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Genetic diversity and hybridisation estimates of *Arctocephalus tropicalis* and *A. gazella* from Marion Island

BY

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ABSTRACT

In this study, hypervariable region I (HVRI) of the mitochondrial DNA (mtDNA) control region, and five microsatellite loci were used to assess genetic variability and the extent of hybridization between the two fur seals (*Arctocephalus tropicalis* and *A. gazella*), that occur on Sub-Antarctic Marion Island. Both species were harvested during the 18th and 19th centuries, leading to a reduction in population size and the extinction of *A. gazella* at some localities. Whilst both species have recovered and are increasing in size, it is not clear to what extent sealing has affected genetic variation, although a more pronounced effect would be expected for *A. gazella*, given the more intensive harvesting of this species. The current study confirmed this hypothesis and revealed that *A. gazella* had a nucleotide diversity of 2.9 % whilst for *A. tropicalis* it was 4.2 %, across the HRVI mtDNA region sequenced. For microsatellite DNA, genetic variation in *A. tropicalis* was higher than in *A. gazella* in terms of the total number of alleles detected and the level of heterozygosity (H_E=0.875, H_O=0.845, mean number of alleles=13.6 and H_E=0.799, H_O=0.781, mean number of alleles=13, respectively). Diversity in both species is among the highest recorded in pinnipeds to date, and suggests that sealing did not overly affect the levels of genetic variation in these species.

In terms of population structure, *A. tropicalis* show a high level of population structure, as indicated by the Φ_{ST} of 0.32 between Marion and Gough Island. Furthermore, the *A. tropicalis* haplotype tree comprising individuals from Marion, Iles Crozet, Gough, and Amsterdam islands, recovered three divergent evolutionary lineages with bootstrap values of 86% and 98%, for two of these lineages, indicating strong genetic structure and independent evolution. Shared haplotypes between Marion and other islands confirmed genetic exchanges, whilst the grouping of Marion and Gough Islands together is indicative of regular migration between these two islands. For *A. gazella*, the haplotype tree recovered numerous instances of grouping of individuals from Marion and Bouvetøya Islands confirming the hypothesis Bouvetøya is likely source of immigrants to Marion Island. This was further confirmed by low population differentiation between these two islands (F_{ST}= 0.062 and Φ_{ST} of 0.08).

The level of hybridization between these species was low at Marion Island with only one hybrid being detected among the 134 animals for which mtDNA data were generated, corresponding to 0.75%. The same individual was identified as a hybrid, following microsatellite profiling of 146 animals, corresponding to a hybridization estimate of 0.68 %. This hybrid individual was classified phenotypically as *A. gazella* and genotypically was

shown to have *A. tropicalis* ancestry. This level of hybridization is low compared to the other islands where the two species co-occur. However as the samples used in this study were primarily collected from species-specific sites, this may be an underestimate, and the studies focusing on sites where they are known to occur symaptrically, may yield higher estimates.

LIST OF ABBREVIATIONS

%	percentage
&	and
(ν)	migration rate
*	asterisk sign
<	less than
>	greater than
±	plus/minus
°C	degrees celsius
µ	micro
A	adenine
AIC	Akaike Information Criterion
AMOVA	Analysis of Molecular Variance
APF	Antarctic Polar Front
bp	base pair
C	cytosine
CHROMAS	sequence alignment program
cm	centimetre
DAPSA	DNA and protein sequence analysis
D-loop	mitochondrial displacement loop
DMSO	dimethylsulphoxide
DnaSP	DNA Sequence Polymorphism
DNTP	deoxynucleotide triphosphate
Ds	double-stranded
F_1	first generation hybrid
Fig.	figure
F_{IS}	heterozygote deficit
F_{ST}	measure of differentiation
FSTAT	a computer package for PCs estimating and testing gene diversities and differentiation statistics from codominant genetic markers
Fu and Li	neutrality statistic
G	guanine
GENEPOP	Population Genetic Software for Exact tests and Ecumenicism
H	Haplotype diversity
H_E	expected heterozygosity
H_O	observed heterozygosity
HRV	hypervariable region
HWE	Hardy-Weinberg equilibrium
IAM	infinite allele model
K	number of alleles
K	number of populations
Kb	kilobase
kg	kilogram
km	kilometre
l	litre
LD	Linkage disequilibrium
M	molarity
MCMC	Markov chain Monte Carlo
ME	Minimum evolution

MEGA	Molecular Evolutionary Genetics Analysis
mg	milligram
$MgCl_2$	magnesium chloride
mtDNA	mitochondrial DNA
n	number
n	number of nucleotide sequences
No.	number
PCR	polymerase chain reaction
PI	Parsimony Informative
pM	picomole
POPINFO	population information
p-value	probability
Q value	membership coefficient
r	Harpending's raggedness index
R	ratio
RFLPs	restriction fragment length polymorphisms
Rase	ribonuclease
R_S	allellic richness
S.E.	Standard Error
sec	seconds
Si	silent-variable substitutions
SMM	stepwise mutation model
SSRs	simple sequence repeats
STRs	short tandem repeats
Sv	replacement-variable substitutions
T	thymine
Tajimas'D	population growth or decline statistics
Taq	*Thermus aquaticus*
TCS	a program for estimating gene genealogies within a population (Phylogenetic network estimation using statistical parsimony)
U	units
X_i	frequencies of unique alleles
α	alpha
θ	theta
π	nucleotide diversity
$\mathbf{\Phi}_{ST}$	analogue of Wright's fixation index F_{ST}

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CONFERENCE PRESENTATIONS

The results of this study have been presented as a poster at a national conference:

Maboko, V. J., Bastos, A. D. and Bester, M. N. Diversity and hybridization estimates in Antarctic (*A. gazella*) and subantarctic (*A. tropicalis*) fur seals from Marion Island based on mitochondrial DNA and microsatellites. *The Zoological Society of Southern Africa*, Potchestroom, 8-12 July 2007.

The results of this study have been presented as a poster at an international conference:

Maboko, V. J., Bastos, A. D., Maswime, T. A., Hoelzel, R. A. and Bester, M. N Diversity and hybridization estimates in Antarctic (*A. gazella*) and subantarctic (*A. tropicalis*) fur seals from Marion Island based on mitochondrial DNA and microsatellite analyses. *The 17th Biennial Conference on the Biology of Marine* Mammals, Cape Town, 29 November-3 December 2007.

CHAPTER 1

GENERAL INTRODUCTION

1.1 TAXONOMY

The Order Carnivora consists of eight families, three of which are aquatic members and belong to the suborder Pinnipedia (Jefferson *et al*. 1993). The 34 pinniped species classified within this suborder were assigned to three families, namely the Otariidae (comprising 14 species), and the closely related Phocidae (comprising 19 species), as well as the Odobenidae, which comprises a single living species, the walrus (Jefferson *et al*. 1993). More recently, 16 species were assigned to the Family Otariidae, which comprises two sub-families, namely the Arctocephalinae (fur seals) and Otariinae (sea lions) (Rice 1998). These two sub-families are characterized by dense fur intermixed with stiff guard hairs and external ears (Bonner 1994). Whilst some controversy remains as to whether the Pinnipedia are monophyletic or diphyletic (Arnason *et al*. 2002, 2006; Scheffer 1958), all species share the common requirement of needing to return to a solid substrate to bear young.

The Arctocephalinae comprises the northern fur seal (*Callorhinus ursinus*) (Linnaeus 1758) and the southern fur seals of which there are eight species belonging to the genus *Arctocephalus* (Geoffroy Saint-Hilaire 1826). These species are primarily found in the southern hemisphere (Repenning *et al*. 1971; Rice 1998). The Southern fur seal (*Arctocephalus australis*) gave rise to a number of closely related species (Berta and Sumich 1999), of which one, the Antarctic fur seal (*Arctocephalus gazella*) is the only eared seal that lives in polar waters (Repenning *et al*. 1971). A*rctocephalus gazella*, was initially called the Kerguelen fur seal as a female individual was collected on Iles Kerguelen in 1874 (Peters 1875), but was later named the Antarctic fur seal when it was found that they were widely distributed around Antarctica (King 1983). Some authors considered *A. tropicalis* (Gray 1872) and *A. gazella* to be the same species (Sivertsen 1954; Scheffer 1958), referring to both as *A. gazella*. However, others (King 1959a, b) considered them to be subspecies of *A. tropicalis* based on their cranial and dental characters. Under this classification scheme, *Arctocephalus tropicalis tropicalis* was denoted as the northern subspecies and *A. tropicalis gazella* the southern subspecies. Repenning *et al*. (1971) elevated the two taxa to full species status, recognizing *A. tropicalis* (Gray 1872) as the Amsterdam fur seal occurring north of

the Antarctic Convergence and *A. gazella* (Peters 1875) as the Kerguelen fur seal occurring south of the convergence. Now *A. gazella* is termed the Antarctic fur seal as it is distributed in Antarctic waters whilst *A. tropicalis* is referred to as the subantarctic fur seal as it is found on islands just north of the Antarctic convergence (King 1983).

1.2 DISTRIBUTION

More than 99% of the World's population of *A. gazella* breed on islands predominantly to the south of the Antarctic Polar Front (APF), as far north as Iles Crozet but its range has recently extended as far east as Macquarie Island and also westwards to Heard Island and eastwards to the vicinity of the Antarctic Peninsula (Hofmeyr *et al*. 1997) (Fig.1.1). *A. tropicalis* breed on islands to the north of the APF with large numbers occurring at Gough, Amsterdam and the Prince Edward Islands (Hofmeyr *et al.* 1997) (Fig. 1.1).

There are three islands close to the APF (Fig. 1.1) at which *A. gazella* and *A. tropicalis* occur sympatrically, namely Marion Island (which is part of Prince Edward Island Archipelago), Iles Crozet and Macquarie Island (Kerley 1984; Goldsworthy *et al*. 1997; Wynen *et al*. 2000). Condy (1978) speculated that hybridization was occurring between these two species on Marion Island based on the presence of adult males with external characteristics of both species and observations of breeding harems containing both *A. tropicalis* and *A. gazella.* As *A. gazella* pups appeared to be born and weaned earlier than *A. tropicalis* pups, Condy (1978) also suggested that temporal differences and other ecological and genetic processes were apparently limiting the hybridization between the two fur seal species.

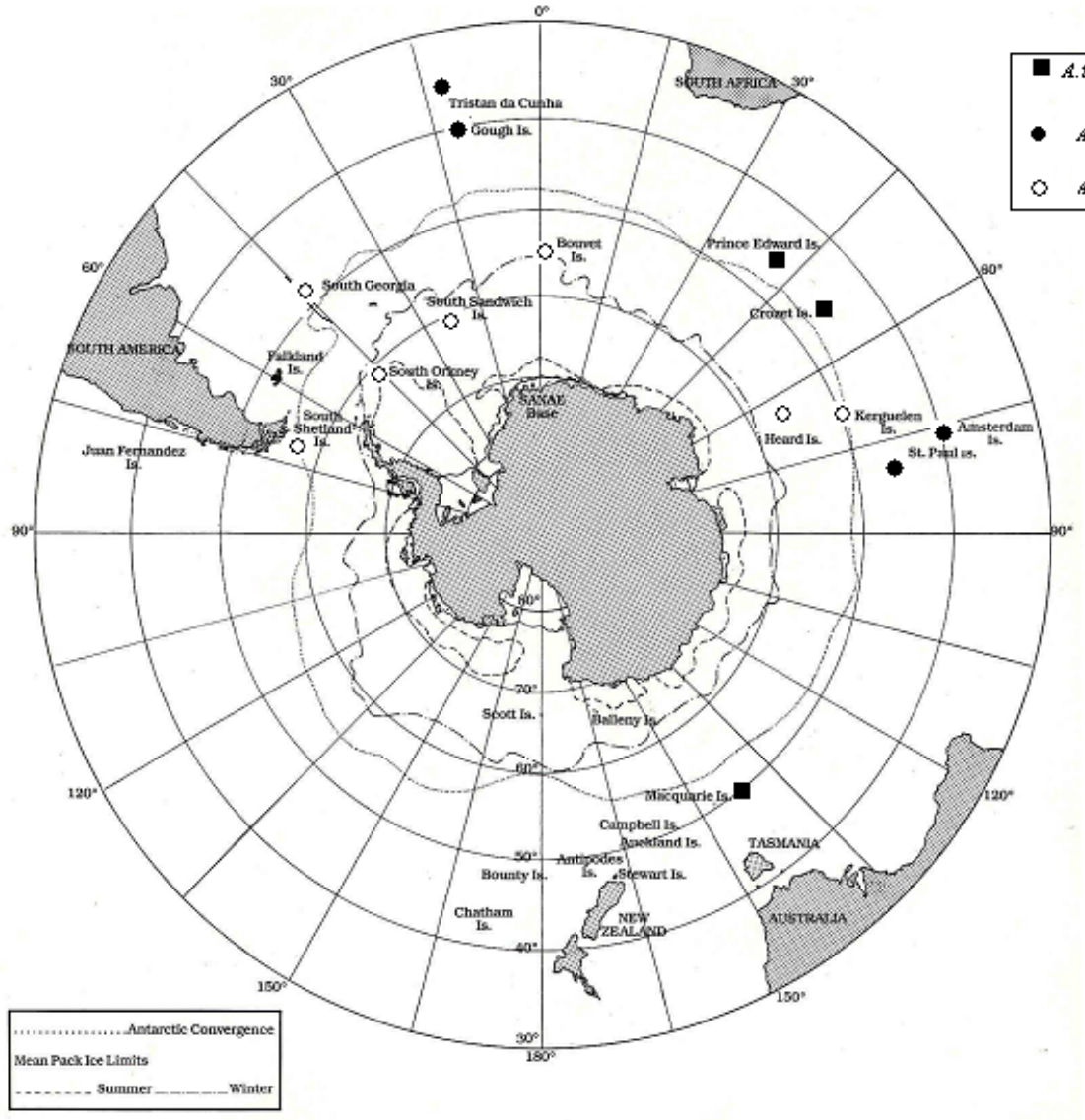


Fig. 1.1: Map indicating those islands where fur seals (*A. tropicalis* and *A. gazella*) breed exclusively and sympatrically (Map adapted from original by John Cox, Australian Antarctic Division). *Arctocephalus tropicalis* breed on the Prince Edward Islands, Gough, Amsterdam, St Paul, Macquarie and Tristan da Cunha islands and Iles Crozet, whilst *A. gazella* breed on the Prince Edward Islands, South Georgia, South Sandwich, Bouvetøya, South Shetland, Heard, and Macquarie islands, and Iles Kerguelen and Iles Crozet. These species occur sympatrically on the Prince Edward Islands, Iles Crozet, and Macquarie Island.

Breeding aggregations are mostly allopatric at Marion Island and that makes it difficult to assess the level of hybridization (Condy 1978; Wilkinson and Bester 1990). Condy (1978) also reported that despite the temporal isolating mechanism, successful interspecific matings do occur and hybrid pups are produced occasionally. The hybrid pups are however difficult to distinguish from pure-bred pups of either parental species, since they are all black in color and approximately the same size (Bester and Wilkinson 1989). However, adult hybrid bulls are most easily identified because they occupy conspicuous positions within the breeding colony and possess a clearly intermediate appearance between the two species. The guttural challenges produced by these hybrid bulls are typical of either parental species (St. Clair Hill *et al.* 2001; Page *et al.* 2001).

Hybridisation is predicted to occur at low levels on Marion Island as there is limited opportunity for heterospecific mating due to the low degree of sympatry at individual sites. Hybrid adult males have however been seen at several beaches such as Cape Davis, Good Hope Bay, and Sealers' Cave from May 2003 to May 2004 (Fig. 1.2), and four putative hybrid pups were observed at Sealers' Beaches (Cape Davis), Sickle Cove and Sealers' Cave. However, their classification was uncertain (Hofmeyr *et al*. 2006).

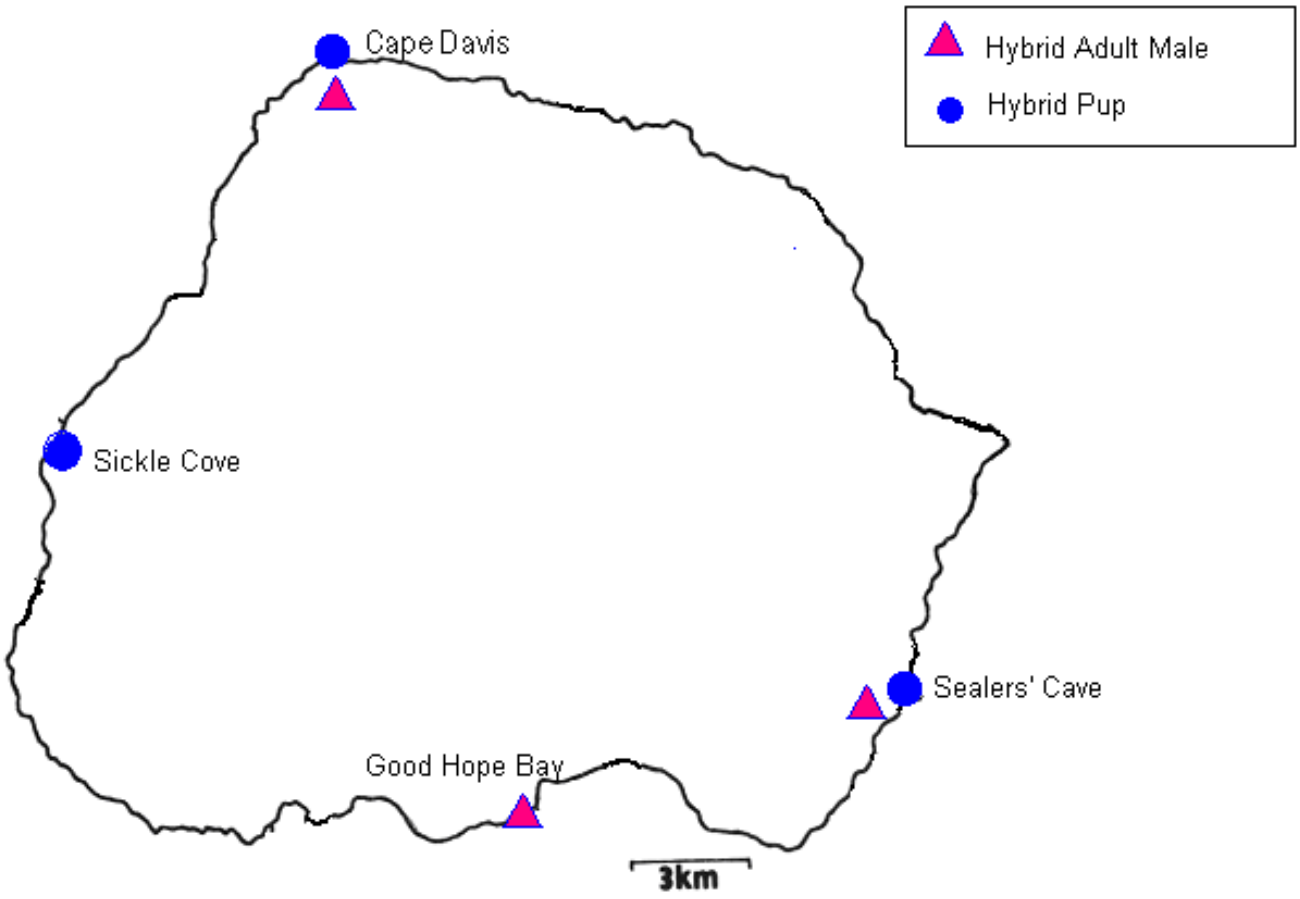


Fig. 1.2: Map showing beaches at Marion Island (46°54′S, 37°45′E) where hybrid adult males and hybrid pups have been observed (Hofmeyr *et al*. 2006). Beaches where hybrid adult males have been sighted include: Good Hope Bay, Cape Davis, Sealers' Cave and are denoted by a triangle, whilst hybrid pups have been sighted at Cape Davis, Sickle Cove and Sealers' Cave (indicated by a circle).

