Island. *Australian Journal of Science* **21:** 87-88.

Csordas, S.E. and S.E. Ingham (1965) The New Zealand fur seal, *Arctocephalus forsteri* (Lesson), at Macquarie Island, 1949-64. *CSIRO Wildlife Research* **10:** 83-99.

Cummings, M.P., S.P. Otto and J. Wakeley (1995) Sampling properties of DNA sequence data in phylogenetic analysis. *Molecular Biology and Evolution* **12:** 814-822.

Cumpston, J.S. (1968) *Macquarie Island.* Antarctic Division, Department of External Affairs, Melbourne.

De Villiers, R.F. and G.J.B. Ross (1976) Notes on numbers and distribution of fur seals, *Arctocephalus tropicalis* (Gray) on Marion and Prince Edward Islands, Southern Ocean. *Journal of Mammalogy* **57:** 595-600.

Dizon, A.E., S.J. Chivers, and W.F. Perrin (1997) *Molecular Genetics of Marine Mammals.* Society for Marine Mammalogy, Lawrence, KS.

Excoffier, L., P. Smouse and J.M. Quattro (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* **131:** 479-491.

Felsenstein, J. (1993) PHYLIP (Phylogeny Inference Package) version 3.5c. Distributed by the author. Department of Genetics, University of Washington, Seattle.

Fevoden, S.E. and L. Sømme (1976) Observations on birds and seals at Bouvetøya. *Norsk Polarinstitutt rbok* **1976:** 367-371.

Fischer, W.J., W.A. Koch and A. Elepfandt (2000) Sympatry and hybridization between the clawed frogs *Xenopus laevis laevis* and *Xenopus muelleri* (Pipidae). *Journal of Zoology, London* **252**: 99-107

Fitzsimmons N.N , C. Moritz and S.S Moore (1995) Conservation and dynamics of microsatellite loci over 300 million years of marine turtle evolution. *Molecular Biology and Evolution* **12**: 432-440

Fleischer, L.A. (1987) Guadelupe fur seal, *Arctocephalus townsendi*. In: *Status, Biology and Ecology of Fur Seals, Proceedings of an International Symposium and Workshop* (ed. J.P. Croxall and R.L. Gentry), pp. 43-48. Cambridge, England.

Gaggiotti, O.E., O. Lange, K. Rassman and C. Gliddons (1999) A comparison of two indirect methods for estimating average levels of gene flow using microsatellite data. *Molecular Ecology* **8**:1513-1520.

Gemmell, N.J., T.M. Burg, I.L. Boyd and W. Amos (2001) Low reproductive success in territorial male Antarctic fur seals (*Arctocephalus gazella*) suggests the existence of alternative mating strategies. *Molecular Ecology* **10**: 451-460.

Gemmell, N.J., P.J. Allen, S.J. Goodman and J.Z. Reed (1997) Inter-specific microsatellite markers for the study of pinniped populations. *Molecular Ecology* **6**: 661-666.

Goldsworthy, S D (1992) Maternal Care in Three Species of Southern Fur Seal (*Arctocephalus* spp). Department of Ecology and Evolutionary Biology. Melbourne, Monash University.

Goldsworthy, S D , J Francis, D Boness and R Fleischer (2000) Variation in the mitochondrial control region in the Juan Fernandez fur seal (*Arctocephalus philippii*). *Journal of Heredity* **91**: 371-377.

Goldsworthy, S.D., D.J. Boness and R.C. Fleischer (1999) Mate choice among sympatric fur seals: female preference for conphenotypic males. *Behavioural Ecology and Sociobiology* **45**: 253-267.

Goldsworthy, S.D., D. Pemberton and R.M. Warneke (1997) Field identification of Australian and New Zealand fur seals, *Arctocephalus* spp., based on external features. In: *Marine Mammal Research in the Southern Hemisphere* (ed. M. Hindell, and K. Kemper), pp. 63-71 Surrey Beatty & Sons, Sydney.

Goldsworthy, S.D. and P.D. Shaughnessy (1989) Subantarctic fur seals *Arctocephalus tropicalis* at Heard Island. *Polar Biology* **9**: 337-339.

Goldsworthy, S.D., J Francis, D. Boness and R. Fleischer (2000) Variation in the mitochondrial control region in the Juan Fernandez fur seal (*Arctocephalus philippii*). *Journal of Heredity* **91**: 371-377.

Goodman, S J (1997a) Dinucleotide repeat polymorphisms at seven anonymous microsatellite loci cloned from the European harbour seal (*Phoca vitulina vitulina*) *Animal Genetics* **28**: 308-322.

Goodman, S.J. (1997b) Rst Calc: a collection of computer programs for calculating estimates of genetic differentiation from microsatellite data and determining their significance. *Molecular Ecology* **6**: 881-885.

Goodman, S.J. (1998) Patterns of extensive genetic differentiation and variation among European harbor seals (*Phoca vitulina vitulina*) revealed using microsatellite DNA polymorphisms. *Molecular Biology and Evolution* **15:** 104-118.

Graybeal, A. (1998) Is it better to add taxa or characters to a difficult phylogenetic problem? *Systematic Biology* **47:** 9-17.

Green, K., H.R. Burton and R. Williams (1989) The diet of Antarctic fur seals *Arctocephalus gazella* (Peters) during the breeding season at Heard Island. *Antarctic Science* **1:** 317-324.

Green, K., R Williams, K.A Handasyde, H R. Burton and P.D. Shaughnessy (1990) Interspecific and intraspecific differences in the diet of fur seals, *Arctocephalus* species (Pinnipedia: Otariidae) at Macquarie Island. *Australian Mammalogy* **13:** 193-200.

Grewe, P.M., C.C. Krueger, C.F. Aquadro, E. Bermingham, H.L. Kincaid, and B. May (1993) Mitochondrial DNA variation among lake trout (*Salvelinus namaycush*) strains stocked into Lake Ontario. *Canadian Journal of Fisheries and Aquatic Science* **50:** 2397-2403.

Guinet, C., P. Jouventin and J.-Y. Georges (1994) Long term population changes of fur seals *Arctocephalus gazella* and *Arctocephalus tropicalis* on subantarctic (Crozet) and subtropical (St. Paul and Amsterdam) islands and their possible relationship to El Nino Southern Oscillation. *Antarctic Science* **6:** 473-478.

Harpending, H.C., M.A. Batzer, M. Gurven L.B. Jorde, A.R. Rogers and S.T. Sherry (1998) Genetic traces of ancient demography. *Proceedings of the National Academy of Science, USA* **95:** 1961-1967.

Headland, R. (1984) *The Island of South Georgia*. Cambridge University Press, Cambridge.

Hewitt, G M (2001) Speciation, hybrid zones and phylogeography - or seeing genes in space and time. *Molecular Ecology* **10:** 537-549.

Hillis, D M., and J.P. Huelsenbeck (1992) Signal, noise and reliability in molecular phylogenetic analyses. *Journal of Heredity* **83:** 189-195.

Hillis, D.M. and C. Moritz (1990) *Molecular Systematics*. Sinauer Associates, Sunderland.

Hindell, M.A. and C.R. McMahon (2000) Long distance movement of a southern elephant seal (*Mirounga leonina*) from Macquarie Island to Peter 1ØY. *Marine Mammal Science* **16:** 504-507.

Hindell, M. and C. Kemper (1997) *Marine Mammal Research in the Southern Hemisphere*. Surrey Beatty and Sons, Sydney.

Hoelzel, A.R., J. Halley, S.J. O'Brien C. Campagna, T Arnbom, B. Le Boeuf, K. Ralls and G.A. Dover (1993) Elephant seal genetic variation and the use of simulation models to investigate historical population bottlenecks. *Journal of Heredity* **84:** 443-449.

Hofmeyr, G.J.G., M.N. Bester and F.C. Jonker (1997) Changes in population sizes and distribution of fur seals at Marion Island. *Polar Biology* **17:** 150-158.

Holdgate, M.W , P.J. Tilbrook and R.W. Vaughan (1968) The biology of Bouvetøya. *British Antarctic Survey Bulletin* **15:** 1-7.

Hubbs, C.L. (1956) Back from oblivion. Guadelupe fur seal: still a living species. *Pacific Discovery Californian Academy of Science* **9:** 14-21.

Hubbs, C.L. and K.S. Norris (1971) Original teeming abundance, supposed extinction, and survival of the Juan Fernandez fur seal. *Antarctic Research Series* **18:** 35-51.

Isaksen, K., G.J.G. Hofmeyr, B.M. Dyer, A. Naestvold, F. Mehlum, I. Gjertz, V. Bakken and O. Huyser (1997) *Preliminary results from CEMP-monitoring of Antarctic fur seals, chinstrap penguins and macaroni penguins at Bouvetøya 1996/1997.* Working Group Paper submitted to CCAMLR WG-EMM 1997. Agenda Item Number 2.iii.

Jarne, P. and P.J.L. Lagoda (1996) Microsatellites, from molecules to populations and back. *Trends in Ecology and Evolution* **11:** 424-429.