1.3 IMPACT OF SEALING

Many natural populations have experienced well-documented human-induced declines. Marine mammals, including pinnipeds, have suffered particularly extreme reductions in population size from the impacts of over-hunting in previous centuries (Bonnell and Selander 1974; Busch 1985; Hoelzel *et al*. 1993). *Arctocephalus gazella* and *A. tropicalis* experienced substantial declines in the number and size of their populations throughout the Southern Ocean due to commercial sealing during 18th and 19th centuries (Bonner and Laws 1964), to the extent that both species experienced local extinction at some sites (Wynen *et al*. 2000).

As major populations of *A. tropicalis* occurred on islands north of the APF, which did not support large populations of elephant seals, sealers probably only visited them when fur seal numbers were large enough to ensure an economic return (Shaughnessy 1982; Roux 1987). Fur sealing was most intense at islands that were associated with large populations of intensively harvested marine mammals such as the southern elephant seal *Mirounga leonina* (Linnaeus 1758) and southern right whale *Eubalaena australis* (Desmoulins 1822) (Rand 1956, Bonner and Laws 1964). *Arctocephalus gazella* was more severely exploited than *A. tropicalis* and became locally extinct on islands south or just north of the APF that had large populations of elephant seals. Sealing at Marion Island continued sporadically until 1931 (Marsh 1948).

Arctocephalus tropicalis survived this exploitation and was believed to be the only fur seal species occurring at Marion Island until one of the skulls collected in 1951 was found to be that of *A. gazella* (Rand 1956). The recovery of this skull suggests that *A. gazella* might have been present at Marion Island prior to sealing and that the intensive harvesting of seals led to the localized extinction of this species. Alternatively, it may have been a recent migrant from elsewhere, most probably from the Scotia Arc islands (Wynen *et al*. 2000; De Bruyn *et al*. 2007) or even Bouvetoya (Hofmeyr *et al*. 2005).

Molecular genetic markers can be used to test the predictions of population genetic theory in both laboratory studies and natural populations (Hoelzel *et al*. 1993; Leberg 1992; Tarr *et al.* 1998). Population genetic theory makes clear quantitative predictions in genetic variability expected from population declines or bottlenecks (Nei *et al.* 1975). Populations that are reduced to small size and subsequently experience rapid growth lose relatively less variation

than populations that do not recover rapidly (Maruyama and Fuerst 1985). The fur seal population at Marion Island has rapidly increased in size since 1989 and *A. tropicalis* may have reached carrying capacity (Wilkinson and Bester 1990; Hofmeyr *et al.* 1997, 2006). The severity and duration of the Marion Island population bottlenecks are not known. However, from the documented recolonization of islands across their former range and increasing population size, both species appear to be recovering well (Wynen *et al*. 2000) across their distributional range which includes the Prince Edward Islands (Bester *et al*. 2003; Hofmeyr *et al*. 2006).

Molecular data suggests that the females of *A. tropicalis* from Marion Island, and to a lesser extent Ile Amsterdam, probably recolonized Macquarie Island and Iles Crozet (Wynen *et al*. 2000). In contrast, the recent establishment of *A. gazella* on Marion Island is believed to have occurred owing to migrants from other islands (Hofmeyr *et al*. 2006; De Bruyn *et al*. 2007). Two geographical regions are considered to be source populations, namely a western region consisting of populations from South Georgia and Bouvetøya, which also serve as source populations for the South Shetlands and Heard Island, and an eastern region containing the populations of Iles Kerguelen and Macquarie Island (Wynen *et al*. 2000).

During 2003/4, a census of *A. tropicalis* and *A. gazella* populations at Marion Island an estimated 150 000 and 5 800 individuals of each species, respectively were found to be present (Hofmeyr *et al.* 2006). This translates into an *A. tropicalis* population growth rate of 5.3 % over the past 15 years, and a mean annual rate of 14.8 % over the past 21 years for *A. gazella* (Hofmeyr *et al.* 2006). At this stage, *A. gazella* is believed to be in the rapid recolonization phase of population growth, whilst *A. tropicalis* seems to be approaching carrying capacity (Hofmeyr *et al*. 2006).

1.4 REPRODUCTIVE BIOLOGY

1.4.1 Breeding Strategies

Otariids (eared seals) are highly polygynous (Bartholomew 1970). They breed at high densities at traditional sites where females come ashore to give birth and mate. Mating systems differ between species and populations, but males commonly defend territories at rookeries where females give birth (King 1983, Gentry 1997). In polygynous mating systems, a handful of dominant males monopolize matings, which leads to many males being excluded from the breeding population. In otariids that exhibit resource defence polygyny, females are relatively sedentary between the time of parturition and oestrus (Boness 1991). Traditional models of pinniped breeding behaviour have emphasised terrestrial mating. Pinnipeds have diverse mating systems, and they are classified based on breeding habitat (Riedman 1990). Some breed on land, some on pack ice and some on ice attached to land (fast ice). Fur seals are among the seals that breed on land. Breeding territories have a strong consequence in determining the level of pinniped polygyny because a reliable breeding environment promotes female gregariousness (Bartholomew 1970).

Fur seals are highly polygynous and sexually dimorphic with males being up to four times heavier than females (Riedman 1990). Typically, dominant bulls vigorously defend discrete territories in breeding rookeries within which harems of up to 10 female seals give birth to and rear their pups (Bonner 1968; McCann 1980; Arnould and Duck 1997). Fighting is common between territorial males and holding a territory is costly. For example, a male can lose up to 50 % of his body weight during the 60 days of territory tenure (McCann 1980). In areas where *A. tropicalis* and *A. gazella* co-occur, breeding harems contain females of both species and thus present the perfect opportunity for interspecific matings to occur (Condy 1978; Kerley 1983b).

Both species show similar hauling out patterns with summer (breeding) and autumn (moulting) peaks in numbers ashore, corresponding to December and March, respectively (Bester 1981, Kerley 1983c). However, the seasonal haulout patterns of the two species differ slightly. *Arctocephalus gazella* females give birth approximately ten days earlier than *A. tropicalis* females at Marion Island and their median birth dates (calculated indirectly using a simplified probit analysis) were 6 and 17 December respectively (Kerley 1983a), with little

variation between years (Hofmeyr *et al*. 2006). Because the breeding periods of the two species overlap, the differences in seasonal breeding cycles are not adequate to avoid hybridization (Kerley 1983a). Adult males of both species are ashore while females of both species are in oestrus.

1.4.2 Maternal Strategies

Attendance behaviour is measured as activities such as number and duration of visits to shore from birth until weaning, number and length of trips to sea, changes in trips as a function of age and suckling period frequency while ashore (Gentry and Kooyman 1986). The pattern reveals food availability, environmental fluctuations and differences in conditions at populations and sites (McCann 1987). These activities end when the pups are weaned, which usually occurs at around 4 months in *A. gazella* and 10 months in *A. tropicalis* (Condy 1978; Kerley 1984). *Arctocephalus gazella* pups grow faster than *A. tropicalis* pups (Kerley 1985). Generally, fur seal mothers have longer foraging trips when the foraging conditions are poor and that results in slow pup growth (Trillmich *et al*. 1991; Boyd *et al*. 1997; McCafferty *et al*. 1998). *Arctocephalus gazella* females visit the shore for a short period to feed the pup before returning to the sea as soon as their milk is depleted because their pups' demand is high as they grow very fast. In contrast, *A. tropicalis* spend a longer time onshore owing to the lesser demand by pups, which grow more slowly (Bester and Bartlett 1990). The maternal adaptations of both *A. gazella* and *A. tropicalis* at mid-latitude Marion Island (Kerley 1985) agree with those of their conspecifics at the major population centres in higher (South Georgia - 62°S) and lower (Gough Island - 40°S) latitudes (Bester 1981, Gentry and Kooyman 1986) respectively, and appear genetically fixed.

1.5 GENETIC STUDIES ON FUR SEALS

Of the various possible genetic problems that face a declining population, loss of genetic variability and inbreeding depression have, historically received most attention (O'Brien, 1994). Wynen *et al*. (2000) conducted a molecular study on fur seals in the Southern Ocean including Marion Island and reported that there is lower genetic variation and population structure in *A. gazella* compared with *A. tropicalis*. The nucleotide diversity of *A. tropicalis*

was found to be 4.8 % and that of *A. gazella* was found to be 3.2 %, based on data generated for 20 individuals of each species from Marion Island (Wynen *et al*. 2000). Hybridisation between the species was not assessed.

Natural hybridization occurs rarely in mammals (Gray 1971), although it can be widespread in other taxonomic groups (Hubbs 1955; Grant and Grant 1992; Arnold 1997). Hybridization events in mammalian species have almost always involved disturbed habitats where one population is in decline (Carr *et al*. 1986, Lehman *et al*. 1991). In a study by Lancaster *et al*. (2006) on fur seals, mtDNA control region data (RFLP) and 10 microsatellite loci revealed that hybridization takes place at Macquarie Island, where three species of fur seals, *A. tropicalis*, *A. gazella* and *A. forsteri*, the New Zealand fur seal (Lesson 1828), occur sympatrically. This study detected high levels of hybridization (17-30 %) involving all three species, although the New Zealand fur seals were not expected to be part of this hybridisation due to absence of females and males not being in control of the territories during the breeding season. Four categories of hybrids were detected, namely: Antarctic-subantarctic (A-S), Antarctic-New Zealand (A-NZ), subantarctic-New Zealand (S-NZ) and Antarctic-subantarctic-New Zealand (A-S-NZ). There seems to be reproductive success, as over 50 % of New Zealand hybrids and 43 % of Antarctic-subantarctic hybrids were not F_1. Lancaster *et al.* (2007) also revealed the lower reproductive success of hybrid males and the fitness costs of hybridization. In this study, RFLP profiles of the mitochondrial control region and a panel of nine microsatellites revealed no relationship between male reproductive success and the three genetic measures of outbreeding. Hybrid males were found to have lower reproductive success than pure species males and this was suggested as being a disadvantage of hybridization.

In another study on hybridization between *A. tropicalis* and *A. gazella* at Iles Crozet, mtDNA and microsatellites markers were compared for the identification of hybrids (Kingston and Gwilliam 2007). Hybridization was low with hybrid genotypes comprising just 1 % of the population, whilst 2.4 % were backcrosses to one of the parental species. With respect to hybridization estimates between fur seal species at Marion Island, no genetic estimates are available. However, in a study done on skull morphometrics of male *A. gazella* and male *A. tropicalis* and their interspecific hybrids (Kerley and Robinson 1987), hybrids were found to be intermediate between the two parental species in their cranial morphology, although the cranial morphometrics of some hybrids tended towards one of the parent species.

1.6 POPULATION GENETIC STUDIES

Recent studies investigating population structure and genetic diversity in natural populations have relied heavily on molecular techniques such as DNA sequencing, allozymes and restriction fragment length polymorphisms (RFLPs). The number of studies employing both nuclear and mitochondrial DNA (mtDNA) markers has, however, increased in recent years (Karl *et al*. 1992, Degnan 1993; Abernathy 1994, Gottelli *et al*. 1994; Palumbi and Baker 1994; Pope *et al*. 1996; Berubé *et al*. 1998; Simonsen *et al*. 1998) as more detailed information can be obtained by simultaneously evaluating both genome targets.

Microsatellite markers are useful in population genetic studies of pinniped species, especially in conjunction with mtDNA, but wide ranging surveys of broadly distributed species are limited. The desirable properties that favour microsatellites in population studies include a Mendelian codominant mode of inheritance, high polymorphism, ease of use and scoring, selective neutrality, and a high mutation rate (10^{-4} to 10^{-5})**.** Furthermore, the analysis of nuclear markers such as microsatellites provides both parental and maternal information (Hughes and Queller 1993). Owing to maternal inheritance and the relatively fast evolutionary rate of its 37 genes, mitochondrial DNA (mtDNA) has been used to provide insights into population genetic structure, gene flow, hybridization, biogeography, and phylogenetic relationships of various animals (Avise *et al.* 1986; Moritz *et al.* 1987; Sang *et al.* 1994; Taylor and Dodson 1994; Jean *et al.* 1995).

1.7 STUDY AREA

Marion Island (46°54′S, 37°45′E) is located within the Prince Edward Island group approximately 2300 kilometers south east of Cape Town, South Africa. Marion Island and Prince Edward Island together form the Prince Edward archipelago. These islands are situated in the Southern Indian Ocean, where they were formed some 1.2 million years ago in a series of volcanic events (Chown and Hänel 1998). They lie 950 km to the west of the closest landmass, Iles Crozet. Marion Island is 290 km^2 in area, roughly oval in shape with a maximum altitude of 1230m (Jan Smuts [Mascarin] peak). It measures 24 km from west to east and 17 km from north to south, and has a circumference of roughly 90 km (Wilkinson

1992). Most of the island's coastline is irregular in shape due to the Island's volcanic origin and comprises cliffs as high as 15m. These irregularities have been smoothed over time resulting in open bays with stony beaches along the base of the cliffs (Wilkinson 1992), providing suitable haul-out sites for *A. gazella* and *A. tropicalis*.

1.8 AIMS AND OBJECTIVES

Whilst studies on hybridization in fur seals have been conducted at other study sites such as Macquarie Island (Goldsworthy *et al*. 1999; Lancaster *et al.* 2006, 2007) and Iles Crozet (Kingston and Gwilliam 2007), the level of hybridization between Marion Island's fur seals has not been genetically assessed. Analyses of both mtDNA and nuclear markers such as microsatellites will be used in the current study to estimate hybridization and to assess population structure. The mtDNA information will most likely underestimate the level of hybridization because of its strictly maternal inheritance, which essentially means that hybrids will only be detected when a conflict between phenotype and this maternal marker is observed. Microsatellites should however provide a better hybridization estimate as they are bi-parentally inherited.

The aims of this study are the following:

- To obtain more accurate baseline estimates of the level of genetic diversity in *A. tropicalis* and *A. gazella* populations at Marion Island through the genetic characterization of numerous individuals with two molecular markers.
- To assess the level of hybridization between the two species using both mtDNA and microsatellite data.

CHAPTER 2
MITOCHONDRIAL DNA SEQUENCE ANALYSIS

2.1 INTRODUCTION

2.1.1 mtDNA

Mitochondrial DNA (mtDNA) is known as the female molecular analogue of male surname transmission. Little to no detectable paternal contribution of mtDNA occurs and as a result, paternal contribution of mitochondria to the offspring is estimated to be 1-5 % of the total DNA, as it is generally not detected (Avise and Lansman 1983). The maternal inheritance of mitochondria in animals and plants is through the egg cytoplasm, as the sperm cell usually does not have sufficient cytoplasmic space for organelles. With this maternal form of inheritance, mtDNA is inherited as a single non-recombining locus (at least in higher animals), and in mammals evolves about tenfold faster than nuclear DNA. This results in mtDNA being an extremely valuable study tool in molecular population genetics and systematics (Brown *et al*. 1982).

The mitochondrial genome has a small, non-coding region called the control region, which constitutes around 7 % of the total genome. Because of its high mutation rate, it has become a popular marker for population studies, as this region is sufficiently variable that relatively short sequences can resolve differences between closely related taxa. The D-loop region which is situated within the control region has a number of peculiar features in mammals, including: strong-rate heterogeneity among sites, the presence of tandem repeated elements, a high frequency of insertion/deletion events in some tracts, and lineage specificity (Saccone *et al.* 1991; Sbisa *et al.* 1997; Pesole *et al.* 1999).

The mtDNA genome includes other more slowly evolving regions that are useful for higher-level phylogenetic studies, while the more rapidly evolving regions, such as D-loop are useful for population level studies (Stevens *et al*. 1989, Baker *et al*. 1993, Arnason and Gullberg 1996). As this genome in most animals is inherited maternally, discrepancies in some phylogenies have been noticed when using both mtDNA and nuclear DNA to make inferences. This bias usually occurs in animals that show sex-

biased dispersal patterns, such as turtles (Sauropsida) (Bowen *et al*. 1992), humpback whales (*Megaptera novaeangliae*) (Borowski 1781) (Palumbi and Baker 1994) and white-eyed birds (genus Zosteropidae) (Degnan 1993).

Mitochondrial DNA (mtDNA) studies have been useful in documenting introgression in a number of mammalian species in the wild, confirming the occurrence of past hybridization events (Ferris *et al*. 1983, Carr *et al*. 1986, Tegelström 1987, Lehman *et al*. 1991). However, Pamella and co-workers (2004) commented that documentation of mammalian hybrids occurs infrequently because of the challenges inherent in locating and accurately identifying them. The use of mtDNA analysis in hybridization studies is useful for determining the occurrence of assortative mating or sex-specific viability differences (Avise 1994).

The mtDNA genome appears to be prone to introgression and molecular leakage (Ballard and Whitlock 2004). The mtDNA molecule of one species can totally replace the mtDNA of another species, in some populations without any discernible nuclear introgression, e.g. mtDNA in a population of brook trout in Lake Alain in Québec is identical to the Québec Arctic genotype, yet the brook trout are morphologically indistinguishable from normal brook trout and have diagnostic brook trout alleles at nuclear loci (Bernatchez *et al*. 1995).

2.1.2 Pinniped studies making use of the mtDNA control region

A study on post-sealing genetic variation of *A. tropicalis* and *A. gazella* was carried out on Southern Ocean island populations using the mtDNA control region. At Marion Island, the level of diversity for 20 *A. tropicalis* was found to be 4,5% whilst for 20 *A. gazella* it was 3,4%. For 20 *A. tropicalis* from Iles Crozet, diversity was 4,4% and 2,9% for the same number of *A. gazella* individuals, whilst at Macquarie Island the diversity was 4,6% for 17 *A. tropicalis* and 2,1% for 20 *A. gazella* (Wynen *et al*. 2000).

In the current study, the mtDNA marker will be used to determine the level of genetic diversity of the two fur seal species, *A. tropicalis* and *A. gazella* at Marion Island, which experienced a population bottleneck due to the intensive sealing that took place

during the 18th and 19th centuries. As these two species occur sympatrically on some beaches at Marion Island, and as hybrids have been recorded here previously (Condy 1978, Kerley 1984, Hofmeyr *et al*. 2006) and elsewhere (Goldsworthy 1999), the mtDNA sequences generated in this study will also provide a first molecular estimate of the level of hybridization that occurs between these species at the Prince Edward Islands.

2.2 MATERIALS AND METHODS

2.2.1 Sample Collection

From April to August of 2003, tissue samples were collected for mtDNA and microsatellite DNA analyses from *A. tropicalis* and *A. gazella* pups on Marion Island. A total of 76 *A. tropicalis* individuals were sampled at seven sites, whilst for *A. gazella*, 58 individuals were sampled at four beaches on Marion Island (Fig. 2.1). Species identification of pups was based on phenotypic appearance (Bester & Wilkinson 1989) and on breeding colony beach type, as these species display marked differences in habitat types utilised (Condy 1978). All animals were sexed in the field. Pups were caught and restrained by hand (Erickson & Bester 1993) and two standard biopsy techniques were used for taking biopsies either from the trailing edge of the left foreflipper (Shaughnessy *et al*. 1993, Wynen *et al*. 2000) or by slicing off the tip of a fleshy extension of a left hindflipper. Sampling equipment was washed with ethanol between each biopsy. Pups were immediately released after taking the skin biopsy and the biopsies were then stored in salt-saturated 20% dimethylsulphoxide (DMSO) solution at room temperature (Amos and Hoelzel 1991, Wynen *et al*. 2000). The salt has preserving properties (denatures proteins) while DMSO enhances cell membrane permeability.

2.2.2 Mitochondrial control region extraction, amplification and sequencing

DNA was isolated from approximately 25 mg of tissue using the Roche High Pure PCR Template Preparation kit and the extraction protocol prescribed by the supplier for mammalian tissue. A fragment of approximately 460 base pairs (bp) of the mitochondrial displacement loop (D-loop) region was amplified by polymerase chain reaction (PCR) amplification, with universal primers L15925 (5'-TACACTGGTCTTGTAAACC-3') and H16499 (5'-CCTGAAGTAGGAACCAGAT-3'), modified from those reported by Kocher and co-workers (1989). PCR reactions were set up in a laminar flow hood to minimize the risk of contamination, and a negative control, comprising all components except DNA, was always included to control for reagent contamination.

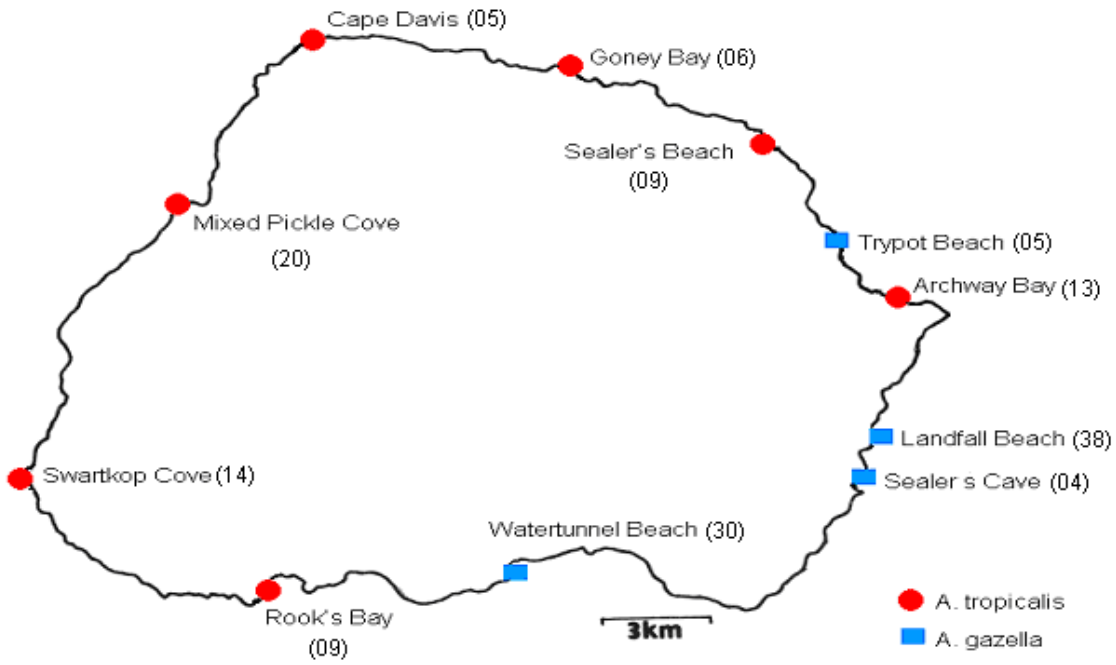


Fig. 2.1: Map showing sampling localities at Marion Island (46°54′S, 37°45′E). Sampling localities for *A. tropicalis* are Archway Bay, Sealer's Beach, Mixed Pickle Cove, Cape Davis, Goney Bay, Swartkop Cove and Rook's Bay. For *A. gazella* samples were collected from Watertunnel Beach, Trypot Beach, Landfall Beach and Sealer's Cave. The number of individuals sampled at each beach is denoted in brackets at each sampling site.

Genomic amplifications were performed in a final volume of 50 µl containing 1X buffer, 0.2 µM dNTP, 0.4 µM of each primer, 1U of *Taq* polymerase (BioTools) and approximately 100ng of genomic DNA. Thermal cycling was performed using a Perkin-Elmer thermocycler (model 2720) with all denaturation steps being performed at 96°C for 10 sec, and all extension steps at 72°C for 1 min. Primer annealing followed a 'touch-down' approach with the first two cycles being performed at 50°C for 35 s; followed by 13 cycles at 49°C for 30 s, with the remaining 25 cycles being performed at 48°C for 30 s. A final extension step of 72°C for 1 min was performed at the end of the 40 cycles comprising a denaturation, primer-annealing and extension step. The amplification products were evaluated by 1.5 % agarose gel electrophoresis against a 100 bp molecular weight marker (Promega). Products of the expected ~500bp size were purified directly from the tube using the Roche PCR product purification kit, as prescribed by the supplier. Following removal of unincorporated primers and dNTPs, cycle sequencing of purified amplicons, with each of the PCR amplification primers was performed using the ABI PRISM Big dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, 1995), in separate reactions, at an annealing temperature of 48°C in a final reaction volume of 10 µl. All reactions were precipitated by sodium acetate-mediated ethanol precipitation of double-stranded DNA (dsDNA). Amplified fragments were separated on an ABI 3100 Genetic Analyser (Applied Biosystems).

2.2.3 Control Region Sequence Analysis

The CHROMAS program (version 1.43, McCarthy 1997) was used to view and edit sequences, prior to being exported as text files to an alignment programme. Sense and antisense strand sequences were aligned to one another in DAPSA (version 4.91, Harley 2001) in order to verify the sequences of both strands and to generate a contiguous full-length sequence. A nucleotide similarity search was performed for each sequence finalized in this manner by blasting it against all available sequences within the Genbank database (www.ncbi.nlm.nih.gov/blast). This was done to verify gene and species identity and to obtain homologous sequences for phylogenetic analyses purposes. The sequences generated for *Arctocephalus* individuals from Marion Island were aligned with the Clustal programme included in MEGA version 3.1 (Kumar *et al*. 2004).

2.2.3.1 Gene Trees for each Arctocephalus species

Two different datasets were compiled for each species. For *A. tropicalis*, a Marion Island-only dataset (N=96) that contained all 76 *A. tropicalis* sequences generated *de novo* in this study plus the 20 reported previously (Wynen *et al*. 2000) was compiled. In addition, a combined all-island dataset that included the 96 Marion Island sequences as well as 20 sequences from the Gough islands (Wynen *et al*. 2000) and five additional sequences from Ile Amsterdam and Iles Crozet (Wynen *et al*. 2001) , that are available on Genbank, were subsequently added bringing the sample size of the *A. tropicalis* dataset to 121 individuals. Similarly, for *A. gazella*, a Marion-only dataset (N=78) was compiled with sequences generated in this study alone (N=58) and 20 generated previously (Wynen *et al*. 2000). The second, all-island *A. gazella* dataset, comprised the 78 Marion Island sequences, 20 Bouvetøya sequences that were included in the Wynen *et al*. (2000) study and a further 5 (AF384376-80) from Bouvetøya (Wynen *et al*. 2001), bringing the sample size of the latter dataset to 103 individuals. Minimum evolution (ME) trees were constructed using each of the Marion Island-only and combined all-island datasets detailed previously, in MEGA version 3.1 (Kumar *et al*. 2001). Nodal support was assessed by 10 000 bootstrap replications, whilst sensitivity of each dataset to model assumptions was evaluated by comparing the tree obtained with uncorrected p-distances, with that inferred using the parameter-rich model in MEGA that most closely matched the best-fit model selected under the Akaike Information Criterion (AIC) in ModelTest version 3.06 (Posada and Crandall 1998).

2.2.3.2 Haplotype trees

The complete dataset p-distance trees were used to identify unique haplotypes and aided in removing identical sequences so that haplotype trees could be constructed. To this end, three datasets were compiled, namely (i) an *A. gazella* haplotype dataset (ii) an *A. tropicalis* haplotype dataset, and (iii) a combined *A. gazella* and *A. tropicalis* haplotype dataset. ModelTest version 3.06 (Posada and Crandall 1998) was again used to identify the model of sequence evolution that best fitted each of the three haplotype datasets, and to guide model selection in MEGA version 3.1.

2.2.3.3 Population comparisons

Determination of population statistics was performed using the DNA Sequence Polymorphism (DnaSP) version 3.5.1 programme (Rozas and Rozas 1999), and included estimations of (i) nucleotide diversity, (ii) gene (haplotype) diversity and other parameters such as (iii) Tajimas' D statistic (Tajima 1989), which evaluates the possibility that the population tested has undergone a bottleneck. TCS (Clement *et al*. 2000) was used to determine the number of haplotypes and also to confirm the haplotypes revealed by the complete dataset p-distance trees. In order to assess within-species population structure, analysis of molecular variance (AMOVA; Excoffier *et al*. 1992) was performed in Arlequin version 2.000 (Schneider *et al*. 2000). F_{ST} was calculated from haplotype frequencies in order to compare populations (Wright 1951) and overall Φ_{ST} was calculated based on sequence data and sequence haplotype frequencies using the Tamura-Nei model with a gamma distribution of 0.48. (Weir and Cockerham 1984, Weir 1990). A statistical test, Harpending's Raggedness index (*r*) (Harpending *et al*. 1993) for detecting population growth based on mismatch distribution was calculated using Arlequin (Schneider *et al*. 2000) and graphs were plotted in Excel.

2.3 RESULTS

2.3.1 Intra-specific statistics

2.3.1.1 A. tropicalis

Due to the short sequence length of available mtDNA control region sequences in the Genbank database, the homologous Marion-only dataset comprising 96 *A. tropicalis* individuals (the 76 generated in this study and the 20 generated previously by Wynen *et al.* 2000) from Marion Island was reduced to just 312 nucleotides (nt). For the Gough-Marion dataset which comprised 116 individuals (96 from Marion Island and 20 from Gough Island), 26 unique haplotypes were recovered (Table 2.2 and Fig. 2.7). Alignment of haplotype sequences revealed the presence of 258 conserved sites, 44 variable sites (Fig. 2.2), 36 parsimony informative sites and 8 singletons (Table 2.1). For the Marion Island only dataset comprising 96 individuals, 17 unique haplotypes were recovered and alignment of these sequences revealed the presence of 262 conserved sites, 40 variable sites, 33 parsimony informative sites and 7 singletons (Table 2.1). Gene (haplotype) diversity for Marion and Gough populations combined

was 0.869 and for the Marion Island only population it was 0.821. Nucleotide diversity (π) which provides a measure of genetic variation within the control region characterized in this study was 0.046 (4.6 %) for the combined dataset comprising Marion and Gough individuals, whilst for individuals exclusively from Marion Island it was 0.042 (4.2 %). The Tajimas'D test for the combined Marion and Gough populations was positive, with a value of 1.960, but was not significant (0.10>p>0.05). For the Marion Island only dataset, a positive value of 1.926 was obtained (0.10>p>0.05) which was not significant. For the Marion Island population, Harpending's Raggedness (r) based on mismatch distribution had a value of 0.053. The observed pairwise and expected model differed significantly (p= 0.000) (Fig. 2.9a). Similarly, for the Gough Island population, Harpending's Raggedness (r) = 0.126, the observed pairwise and expected model differed significantly (p= 0.020) (Fig. 2.9b). F_{ST} (Wright 1951) indicating the degree of differentiation between Marion and Gough populations was 0.168. An overall Φ_{ST} value of 0.320 was obtained for the *A. tropicalis* combined dataset (Marion and Gough islands), whilst for Marion Island alone it was 0.319.

2.3.1.2 A. gazella

A homologous 312 bp fragment was obtained for 98 *A. gazella* individuals after combining the 58 sequences generated in this study from Marion Island, with the 40 previously generated for Marion Island and Bouvetøya (Wynen *et al*. 2000). The number of unique haplotypes recovered from this combined dataset was 25 (Table 2.3 and Fig. 2.8). Sequence alignment of all individuals (Marion and Bouvetøya) revealed the presence of 260 conserved sites, 49 variable sites, 39 parsimony informative sites and 10 singletons. For the Marion Island only dataset (N=78) , 19 unique haplotypes were recovered and sequence alignment revealed the presence of 264 conserved sites, 43 variable sites (Fig.2.3), 30 parsimony informative sites and 13 singletons. Haplotype (gene) diversity was 0.827 for Marion and Bouvetøya populations and for the Marion Island population only it was 0.797, whilst nucleotide diversity was 0.033 (3.3%) for the combined (Marion and Bouvetøya populations) dataset, and 0.029 (2.9%) for the Marion Island population (Table 2.1). Tajima's D for the combined Marion and Bouvetøya populations had a positive, non-significant value of 0.085 (p>0.10) whilst for the Marion Island population a negative value of –0.138 (p>0.10) was recovered which was not significant. For the Marion Island population, Harpending's Raggedness index (r) based

on mismatch distribution had a value of 0.011 and the observed pairwise and expected model did not differ significantly (p= 0.290) (Fig. 2.9c). For the Bouvetøya Island population, Harpending's Raggedness index value was 0.055, and the observed pairwise and expected model did not differ significantly (p= 0.060) (Fig. 2.9d). F_{ST} between Marion and Bouvetøya populations was 0.062. An overall Φ_{ST} value for *A. gazella* from both Marion and Bouvetøya islands was 0.079 and for Marion Island only it was 0.082.

2.3.2 Phylogenetic Analyses

2.3.2.1 A. tropicalis

To determine relatedness of individuals from different geographical areas, a Minimum Evolution haplotype tree was inferred using the Tamura–Nei model of sequence evolution and a gamma distribution shape parameter of 0.551 (Fig. 2.4). Data of a total of 121 individuals, reduced to 26 unique haplotypes were used to construct the tree and the New Zealand fur seal (*Arctocephalus forsteri*; Genbank accession No. AFU03576) was used as an outgroup. Twenty-six haplotypes of which 17 were from Marion Island, seven from Gough Island and two from Ile Amsterdam were included in the data set, as was one male individual from Marion Island (GZ23 from Sealer's Cave) which had an *A. tropicalis* mtDNA control region but presented phenotypically as *A. gazella* was included.

From the tree, three distinct lineages were recovered with varying levels of bootstrap support. The first lineage (denoted I) had 46 % bootstrap support, whilst lineages II and III had 98 % and 86 %, respectively. Lineage I, which collapses into a polytomy, comprises haplotypes from Gough, Marion and Amsterdam islands, with most of these being from Marion and Gough islands. The haplotypes within monophyletic lineage II comprised individuals from all islands except individuals from Ile Amsterdam (Fig. 2.4). The haplotypes within monophyletic lineage III comprised individuals from all three islands (Fig. 2.4). Lineage I comprised 12 haplotypes representing 31 individuals, Lineage II comprised five haplotypes recovered from 39 individuals and Lineage III comprised nine haplotypes from 51 individuals. Marion Island shared haplotypes with the other three islands (Gough Island, Iles Crozet, and Ile Amsterdam) (Table 2.2), but despite more data being available from Gough Island, only one shared Marion-Gough haplotype was recovered. There were two shared haplotypes between Marion and

Amsterdam islands, and one shared haplotype between Iles Crozet and Marion Island. No shared haplotypes among the Gough Island, Iles Crozet and Ile Amsterdam populations were observed (Table 2.2).

2.3.2.2 A. gazella

The starting dataset of 103 individuals of *A. gazella* was reduced to a haplotype dataset containing 25 unique sequences, of which 19 were from Marion Island and 6 from Bouvetøya. A Minimum Evolution tree was inferred using the Tamura–Nei model of sequence evolution with gamma distribution shape parameter of 0.481. Nodal support was assessed by bootstrap resampling with 10 000 replicates (Fig. 2.5). The same *A. forsteri* sequence used as an outgroup in the *A. tropicalis* analyses, was again included for outgroup purposes. The haplotype tree of *A. gazella* revealed two lineages denoted I and II in Fig. 2.5 that contained haplotypes from Marion and Bouvetøya. No Marion-only haplotype lineages or Bouvetøya-only haplotype lineages were recovered. Lineage I comprised 23 haplotypes recovered from 94 individuals (with 90 % bootstrap support) whilst lineage II had 2 haplotypes from 9 individuals (supported by 99 % of the bootstrap replicates). Marion Island and Bouvetøya shared 5 haplotypes (Table 2.3); Fig. 2.5).

2.3.2.3 Combined *A. gazella* and *A. tropicalis* data set

The mtDNA sequences of 116 individuals of *A. tropicalis* (96 from Marion and 20 from Gough) and 98 individuals of *A. gazella* (78 from Marion and 20 from Bouvetøya) together with sequences from 5 individuals each of *A. tropicalis* and *A. gazella* downloaded from the Genbank (Wynen *et al.* 2001), were used to compile a haplotype dataset. This haplotype dataset comprising 51 haplotypes (25 were from *A. gazella* and 26 from *A. tropicalis*) was used for phylogenetic analysis of the combined dataset (Fig 2.6). A Minimum Evolution tree was constructed using the Tamura-Nei model of sequence evolution, with 10 000 bootstrap replications being performed to assess nodal support (Fig. 2.6), and the same *A. forsteri* sequence used for the previous haplotype trees, was again included as an outgroup. The *A. tropicalis* mtDNA origin of an individual classified phenotypically as *A. gazella* (denoted GZ23 (HT26) in the tree)

was again evident. No further evidence of hybridization was observed with this maternally inherited mitochondrial genome region.

```
          11111111 1111111122 2222222222 2222222222 2233
         3401133777 7789999900 0111111223 3334445667 8900
         1223489123 4730248914 9024678131 3670577097 0248
HT07     CTTTCAGATA GGTATAGGTA TCTGCTACCC CAAGTGTAGG CGGG
HT01     ........C. .......... CT....G... T....A.... ..A.
HT02     ...C....C. .......... CT....G... T....A.... T.A.
HT03     ........C. .......... CT....G... T....A.... ....
HT06     ........C. A......... CT........ T....A.... ..A.
HT08     ........CG ......A..T ......G... .....A.... ..A.
HT15     .C.....GCG AA..CG.... ...A...... .GG.CACGA. ..AA
HT20     .......... A......AC. ...A..GTTT TG.ACA..A. ...A
HT11     T......G.. A......... .T.A...... .....A.G.. ..A.
HT24     .........G A..G...A.. C..A..GTTT TG.ACA..A. ...A
HT25     .........G A..G...A.. C..ATCGTTT TG.ACA..A. ...A
HT21     .......... A......AC. C..A..GTTT TG.ACA..A. ...A
HT22     .......... A..G...A.. C..AT.GTTT TG.ACA..A. ...A
HT26     ...CT....G A..G...A.. C..ATCGTTT TG.ACA..A. ...A
HT14     .C.....GCG AA.GCG.... ...A...... .GG.CACGA. ..AA
HT16     .C.....GCG AA..CG.... ..CA...... .GG.CACGA. .TAA
HT18     .......... A......AC. C..A..GTTT TG.ACA..A. .T.A
HT12     T......G.. A......... .T.A...... .....A.G.. .TA.
HT19     .......... A......AC. ...A..GTTT TG.ACA..AA .T.A
HT17     .CA....GCG AA..CG.... ...A...... .GG.CACGAA .TAA
HT13     .C...G.G.G AA..CG.... ...A...... TGG.CACGA. ..AA
HT10     T......... A.C....... CT.A...... .....A.... ....
HT23     .......... ...G...A.. C..AT.GTTT TG.ACA..A. ...A
HT09     ......A.CG ......A..T ......G... .....A.G.. ..A.
```

Fig. 2.2: Alignment of *A. tropicalis* haplotype sequences from Marion and Gough islands indicating the 44 variable sites identified from the homologous mtDNA control region characterized in this study.

```
          1111111 1111111111 2222222222 2222222222 222222233
         340111233 4778888999 1111111112 2333444445 566678900
         9682245889 3230235012 0123456783 6347025893 702971648
HG10     TTTC-TCGAA TTAGCTTAAT TCTGACTCAC CCTGAGTCTC CAGAATCGA
HG01     ....-.T..G ........G. .T........ ......C... .........
HG02     C...-.T..G ........G. .T........ ......C... .........
HG03     ....-.T..G ........G. .T........ T.....C.C. .........
HG04     ....-.T.GG ...A....G. .......... ......CT.. .........
Hg05     ....-.T... ...A....G. .......... ......CT.. .........
HG06     ....-.T..G .......... .......... ......C... .........
HG07     ....-....G ........GC .......... .TC...C... .........
HG08     ....-.T..T ........GC .....T.... ..C...C..T .G.......
HG09     ....-.T..G ........GC .......... ..C...C..T .G.......
HG11     ....-.T... .......... .......... ......C... .........
HG12     ....-...G. .......... .......... ..C...C... ....G....
HG13     ....-...G. ....T...G. ...A...... ..C...C... .......A.
HG14     ....-...G. ........G. ..C....... ......C... .........
HG15     ....-.T.G. ....T....C ......C... .TC...C... .........
HG16     ....-.T..G ....TC.... .......... ..C...C... .........
HG17     ....-.T..G ....T..G.. ....G..... ..C...C... T.....TA.
HG18     ....-.T... ....T..G.. ....G..... ..C...C... T........
HG19     .C..TC.A.G .........C ...A.....T TT....C... ....G..A.
HG20     .C..CC.A.G .........C ...A.....T TT....C... ....G..A.
HG21     ....-..... C.G.T....C .......... T.....C.C. .........
HG22     ....-..... C.G.T....C ........G. T.....C.C. .........
HG23     ....-..... C.G.T.C..C ........G. T..AG..... T.AGG...G
HG24     ..CTC....G .C..TC.... CT..GTCTC. TT....C... ...G.C...
HG25     ..CTC....G .C..TC.... CT..GTCTC. TT...AC... ...G.C...
```

Fig. 2.3: Nucleotide sequence alignment of *A. gazella* haplotype sequences from Marion and Bouvetøya islands, indicating the 49 variable sites identified in the mtDNA control region characterized in this study.