Jouventin, P., J.C. Stahl and H. Weimerskirch (1982) La recolonisation des Iles Crozet par les otaries (*Arctocephalus tropicalis* et *A. gazella*). *Mammalia* **46:** 505-514.

Kallersjo, M., V. A. Albert and J. S. Farris (1999) Homoplay increases phylogenetic structure. *Cladistics* **15:** 91-93.

Kappe, A.L. (1998) *Detecting Genetic Variation: Application of Molecular Techniques in Conservation Biology.* PhD Thesis, University of Groningen, The Netherlands.

Kerley, G.I.H. (1987) *Arctocephalus tropicalis* on the Prince Edward Islands. In: *Status, Biology and Ecology of Fur Seals, Proceedings of an International Symposium and Workshop* (ed. J.P. Croxall and R.L. Gentry), pp. 61-64. Cambridge, England.

Kerley, G.I.H. (1985) Pup growth in the fur seals *Arctocephalus tropicalis* and *A. gazella* on Marion Island. *Journal of Zoology, London A* **205:** 315-324.

Kerley, G.I.H. (1983a) Comparison of seasonal haul-out patterns of fur seals *Arctocephalus tropicalis* and *A. gazella* on subantarctic Marion Island. *South African Journal of Wildlife Research* **13:** 71-77.

Kerley, G.I.H. (1983b) Relative population sizes and trends, and hybridisation of fur seals *Arctocephalus tropicalis* and *A. gazella* at the Prince Edward Islands, Southern Ocean. *South African Journal of Zoology* **18:** 388-392.

Kerley, G.I.H. and T.J. Robinson (1987) Skull morphometrics of male Antarctic and subantarctic fur seals, *Arctocephalus gazella* and *A. tropicalis* and their interspecific hybrids In: *Status, Biology and Ecology of Fur Seals*, (ed. J.P. Croxall and R.L. Gentry), pp. 121-131. Cambridge, England.

Kim, K.C., C.A. Repenning and G.V. Morejohn (1975) Specific antiquity of the sucking lice and evolution of otariid seals. *Rapports et Proces-Verbaux des Reunions/Conseil Permanent International Pour l'Exploration de la Mer* **169:** 544-549.

Kimura, M. (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* **16:** 111-120.

King, J.E. (1983) *Seals of the World.* University of Queensland Press, St Lucia.

King, J.E. (1978) On the specific name of the southern sea lion (Pinnipedia, Otariidae). *Journal of Mammalogy* **59:** 861-863.

King, J.E. (1969) The identity of the fur seals of Australia. *Australian Journal of Zoology* **17:** 841-853.

King, J.E. (1960) Sea lions of the genera *Neophoca* and *Phocarctos*. *Mammalia* **24:** 445-456.

King, J.E. (1959). The northern and southern populations of *Arctocephalus gazella*. *Mammalia* **23:** 19-40.

Kocher, T.D., W.K. Thomas, A. Meyer, S.V. Edwards, S. Paabo, F.X. Villablanca and A.C. Wilson (1989) Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proceedings of the National Academy of Science USA* **86:** 6196-6200.

Kovacs, K.M., C. Lydersen, M.O. Hammill, B.N. white, P.J. Wilson and S. Malik (1997) A harp seal x hooded seal hybrid. *Marine Mammal Science* **13:** 460-468.

Kretzmann, M.B., W.G. Gilmartin, A. Meyer G.P. Zegers, S.R. Fain, B.F. Taylor and D.P. Costa (1997) Low genetic variability in the Hawaiian monk seal. *Conservation Biology* **11:** 482-490.

Kumar, S., K. Tamura and M. Nei (1993) *MEGA: Molecular Evolutionary Genetics Analysis*, version 1.0. The Pennsylvania State University, PA.

Laws, R.M. (1977) Seals and whales of the Southern Ocean. *Philosophical Transactions of the Royal Society of London, B* **279:** 81-96.

Laws, R.M. (1973) Population increase of fur seals at South Georgia. *Polar Record* **16:** 856-858.

Lento, G.M. (1995) *Molecular Systematics and Population Genetic Studies of Pinnipeds: Phylogenies of our Fin-Footed Friends and their Surreptitious "Species" Status*. PhD Thesis, Victoria University of Wellington.

Lento, G M., M Haddon, G.K. Chambers and C.S. Baker (1997) Genetic variation of southern hemisphere fur seals (*Arctocephalus* spp.): investigation of population structure and species identity. *Journal of Heredity* **88:** 202-208.

Lento, G. M., R. E. Hickson, G. K. Chambers and D. Penny (1995) Use of spectral analysis to test hypotheses on the origin of pinnipeds. *Molecular Biology and Evolution* **12:** 28-52.

Lento, G.M., R.H. Mattlin, G.K. Chambers and C.S. Baker (1994) Geographic distribution of mitochondrial cytochrome *b* DNA haplotypes in New Zealand fur seals (*Arctocephalus forsteri*). *Canadian Journal of Zoology* **72:** 293-299.

Ling, J.K. (1999) Exploitation of fur seals and sea lions from Australia, New Zealand and adjacent subantarctic islands during the eighteenth, nineteenth and twentieth centuries. *Australian Zoologist* **31:** 323-343.

Lu, G., D.J. Basley and L. Bernatchez (2001) Contrasting patterns of mitochondrial DNA and microsatellite introgressive hybridization between lineages of lake whitefish (*Coregonus clupeaformis*); relevance for speciation. *Molecular Ecology* **10:** 965-985.

Lunn, N.J. and I.L. Boyd (1991) Pupping-site fidelity of Antarctic fur seals at Bird Island, South Georgia. *Journal of Mammalogy* **72:** 202-206.

Maldonado, J.E., F. Orta-Davila, B.S. Stewart, E. Geffen and R.K. Wayne (1995) Intraspecific genetic differentiation in California sea lions (*Zalophus californianus*) from southern California and the Gulf of California. *Marine Mammal Science* **11:** 46-58.

Masuda, R., and M. Yoshida (1994) A molecular phylogeny of the Family Mustelidae (Mammalia, Carnivora), based on comparison of mitochondrial cytochrome b nucleotide sequences. *Zoological Science* **11:** 605-612.

McCann, T.S. and D.W. Doidge (1987) Antarctic fur seal, *Arctocephalus gazella*. In: *Status, Biology and Ecology of Fur Seals, Proceedings of an International Symposium and Workshop* (ed. J.P. Croxall and R.L. Gentry), pp. 5-8. Cambridge, England.

McElroy, D., P. Moran, E. Bermingham and I. Kornfield (1992) REAP: an integrated environment for the manipulation and phylogenetic analysis of restriction data. *Journal of Heredity* **83:** 157-158.

Miller, M.P. (1997) *Tools for Population Genetics Analyses (TFPGA).* http://herb.bio.nau.edu/~miller/

Miyazaki, S., H. Horikawa, N. Kohno K. Hirota, M. Kimura, Y. Hasegawa, Y. Tomida, L.G. Barnes and C.E. Ray (1994) Summary of the fossil record of pinnipeds of Japan, and comparisons with that from the eastern North Pacific. *The Island Arc* **3:** 361-372.

Morejohn, G.V (1975) A phylogeny of otariid seals based on morphology of the baculum. *Rapports et Proces-Verbaux des Reunions/Conseil Permanent International Pour l'Exploration de la Mer* **169:** 49-56.

Moritz, C. (1994a) Defining 'Evolutionary Significant Units' for conservation. *Trends in Ecology and Evolution* **9:** 373-375.

Moritz, C. (1994b) Application of mitochondrial DNA analysis in conservation: a critical review. *Molecular Ecology* **3:** 401-411.

Mukai, T., K. Naruse, T. Sato, A. Shima and M. Morisawa (1997) Multiregional introgressions inferred from the mitochondrial DNA phylogeny of a hybridizing species complex of gobiid fishes, genus *Tridentiger*. *Molecular Biology and Evolution* **14:** 1258-1265.

Nei, M. (1987) *Molecular Evolutionary Genetics*. Columbia University Press, New York.

Nei, M., T. Maruyama and R. Chakraborty (1975) The bottleneck effect and genetic variability in populations. *Evolution* **29:** 1-10.

Olstad, O. (1929) Trekk fra sydishavets dyreliv. *Norsk Geologisk Tidsskrift* **2:** 511-534.

Paetkau, D., W. Calvert, I. Stirling and C. Strobeck (1995) Microsatellite analysis of population structure in Canadian polar bears. *Molecular Ecology* **4:** 347-354.

Paetkau, D and C Strobeck (1994) Microsatellite analysis of genetic variation in black bear populations. *Molecular Ecology* **3:** 489-495.

Page, B., S.D. Goldsworthy and M.A. Hindell (2001) Vocal traits of hybrid fur seals: intermediate to their parental species. *Animal Behaviour* **61:** 959-967.

Payne, M.R. (1979) Fur seals *Arctocephalus tropicalis* and *A. gazella* crossing the Antarctic convergence at South Georgia. *Mammalia* **43:** 93-98.