Table 2.1: Sequence and population statistic parameters for Marion-only and for Marion-Gough (*A. tropicalis* combined) and Marion-Bouvetøya (*A. gazella* combined)

Statistics	*A. tropicalis* (Marion-only)	*A. tropicalis* (Combined)	*A. gazella* (Marion-only)	*A. gazella* (Combined)
No. of individuals	96	116	78	98
AT%	58.8	58.7	59.3	59.3
Variable sites	40	44	43	49
Conserved sites	262	258	264	260
PI sites	33	36	30	39
Singleton sites	7	8	13	10
No. of haplotypes	17	26	19	25
Haplotype diversity (h)	0.821	0.869	0.797	0.827
R=si/sv		21.0		31.4
Gamma distribution		0.551		0.481
Nucleotide diversity (π)	0.042	0.046	0.029	0.033
Tajima's D	1.926	1.960	-0.138	0.085
Φ_{ST}	0.320	0.319	0.082	0.079
F_{ST} (θ)		0.168		0.062
Fu and Li's test	6.314	3.244	0.789	0.561

PI: Parsimony informative

Table 2.2: Number of *A. tropicalis* haplotypes, names of individuals that fall under each haplotype and their place of origin.

Haplotype designation	Number of individuals	Sample names	Island of origin
HT 01	11	TR24, TR25, TR56, GI16, GI13, GI15, GI19, GI9, GI11, GI10, GI2	Marion (n=3), Gough (n=8)
HT 02	2	GI3, GI8	Gough (n=2)
HT 03	1	GI7	Gough (n=1)
HT 04	1	AF384381	Ile Amsterdam(n=1)
HT 05	1	AF384385	Ile Amsterdam(n=1)
HT 06	1	GI12	Gough (n=1)
HT 07	2	TR1, TR12	Marion (n=2)
HT 08	3	TR15, TR 17, TR 20	Marion (n=3)
HT 09	2	GI14, GI18	Gough (n=2)
HT 10	3	GI4, GI6, GI17	Gough (n=3)
HT 11	3	TR16, TR26, TR27	Marion (n=3)
HT 12	1	MA62	Marion (n=1)
HT 13	2	GI1, GI20	Gough (n=2)
HT 14	1	TR65	Marion (n=1)
HT 15	34	TR2, TR42, TR9, TR30, TR44, TR33, TR60, TR73, TR40, TR47, TR66, TR71, TR45, TR74, MA61, MA63, TR32, MA31, TR21, MA70, TR13, TR8, TR70, TR54, MA71, TR35, TR48, MA40, TR4, MA34, MA74, TR10, TR23, AF384384	Marion (n=33), Iles Crozet (n=1)
HT 16	1	MA36	Marion (n=1)
HT 17	1	MA73	Marion (n=1)
HT 18	1	MA38	Marion (n=1)
HT 19	1	MA72	Marion (n=1)
HT 20	7	TR3, TR36, TR29, TR31, TR11, TR14, TR43	Marion (n=7)
HT 21	12	TR39, MA57, MA58, TR67, MA60, TR61, TR6, TR7, TR22, TR5, TR64, AF384383	Marion (n=11) Ile Amsterdam (n=1)
HT 22	2	TR52, TR53	Marion (n=2)
HT 23	1	GI5	Gough (n=1)
HT 24	6	TR18, MA33, TR34, TR38, TR19, AF384382	Marion (n=5) Ile Amsterdam (n=1)
HT 25	20	TR28, MA32, MA39, TR72, TR51, TR55, TR68, TR59, MA35, TR37, TR58, TR49, TR50, TR46, TR57, TR63, TR75, TR69, TR41, TR62	Marion (n=20)
HT 26	1	GZ23	Marion (n=1)

Table 2.3: Number of *A. gazella* haplotypes, names of individuals that fall under each haplotype and their place of origin

Haplotype designation	Number of individuals	Sample names	Island of origin
HG 01	10	GZ3, GZ18, GE, GZ21, LV, LF, LI, LD, MA2G, AF384377	Marion (n=9), Bouvetøya (n=1)
HG 02	3	BI6, BI8, BI10	Bouvetøya (n=3)
HG 03	1	BI2	Bouvetøya (n=1)
HG 04	7	GZ7, WR, WE, LG, MA9G, BI12, BI16	Marion (n=5), Bouvetøya (n=2)
HG 05	1	BI11	Bouvetøya (n=1)
HG 06	1	GZ16	Marion (n=1)
HG 07	1	LK	Marion (n=1)
HG 08	1	GZ5	Marion (n=1)
HG 09	10	GZ8, WL, LR, GZ27, LZ, GZ9, MA11G, MA17G, BI5, AF384380	Marion (n=8), Bouvetøya (n=2)
HG 10	2	GZ2, GZ25	Marion (n=2)
HG 11	2	GZ13, WI	Marion (n=2)
HG 12	2	GZ24, MA5G	Marion (n=2)
HG 13	1	LB	Marion (n=1)
HG 14	1	LS	Marion (n=1)
HG 15	2	GZ14, GC	Marion (n=2)
HG 16	1	MA4G	Marion (n=1)
HG 17	3	GZ26, WQ, LC	Marion (n=3)
HG 18	1	BI15	Bouvetøya (n=1)
HG 19	2	MA6G, MA8G	Marion (n=2)
HG 20	2	BI3, AF384378	Bouvetøya (n=2)
HG 21	1	GZ20	Marion (n=1)
HG 22	38	GZ6, GZ4, MA14G, MA19G, MA16G, MA20G, MA3G, MA1G, MA7G, MA15G, MA10G, GB, LN, GZ10, GD, LY, LQ, LH, WK, WP, GZ11, GZ15, GZ19, LP, LW, GZ17, WT, MA12G, GZ12, GA, GZ1, GZ22, WO, BI1G, BI13G, BI14G, BI7G, AF384376	Marion (n=33) Bouvetøya (n=5)
HG 23	1	BI17	Bouvetøya (n=1)
HG 24	1	WJ	Marion (n=1)
HG 25	8	MA13G, MA21G, BI19, BI9, BI18, BI20, BI4, AF384379	Marion (n=2), Bouvetøya (n=6)

Blue= Exclusive to Marion Island

Red= Exclusive to Gough Island

Green= Exclusive to Ile Amsterdam (Genbank)

HT26*=Hybrid

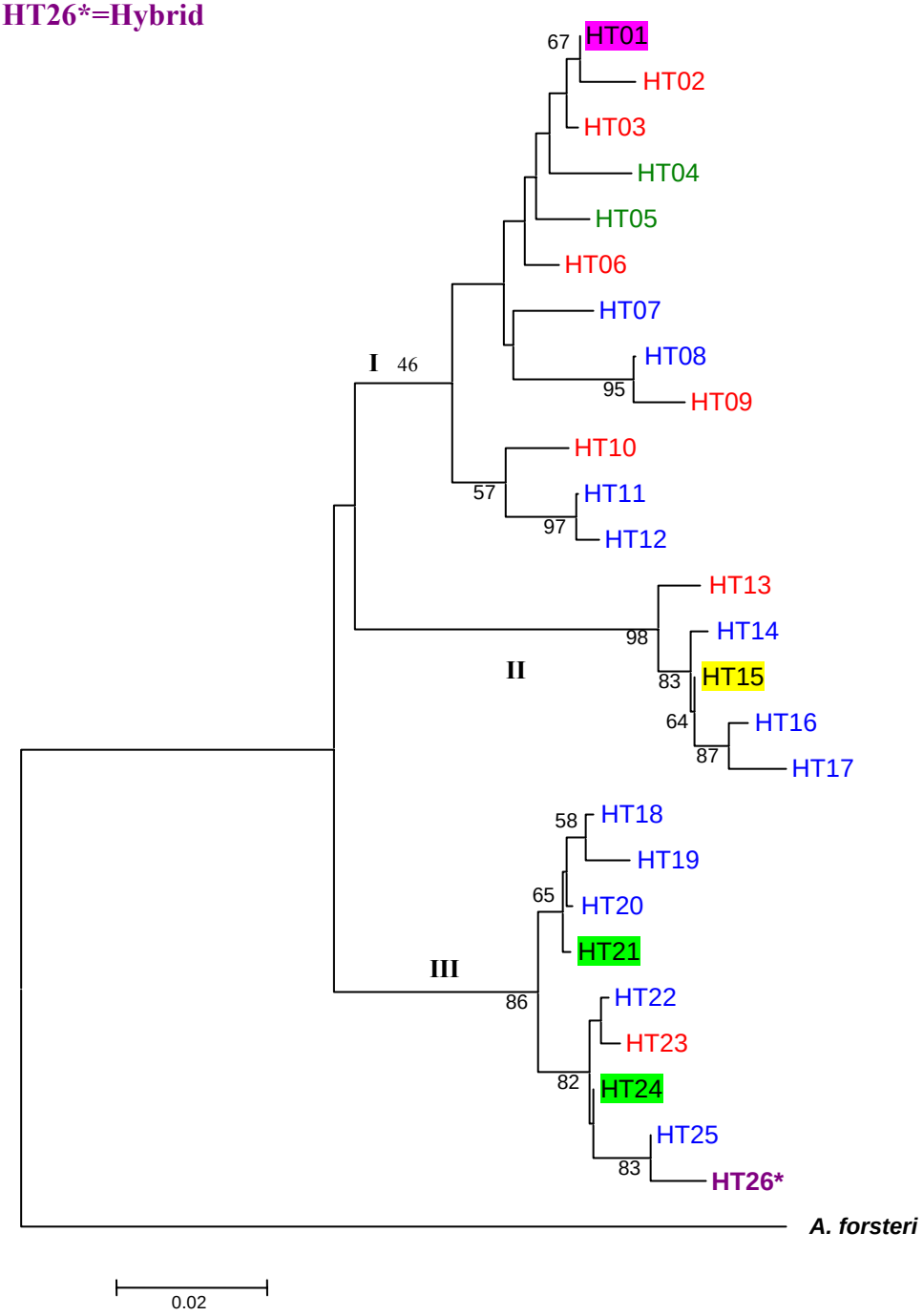


Fig. 2.4: Minimum evolution tree depicting *A. tropicalis* mtDNA haplotype relationships of individuals from Marion, Gough and Amsterdam islands. Bootstrap values obtained following 10 000 replications and > 40 are indicated next to the relevant nodes. Pink shaded haplotypes indicate those shared between Marion and Gough, yellow shading indicates Marion-Iles Crozet shared haplotypes and green shading indicates Marion-Amsterdam shared haplotypes.

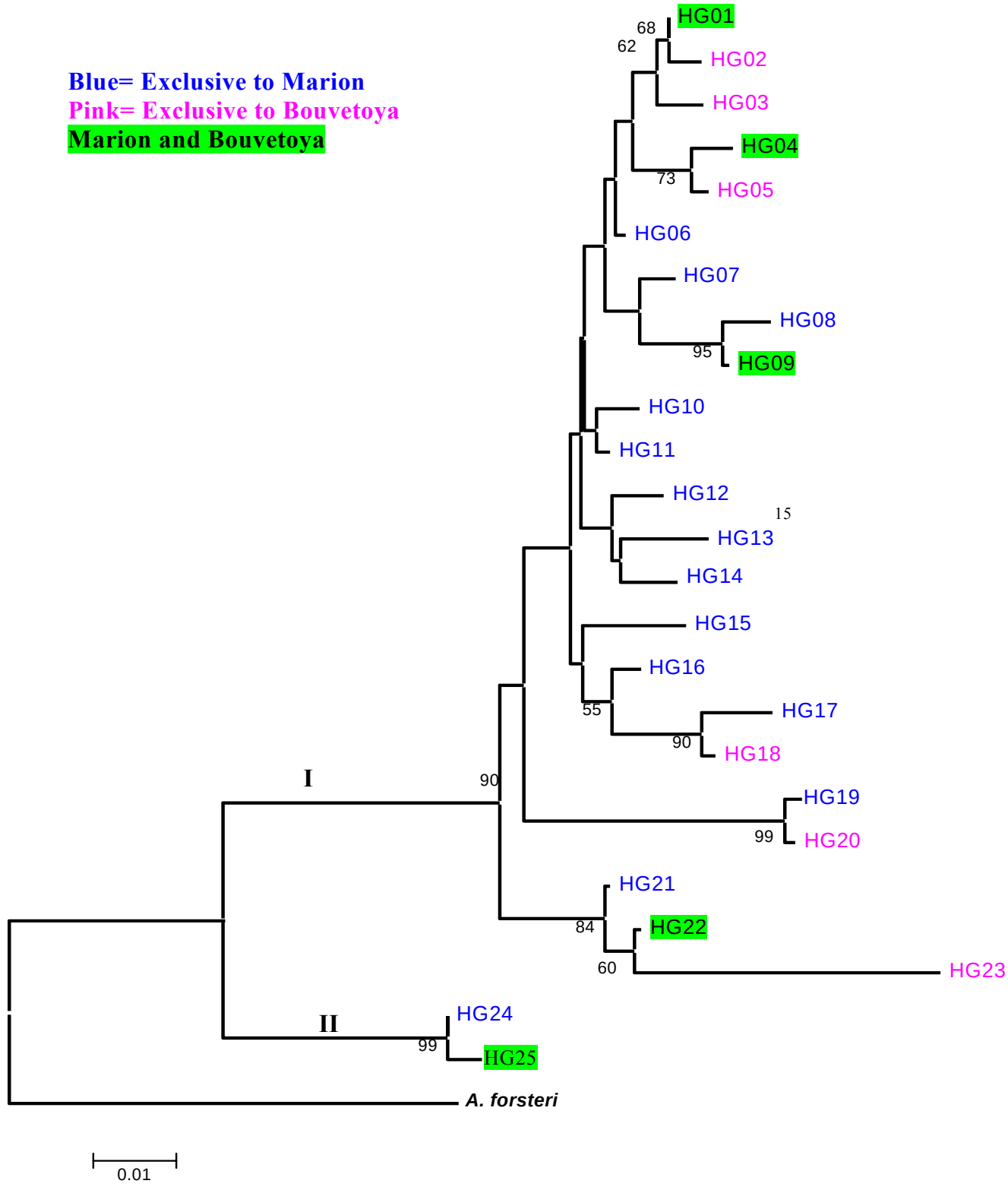


Fig. 2.5: Minimum evolution tree depicting *A. gazella* mtDNA haplotype relationships of individuals from Marion Island and Bouvetøya. Bootstrap values obtained following 10 000 replications and > 50 are indicated next to the relevant nodes. Green shaded haplotypes indicate those shared between Marion Island and Bouvetøya.

Blue=Marion

Red=Gough

Green=Amsterdam

Pink=Bouventoya

HT26*=Hybrid

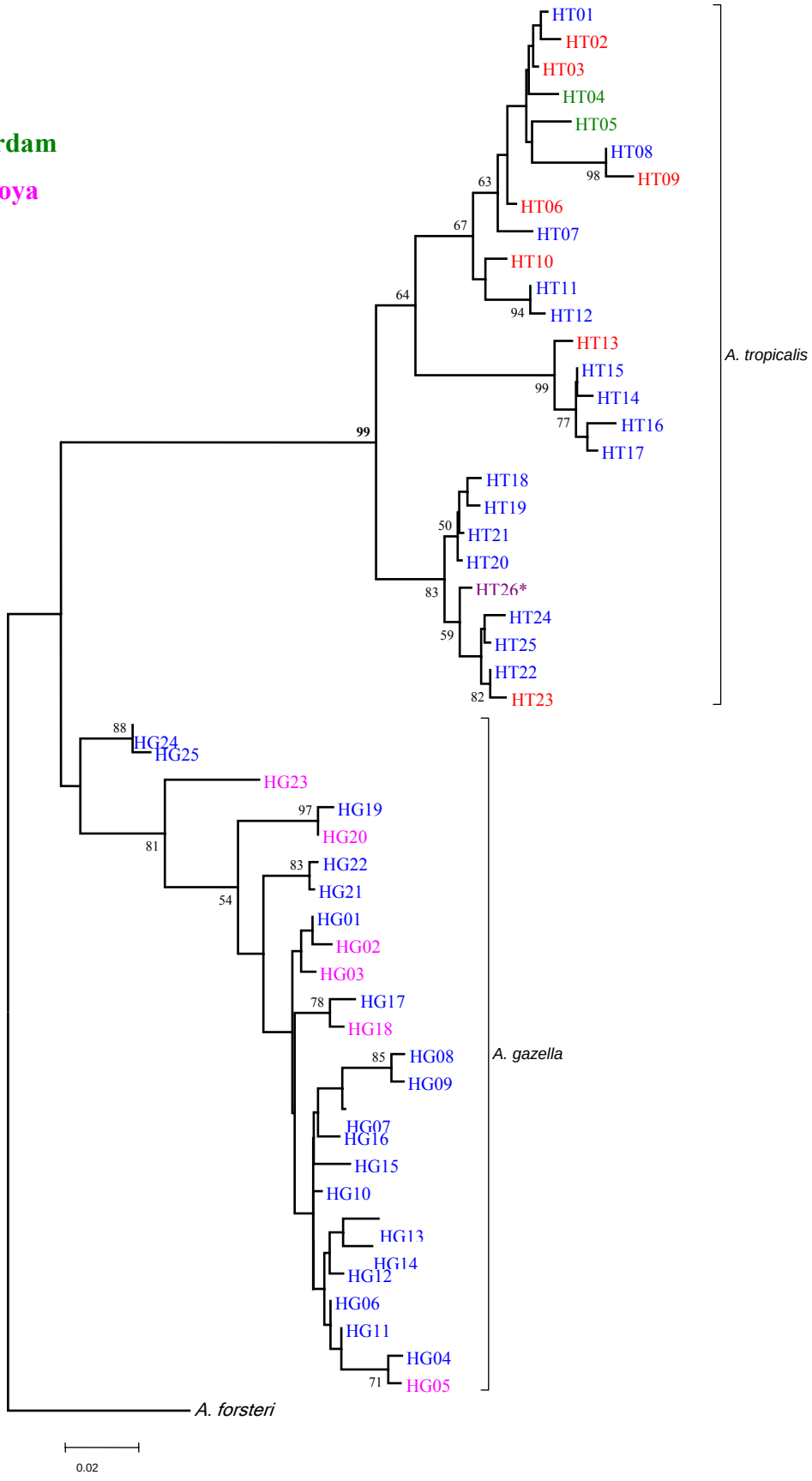


Fig. 2.6: Minimum Evolution tree depicting *A. tropicalis* and *A. gazella* mtDNA haplotypes. *A. tropicalis* haplotypes are comprised of individuals from Marion, Gough and Amsterdam islands whilst *A. gazella* haplotypes are comprised of individuals from Marion Island and Bouvetøya. The haplotype with an asterisk sign (HT26*) indicates the hybrid that has a matrilineal *A. tropicalis* mtDNA control region but which is phenotypically *A. gazella.*

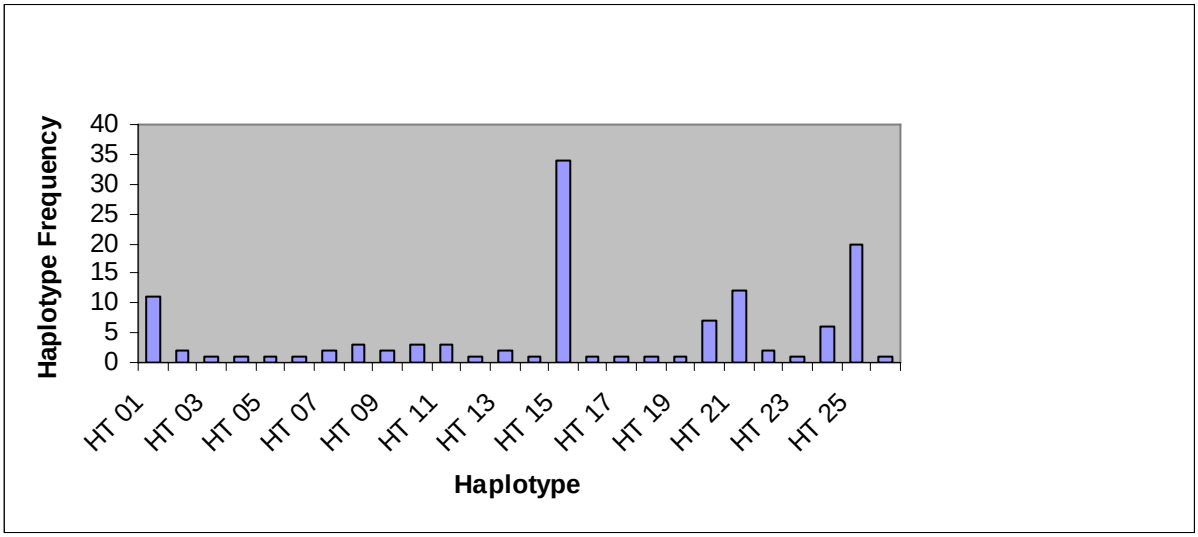


Fig. 2.7: Graph depicting the frequency with which each of the 26 *A. tropicalis* haplotypes occurs in the Marion Island dataset.

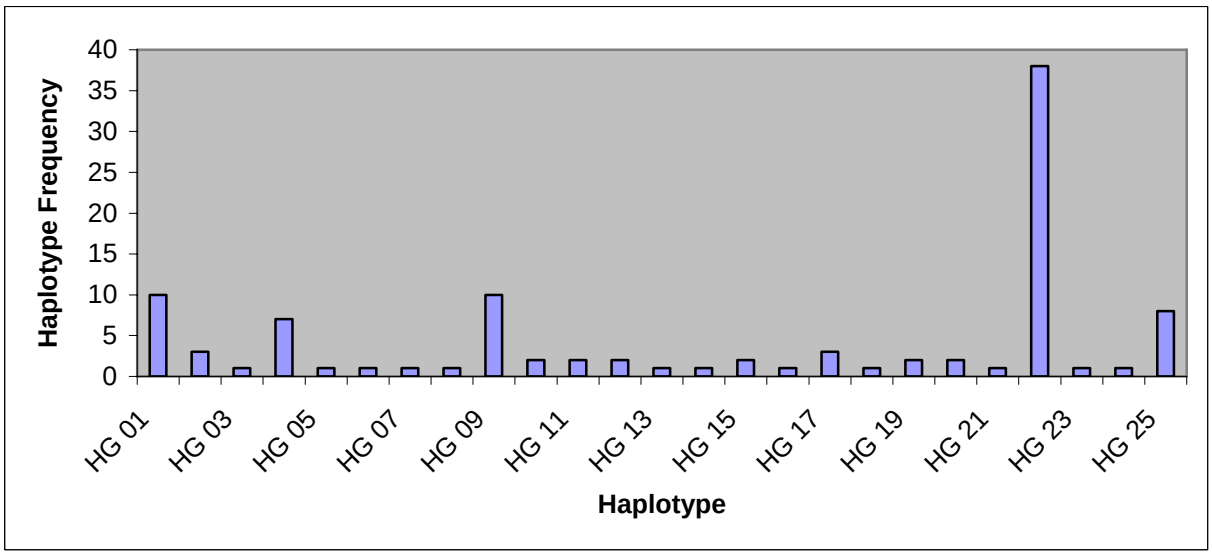


Fig. 2.8: Graph depicting the frequency with which each of the 25 *A. gazella* haplotypes occurs in the Marion Island dataset generated in this study.

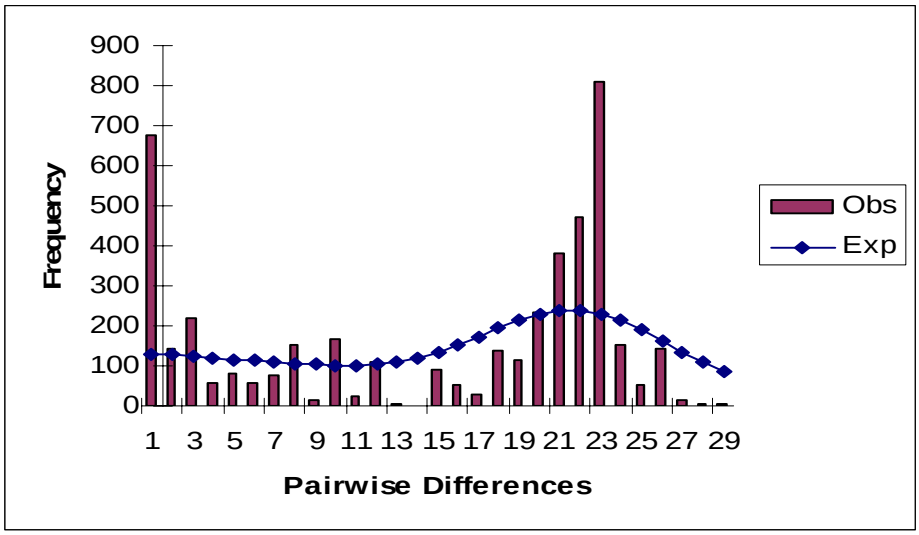


Fig. 2.9a: Mismatch distribution for *A. tropicalis* population on Marion Island. Harpending's raggedness index (r) = 0.053. The observed pairwise and expected model differ significantly ($P < 0.001$).

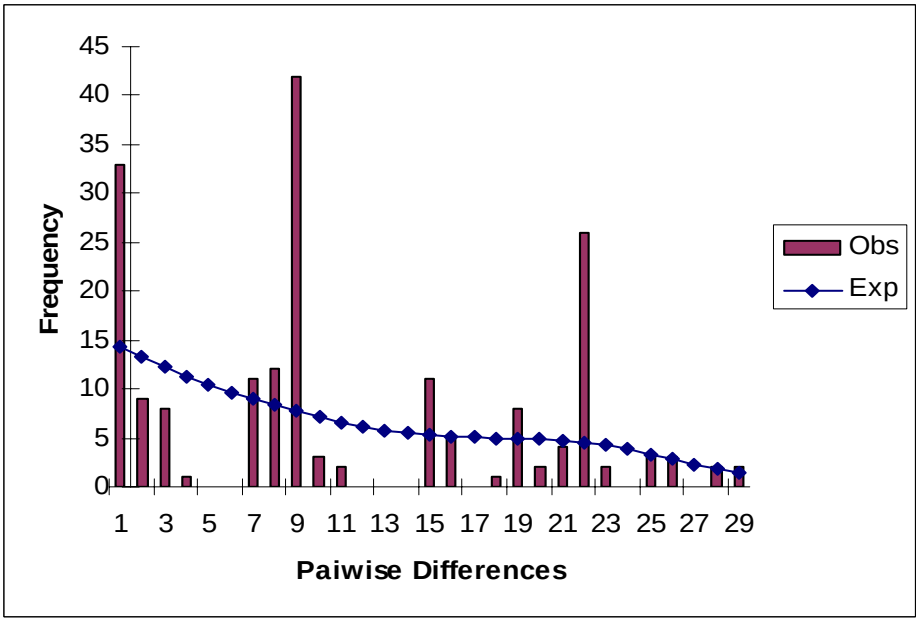


Fig. 2.9b: Mismatch distribution for *A. tropicalis* population on Gough Island. Harpending's Raggedness index (r) = 0.126. The observed pairwise and expected model differ significantly ($P = 0.020$).

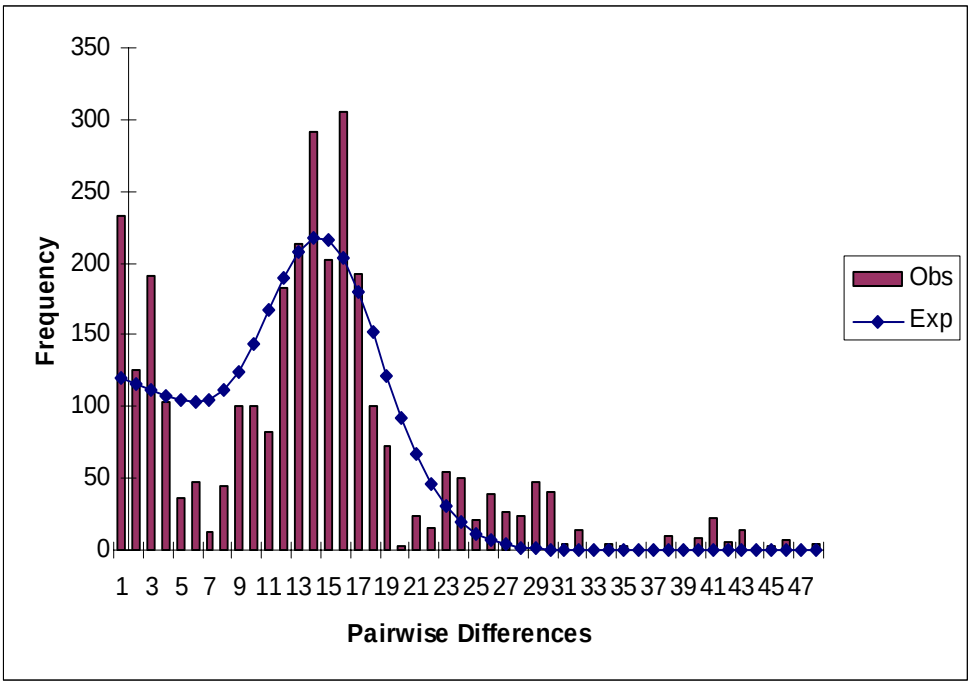


Fig. 2.9c: Mismatch distribution for *A. gazella* population on Marion Island. Harpending's Raggedness index (r) = 0.011. The observed pairwise and expected model do not differ significantly (P = 0.290).

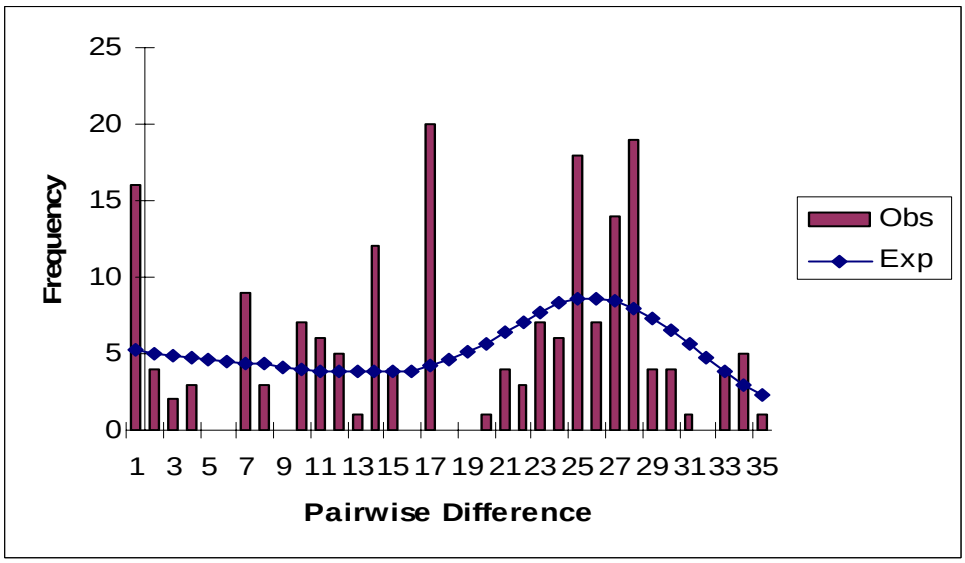


Fig. 2.9d: Mismatch distribution for *A. gazella* population on Bouvetøya Island. Harpending's Raggedness index (r) = 0.055. The observed pairwise and expected model do not differ significantly (P = 0.060).

2.4 DISCUSSION

2.4.1 Genetic variability

The results from the current study indicate that the effects of seal harvesting were not overly severe for the two *Arctocephalus* fur seal species at Marion Island, as the mtDNA nucleotide diversity estimates for the combined datasets of both *A. gazella* (Marion and Bouventøya) and *A. tropicalis* (Marion and Gough islands) were relatively high, 3.3 % and 4.6 %, respectively, when compared to those of other fur seal species. These levels of genetic variation are only marginally higher and lower, respectively than those reported previously by Wynen and co-workers (2000), for the same fur seal species. When comparing the previously published estimates for these two seal species with those of other fur seal species that underwent severe population reductions due to sealing, namely *A. forsteri* π=5.1 % > *A. tropicalis*, π=4.8 % > *A. gazella* π=3.2 % > *A. phillippii* π=3.0 % > *A. townsendi* π=2.5 % (Wynen *et al*. 2000, Goldsworthy *et al*. 2000, Weber *et al*. 2004), it is clear that the values of both *A. tropicalis* and *A. gazella* determined during this study fall within the upper part of the range. When these *Arctocephalus* diversity values are contrasted with those of the northern elephant seal (*Mirounga angustirostris*) (Gill 1866) and Hawaiian monk seal (*Monachus schauinslandi*) (Matschie 1905) which were also extensively harvested and which have nucleotide diversity values of 0.43 % and 0.7 % respectively, for the mitochondrial control region (Hoelzel *et al*. 1993), it is clear that *Arctocephalus* species have moderate to high levels of diversity. Nucleotide diversity of the combined datasets of 3.3 % for *A. gaze*lla is markedly lower than that of *A. tropicalis* (4.6 %) in this study. A similar trend was observed when the nucleotide diversity of *A. tropicalis* and *A. gazella* from Marion Island only was compared, 4.2 % and 2.9 %, respectively. These differences in diversity values between *A. tropicalis* and *A. gazella* are most likely attributable to the more intensive harvesting of *A. gazella* (Wynen *et al*. 2000). The greater degree of *A. gazella* exploitation may be due to differences in habitat preference. *A. gazella* individuals prefer to breed on open vegetation areas causing them to be more exposed to sealers while *A. tropicalis* prefer rocky areas and caves (Condy 1978). Despite the differences in beach preferences of these Marion congenerics, the level of genetic variation of both species is relatively high when compared with other marine mammals that experienced bottlenecks due to human exploitation.

Differences in genetic variation have previously been noted in species that have undergone drastic population reductions. Some species have low genetic variability while recovering from bottlenecks (Ellegren *et al*. 1993) while other species that have undergone a drastic population reduction have high levels of genetic variabily (Carson 1990, Dinerstein and McCracken 1990, Robinson *et al*. 1993). Post-sealing populations of *A. tropicalis* and *A. gazella* are recovering rapidly (Wynen *et al*. 2000; Hofmeyr *et al*. 2006) and the genetic data indicate migration between islands which may explain why their levels of genetic variation are not overly reduced. Genetic variability of species is an important parameter to assess because once it becomes significantly decreased, it can negatively impact on reproductive success and lead to reduced disease resistance (O'Brien *et al*. 1985, Allendorf and Leary 1986, O'Brien and Evermann 1988) in the short term, and to the inability of a species adapting to environmental changes in the long run. Positive, non-significant values of Tajima's D that were obtained for *A. tropicalis* (Marion Island only), *A. gazella* (Bouvetøya only), *A. tropicalis* (Marion and Gough islands combined) and *A. gazella* (Marion Island and Bouvetøya combined) suggest that there is an excess of high-frequency mutations, as would be expected after a population contraction or under balancing selection (Tajima 1989). The *A. tropicalis* population from Gough Island alone and the *A. gazella* population from Marion Island alone had negative values of Tajima's D, indicating a historical growth or expansion mode and also suggesting an excess of low-frequency mutations, as would be expected after a population expansion. When comparing results from Harpending's Raggedness index (r) and Tajima's D tests, both tests indicate population contraction for the Marion Island *A. tropicalis* and a population expansion for the *A. gazella* population from Marion Island. However, contradictory results were obtained for the *A. tropicalis* population from Gough Island since the Harpending's Raggedness test showed population contraction whilst Tajima's D showed population expansion. Similarly, contradictory results were obtained for the *A. gazella* population on Bouvetøya as Harpending's Raggedness index indicated a population expansion whilst Tajima's D showed a population contraction. It should, however, be noted that several statistical tests for detecting population growth differ in their power of analysis. Harpending's Raggedness index is among the most powerful statistics whilst Tajima's D has less power (Ramos-Onsins and Rozas 2002). Harpending's Raggedness index is based on mismatch distribution which is known to be conservative because it utilizes less

information in the collected data (Felsenstein 1992). Due to contradictory of the Harpending's Raggedness and Tajima's D in some cases in this study, greater weight is given to the results of the raggedness test as it is considered more powerful than Tajima's D. The raggedness test also corresponds well with the results of the study done by (Hofmeyr *et al*. 2006) where it was shown that *A. gazella* seems to be in the rapid recolonization phase of population growth (mean annual rate of 14.8 % over the past 21 years) whilst *A. tropicalis* has approached its carrying capacity (mean annual rate of 5.3 % over the past 15 years). The raggedness test also supports the study done by (Hofmeyr *et al*. 2005) on Bouvetøya which revealed that *A. gazella* is increasing in population size.

2.4.2 Population Structure

From the haplotype trees (Fig. 2.4 and Fig. 2.6) it is clear that *A. tropicalis* has three divergent evolutionary lineages (labelled I-III in Fig. 2.4), with bootstrap values being 46/98/86 for each lineage in the *A. tropicalis* only haplotype tree (Fig. 2.4), and 67/99/83 in the combined *A. tropicalis/A. gazella* haplotype tree (Fig. 2.6). The fairly high bootstrap support for the three discrete lineages (particularly in Fig. 2.6) indicates strong genetic structure and historical evidence of independent evolution. However, the individuals within each of these clades do not group according to their current geographical sampling locations, pointing to recent migration and genetic exchanges, particularly between Marion and Gough islands which always appear together within the different lineages. These exchanges between these two islands may have been precipitated by the sealing that occurred during the 18th and 19th century and is certainly possible, as these two islands are separated from each other by 1071 km of ocean, a distance that falls within the documented dispersal range of the species (Bester 1989, David *et al.* 1993, Goldsworthy and Shaughnessy 1989, Hofmeyr *et al.* 2006), and forging range of lactating females with dependant pups ashore (Georges *et al.* 2000; Osborne *et al.* 2002). The shared haplotype between Iles Crozet and Marion Island populations confirms previous suggestions (Wynen *et al*. 2000) that Marion Island is the major source of the Iles Crozet *A. tropicalis* population. The relatively close proximity of Marion and Gough islands is similar to the distance separating Marion Island and Ile Amsterdam, which could explain the links between Marion Island and each of these two

Islands. On the other hand, the lack of shared haplotypes between Ile Amsterdam and Gough Island, despite at least one tagged 5-year-old subadult male (from Ile Amsterdam) turning up at Gough Island 5000 km to the west (Hänel *et al.* 2005), supports the notion that migration of *A. tropicalis* between islands is relatively recent. There is also no evidence of Marion Island being used as a stepping-stone between the two islands (this study). However, it should be noted that only four individuals from Amsterdam Island were available in the Genbank for comparisons, so the apparent lack of shared haplotypes may well be due to inadequate sampling.

Shared haplotypes between islands confirms gene flow and migration. Fur seals are said to be philopatric (Riedman 1990) even if they can disperse long distances (Bester 1989, Hofmeyr *et al.* 2005, De Bruyn *et al.* 2007, Ferreira *et al.* 2008). Species with high female philopatry are considered to have low gene flow between populations (Wynen *et al*. 2000). *Arctocephalus tropicalis* shows high levels of population structure as confirmed by the Φ_{ST} of 0.320 between Marion and Gough islands. However, the difference between the two islands was moderately low (F_{ST}=0.168). This was confirmed by one haplotype shared between them, and shows that despite the islands being closer to each other and expecting the species to be more similar, the genetic make up of the species occurring on both islands differ a little.