Payne, M.R. (1977) Growth of a fur seal population. *Philosophical Transactions of the Royal Society of London, B.* **279:** 67-79.

Pemberton, J.M., J. Slate, D.R. Bancroft and J.A. Barrett (1995) Nonamplifying alleles at microsatellite loci: a caution for parentage and population studies. *Molecular Ecology* **4:** 249-252.

Primmer, C.R., A.P. Moller and H. Ellegren (1996) A wide-ranging survey of cross species microsatellite amplification in birds. *Molecular Ecology* **5:**365-378.

Rand, R.W. (1956) Notes on the Marion Island fur seal. *Proceedings of the Zoological Society of London* **126:** 65-82.

Raymond, M. and F. Rousset (1995) GENEPOP (version 1.2); population genetics software for exact tests and ecumenicism. *Journal of Heredity* **86:** 248-249.

Repenning, C.A. (1976) Adaptive evolution of sea lions and walruses. *Systematic Zoology* **25:** 375-390.

Repenning, C.A., C.E. Ray and D. Grigorescu (1979) Pinniped biogeography. In: *Historical Biogeography, Plate Tectonics, and the Changing Environment* (ed. J. Gray, and A.J. Boucot), pp. 357-369 Oregon State University Press.

Repenning, C.A., R.S. Peterson and C.L. Hubbs (1971) Contributions to the systematics of the southern fur seals, with particular reference to the Juan Fernandez and Guadelupe species. In: *Antarctic Pinnipedia* (ed. W.E. Burt), pp. 1-52 American Geophysical Union, Washington DC.

Reynolds, J.E., D.K. Odell and S.A. Rommel (1999) Marine mammals of the world. In: *Biology of Marine Mammals* (ed. J.E. Reynolds, and S.A. Rommel), pp. 1-14 Melbourne University Press, Melbourne.

Rhymer, J.M. and D. Simberloff (1996) Extinction by hybridization and introgression. *Annual Reviews in Ecology and Systematics* **27:** 83-109.

Rice, D.W. (1998) *Marine Mammals of the World: Systematics and Distribution*. Society for Marine Mammalogy, Special Publication no. 4, Lawrence, KS.

Rice, W.R. (1989) Analysing tables of statistical tests. *Evolution* **43:** 223-225.

Richards, R. (1994) "The upland seal" of the Antipodes and Macquarie Islands: a historian's perspective. *Journal of the Royal Society of New Zealand* **24:** 289-295.

Riedman, M. (1990) *The Pinnipeds: Seals, Sea Lions and Walruses*. University of California Press, Berkeley.

Roberts, B. (1950) Historical notes on Heard and McDonald Islands. *Polar Record* **5:** 580-584.

Robinson, S., L. Wynen and S.D. Goldsworthy (1999) The impact of predation by a Hooker's sea lion (*Phocarctos hookeri*) on a small population of fur seals (*Arctocephalus* spp.) at Macquarie Island. *Marine Mammal Science* **15:** 888-893.

Roff, D.A. and P. Bentzen (1989) The statistical analysis of mitochondrial DNA polymorphisms: χ^2 and the problem of small samples. *Molecular Biology and Evolution* **6:** 539-545.

Rousset, F. (1997) Genetic differentiation and estimation of gene flow from F-statistics under isolation by distance. *Genetics* **145:** 1219-1228.

Roux, J.-P. (1987a) Recolonization process in the subantarctic fur seal, *Arctocephalus tropicalis* on Amsterdam Island. In: *Status, Biology and Ecology of Fur Seals, Proceedings of an International Symposium and Workshop* (ed. J.P. Croxall and R.L. Gentry), pp. 189-194. Cambridge, England.

Roux, J.-P. (1987b) Subantarctic fur seal, *Arctocephalus tropicalis* in French subantarctic territories. In: *Status, Biology and Ecology of Fur Seals, Proceedings of an International Symposium and Workshop* (ed. J.P. Croxall and R.L. Gentry), pp. 79-81. Cambridge, England.

Roy, M.S., E. Geffen, D. Smith, E.A. Ostrander and R.K. Wayne (1994) Patterns of differentiation and hybridization in North American wolflike canids, revealed by analysis of microsatellite loci. *Molecular Biology and Evolution* **11:** 553-570.

Rozas, J. and R. Rozas (1997) DnaSP version 2.0: A novel software package for extensive molecular population genetics analysis. *Computer Applications in the Biosciences* **13:** 307-311

Rzhetsky, A., and M. Nei (1993) Theoretical foundations of the minimum-evolution method of phylogenetic inference. *Molecular Biology and Evolution* **10:** 1073-1095.

Rzhetsky, A., and M. Nei (1992) A simple method for estimating and testing minimum-evolution trees. *Molecular Biology and Evolution* **9:** 945-967.

Saitou, N. and M. Nei (1987) The Neighbor-Joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4:** 406-425.

Sarich, V.M. (1969) Pinniped phylogeny. *Systematic Zoology* **18:** 416-422.

Scheffer, V.B. (1958) *Seals Sea Lions and Walruses: A Review of the Pinnipedia*. Stanford University Press, Stanford.

Schneider, S., J.-M. Kueffer, D. Roessli and L. Excoffier (1997) *Arlequin*. Genetics and Biometry Laboratory, University of Geneva, Switzerland, Geneva.

Shaughnessy, P.D. (1982) The status of the Amsterdam Island fur seal. In: *Mammals in the Seas: FAO Fisheries series no.5*, pp. 411-421. FAO, Rome

Shaughnessy, P.D. (1970) Serum protein variation in southern fur seals, *Arctocephalus* spp. in relation to their taxonomy. *Australian Journal of Zoology* **18:** 331-343.

Shaughnessy, P.D., E. Erb and K. Green (1998) Continued increase in the population of Antarctic fur seals, *Arctocephalus gazella*, at Heard Island, Southern Ocean. *Marine Mammal Science* **14:** 384-389.

Shaughnessy, P.D., G.L. Shaughnessy and L. Fletcher (1988) Recovery of the fur seal population at Macquarie Island. *Papers and Proceedings of the Royal Society of Tasmania* **122:** 177-187.

Shaughnessy, P.D. and L. Fletcher (1987) Fur seals, *Arctocephalus* spp. at Macquarie Island. In: *Status, Biology and Ecology of Fur Seals, Proceedings of an International Symposium and Workshop* (ed. J.P. Croxall and R.L. Gentry), pp. 177-188. Cambridge, England.

Shaughnessy, P.D. and H.R. Burton (1986) Fur seals *Arctocephalus* spp. at Mawson station, Antarctica, and in the Southern Ocean. *Polar Record* **23:** 79-81.

Sivertsen, E. (1954) A survey of the eared seals (Family Otariidae) with remarks on the Antarctic seals collected by M/K Norvegia in 1928-1929. Det Norske Videnskaps-Akademi i Oslo.

Slade, R W (1997) Genetic studies of the southern elephant seal *Mirounga leonina*. In: *Marine Mammal Research in the Southern Hemisphere* (ed M. Hindell, and K. Kemper), pp. 11-29. Surrey Beatty & Sons, Sydney.

Slade, R.W., C. Moritz and A. Heideman (1994) Multiple nuclear-gene phylogenies: applications to pinnipeds and comparison with a mitochondrial DNA gene phylogeny. *Molecular Biology and Evolution* **11:** 341-356.

Slatkin, M. (1995) A measure of population subdivision based on microsatellite allele frequencies. *Genetics* **139:** 457-462.

Slip, D.J. (1997) Foraging Ecology of Southern Elephant Seals from Heard Island. PhD Thesis, School of Zoology, University of Tasmania.

Smale, L., S. Nunes and K.E. Holekamp (1997) Sexually dimorphic dispersal in mammals: patterns, causes and consequences. *Advances in the Study of Behavior* **26:** 181-250.

Soltis, D.E., P.S. Soltis, M.E. Mort, M.W. Chase, V. Savolainen, S.B. Hoot and C.M. Morton (1998) Inferring complex phylogenies using parsimony: an empirical approach using three large DNA data sets for angiosperms. *Systematic Biology* **47:** 32-42.

Stewart, B S , P K. Yochem, R.L. De Long and G A Antonelis (1987) Interactions between Guadalupe fur seals and California sea lions at San Nicholas and San Miguel Islands, California. In *Status, Biology and Ecology of Fur Seals.* (ed. J.P. Croxall and R.L. Gentry), pp. 103-106. Cambridge, England.

Stirling, I., and R.M. Warneke (1971) Implications of a comparison of the airborne vocalizations and some aspects of the behaviour of the two Australian fur seals, *Arctocephalus* spp., on the evolution and present taxonomy of the genus. *Australian Journal of Zoology* **19:** 227-241.

Swofford, D.L. (1999) *"PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods).* version 4.". Sinauer Associates, Sunderland, Ma.

Tajima, F. (1983) Evolutionary relationship of DNA sequences in finite populations. *Genetics* **1983:** 437-460.