The *A. gazella* haplotype tree (Fig 2.5), revealed that Marion Island and Bouvetøya populations share a common evolutionary history, as their haplotypes were present in each of the two highly supported lineages recovered. Both populations receive dispersing (leucistic) animals from Scotia Arc islands, most probably South Georgia (Hofmeyr *et al.* 2005, De Bruyn *et al.* 2007). The two island populations shared five haplotypes pointing to more recent migration (Table 2.3), confirming the suggestion of Wynen and co-workers (2000) that Bouvetøya is probably the source of immigrants to Marion Island. This was supported by the low F_{ST} of 0.062 indicating that there is no difference between the *A. gazella* populations from these two islands. Further, Marion Island and Bouvetøya haplotypes grouped together with high bootstrap support (ranging from 60 % to 99 %). *Arctocephalus gazella* also showed a low population structure with a Φ_{ST} of 0.082 between Marion Island and Bouvetøya and that is indicative of this species being more intensively harvested during the 18th and 19th centuries.

When constructing the tree of both the species (Fig. 2.6), the individual with A. *tropicalis* mitochondrial DNA but which phenotypically was classified as an *A. gazella* (GZ23-HT26), grouped within the *A. tropicalis* clade. The grouping of this hybrid individual with other *A. tropicalis* haplotypes, predominantly from Marion Island (bootstrap support of 83 %), confirms the presence of matrilineally-derived *A. tropicalis* DNA, i.e. that in the past an *A. tropicalis* female bred with an *A. gazella* male. This confirms that hybridization has occurred at Marion Island but at a very low level with only one hybrid individual being detected in the 174 individuals (0.57 %) genetically characterised. High frequencies of backcrossed or introgressed individuals show a high potential for interspecific gene flow (Chung *et al*. 2005) and a correlation between the number of individuals in an area and the frequency of hybridization of fur seal population was shown by Hofmeyr *et al.* (1997). The results presented here represent the first genetic confirmation of hybridization between these two fur seal species at Marion Island, *albeit* at a very low level.

CHAPTER 3
MICROSATELLITE ANALYSIS

3.1. INTRODUCTION

Microsatellites, also called Simple Sequence Repeats (SSRs) or Short Tandem Repeats (STRs), are tandem repeats that are between two and six base pairs long such as (CA)n or (ATT)n (Beckmann & Weber 1992). STRs occur approximately every 10 kb throughout the eukaryotic genome and are highly polymorphic (Tautz 1989). Polymorphism is caused by the difference in the number of repeat units present, and most likely arises through slipped-strand mispairing (Schlotterer and Tautz 1992). Microsatellites are found embedded in unique DNA sequences and they generally have a total length of less than 100 bp (Tautz 1989). This enables polymerase chain reaction (PCR) amplification with primers designed to target the flanking unique sequence (Tautz 1989), often in an across-species manner. Microsatellite repeats consist of three main families, namely pure (for example $(GT)_{15}$), compound (for example $(GT)_7(AT)_5$) and interrupted (for example $(GT)_4ATCT(GT)_3$). The pure microsatellites display higher levels of size polymorphism than the compound and interrupted families (Jarne and Lagoda 1996), and microsatellites containing either GT or CA dinucleotide repeats are the most common type found in mammals. In plants the most common microsatellite type is AT although microsatellites are generally not as abundant in plants as they are in mammals (Lagercrantz *et al*. 1993).

Microsatellites have both advantages and disadvantages. The major advantages include that they provide a large number of genetic loci for analysis (Hughes and Queller 1993), they give multiple independent genealogies for population-level studies, and they have high evolutionary rates (10^{-4} to 10^{-5} mutations per generation) (Hughes and Queller 1993). The disadvantages include the occurrence of null alleles, the possibility of an ascertainment bias and the fact that the underlying mutational process is not clearly understood (Callen *et al*. 1993, Ellegren *et al*. 1995, Rubinsztein *et al.* 1995, Crawford *et al*. 1998). A microsatellite null allele is an allele, which fails to amplify via the PCR, leading to inflated estimates of homozygosity at that particular locus. PCR failure can arise due to variation in nucleotide sequences within the flanking region that hinder the annealing of the primer to the template DNA during the performance of PCR. Null alleles can also arise from size-associated amplification bias where preferential amplification of shorter alleles occurs due to

inconsistencies in the quality and quantity of the DNA template or due to slippage during amplification (Gagneux *et al*. 1997, Wattier *et al*. 1998, Shinde *et al*. 2003). Genotypic errors such as null alleles can lead to deviations from Hardy-Weinberg equilibrium (HWE) resulting in biased population genetic analyses (van Oosterhout *et al*. 2004).

Microsatellites provide useful information for a number of aspects that include: population genetic studies (including the structure and intraspecific relationships of species), parentage and kinship, forensics, and gene mapping. Demographic history of populations must be well known in order to make proper decisions about a population. For example, there may be concerns regarding the level of inbreeding within a population, the ratio of effective population size to census size, and the effective population size itself (Lande and Barrowclough 1987). By genetically characterizing a subset of a population, insights into all of these estimates can be provided through the analyses of microsatellite data.

Markers do not all provide equal information at the different levels of genetic variability. In relatedness, kinship and assignment studies, loci with more alleles provide more information and allow for more accurate assessments. However, very advantage can be problematic when determining population structure, as standard analyses of population subdivision can be confounded by loci with large numbers of alleles (O'Reilly *et al*. 2004, Olsen *et al*. 2004) and large sample sizes will be required to assess population allele frequencies accurately. Microsatellites are valuable for population genetic studies for two main reasons. Firstly, microsatellite loci generally generate numbers of alleles in excess to that found with allozymes or restriction fragment length polymorphisms (RFLPs). Secondly, microsatellites have higher mutation rates than that found in non-repetitive, noncoding DNA, with current estimates ranging from $1x10^{-3}$ to $4.5x10^{-5}$ spontaneous mutations per microsatellite locus (Dietrich *et al*. 1992, Weissenbach *et al*. 1992).

Genetic markers should not to be treated as being absolutely diagnostic for differentiating between species, the reason being the impossibility in finding the effects of heterogeneity or ancient introgression between sympatric species (Goodman *et al*. 1999). Markers used for the identification of hybrids need not be diagnostic for the entire species as hybridization occurs between local populations. The number of diagnostic loci detected makes the rapid identification and precise classification of hybrids possible through genetic analyses (Morizot *et al*. 1991) and also assists with long-term monitoring of introgression (Tranah *et al*. 2003).

Co-dominant markers are preferred because they allow for the identification of homologous versions of a particular gene (different alleles at the same locus), which makes it possible to recognize heterozygotes and furthermore permits the estimation of allele frequencies. However, a large number of dominant markers can also be used for overcoming difficulties associated with the recognition of heterozygote genotypes. Microsatellites are highly reliable, co-dominant markers (Webster *et al*. 2002).

Several new statistical methods have been introduced following the development of polymorphic and abundant microsatellite markers (Hansen *et al*. 2001). Recent model-based Bayesian statistical techniques are used to resolve problems associated with detecting hybridization and hybrid individuals in contact zones of populations or species and utilize microsatellites, as they are molecular markers with high levels of polymorphism. These methods have been used in different scenarios for hybridization identification (Beaumont *et al*. 2001, Randi *et al*. 2001, Hansen 2002, Randi and Lucchini 2002, Pierpaoli *et al*. 2003) and also for assessing introgression (Susnik *et al*. 2004). The two programs that use Bayesian approaches for the identification of hybrid individuals but which vary in their approach are: STRUCTURE (Prichard *et al*. 2000, Falush *et al*. 2003) which is the method assigning probabilities for an individual to have recent ancestry in two or more populations, and NEWHYBRIDS (Anderson and Thompson 2002), which estimates the probability of individuals belonging to distinct hybrid or purebred classes.

A study by Vähä & Primmer (2006) in which the performance of the two methods for detecting hybrid F_1 and backcross individuals having different levels of parental population genetic divergence (F_{ST} = 0.03, 0.06, 0.12 or 0.21) was assessed under different scenarios which included differences in the number of loci (6, 12, 24 or 48) and in hybridization levels (10 % and 1 %). They found that with STRUCTURE the ability to identify hybrids dropped from 97.5 % at high hybridization levels to 96.0 % in the case of low hybridization and that the method was also less sensitive to the proportion of hybrids included in the sample. In NEWHYBRIDS the hybrid identification was reduced from 96.5 % in high hybridization to 91.5 % in the low hybridization scenario and this method worked better with the presence of individuals from both backcross and F_1 hybrid classes in the sample. Vähä and Primme (2006) also noted that even in cases where the divergence between parental populations was high, the ability to separate backcrosses from purebred parental individuals would require a genotyping effort of at least 48 loci.

Microsatellite loci that have been used in population genetic studies of pinniped species reveal different amounts of nuclear gene differences, measured as the number of alleles present per locus and the number of polymorphic loci present per species (Robert *et al*. 2004). When seven microsatellite loci isolated from the harbour seal (*Phoca vitulina*) (Linnaeus 1758) were applied to seven pinniped species, namely *P. vitulina*, the harp seal (*Pagophilus groenlandica*) (Erxleben 1777), the grey seal (*Halichoerus grypus*) (Fabricius 1791), the hooded seal (*Cystophora cristata*) (Erxleben 1777), *Arctocephalus gazella*, *A. tropicalis* and the New Zealand fur seal (*A. frosteri*) (Coltman *et al*. 1996), it was revealed that the number of alleles per locus ranged from 1 to 7 in these species. Twenty microsatellite loci isolated from the grey seal, harbour seal and the South American fur seal (*A. australis*) (Zimmermann 1783) were subsequently evaluated in 18 pinniped species (Gemmell *et al*. 1997) comprising three families, namely the Phocidae, Odobenidae and Otariidae (two sea lions and three fur seal species), and was inclusive of *A. gazella*. In the latter study it was shown that for each pinniped species, the number of polymorphic loci ranged from 3 to 19, with a mean of 13 (Gemmell *et al*. 1997).

Microsatellites are frequently useful for uncovering more recent population variations as divergences occurring in the more distant past are frequently obscured by recombination and back mutation in microsatellite markers (DiRienzo *et al*. 1994). This means that the chance of a microsatellite mutation producing an allele that is already present in the population (and therefore undetectable) is higher than for mtDNA, where new mutations often result in the creation of a new haplotype. The highly variable stepwise mutating microsatellite markers are often homoplasious (van Oppen *et al*. 2000). Homoplasy occurs when different copies of a locus are identical in state (have identical size), but not identical by descent (Estoup *et al*. 2002). This is referred to as size homoplasy. In microsatellites, most mutations appear to involve the gain and loss of a single repeat unit (Weber and Wong 1993) leading to high levels of homoplasy. In the stepwise mutation model (SMM, Kimura and Ohta 1978) microsatellite alleles mutate due to the loss or gain of a single repeat unit and alleles can mutate to an allelic state which is already present in the population. In contrast, in the infinite allele model (IAM, Kimura and Crow 1964) mutations can arise from the loss or gain of any number of repeats and will always result in new alleles not previously sampled in a population (IAM). In the SMM (Kimura and Ohta 1978) it is assumed that homoplasy can arise whilst homoplasy is not accommodated in the IAM of Kimura and Crow (1964).

Homoplasy leads to the underestimation of the total amount of variation and genetic distance and to the overestimation of resemblance among populations. Available data indicate that microsatellite evolution does not strictly occur according to either the SMM or IAM. Microsatellites (1 to 2 bp repeat unit) and short tandem repeats (STR) (3 to 5 bp repeat unit) appear to mutate in terms of SMM whilst minisatellites (15 to 70 bp repeat unit) deviate towards IAM (Shriver *et al*. 1993). This suggests that the most accurate model of mutation in microsatellites lies between the two models (Shriver *et al*. 1993). A common assumption of both models is that changes in tandem repeat number are considered to be the only sources of size variation (Symonds and Lloyd 2003).

Hybridization is a phenomenon that successfully engages the natural mating between individuals from two populations that are differentiated by one or more heritable characters (Arnold 1997). Hybridization and secondary contact in most taxa result from anthropogenic influences. Modifications such as introductions of non-native taxa result in changes to the global distribution of many taxa, often at the expense of regional and endemic native populations or species (Olden *et al*. 2004). Through hybridization, the genetic intergrity can be pressurized and that leads to homogenization of gene pools, outbreeding depression and even hybrid speciation (Avise 1994, Arnold 1997, Frankham *et al*. 2002). In natural populations, harvesting can impact on the genetic composition and gene flow and that might lead to a reduction in population size and also extinction.

The current study is aimed at detecting hybridization between *A. tropicalis* and *A. gazella* using nuclear microsatellite markers. Hybridization has previously been suspected on Marion Island from observations at several beaches (Condy 1978, Hofmeyr *et al*. 2006) and from a skull morphometrics study (Kerley and Robinson 1987). However, genetic-based estimates are lacking for these populations. The use of genetic markers is important in hybrid detection (Busack and Gall 1981, Allendorf and Leary 1988) as it is not easy to detect hybrids even when distinct morphological differences exist between species. For example, rainbow and cutthroat trout populations rarely display intermediate phenotypes, even in F_1 hybrids (Leary *et al*. 1983, Hawkins 1997).

With respect to the two fur seal species that form the basis of this study, whilst the adults of each species are readily distinguishable from each other on the basis of morphology, the pups and hybrids are not (Table 3.1). The aim of this study was to assess the levels of

hybridisation between the two Marion Island fur seal species using a genetic approach and was achieved using DNA extracted from pup skin biopsies, as template for amplification of microsatellites isolated from non-target seal species.

3.2 MATERIALS AND METHODS

3.2.1 Study site and sample size

The current study was conducted with *Arctocephalus* DNA samples from Marion Island only. All seal pups were predominantly sampled from species-exclusive beaches at Marion Island, with pups being classified as *A. tropicalis* or *A. gazella* based on breeding localities where they are known to occur exclusively (Condy 1978, Bester and Wilkinson 1989), their physical appearance (Bester and Wilkinson 1989) and also on the physical appearance and external features of the adults present at these beaches (Table 3.1). Skin biopsies of 69 individuals of *A. tropicalis* and 77 individuals of *A. gazella* were used for microsatellite genotyping (Fig. 3.1).

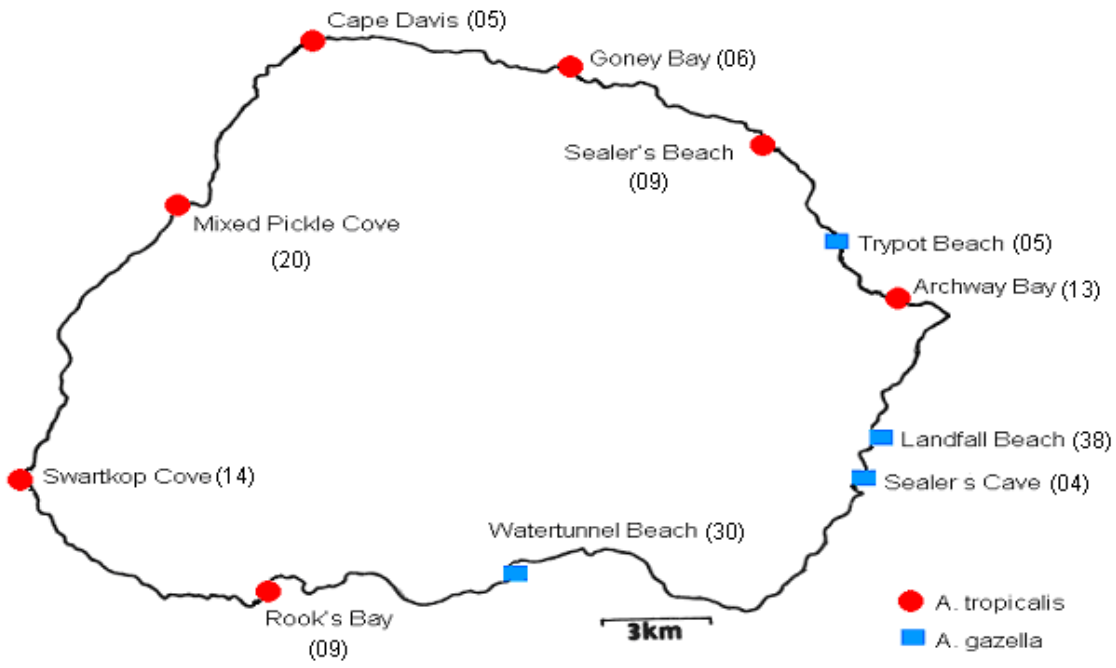


Fig. 3.1: Map indicating the 11 sampling localities at Marion Island (46°54′S, 37°45′E) at which *A. tropicalis* and *A. gazella* pups were sampled. The number of individuals sampled per locality is indicated in brackets next to each locality.

Table 3.1: The external features used to distinguish between *A. tropicalis* and *A. gazella* (Payne 1977; Condy 1978; Bester and Wilkinson 1989; Goldsworthy *et al*. 1999; St Clair Hill *et al*. 2001; Kingston 2006).

Age/sex class	***A. tropicalis***	***A. gazella***
Pups		
Colour	Black	Black
Body size	Difficult to distinguish	Difficult to distinguish
Adults		
Pelage: females	Yellow chest and throat, back and sides are grey to dark brown and black	Creamy throat and chest, back and sides are grey to brownish
Pelage: males	Similar to females but with prominent crest of black hair, raised when agitated	Similar to females, uniformly grey with no crest
Body length: females	116.3±6.2 cm	118.3±6.6 cm
Body length: males	156.7±9.4 cm	168.6±12.2 cm
Body size: females	Up to 50kg, compact and sturdy bodies	Up to 50kg bodies more slender
Body size: males	Up to 140kg, short and compact appearance	Over 200kg, longer less bulky bodies
Ears	Short black fleshy ears, no hair covering, sometimes just protrude above the fur	Long visible ears often covered with pale hair often white on tip
Eyes	Large oval eyes, eye lids with no hair on them	Smaller and narrower eyes, eyelids covered by short white hair
Flippers	Short broad flippers, easy to move in rugged irregular beaches	Long and slender flippers, movement on rugged areas seem to be awkward
Pup attraction call of females	Mournful wail typically without trilling	High pitched often with trilling components

3.2.2 Optimization of microsatellite typing

Genomic DNA was extracted from skin biopsy samples of pups using the Roche extraction protocol. Nine published microsatellite primers known to successfully amplify pinniped and phocid species' loci were initially screened for polymorphism in both *A. gazella* and *A. tropicalis* (Coltman *et al*. 1999; Gemmell *et al*. 1997; Goodman 1997; Slade *et al*. 1998; Hoelzel *et al*. 1999). Biotools *Taq* polymerase, with varying concentrations of $MgCl_2$ was used to optimise reaction conditions when screening for polymorphism, but was subsequently abandoned due to the low amplification and polymorphism detection success-rate. Subsequent reaction optimization and screening for polymorphism was performed with the QIAGEN® Multiplex PCR kit. This multiplex genotyping approach was less time-consuming as it permitted the co-amplification of numerous loci simultaneously, and because a single buffer that contains a mixture of all required reagents is supplied, with only primers and genomic DNA to be added. By using the multiplex PCR kit in monoplex format for polymorphism detection, it was determined that five loci out of the nine screened were polymorphic. This panel of polymorphic loci (Table 3.2) consisted of two loci isolated from grey seals *H. grypus* (Hg6.3 and Hg8.10, with reported allele size ranges of 215-225bp and 175-195bp, respectively), one from the harbour seal *P. vitulina* (Pv9 with an allele size range of 162-170bp), one from the southern elephant seal *M. leonina* (M11a, with an allele size range of 141-151) and one from *Leptonychotes weddellii* (Lesson 1826) (Lw10, with an allele size range of 106-130bp). Consistently good amplification was obtained for these five loci and the allele sizes were all within the expected size range reported for other pinniped species. Although this 5-locus panel is at the lower size limit it still provides a means for preliminary assessment of population genetic structure of *Arctocephalus* from Marion Island and for detecting hybridization. An initial subset of samples was used to assess polymorphism and to optimise the reaction conditions, with all remaining untyped samples subsequently being genotyped using the panel of five polymorphic dinucleotide repeat microsatellite loci, detailed in Table 3.2.

3.2.3 Microsatellite PCR amplification and genotyping

QIAGEN® Multiplex PCR amplifications of the 5-locus microsatellite panel were performed in a final reaction volume of 7 µl containing 3.5 µl of Multiplex PCR Master Mix, 17.5 pM of each primer and 0.5µl of extracted DNA and supplier-provided

RNase-free water. Thermal cycling was carried out on a Perkin-Elmer thermocycler (model 1994) as follows: an initial denaturation step at 95ºC for 15 min to activate the polymerase enzyme, followed by 34 cycles at 94°C for 30s, annealing at 57ºC for 90s, extension at 72ºC for 60s, followed by a final, prolonged extension at 60ºC for 30min. Amplification products were visualized by electrophoresis on a 2 % agarose gel and all reactions producing faint or unclear bands were repeated. Selected amplified PCR fragments were titrated to determine the best dilution for each locus. For *A. tropicalis*, Lw 10 and Hg 8.10 were diluted 1 in 10 (1µl of each locus amplicon + 9µl of distilled water) and M11a, Pv9 and Hg 6.3 were diluted 1 in 50 (1µl of each locus amplicon + 49µl of distilled water). For *A. gazella*, only M11a was diluted 1 in 10 whilst Lw 10, Pv 9, Hg 8.10 and Hg 6.3 were diluted 1 in 25. One microlitre (1µl) of each of the diluted fragments was added to 10µl of 500LIZ™ formamide mix (14:1000). The samples were then denatured at 95ºC for 3 min before being run on an ABI P$_{RISM}$® 3100 Genetic Analizer (Applied Biosystems, Forster City, USA). The program GeneMapper™ Software version 3.0 (Applied Biosystems, Forster City, USA) was used to view and assign allele sizes to the peaks. All amplified PCR products were analysed in the same way.

Table 3.2: Primer sequences of the 5 polymorphic loci analysed, their fluorescent labels and expected allelic size range. Original citations of the sequences: *Allen *et al*. (1995), ** Hoelzel *et al.* (2001), and ***Davis *et al.* (2002).

Locus	Primer Sequence	Allelic Size Range	Label	Colour
Hg 6.3*	F: CAG GGG ACC TGA GTG CTT ATG	215-225	Ned	Yellow
	R: GAC CCA GCA TCA GAA CTC AAG			
Hg 8.10*	F: AAT TCT GAA GCA GCC CAA G	175-195	Pet	Red
	R: GAA TTC TTT TCT AGC ATA GGT TG			
M11a**	F: TGT TTC CCA GTT TTA CCA	141-151	Ned	Yellow
	R: TAC ATT CAC AAG GCT CAA			
Pv 9*	F: TAG TGT TTG GAA ATG AGT TGG CA	162-170	Vic	Green
	R: ACT GAT CCT TGT GAA TCC CAG C			
Lw 10***	F: AAC ACT AGC CCT GAC TTC	106-130	Pet	Red
	R: TTA CAG AGC AGG AGT TCA			

3.2.4 Methods of Analysis

3.2.4.1 Microsatellite descriptive statistics

A total of 77 individuals of *A. tropicalis* and 69 of *A. gazella* from Marion Island were used in the analyses**.** The MICRO-CHECKER program was used to check for genotype-scoring errors such as stutter bands, short allele dominance and null alleles (alleles not amplifying due to mutations in the PCR primer sites). In this program, the null allele frequencies are estimated and adjustment of allele and genotype frequencies of the amplified locus is performed prior to their utilization in other genetic analysis programmes. The calculations of the standard genetic variability statistics such as the number of alleles per locus, which provides a measure of allelic richness for both *A.*

tropicalis and *A. gazella*, were done in FSTAT version 2.9.3 (Goudet 2001). Genetix version 4.03 (Belkhir *et al*. 1997) was employed for calculating gene diversity, expected heterozygosity (H_E) and observed heterozygosity (H_O). Deviations from HWE for each locus were estimated by calculating F_{IS} (Weir & Cockerham 1984) in GENEPOP 3.1 (Raymond and Rousset 1995). Consistently large F_{IS} values across loci indicate the occurrence of inbreeding in a population (Pemberton *et al.* 1995). The genetic differentiation (F_{ST}) between the two species was also calculated in Genepop 3.1. Possible deviations from HWE were determined by calculating exact significance probabilities according to the procedure described by Louis and Dempster (1987) in GENEPOP 3.1 (Raymond and Rousset 1995). This software was also employed to determine linkage disequilibrium (non-random association between alleles) between pairs of loci with Fisher's exact test with the significance level set at 0.05. For the above two tests, the Markov chain method was used and the dememorization number set to 1000, the number of batches to 100 and the number of iterations per batch to 1000.

3.2.4.2 Hybrid identification

Because it is not easy to distinguish hybrid pups from pure *A. tropicalis* and *A. gazella* pups due to their similarity in colour and size (Condy 1978, Hofmeyr *et al.* 2006), genetic estimates of hybridization were attempted. The Bayesian clustering method executed in STRUCTURE version 2.0 (Prichard *et al*. 2000) was used to genetically detect inter-specific hybrids from the microsatellite data generated in this study. This Bayesian method assigns probabilities for individuals to have recent ancestry in two or more populations. The assignment of individuals to populations also solves the problem of bias that can potentially be introduced due to misidentifications based on physical appearance (phenotype). In the first run, the admixture model was adopted as it was thought that there might be hybridization occurring between the *A. gazella* and *A. tropicalis* populations, and the analysis was performed using the correlated frequency model. The alpha parameter (α), describing the rate of convergence of the Markov chain with an initial value of 1.0, was checked to ensure that burn-in and run lengths were sufficient. The allele frequency prior was set to one. Q (as an estimated membership coefficient for an individual in each cluster) was utilized as a metric of individual assignments and their emergent characteristics. A q-value of 0.10 was set as

recommended by Vähä and Primmer (2006) as the efficient threshold to differentiate purebred individuals from hybrid individuals. Under these conditions, individuals having a q-value greater than 90 % (q > 0.9) fall under the presumed parental population and individuals with q < 0.9 are classified as putative hybrids (Vähä and Primmer 2006). In the second run, the POPINFO option was flagged for the reference individuals. This prior population information model reveals whether an individual is an immigrant or has immigrant ancestry in recent generations (Prichard *et al*. 2000). The model reveals if the individuals have the possibility of being in the assumed population whilst having ancestry in the other populations. In this run, three different intergroup migration rates were assumed, namely 0.01, 0.05 and 0.1 and the length of burn-in was set at 100 000 for all the migration rates considered. The number of MCMC replicates after burn-in was 500 000 for migration rates (ν) 0.01 and 0.1, and 1 000 000 for 0.05. The reason for using different migration rates was to assess the accuracy and consistency of the results. Before the runs, the K value was set to 2 for each species to be represented and three iterations were run. Different analyses were done to evaluate the consistency of results, as suggested by Prichard *et al*. (2000).

3.3 RESULTS

3.3.1 Conformance to Hardy-Weinberg equilibrium (HWE)

Microsatellites alleles were scored for 5 loci: Hg 6.3, Hg 8.10, M11a, Pv9, and Lw10. Using the Microchecker program, it was found that none of the loci displayed evidence of null alleles and that mis-scoring due either to stutter bands or short allele dominance appears not to be a factor for concern (results not shown). The number of individuals analysed and deviations due to heterozygote deficit (F_{IS}) for each locus within *A. tropicalis* and *A. gazella* populations are provided in Table 3.3. The overall inbreeding coefficients (F_{IS}) of *A. tropicalis* and *A. gazella* were identical (0.029 each) and were not significant (P=0.138, Wilcoxon matched-pair test). In the *A. tropicalis* population, a per-locus analysis indicated no deviation from HWE due to heterozygote deficit (Table 3.3). However, in the *A. gazella* population one locus (M11a), whilst not deviating from HWE did show a significant heterozygote deficit (P=0.001), due either to selection or inbreeding or possibly due to null alleles. The latter is probable as the microsatellite primers used in this study were not *Arctocephalus*-specific (Table 3.3).

3.3.2 Linkage disequilibrium

No pairwise linkage disequilibrium (LD) among the 5 loci was observed for either the *A. tropicalis* or the *A. gazella* populations (Table 3.4), confirming that there is no non-random association of alleles and that independent assortment occurs across all loci. For this reason, all loci were retained for subsequent analyses.

Table 3.3: Single-locus evaluation of heterozygote deficit (F_{IS}) in the 69 *A. tropicalis* and 77 *A. gazella* individuals sampled from Marion Island. The complete enumeration method (Louis and Dempster 1987) was used to calculate an exact P-value for the five loci. Significant p-values (denoted by a *) indicate a heterozygote deficit, whilst HWE (P) indicates the unbiased estimates of Hardy-Weinberg Equilibrium exact P-values for each locus.

	***A. tropicalis* (n=69)**			***A. gazella* (n=77)**		
Locus	**F_{IS}**	**(P- value)**	**HWE (P)**	**F_{IS}**	**(P-value)**	**HWE (P)**
Hg 6.3	-0.03	0.576	0.77	-0.02	0.379	0.31
Hg 8.10	0.00	0.110	0.12	0.08	0.354	0.41
M11a	-0.06	0.977	0.61	0.07	**0.001***	0.13
Pv9	0.01	0.549	0.90	-0.03	0.622	0.26
Lw10	-0.06	0.827	0.35	0.06	0.1302	0.22
Overall	**-0.029**			**0.029**		

3.3.3 Genetic differentiation

The F_{ST} between *A. tropicalis* and *A. gazella* populations was 0.097 (P<0.001). This F_{ST} value indicates that despite very few loci being characterized, the 5 loci used in this study were able to uncover moderate levels of genetic differentiation between the species indicating that they may be useful for detecting hybridisation.

3.3.4 Microsatellite descriptive statistics and variability

The number of alleles, number and frequency of unique alleles, observed heterozygosity (H_O) and expected heterozygosity (H_E) of *A. tropicalis* and *A.gazella* are summarized in

Table 3.5 and 3.6, respectively. In *A. tropicalis*, all loci were polymorphic with a mean of 13.6 alleles (range: 8-21) per locus and an H_O value of 0.875 (range, 0.768-0.926). In *A. gazella*, the mean number of alleles was 13 (range: 4-21) with an H_O value of 0.781 (range, 0.455-0.870) (Tables 3.5 and 3.6). The number of alleles present for the polymorphic loci and their frequencies are shown graphically in Fig. 3.2. The most variable locus in *A. tropicalis* was Hg 8.10 with 21 alleles and the least variable was Pv9 with 8 alleles. In *A. gazella*, the most variable locus was M11a with 21 alleles and the least variable was Hg 8.10 with just 4 alleles. The average genetic diversity across loci was high, with a mean value of 0.845 (Table 3.5) and 0.799 (Table 3.6) for *A. tropicalis* and *A. gazella*, respectively. The ratio of observed/expected heterozygosity (H_O/H_E) for *A. tropicalis* was 0.875/0.845 (1.036) and higher than that of *A. gazella* at 0.781/0.799 (0.977). A mean of 4.8 unique alleles were observed in *A. tropicalis* and a mean of 4.2 in *A. gazella*.

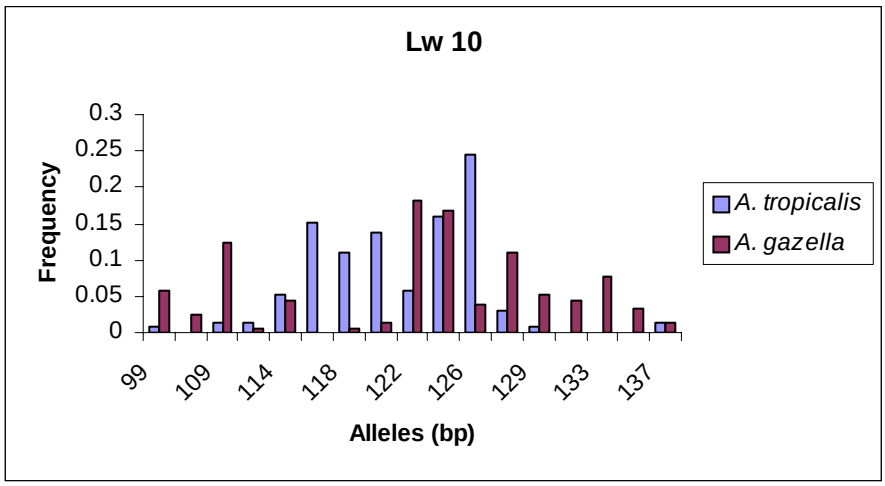
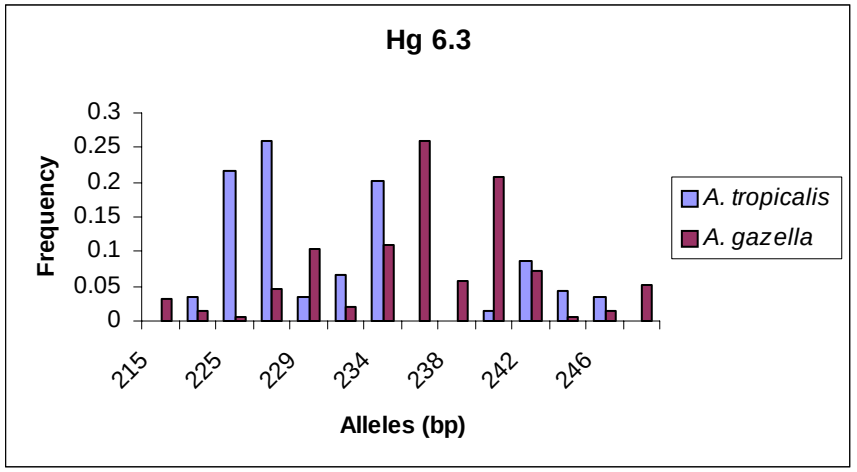
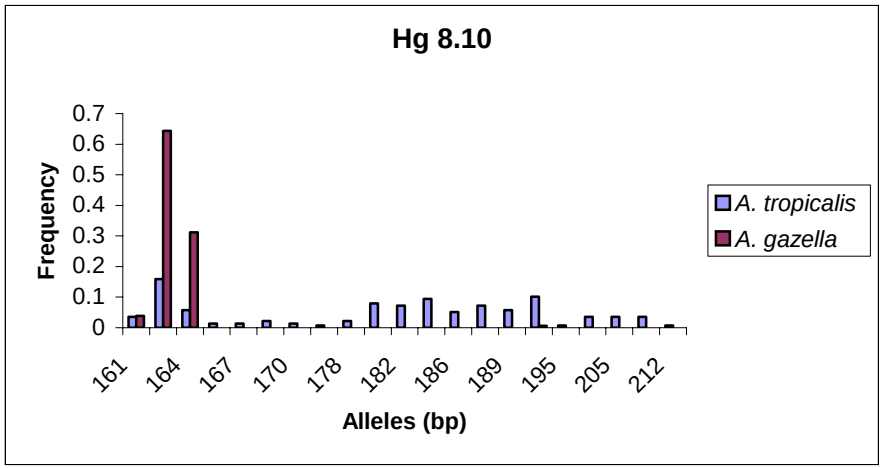
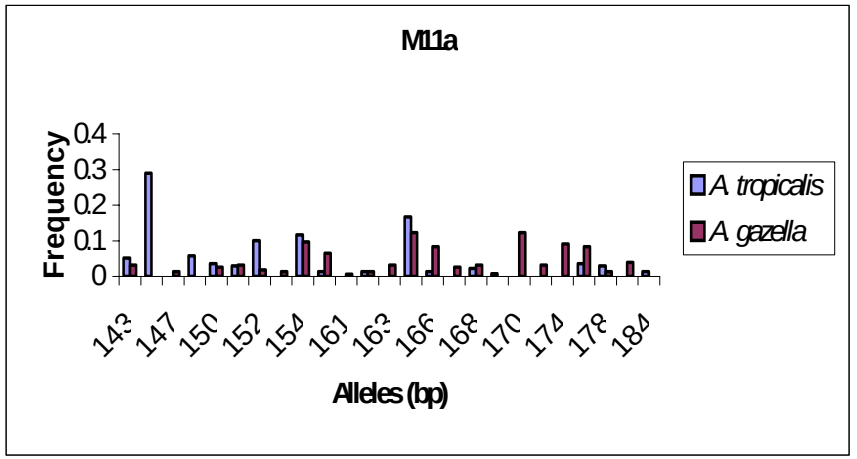


Fig. 3.2: Allele frequencies of the five polymorphic loci in *A. tropicalis* and *A. gazella* from Marion Island (Lw10, this page, and Hg 6.3, Hg 8.10, M11a, Pv9 on the next page).


Hg 6.3
Frequency
0.3
0.25
0.2
0.15
0.1
0.05
0
215
225
229
234
238
242
246
Alleles (bp)
A. tropicalis
A. gazella


Hg 8.10
Frequency
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
161
164
167
170
178
182
186
189
195
205
212
Alleles (bp)
A. tropicalis
A. gazella


M11a
Frequency
0.4
0.3
0.2
0.1
0
Alleles (bp)
A tropicalis
A gazella

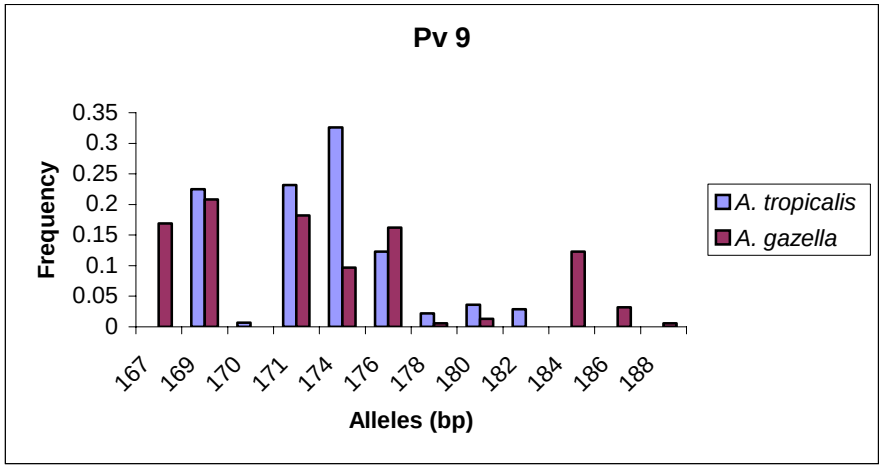

Pv 9
Frequency
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
167
169
170
171
174
176
178
180
182
184
186
188
Alleles (bp)
A. tropicalis
A. gazella

Table 3.4: Probabilities of genotypic linkage disequilibrium (LD) from pairwise comparisons of the five loci characterised for the *A. tropicalis* and *A. gazella* Marion Island populations, as calculated in GENEPOP.

Population	Locus Pair	P-value	S.E
A. tropicalis	Hg8.10 & Hg6.3	0.140	0.034
A. tropicalis	Hg8.10 & Pv9	0.118	0.030
A. tropicalis	Hg6.3 & Pv9	0.360	0.042
A. tropicalis	Hg8.10 & M11a	0.494	0.050
A. tropicalis	Hg6.3 & M11a	0.131	0.032
A. tropicalis	Pv9 & M11a	0.411	0.045
A. tropicalis	Hg8.10 & LW10	0.240	0.042
A. tropicalis	Hg6.3 & LW10	0.093	0.028
A. tropicalis	Pv9 & LW10	0.756	0.038
A. tropicalis	M11a & LW10	1.000	0.000
A. gazella	Hg8.10 & Hg6.3	0.052	0.015
A. gazella	Hg8.10 & Pv9	0.602	0.030
A. gazella	Hg6.3 & Pv9	0.568	0.048
A. gazella	Hg8.10 & M11a	0.483	0.038
A. gazella	Hg6.3 & M11a	1.000	0.000
A. gazella	Pv9 & M11a	1.000	0.000
A. gazella	Hg8.10 & LW10	0.841	0.028
A. gazella	Hg6.3 & LW10	0.184	0.037
A. gazella	Pv9 & LW10	0.200	0.038
A. gazella	M11a & LW10	0.213	0.041

S.E.: Standard Error

Table 3.5: Number of alleles (K), frequencies of unique alleles (X_i), gene diversity expressed as allelic richness (Rs), Expected heterozygosity (H_E) and observed heterozygosity (H_O) in *A. tropicalis* for 5 microsatellite loci.