Takezaki, N., A. Rzhetsky and M. Nei (1995) Phylogenetic test of the molecular clock and linearized trees. *Molecular Biology and Evolution* **12:** 823-833.

Thompson, J.D., D.G. Higgins and T.J. Gibson (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22:** 4673-4680.

Torres, D. and A. Aguayo (1984) Presence of *Arctocephalus tropicalis* (Gray 1872) at the Juan Fernandez Archipelago, Chile. *Acta Zoologica Fennica* **172:** 133-134.

Trillmich, F., and P. Majluf (1981) First observations on colony structure, behaviour, and vocal repertoire of the South American fur seal (*Arctocephalus australis* Zimmermann, 1783) in Peru. *Zeitschrift fur Saugetierkunde* **46:** 310-322.

Vrana, P.B., M.C. Milinkovitch, J.R. Powell and W.C. Wheeler (1994) Higher level relationships of the Arctoid Carnivora based on sequence data and 'total evidence'. *Molecular Phylogenetics and Evolution* **3:** 47-58.

Wace, N.M. and M.W. Holdgate (1976) *Man and Nature in the Tristan da Cunha Islands.* IUCN Publications, Morges.

Weir, B.S. (1990) *Genetic Data Analysis.* Sinauer Associates, Inc., Sunderland, MA.

Weir, B.S. and C.C. Cockerham (1984). Estimating F-statistics for the analysis of population structure. *Evolution* **38:** 1358-1370.

Wheeler, W.C. (1992) Extinction, sampling and molecular phylogenetics. In: *Extinction and Phylogeny* (ed. M.J. Novacek and Q.D. Wheeler), pp 205-215. Columbia University Press, N Y.

Wickens, P. and A.E. York (1997) Comparative population dynamics of fur seals. *Marine Mammal Science* **13:** 241-292.

Wynen, L.P., S.D. Goldsworthy, S.J. Insley, M. Adams, J.W. Bickham, J. Francis, J.P. Gallo, A.R. Hoelzel, P. Majluf, R.W.G. White and R.W. Slade (2001) Phylogenetic relationships within the eared seals (Otariidae: Carnivora), with implications for the historical biogeography of the family. *Molecular Phylogenetics and Evolution* **21:** 270-284.

Wynen, L.P., S.D. Goldsworthy, C. Guinet, M.N. Bester, I.L. Boyd, I. Gjertz, G.J.G. Hofmeyr, R.W.G. White and R.W. Slade (2000) Post-sealing genetic variation and population structure of two species of fur seal (*Arctocephalus gazella* and *A. tropicalis*). *Molecular Ecology* **9:** 299-314.

Wynen, L.P., S.D. Goldsworthy, R.W.G. White and R.W. Slade (in review) Genetic variation and population structure in two fur seal species, *Arctocephalus* spp. *Journal of Heredity*.

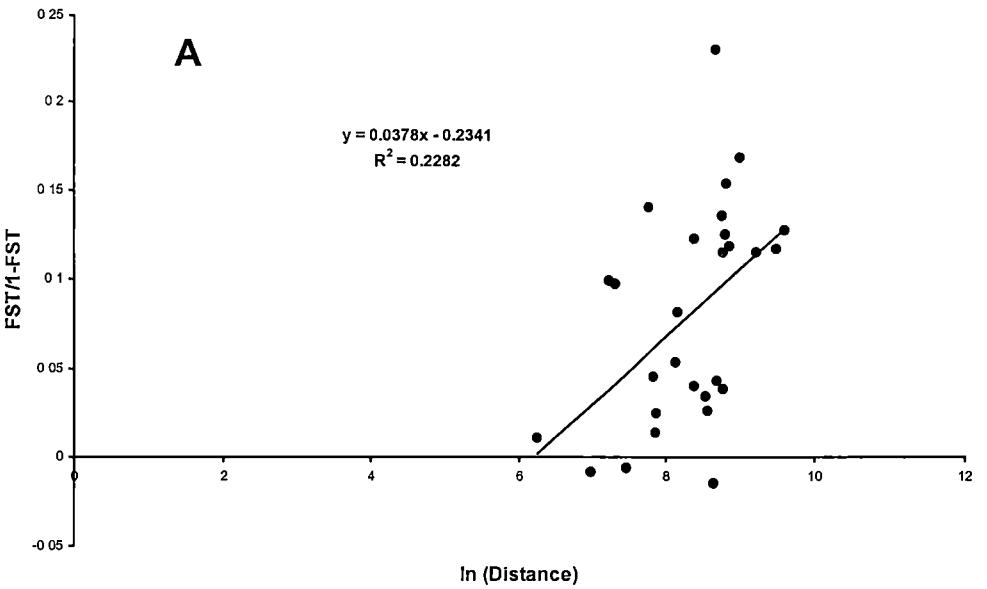
Wynen, L.P., M.A. Hindell, S.D. Goldsworthy and R. Slade (in prep) Hybridisation between fur seal species (*Arctocephalus* spp.) at Macquarie Island: a comparative analysis of molecular and field based techniques for species identification.

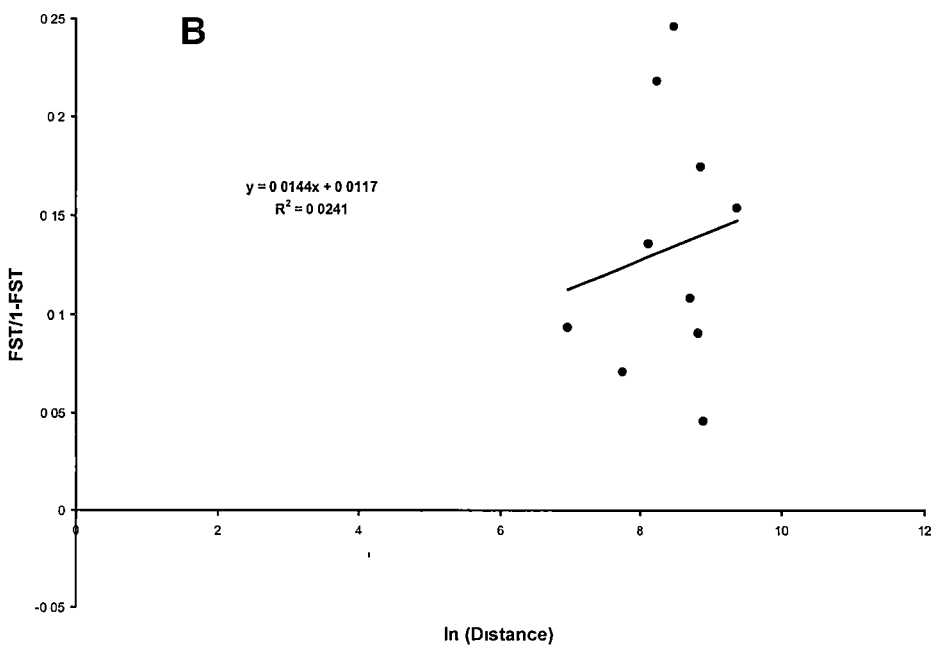
Zar, J.H. (1996) *Biostatistical Analysis* Prentice Hall International Inc, Upper Saddle River, N.J.

Appendix 1

Isolation by distance plots for Antarctic fur seal populations (A) and Subantarctic fur seal populations (B).

A





Appendix 2

Allele frequency (%), number of individuals sampled (n) and allele distributions for each population within the Antarctic fur seal (AFS) and the subantarctic fur seal (SAFS), as well as for these species and the New Zealand fur seal (NZFS) overall. Blank spaces indicate an allele frequency of zero. *no population data are presented for 10E4 due to the small sample size. Alleles observed in only one species denoted by shading as follows: AFS, SAFS and NZFS.

	SAFS				AFS							Species		
	AI	CI	GI	MA	BI	CI	HI	KI	MA	SG	SS	SAFS	AFS	NZFS
Hg1.4														
n	**20**	**19**	**20**	**17**	**20**	**20**	**19**	**20**	**20**	**20**	**18**	**76**	**137**	**16**
189						10.0			2.5		8.3		2.9	
191		5.3									2.8	1.3	0.4	
193	5.0	2.6	7.5		5.0	15.0	2.6	2.5	2.5	5.0		3.9	4.7	9.4
195	2.5	21.0	12.5	8.8								11.2		12.5
197	10.0	39.5	25.0	73.5								35.6		24.9
199	7.5	5.3	15.0					2.5	2.5		5.6	7.2	1.5	9.4
201	15.0	5.3	20.0		5.0	17.5		2.5	15.0	17.5	8.3	10.5	9.5	18.8
203	45.0	21.0	15.0	5.9	32.5	7.5	21.1	20.0	17.5	22.5	19.4	22.4	20.1	12.5
205	2.5		2.5	11.8	47.5	42.5	42.1	40.0	55.0	47.5	38.9	3.9	44.8	12.5
207			2.5		10.0	2.5	5.3	20.0	5.0	7.5	16.7	0.7	9.5	
209						5.0	28.9	12.5					6.6	
211	12.5											3.3		
4A3														
n	**20**	**19**	**20**	**17**	**20**	**20**	**20**	**20**	**20**	**20**	**18**	**76**	**138**	**16**
142							7.5	2.5			8.3		2.5	15.6
144					40.0	35.0	45.0	67.5	27.5	57.5	22.2		42.4	31.3
146		5.3			25.0	35.0	22.5	15.0	47.5	15.0	27.8	1 3	26.8	25.0
148			2.5		20.0	17.5	10.0	5.0	17.5	17 5	16.7	0.7	14.9	25.0
150			15.0	2.9	5.0				2.5		19.4	4.6	3.6	3.1
152	20.0	7.9	20.0	11.8						2.5		15.1	0.4	
154	15.0	13.2	5.0	11.8	10.0					2.5		11.2	1.8	
156	37.5	39.4	7.5	35.4		12.5	15.0	10.0	5.0	5.0	5.6	29.6	7.6	
158	27.5	15.8	20.0	17.6								20.4		
160		5.3	22.5	8.8								9.2		
162		2.6	7.5	8.8								4.6		
164		10.5		2.9								3.3		

	SAFS				AFS							Species		
	AI	CI	GI	MA	BI	CI	HI	KI	MA	SG	SS	SAFS	AFS	NZFS
Pvc19														
n	**20**	**19**	**20**	**17**	**20**	**20**	**20**	**20**	**20**	**20**	**18**	**76**	**138**	**16**
110					5.0	2.5	7.5	15.0	5.0	7.5	2.8		6.5	
112				14.7	5.0	10.0	10.0	2.5	2.5		8.3	3.3	5.4	9.4
114	15.0	7.9	12.5	26.5	47.5	35.0	20.0	22.5	40.0	50.0	50.0	15.1	37.8	71.8
116	55.0	89.5	72.5	44.1	22.5	35.0	37.5	42.5	32.5	27.5	27.8	65.8	32.2	18.8
118	5.0		5.0		20.0	17.5	25.0	17.5	20.0	15.0	11.1	2 6	18.1	
120	25.0	2.6	10.0	11.8								12.5		
122				2 9								0.7		
3E3														
n	**20**	**19**	**20**	**17**	**20**	**20**	**20**	**20**	**20**	**20**	**18**	**76**	**138**	**16**
153	100	100	100	100								100		
155					100	100	100	100	100	100	100		100	56.3
157														9.4
159														12.5
161														15.6
163														3.1
165														3.1
Pv9														
n	**20**	**19**	**20**	**17**	**20**	**20**	**20**	**20**	**20**	**20**	**18**	**76**	**138**	**16**
168		5.3		2.9	7.5	12.5	22.5	20.0	10.0	20.0	8.3	2.0	14.5	18.8
170	5.0	28.9	15.0	11.8	32.5	25.0	30.0	17.5	32.5	45.0	27.8	15.1	30.2	
172	7.5	5.3	20.0	23.5	25.0	20.0	12.5	10.0	12.5	7.5		13.8	12.7	21.8
174	55.0	23.7	25.0	20.6	15.0	12.5	12.5	15.0	12.5		27.8	31.6	13.4	25.0
176	12.5	31.6	22.5	26.5	12.5	12.5	10.0	10.0	10.0	7.5	8.3	23.0	10.1	12.5
178		2.6	10.0			5.0				7.5	5.6	3.3	2.5	9.4
180	2.5	2.6	7.5	14.7					2.5		5.6	6.6	1.1	9.4
182	17.5							2.5	2.5			4.6	0.7	3.1
184					5.0	12.5	7.5	22 5	12.5	5.0	8.3		10.5	
186					2.5		5.0	2.5	2.5	5.0	8.3		3.6	
188									2.5	2.5			0.7	

	SAFS				AFS							Species		
	AI	CI	GI	MA	BI	CI	HI	KI	MA	SG	SS	SAFS	AFS	NZFS
Pvc78														
n	**20**	**19**	**20**	**17**	**20**	**20**	**20**	**20**	**20**	**20**	**18**	**76**	**138**	**16**
132			5 0	2 9								2.0		
134			2.5									0.7		
136	82 5	57 9	57 5	55.9								63 8		3.1
138			5.0		17.5	7.5	2.5	7.5	17.5	12.5	16.7	1.3	11.6	
140				2.9								0.7		
142	12.5	15.8	12.5	14.7								13.8		
144		7.9	5.0		5.0	2.5	12.5	5.0	5.0	2.5		3.3	4.7	
146		2.6		8.9	7.5	7.5	10.0	17.5	7.5	5.0	16.7	2.6	10.1	3.1
148		2.6		2.9	5.0	7.5	15.0	15.0	10.0	12.5	11.1	1.3	10.9	6.3
150	5.0		5.0		12.5	15.0	12.5	15.0	5.0	17.5	19.3	2.6	13.8	3.1
152		5.3		2.9	22.5	42.5	32 5	27.5	30.0	42.5	25.0	2.0	31.9	3.1
154		5.3		8.9	30.0	15.0	12.5	7.5	25.0	2.5	5.6	3.3	14.1	12.5
156										5.0	5.6		1.4	21.8
158		2.6	7.5									2.6		18.8
160								2.5					0.4	18.8
162						2.5	2.5	2.5					1.1	6.3
164														3.1
Hg6.3														
n	**19**	**19**	**20**	**17**	**20**	**20**	**19**	**20**	**20**	**20**	**18**	**76**	**137**	**16**
216											2.8		0.4	
218					5.0	2.5	7.9	15.0	10.0		2.8		6.2	
222				5.9	2.5				2.5			1.3	0.7	
224							2.6						0.4	
228		23.7	20.0	29.4								18.0		
230	18.4		27.5	35.3	10.0	10.0			15.0	5.0		20.1	5.8	
232	21.1	13.2	2.5	2.9	10.0	12.5	2.6	5.0	7.5	7.5	25.0	10.0	9.9	
234	26.3	21.0	17.5	2.9			2.6	10.0	5.0		2.8	17.3	2.9	
236	23.7		15.0	11.8	10.0	10.0	10 5	7 5	12.5	12.5	2.8	12.7	9.5	21.8
238					27.5	27.5	36.9	25.0	20.0	32.5	8.3		25.6	43.7
240			7.5		5.0	7.5	7.9	7.5	7.5	2.5	5.6	2.0	6.2	15.6
242					27.5	17.5	21.1	20.0	15.0	17.5	25.0		20.4	6.3
244		23.7	5.0	5.9			5.3	5.0	5.0	2.5	8.3	8.7	3.6	6.3
246			5 0		2.5					2.5	11.0	1.3	2.2	6.3
248	10.5	18 4				2.5	2.6				2.8	7 3	1.1	
250				5 9		7.5		5.0		17.5	2.8	1.3	4.7	
252						2.5							0.4	

	SAFS				AFS							Species		
	AI	CI	GI	MA	BI	CI	HI	KI	MA	SG	SS	SAFS	AFS	NZFS
Aa4														
n	**20**	**20**	**19**	**17**	**20**	**20**	**20**	**20**	**20**	**20**	**18**	**76**	**138**	**16**
204										5.0			0.7	
206					35.0	47.5	22.5	37.5	45.0	22.5	25.0		33.7	
208										10.0	2.8		1.8	
214	20.0	2.6	7.5	5.9	15.0	5.0	2.5	5.0	5 0	2.5	13.9	9.2	6.9	
216	72 5	86 9	85.0	94.1	30.0	37.5	50 0	32 5	20.0	37.5	38.9	84.2	35.2	93.7
218	7.5	10 5	7 5		20.0	10.0	25.0	25 0	30.0	22.5	19.4	6.6	21.7	6.3
10E4*														
n												**2**	**12**	**5**
132													29.1	
134												50.0	4.2	
136														10.0
140													4.2	10.0
142													58.3	
144												25.0	4.2	19.9
146														40.2
148														19.9
150												25.0		
Pv11														
n	**5**	**1**	**0**	**7**	**1**	**1**	**8**	**1**	**5**	**4**	**19**	**13**	**38**	**10**
146							18.8						3.9	
148		50 0			100	100	81.2	100	100	100	100	3.8	96.1	
154	10 0			14 3								11.5		
156				14 3								7.7		35.0
158	20.0			42.8								30.8		
160	30.0	50.0		14.3								23.1		15.0
162	20.0			14.3								15.4		
164	20.0											7.7		15.0
166														25.0
168														10.0

	SAFS				AFS							Species		
	Al	Cl	Gl	MA	Bl	Cl	Hl	Kl	MA	SG	SS	SAFS	AFS	NZFS
Hg4.2														
n	20	19	20	17	20	20	20	20	18	19	19	77	136	16
126											8.8		1.1	
128					2.5						11.8		1.9	
130							10.0	2.5					1.9	
132							2.5	12.5	11.1	2.6			4.1	
136														9.4
138														12.5
144	35.0		12.5	2.9								13.2		3.1
146	5.0											1.3		
148		10.5	12.5	29.5	5.0	5.0			5.6			12.5	2.2	3.1
150			12.5	2.9						5.3	11.8	3.9	2.2	
152	7.5	18.4	5.0	11.9	2.5	5.0						10.5	1.1	
154	12.5	10 5	2 5			10.0		2 5	13 8	7 9	8 8	6.6	6.0	
156	27 5	7 9	5 0	5.9	20 0	10 0		10.0	8.3	26.3	8.8	11.8	11.9	
158			7.5		5.0	27.5	7.5	30.0	5.6	7.9	8.8	2.0	13.5	3.1
160	5.0	5 3	10 0	2 9	15.0	7.5	10.0	17.5	5.6	7.9	5.9	5.9	10.1	
162	2.5	2.6	10.0	5.9	5.0	10.0	15 0	7.5	11.1	5.3		5.3	7.8	9.4
164		7.9	2.5	5.9	25.0	5.0	17.5		5.6	15.8	5.9	3.9	10.8	12.5
166			10.0	8.8		7.5	5.0	7.5	8.3	2.6	5.9	4.6	5.2	9.4
168							22.5						3.4	3.1
170											5.9		0.7	12.5
172														9.4
174								2.5	8.3	5.3	5.9		3.0	
176	2.5	21.1	2.5		5.0		7.5					6.6	1.9	
178					7.5	2.5			8.3	2.6			3.0	3.1
180					7.5	7.5			5.6		2.9		3.4	
182	2.5	2.6	2.5	2.9		2.5		2.5				2.6	0.7	
184			2.5	2.9				5.0	2.8			1.3	1.1	6.3
188			2.5				2.5					0.7	0.4	
192				2 9								0.7		
194				5 9						10 5	5.9	1.3	2.2	
196				8.8								2.0		
222		5.3										1.3		3.1
224		7 9										2.0		
230											2.9		0.4	

Appendix 3

Microsatellite genotype and mitochondrial DNA haplotype (mtDNA) of all adult females (AF), adult males (AM) and pups (P) from Macquarie Island where ID is the unique identifier for each individual. Results for six microsatellite loci are shown, in addition to the species identity as determined by the genotype (Gen). Hybrid individuals are those where some alleles were from a different species than that denoted by mtDNA. The loci where these were observed are shaded, and reference to Table 5.3 will identify which are the unexpected alleles.

Class	ID	mtDNA	Pvc19	3E3	Pv9	Aa4	Pvc78	Pv11	Gen
AF	2	G	114,116	155,155	176,184	216,216	146,152	148,148	G
AF	5	G	114,114	155,155	172,176	216,218	152,154	148,148	G
AF	8	G	116,118	155,155	170,184	206,216	146,148	148,148	G
AF	9	G	114,118	155,155	168,174	206,206	148,148	148,148	G
AF	22	G	116,118	155,155	168,170	206,218	148,148	148,148	G
AF	26	G	116,118	155,155	168,170	216,218	148,152	148,168	G
AF	29	G	112,116	155,155	170,172	216,216	148,152	148,148	G
AF	37	G	114,116	155,155	168,170	216,216	146,152	148,148	G
AF	40	G	110,114	155,155	168,172	206,216	146,154	148,148	G
AF	41	G	110,118	155,155	172,184	206,206	148,148	148,148	G
AF	42	G	114,118	155,155	168,172	206,218	146,146	148,148	G
AF	50	G	118,118	155,155	170,172	216,218	146,148	148,168	G
AF	51	G	116,118	155,155	174,174	216,218	152,154	148,148	G
AF	52	G	118,118	155,155	168,172	206,216	148,152	148,148	G
AF	72	G	116,116	155,155	170,174	204,214	146,154	000,000	G
AF	73	G	110,114	155,155	168,168	216,218	138,148	148,148	G
AF	74	G	114,116	155,155	176,184	216,216	146,152	**148,166**	**H**
AF	75	G	112,118	155,155	170,172	216,216	146,152	148,148	G
AF	76	G	110,114	155,155	168,172	206,218	146,162	148,148	G
AF	79	G	110,116	155,155	168,172	216,216	150,152	148,148	G
AF	80	G	110,114	155,155	174,184	216,218	146,152	148,148	G
AF	81	G	116,116	155,155	170,184	216,216	148,154	**148,160**	**H**
AF	92	G	114,116	155,155	168,168	214,218	148,150	000,000	G
AF	93	G	114,116	155,155	170,176	216,218	148,154	**148,160**	**H**
AF	95	T	114,120	153,153	176,176	216,216	136,136	160,164	T
AF	136	G	114,118	155,155	168,172	216,218	148,152	148,148	G
AF	142	G	110,116	155,155	174,184	206,216	148,148	148,148	G
AF	143	G	110,116	155,155	170,180	216,218	150,154	148,148	G
AF	144	G	118,118	155,155	172,174	206,216	146,152	148,148	G
AF	160	T	116,116	**153,155**	174,174	216,216	142,142	158,162	**H**
AF	164	T	116,116	153,153	174,176	216,216	136,148	156,164	T
AF	165	T	116,116	153,153	176,176	216,216	136,136	160,162	T
AF	168	T	116,116	153,153	174,176	216,216	136,152	156,160	T

Class	ID	mtDNA	Pvc19	3E3	Pv9	Aa4	Pvc78	Pv11	Gen
AF	194	T	116,120	153,153	178,182	214,216	136,142	158,164	T
AF	195	G	112,116	155,155	170,174	216,218	144,162	000,000	G
AF	201	G	114,118	155,155	168,168	216,216	146,146	148,148	G
AF	211	G	114,114	153,155	174,176	216,216	136,146	162,162	H
AF	216	T	116,118	153,153	170,176	216,216	142,152	156,160	T
AF	218	T	116,120	153,153	174,182	216,216	142,142	160,164	T
AF	223	T	114,116	153,153	172,182	218,218	136,142	164,164	T
AF	227	T	114,116	153,161	172,174	216,216	154,158	000,000	H
AF	228	T	116,116	153,153	174,174	216,216	136,154	000,000	T
AF	230	G	114,118	155,155	168,174	216,218	146,146	148,148	G
AF	232	G	110,116	155,155	168,176	216,216	144,146	148,148	G
AF	240	T	116,116	153,153	170,174	216,216	136,136	154,158	T
AF	243	G	112,118	155,155	174,184	206,218	152,154	148,148	G
AF	244	G	116,116	153,155	174,176	216,216	136,146	156,168	H
AF	245	G	114,116	155,155	168,176	216,216	146,152	148,148	G
AF	247	G	114,118	155,155	170,174	216,218	146,150	148,168	H
AF	248	G	116,116	155,155	170,180	216,216	136,146	148,148	G
AF	250	G	114,120	153,155	172,182	216,216	136,148	162,164	H
AF	252	G	114,114	155,155	168,168	218,218	148,148	148,148	G
AF	257	G	110,118	155,155	168,170	216,216	152,152	000,000	G
AF	258	G	114,116	155,155	172,176	206,218	148,148	148,148	G
AF	262	G	114,114	155,155	168,172	206,216	146,148	148,148	G
AF	266	G	114,116	155,155	170,184	216,218	148,150	148,148	G
AF	267	G	112,112	155,155	174,184	216,218	148,152	148,148	G
AF	268	G	116,118	155,155	168,176	206,206	146,148	148,148	G
AF	270	G	114,118	155,155	168,176	206,218	148,152	148,148	G
AF	273	T	114,120	153,155	174,176	216,216	142,150	000,000	H
AF	378	G	114,116	155,155	172,176	216,216	146,158	148,168	H
AF	379	G	116,116	155,155	170,172	206,216	148,152	148,148	G
AF	381	G	114,118	155,155	168,174	216,218	148,152	000,000	G
AF	382	G	110,118	155,155	170,172	218,218	146,152	148,148	G
AF	383	G	114,114	155,155	170,172	206,216	146,154	148,168	H
AF	386	G	114,116	155,155	174,174	206,206	148,152	148,148	G
AF	388	G	114,114	155,155	174,176	216,218	146,148	148,148	G
AF	389	G	116,116	155,155	168,172	216,216	146,152	148,148	G
AF	390	G	114,116	155,155	170,178	208,214	150,156	148,148	G
AF	392	G	114,114	155,155	170,170	206,208	148,152	148,148	G
AF	400	G	116,116	155,155	168,172	206,216	148,148	148,148	G
AF	408	G	114,118	155,155	168,172	206,216	148,152	148,148	G

Class	ID	mtDNA	Pvc19	3E3	Pv9	Aa4	Pvc78	Pv11	Gen
AF	409	G	110,114	155,155	168,170	214,218	146,162	148,148	G
AF	412	T	116,116	153,153	172,174	216,216	134,142	160,162	T
AF	414	G	116,118	155,155	170,184	216,218	138,138	148,148	G
AF	498	G	114,116	155,155	168,176	214,216	146,150	148,148	G
AF	504	G	114,116	155,155	184,184	206,206	146,152	148,148	G
AF	636	G	114,116	153,153	170,182	204,216	144,146	148,154	H
AF	640	G	114,114	153,155	174,182	216,216	136,160	000,000	H
AF	682	G	114,116	153,155	172,174	216,216	136,152	148,162	H
AF	693	G	116,116	155,155	172,174	216,216	146,146	000,000	G
AF	694	G	114,120	153,155	174,180	206,216	136,150	148,164	H
AF	721	G	116,118	155,155	168,170	206,216	148,152	148,148	G
AF	738	G	116,116	153,155	170,174	216,216	136,148	148,158	H
AF	823	G	114,118	155,155	174,184	216,216	148,154	148,148	G
AF	831	G	000,000	153,155	174,174	214,216	136,152	000,000	H
AF	854	G	116,116	155,155	176,184	206,216	152,152	148,148	G
AF	864	G	114,118	155,155	172,184	216,216	148,148	148,148	G
AF	865	G	114,116	155,155	168,168	206,216	146,152	148,148	G
AF	868	G	114,116	155,155	172,184	216,216	148,152	148,148	G
AF	872	G	110,116	155,155	168,184	216,218	146,148	148,148	G
AF	874	G	114,118	155,155	168,186	206,206	148,152	148,148	G
AF	886	G	114,114	155,155	174,174	216,216	146,148	148,148	G
AF	888	G	116,120	153,155	174,182	218,218	136,138	148,164	H
AF	906	G	114,116	155,155	168,172	216,218	148,148	148,148	G
AF	999	G	118,118	155,155	168,174	216,218	146,148	000,000	G
AF	1010	G	114,118	155,155	168,170	206,206	146,148	148,148	G
AF	1128	G	114,116	155,155	168,168	206,216	138,152	148,148	G
AF	1129	G	110,116	155,155	168,184	216,218	146,146	148,148	G
AF	1132	?	114,120	153,153	168,172	216,216	136,136	156,158	T
AF	1135	G	114,120	155,155	168,168	216,218	136,152	148,148	H
AF	1142	G	114,116	155,155	168,172	206,216	146,152	148,148	G
AF	1145	G	114,116	155,155	168,184	206,216	138,146	148,148	G
AF	1146	G	114,114	155,155	176,176	216,218	152,152	148,148	G
AF	1148	G	116,116	155,155	168,184	206,218	146,148	148,148	G
AF	1149	G	114,116	155,155	168,176	206,214	150,152	148,148	G
AF	1150	G	118,118	155,155	168,172	218,218	146,148	148,148	G
AF	1151	G	114,114	155,155	170,170	206,216	150,154	148,148	G
AF	1154	G	114,118	155,155	170,170	214,216	150,152	148,148	G

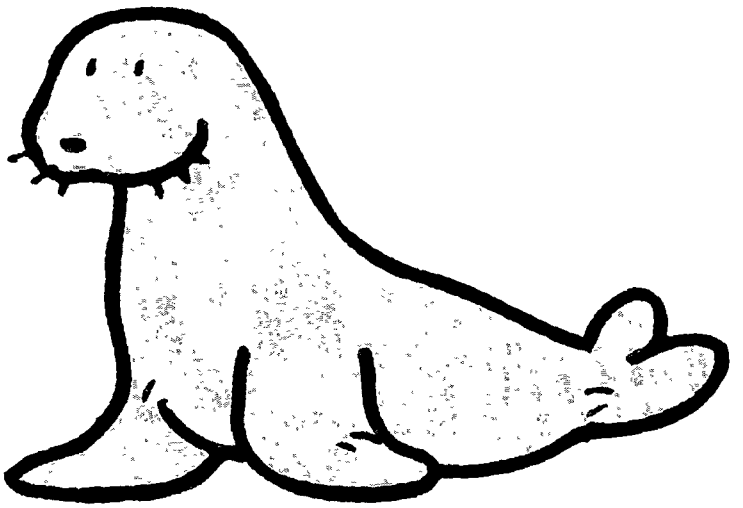
Class	ID	mtDNA	Pvc19	3E3	Pv9	Aa4	Pvc78	Pv11	Gen
AF	1158	G	116,116	155,155	170,174	216,218	146,148	148,148	G
AF	1159	G	114,114	155,155	176,184	206,218	146,148	148,148	G
AF	1175	G	112,116	155,155	172,176	216,216	150,154	148,148	G
AF	1185	G	112,116	155,155	168,172	206,216	148,150	148,148	G
AF	1187	G	114,116	155,155	172,176	206,206	148,148	148,148	G
AM	11	G	116,118	155,155	172,172	216,216	154,160	148,148	G
AM	12	G	116,118	153,155	172,182	216,218	136,162	148,156	H
AM	13	G	116,116	155,155	170,176	206,206	152,154	148,148	G
AM	14	G	116,118	155,155	170,176	206,206	142,152	156,160	H
AM	16	G	112,118	155,155	168,184	216,216	152,154	148,148	G
AM	17	G	114,116	153,155	174,184	216,218	136,152	148,154	H
AM	18	G	110,116	155,155	170,174	216,216	152,160	148,148	G
AM	19	G	114,120	153,155	176,180	216,216	136,162	164,168	H
AM	20	G	116,118	155,155	174,184	206,216	146,148	148,148	G
AM	112	G	118,118	155,155	168,172	216,218	152,154	148,166	H
AM	121	G	114,116	153,155	170,182	206,216	136,148	148,162	H
AM	122	G	116,116	153,155	172,180	216,218	136,152	148,164	H
AM	127	G	114,114	153,155	174,182	206,216	146,148	148,154	H
AM	128	G	116,116	155,155	172,184	216,216	146,146	148,148	G
AM	132	T	116,116	153,153	174,176	216,216	136,158	158,160	T
AM	133	T	116,116	153,153	170,172	214,216	134,136	156,158	T
AM	134	T	114,114	153,153	170,178	216,218	136,150	154,160	T
AM	156	G	114,114	155,155	168,182	216,218	154,158	148,166	H
AM	157	G	114,118	155,161	174,174	216,216	138,158	148,156	H
AM	158	G	114,116	155,155	174,184	206,216	146,152	148,148	G
AM	159	T	116,116	153,153	174,176	216,216	148,148	158,160	T
AM	162	G	112,118	155,155	172,176	216,218	150,162	148,148	G
AM	163	G	114,114	155,155	174,176	216,216	160,162	156,168	H
AM	171	T	116,116	153,153	174,176	216,216	142,146	158,158	T
AM	172	T	114,116	153,153	174,174	216,216	136,150	158,160	T
AM	174	T	114,116	153,155	172,176	216,216	144,156	162,166	H
AM	175	T	114,116	153,153	174,182	216,218	136,142	158,162	T
AM	190	G	110,118	155,155	168,168	206,218	146,148	148,148	G
AM	191	G	114,116	153,155	174,174	216,216	136,160	164,168	H
AM	205	G	116,116	153,155	172,180	216,218	136,152	148,164	H
AM	206	T	114,116	155,155	176,182	216,216	144,152	148,154	H
AM	207	G	116,120	153,155	180,180	216,216	136,158	164,166	H

Class	ID	mtDNA	Pvc19	3E3	Pv9	Aa4	Pvc78	Pv11	Gen
AM	239	T	114,116	153,153	174,182	216,218	136,142	158,162	T
P	776	G	118,120	155,155	172,174	216,216	000,000	148,148	H
P	885	T	114,114	153,153	174,174	216,216	142,142	158,162	T
P	927	G	116,116	155,155	168,184	216,216	148,148	148,148	G
P	928	G	114,116	155,155	172,186	206,216	148,148	148,148	G
P	1013	G	114,114	155,155	170,170	206,208	148,152	148,162	H
P	1014	G	114,116	155,155	176,184	216,216	146,146	148,148	G
P	1015	G	116,118	155,155	170,184	206,206	148,148	148,148	G
P	1016	G	110,114	155,155	168,184	206,206	146,148	148,148	G
P	1017	G	116,116	155,155	168,170	216,216	148,152	148,148	G
P	1018	G	114,116	155,155	168,184	216,218	148,152	148,148	G
P	1019	G	114,116	155,155	174,174	206,216	148,152	148,148	G
P	1020	G	114,116	155,155	168,170	206,216	148,148	148,148	G
P	1021	G	112,114	155,155	172,174	216,216	144,148	148,148	G
P	1022	G	114,116	155,155	172,184	216,216	146,148	148,148	G
P	1023	G	112,114	155,155	174,176	206,218	152,154	148,168	H
P	1024	G	116,118	155,155	168,174	216,216	148,148	148,160	H
P	1025	G	116,116	153,155	170,174	216,216	136,152	148,148	H
P	1026	G	112,116	153,155	172,172	216,216	146,154	148,148	H
P	1027	G	114,118	155,155	170,184	216,216	148,152	148,148	G
P	1028	G	112,118	155,155	168,174	216,216	148,148	148,148	G
P	1029	G	116,116	155,155	170,184	206,218	148,148	148,148	G
P	1030	G	114,116	155,155	168,184	216,216	146,152	148,148	G
P	1031	G	110,118	155,155	168,176	216,216	146,154	148,148	G
P	1032	G	116,116	155,155	168,174	214,216	148,148	148,168	H
P	1033	G	116,116	155,155	168,176	206,206	138,148	148,148	G
P	1034	G	114,116	155,155	168,174	214,216	146,148	148,160	H
P	1035	G	112,118	155,155	168,170	216,216	138,154	148,148	G
P	1036	G	114,116	155,155	174,174	216,216	136,160	148,154	H
P	1037	G	112,114	155,155	170,174	216,218	148,152	148,148	G
P	1038	G	110,116	155,155	172,184	216,216	146,148	148,148	G
P	1039	G	116,118	155,155	170,172	214,218	146,154	166,168	H
P	1040	G	114,116	155,155	168,184	216,216	146,148	148,148	G
P	1041	G	114,116	155,155	168,182	216,216	146,154	148,148	G
P	1042	G	110,116	155,155	168,176	218,218	148,152	148,148	G
P	1043	G	116,118	155,155	168,174	216,218	138,152	148,156	H
P	1044	G	114,116	155,155	172,176	206,218	148,152	148,148	G

Class	ID	mtDNA	Pvc19	3E3	Pv9	Aa4	Pvc78	Pv11	Gen
P	1045	G	116,120	153,153	174,184	216,218	136,146	148,148	H
P	1046	G	110,116	155,155	170,172	216,216	146,152	148,148	G
P	1047	G	116,116	153,155	174,184	216,216	146,146	148,168	H
P	1048	G	116,116	155,155	174,174	206,216	146,148	148,148	G
P	1049	G	114,116	153,155	170,184	216,216	136,152	148,158	H
P	1050	G	114,114	155,155	168,182	216,216	146,158	148,166	H
P	1051	G	116,116	155,155	176,176	206,216	144,152	148,168	H
P	1052	G	112,116	155,155	172,172	216,216	146,152	148,148	G
P	1053	G	114,116	155,155	172,176	214,216	152,162	148,148	G
P	1054	G	114,116	153,155	170,182	216,216	146,162	148,168	H
P	1055	G	116,116	153,155	170,182	216,216	154,162	148,148	H
P	1056	G	116,116	153,155	172,184	216,216	136,152	148,156	H
P	1057	G	116,118	155,155	170,174	216,218	146,152	148,148	G
P	1058	G	114,116	155,155	172,174	216,218	138,148	148,156	H
P	1059	G	114,118	155,155	168,174	206,216	138,154	148,148	G
P	1060	G	114,116	155,155	168,184	216,216	146,148	148,148	G
P	1061	G	112,116	153,155	168,170	216,216	144,154	148,154	H
P	1062	G	116,118	155,155	170,172	216,218	146,148	148,148	G
P	1063	G	116,118	155,155	170,184	218,218	146,148	148,148	G
P	1064	G	112,114	155,155	168,174	216,216	148,152	148,148	G
P	1065	G	114,118	153,153	172,174	216,216	138,152	148,156	H
P	1066	G	114,118	155,155	172,182	216,218	148,158	148,148	G
P	1067	G	116,116	155,155	168,184	206,216	146,146	148,148	G
P	1068	G	116,116	155,155	174,176	206,216	148,152	148,160	H
P	1069	G	114,116	155,155	168,172	216,216	154,158	148,148	G
P	1070	G	116,116	155,155	172,180	216,216	146,146	148,148	G
P	1071	T	116,120	153,153	176,176	216,216	136,136	148,160	H
P	1072	G	116,118	155,155	168,174	206,216	148,148	148,148	G
P	1073	G	114,116	155,155	168,172	206,218	152,154	148,148	G
P	1074	T	114,116	153,153	168,174	216,216	136,136	158,158	T
P	1075	G	114,114	153,153	168,174	216,216	136,154	162,166	H
P	1076	G	116,116	155,155	168,170	206,218	146,148	148,148	G
P	1077	G	116,118	155,155	172,174	216,218	146,148	148,148	G
P	1078	G	114,114	155,155	168,176	206,206	146,148	148,148	G
P	1079	G	112,114	155,155	168,170	216,218	146,158	148,166	H
P	1080	G	114,116	155,155	168,170	206,216	148,152	148,148	G
P	1081	G	114,116	155,155	168,176	206,218	144,148	148,148	G
P	1082	G	116,116	155,155	172,174	206,218	146,148	148,148	G
P	1083	G	116,118	155,155	170,174	216,216	148,158	148,156	H

Class	ID	mtDNA	Pvc19	3E3	Pv9	Aa4	Pvc78	Pv11	Gen
P	1084	G	116,116	155,155	174,184	206,218	146,152	148,148	G
P	1085	G	116,120	**153,153**	174,182	216,216	**136,136**	**148,164**	**H**
P	1086	G	114,114	155,155	170,176	206,216	152,154	148,148	G
P	1087	G	110,112	155,155	168,172	216,218	144,146	148,148	G
P	1088	G	110,114	155,155	172,174	206,216	148,160	**148,156**	**H**
P	1089	G	114,114	**153,155**	170,184	216,216	152,152	**148,154**	**H**
P	1090	G	114,116	155,155	168,184	214,216	146,162	148,148	G
P	1091	G	116,116	155,155	168,172	216,216	146,152	148,148	G
P	1092	G	114,114	155,155	168,176	206,206	148,152	148,148	G
P	1093	G	110,116	155,155	172,176	206,206	154,162	148,148	G
P	1094	G	114,118	155,155	174,188	206,206	138,148	148,148	G
P	1095	G	110,116	155,155	174,176	216,218	148,154	148,148	G
P	1097	G	114,118	155,155	168,174	216,216	146,148	148,148	G
P	1098	G	114,114	155,155	168,174	216,218	138,146	148,148	G
P	1099	G	116,118	155,155	174,184	216,216	148,148	148,148	G
P	1100	G	114,116	155,155	168,180	206,216	152,152	148,148	G
P	1101	G	110,118	155,155	168,184	206,216	148,154	148,148	G
P	1102	G	114,114	155,155	174,184	216,216	138,152	**148,156**	**H**
P	1103	G	**114,120**	**153,155**	170,174	218,218	**136,150**	**164,168**	**H**
P	1104	G	116,114	155,155	168,172	216,218	146,146	148,148	G
P	1105	G	112,116	155,155	168,176	216,216	148,154	148,148	G
P	1106	G	116,118	155,155	170,172	216,218	146,148	148,148	G
P	1107	G	114,116	155,155	168,168	214,218	146,152	148,148	G
P	1108	T	116,116	153,153	172,178	214,216	136,136	158,164	T
P	1109	T	116,116	153,153	174,176	216,216	136,148	156,158	T
P	1110	G	114,116	155,155	168,184	216,216	146,148	148,148	G
P	1111	T	116,120	153,153	174,180	216,216	142,150	158,164	T
P	1112	G	114,116	155,155	176,184	206,218	**136,148**	**148,162**	**H**
P	1113	G	114,118	155,155	172,174	216,216	146,152	148,148	G
P	1114	T	114,116	153,153	172,174	216,216	136,158	156,158	T
P	1115	T	116,120	153,153	174,176	216,216	136,136	158,160	T
P	1116	T	114,116	153,153	174,182	216,218	136,142	158,164	T
P	1117	T	116,116	153,153	174,174	216,216	142,150	160,164	T
P	1118	T	114,116	153,153	174,176	216,216	136,150	158,160	T
P	1119	T	114,120	153,153	174,180	216,216	136,136	160,160	T
P	1120	T	114,120	153,153	176,180	216,216	136,152	160,164	T
P	1121	T	116,116	153,153	174,182	216,218	136,142	158,160	T
P	1122	T	120,120	153,153	172,182	216,218	136,150	164,164	T

Class	ID	mtDNA	Pvc19	3E3	Pv9	Aa4	Pvc78	Pv11	Gen
P	1123	G	112,112	155,155	168,168	216,216	148,154	148,148	G
P	1124	T	114,116	153,153	170,174	216,216	136,136	154,158	T
P	1125	G	114,114	155,155	176,176	206,216	148,160	148,156	H
P	1126	G	114,116	155,155	168,168	216,218	148,152	148,148	G
P	1127	T	116,116	153,153	170,174	216,216	142,152	158,160	T
P	1170	G	114,114	153,155	174,182	216,216	144,148	160,162	H
P	1171	T	114,116	153,153	176,180	216,216	136,158	158,160	T
P	1172	T	116,116	153,153	174,176	216,216	136,158	160,162	T
P	1173	T	116,116	153,153	170,174	206,214	136,136	156,158	H
P	1174	G	114,116	155,155	170,180	216,216	152,158	148,164	H
P	1176	T	116,116	153,153	174,174	216,216	136,142	160,162	T
P	1177	G	114,114	155,155	168,170	208,216	150,158	148,166	H



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