Locus	K	No. of unique alleles	X_i	Allelic richness (R_S)	H_E	H_O
Hg 6.3	10	0	0.000	13.771	0.826	0.855
Hg 8.10	21	17	0.645	3.896	0.922	0.926
M11a	16	4	0.370	20.854	0.853	0.913
Pv 9	8	2	0.036	9.782	0.772	0.768
Lw 10	13	1	0.152	15.772	0.852	0.913
Mean	13.6	4.8	0.241	12.815	0.845	0.875

Table 3.6: Number of alleles (K), frequency of unique alleles (X_i), gene diversity expressed as allelic richness (Rs), expected heterozygosity (H_E) and observed heterozygosity (H_O) in *A. gazella* for 5 microsatellite loci.

Locus	K	No. of unique alleles	X_i	Allelic richness (R_S)	H_E	H_O
Hg 6.3	14	4	0.403	13.920	0.851	0.870
Hg 8.10	4	0	0.000	18.156	0.488	0.455
M11a	21	9	0.377	22.824	0.924	0.870
Pv 9	10	4	0.331	10.743	0.843	0.870
Lw 10	16	4	0.182	16.648	0.891	0.844
Mean	13	4.2	0.259	16.458	0.799	0.781

3.3.5 Hybrid identification using assignment tests

In the first run, where an admixture model was used, almost every individual fell into its phenotypically assigned population, namely either *A. tropicalis* or *A. gazella*, with high probabilities (*A. tropicalis*: mean Q=0.982, range 0.927-0.998 and *A. gazella*: mean Q= 0.983, range 0.902-0.998) (Fig. 3.2). Only one hybrid out of a total of 146 *A. tropicalis* and *A. gazella* individuals screened was detected. This corresponds to a hybrid incidence of 0.68 %**.** This individual was recorded as an *A. gazella* in the field, based on external morphology and sampling locality, but the STRUCTURE program redefined it as a hybrid (Fig. 3.3) based on the relative genetic contribution of each species. This

individual had a q-value of 0.324 whilst the remaining individuals had q-values of 0.9 or more, corresponding to a 90 % or greater probability of belonging to their putative species.

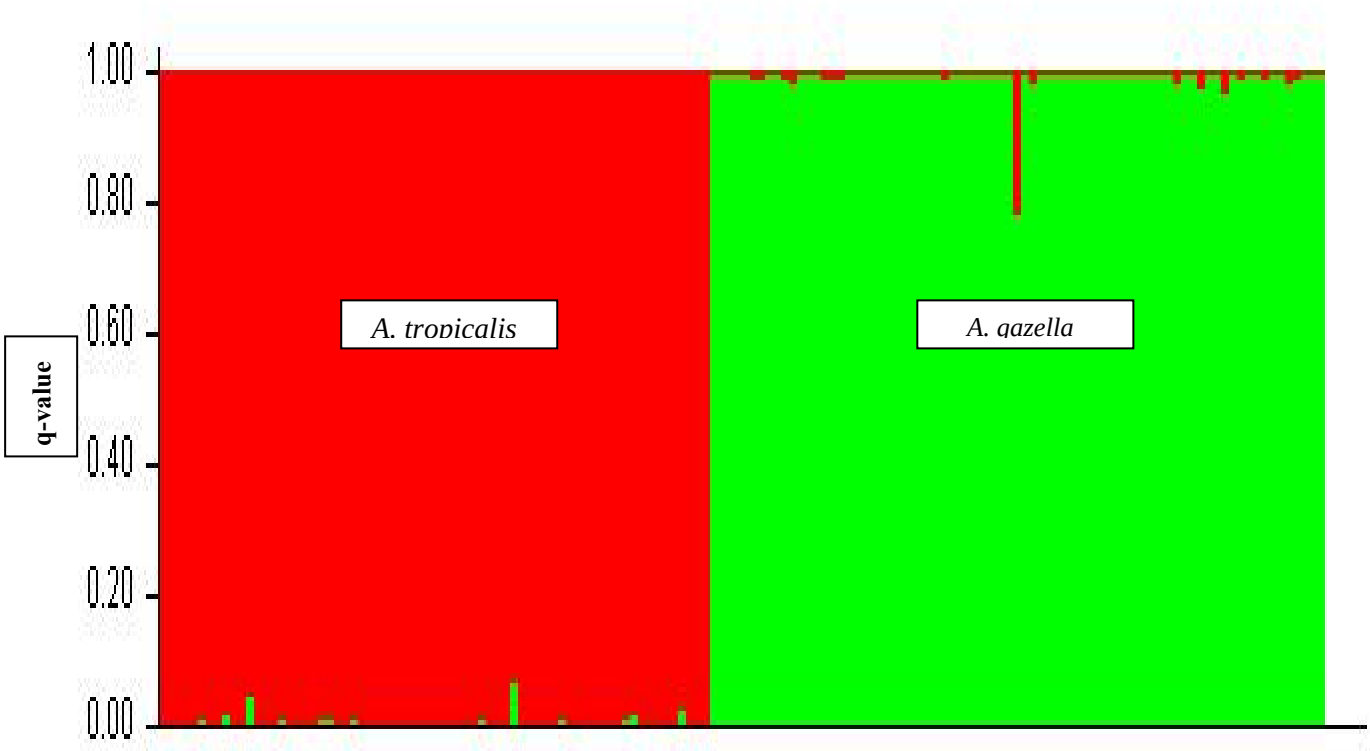


Fig. 3.3: Assignment of all pup individuals of *A. tropicalis* (Red area) and *A. gazella* (Green area) from different beaches in Marion Island to populations by STRUCTURE (K=2). Each bar represents an individual fur seal.

In the second run, where the program was run with prior population information model in STRUCTURE, migration rate (ν) was the most sensitive variable to assess, as a strong conclusion about immigrant ancestry cannot be drawn before considering different migration rates. The different migration rates gave different results, indicating that the amount of information in the data set was insufficient to make a decision (Prichard *et al*. 2000). The migration rate (ν) 0.05 worked best as it was able to detect the hybrid individual (indicated with an asterisk *, Fig. 2.4 and Fig 2.6) indentified in Chapter 2 on the basis of a conflicting morphological-genetic species designation. This migration rate also provided better results in a recent study on hybridization between the two species of fur seals (*A. tropicalis* and *A. gazella*) breeding sympatrically at Iles Crozet (Kingston and Gwilliam 2007). The hybrid with a physical appearance of *A. gazella* showed that it only has a 32 % likelihood that it comes from the *A. gazella* population but a 0 % probability of belonging to the *A. tropicalis* population. Approximately 80 % of the hybrid's nuclear markers were *A. gazella* specific with the remaining 20 % being characteristics of *A. tropicalis* (Fig. 3.3). This indicates that this individual most likely had a single contribution from an *A. tropicalis* ancestor, with the probability of that ancestor being the parent or grandparent, being 0.192 (19 %) and 0.484 (48 %), respectively.

3.4 DISCUSSION

3.4.1 Microsatellite variation

High levels of polymorphism were recovered at the five nuclear microsatellite loci used in this study. The departures from HWE were not significant for all loci in both species but a single locus, M11a, in the *A. gazella* population indicated heterozygote deficiency ($F_{IS,}$ 0.07: P= 0.001). This suggests that this particular locus may be under some sort of selective pressure (Burland 1998, Goodman 1998). Null alleles are also a possibility; however, the MICRO-CHECKER program did not uncover any evidence of null alleles. This software indicates the possiblility of genotyping errors (mainly arising from mis-scoring of stutters), which are subsequently adjusted (removed or rectified) on the basis of allelic and genotypic frequencies of the population, prior to further population genetics analyses.

3.4.2 Genetic diversity

Arctocephalus tropicalis and *A. gazella* are among the pinniped species that experienced a recent population bottleneck due to human exploitation. Population bottlenecks lead to a reduction or loss of genetic variation by sub-sampling of alleles in the population (Hart 1987). Genetic variation in *A. tropicalis* was higher than that observed for *A. gazella* in terms of total number of alleles detected and the level of heterozygosity (Tables 3.5 and 3.6). The reason for this may be because *A. gazella* experienced more intensive harvesting than *A. tropicalis* during the 18th and 19th century (Bonner and Laws 1964). In fact, this species was so heavily exploited that it is believed to have become locally extinct on some islands south of the APF, following sealing (Bonner and Laws 1964) but not at Marion island (north of APF) which was likely out of its distributional range. Habitat preference most likely contributed to the more intensive exploitation as *A. gazella* prefers to breed in open vegetation areas that are easily accessible to sealers (Bester 1982) while *A. tropicalis* prefer to breed in rocky areas (Condy 1978, King 1983, Kerley 1984). The differences in breeding site preference also influences the level of genetic differentiation, as these congenerics have an F_{ST} value of 0.097, which indicates that they exhibit moderate levels of genetic differentiation. In contrast, *A. gazella* had a higher allelic richness than *A. tropicalis*. Estimation of allelic richness is difficult among populations when the sample size is not equal (Leberg 2002). The reason underlying *A. gazella* having a higher allelic richness than *A. tropicalis* may be due to the slightly larger sample size of 77 individuals versus the 69 *A. tropicalis* individuals as well as differences in sampling intensity and the number of sites sampled per species. For *A. tropicalis*, more sites

(7) were sampled (versus 4 for *A. gazella*), making it tempting to speculate that the *A. gazella* higher allelic richness is possibly due to migrants from multiple genetically differentiated islands, rather than migrants from a single source population. However, whilst some studies observed that there is a proportional relationship between allelic richness and sample size, the exact cause for this is not clearly understood (Sjogren and Wyoni 1994, Petit *et al*. 1998, Haavie *et al*. 2000).

Departure from HW proportions is suggestive of amongst others, non-random mating (inbreeding), selection, mutation, migration or the presence of population substructure, all of which lead to Wahlund's effect, or to the presence of null alleles which can also underlie a departure from Hardy-Weinberg expectations (Goodman 1998). The use of microsatellites has increased due to their codominant inheritance and highly polymorphic nature, however, the occurrence of null alleles is one disadvantage since misidentification of alleles can lead to false estimations (Liewlaksaneeyanawin *et al*. 2002). Any microsatellite allele at a locus that fails to amplify to detectable levels through PCR is defined as a null allele. (Dakin and Avise 2004). In *A. tropicalis*, no large inbreeding coefficient (F_{IS}) values were obtained at any one locus. The reflection of heterozygote deficit in M11a in *A. gazella* by a higher F_{IS} value (0.07, p=0.001) might mean that the locus is reflecting the effects of inbreeding. Furthermore the heterozygote deficit is also reflected by a positive F_{IS} value, which may indicate that the locus is under selection. From the observation of F_{IS} values at the other loci, the values were not consistently large or significantly different from zero, suggesting that the heterozygote deficiency is unlikely to be the result of the presence of null alleles. F_{IS} can be used as an indicator of breeding strategies (Chesser 1991 a, b). High values of F_{IS} indicate highly polygynous mating systems (Pope 1992). *A. gazella* is an otariid described behaviourally as a polygynous animal (Bartholomew 1970), where one male defends a territory and mates with many females (Bonner 1968). Territorial males father many pups (Hoffman *et al*. 2003). Genetic structure and mating systems of a species may be affected by extreme site fidelity (Hoffman *et al*. 2006). Most seals, including *A. gazella*, usually return to territories held in previous seasons (Gentry 1998). In mammals, females generally show stronger site fidelity than males (Haley 1994). However, in contrast to this general trend, *A. gazella* males show stronger site fidelity than females (Hoffman *et al*. 2006). Strong fidelity together with natal philopatry can lead to higher levels of homozygosity due to the inbreeding (Hoffman *et al*. 2004) and to reduced fitness.

3.4.3 Hybridization

The two species prefer different areas on Marion Island (Condy 1978, King 1983, Hofmeyr *et al*. 1997, Kerley 1984) and Iles Crozet (Jouventin & Weimerskirch 1990, Guinet *et al*. 1994) and that leads to levels of hybridization between species being low on both islands. At Iles Crozet, hybridization was estimated to be approximately 1 % (Kingston and Gwilliam 2007) while at Marion only small numbers of hybrids were observed based on phenotypic appearance (Hofmeyr *et al*. 2006, Kerley and Robinson 1987). Nevertheless, at Macquarie Island both species haul-out at the same breeding territories resulting in the level of hybridization being high (Shaughnessy 1993) with hybrids comprising as much as 25 % of all pups born each year (Goldsworthy *et al*. 1999). The high level of hybridization at Macquarie Island was also confirmed by Lancaster *et al*. (2006) where hybridization occurred between *A. tropicalis*, *A. gazella* and *A. forsteri* with an estimated 17 to 30 % of all pups being hybrids. No hybridization has been reported from Heard Island due most likely to its recent colonization by *A. tropicalis* (Goldsworthy and Shaughnessy 1989).

From the results obtained in this study, it was confirmed genetically that hybridization levels between *Arctocephalus* species at the Marion Island, part of the Prince Edward Island group, is low, which is in accordance with previous reports estimating the proportion of hybrids to be 0.02 % (Hofmeyr *et al*. 1997). In this study, a male hybrid pup that was classified genetically as *A. gazella* was sampled at a beach dominated by *A. tropicalis*, at Sealer's Cave. The beach at Sealer's Cave is one of the beaches where putative hybrid pups have previously been observed but where definitive classification was uncertain (Hofmeyr *et al*. 2006). Hybridization between *A. tropicalis* and *A. gazella* at Marion Island is set to continue at a slow pace because there are very few locations where the two species breed sympatrically. For most of the breeding beaches they occur allopatrically, and hybrids have only been observed at the following sites: Cape Davis, Good Hope Bay and Sealer's Cave (Hofmeyr *et al*. 2006). On Macquarie Island, the extensive hybridization is suggested to be recent and have been caused by human impacts. However, a decline in the proportion of hybrids over time is expected as it is recovering from human disturbances meaning that there will be reduced migration of hybrid lineages into allopatric populations (Lancaster *et al*. 2006).

3.4.4 Breeding strategies

In addition to spatial separation arising from difference in habitat preference, the two species also have temporal variation in breeding cycles (Kerley 1983a). At sites where the two species occur sympatrically, adult males of both species often compete for breeding territories in order to secure harems. *Arctocephalus gazella* females haul-out first and mate before females of *A. tropicalis*. However, as *A. tropicalis* females normally arrive to mate on shore before the female *A. gazella* return to sea, there is a short temporal overlap in their beach presence and therefore also an opportunity for hybridization with males of the other species. If pregnant *A. tropicalis* arrive, bear young and then come into oestrus, females of both species may be in oestrus at the same time. Due to short temporal overlap on the beaches, it is possible that a displaced *A. tropicalis* female might become incorporated within the harem of a male *A. gazella*.

The lactation strategies of the two species also differ significantly. *Arctocephalus gazella* suckle their offspring for about 4 months, while offspring of *A. tropicalis* suckle for approximately 10 to 11 months (Kerley 1983a, Doidge *et al*. 1986, Bester 1987, Goldsworthy 1992). The premature weaning of hybrid pups of *A. gazella* mothers are therefore likely to be more adversely affected than true-bred *A. gazella* pups, leading to higher pup mortality and a reduced number of hybrids reaching adulthood (Kerley 1985). This in turn would explain the low levels of observed hybrids which are only readily detected in adult animals. However, it has been reported that under favourable environmental conditions the F_1 hybrid individuals can experience high fitness (Grant and Grant 1992, 1998, Lancaster *et al*. 2007).

3.4.5 Habitat Preference

The difference in their habitat preference is perhaps the greatest potential barrier to hybridization on Marion Island. The breeding sites of the two species are separated from one another topographically and geographically and that limits their contact in times of mating and as a result interbreeding is reduced (Hofmeyr *et al*. 1997). *Arctocephalus tropicalis* breed in rocky areas while *A. gazella* prefer vegetated areas behind the landing beaches. However, hybridization is likely to occur on open rocky beaches where their breeding sites overlap (Condy 1978, Kerley 1984, King 1983, Bester 1982).

3.4.6 Species mate-recognition

Species recognition through vocalization is among the factors that play a role when considering the possibility of hybridization between two closely related species breeding sympatrically. The calls of the two species differ (St Clair Hill *et al*. 2001) with *A. tropicalis* males having a louder and lower frequency bark than *A. gazella* males (Page *et al*. 2002). It is expected that the females of each species could utilize these inter-specific differences in male calls to avoid hybridisation (St Clair Hill *et al*. 2001), however, vocalization is not a strong deterrent as hybridisation levels at some islands such as Macquarie Island (Lancaster *et al*. 2006) are very high. Call differences between the two species only correlates the phylogenetic distance between them (Page *et al*. 2002).

The *A. gazella* individual detected by analysis of the microsatellite data as a hybrid was also shown to be a hybrid in the mitochondrial DNA (mtDNA) data analysis (Chapter 2). This individual that was identified as *A. gazella* phenotypically had a maternally inherited mtDNA D-loop region characteristic of that of *A. tropicalis*. According to microsatellite data analyses, this individual contains approximately 20 %: 80 % *A. tropicalis*: *A. gazella* genetic material. Together with the higher probability of a genetic contribution from an *A. tropicalis* grandparent (versus that of a parent), and the strict maternal inheritance of mtDNA, these results are strongly suggestive of an *A. tropicalis* matrilineal grandparent. Hybridization and introgression between species may occur more often than currently recognised (Allendorf and Luikart 2006). Hybridization between species is a natural phenomenon caused by evolutionary processes, however, it can also occur due to anthropogenic disturbances (human-related activities) (Anderson and Hubricht 1938, Seehausen 2004) e.g. habitat modifications and human mediated introductions of non-native taxa (Rhymer and Simberloff 1996).

Current mixing has been recorded for a number of taxa that were historically isolated and is increasing due to human disturbances in recent decades (Allendorf *et al*. 2001, Olden *et al*. 2004). The modification of patterns of water flows may also bring species that were previously isolated geographically into contact and many independent lineages are able to hybridize and exchange genes (introgression) for quite a long time without changing phenotypically (Bush 1994). This might be due to the fact that the exchange of genes between species is often unequal, with gene transfer predominantly from the more common to the more rare species (Dowling *et al*. 1989, Taylor and Herbet 1993, Wayne 1993). It is therefore important to have genetic tools for detecting hybridization at hand. Whilst the number of loci

used in this study were limited to five, the value of these markers for uncovering back-crossed hybrids was clearly demonstrated and should be expanded upon in future.

CHAPTER 4
CONCLUSIONS AND FUTURE PROSPECTS

The loss of genetic variability is unfavourable to a population that needs to adapt or respond to environmental change (Frankel and Soule 1981, Lacy 1987, Lynch *et al*. 1995, Waldick *et al.* 2002). Low levels of genetic variability have been associated with low fitness in populations (Keller *et al*. 1994, Madsen *et al*. 1996, Pastor *et al*. 2004) and loss of genetic variation is expected to occur whenever a population goes through a bottleneck. Subantarctic and Antarctic fur seals (*A. tropicalis* and *A. gazella*) were among the seal species which underwent human-induced population reductions due to the extensive and indiscriminate exploitation that occurred during the 18th and 19th century. The current study aimed to estimate the genetic variability for both populations of these species on Marion Island using both mtDNA and microsatellite markers. Both markers revealed that the genetic diversities of the two species are among the highest diversities recorded for pinniped species formerly exploited by sealers. For the mtDNA control region characterised, the nucleotide diversities were 4,2 % and 2,9 % for *A. tropicalis* and *A. gazella*, respectively. For microsatellite DNA, genetic variation in *A. tropicalis* was higher than in *A. gazella* in terms of the total number of alleles detected and the level of heterozygosity (H_E=0.875/H_O=0.845, mean number of alleles=13.6 and H_E=0.799/H_O=0.781, mean number of alleles=13, respectively) for the five-locus panel used in this study. This suggests that sealing may not have overly affected the level of genetic variation of these species or the two species might not have undergone as severe a population decrease in their areas of distribution as anticipated from historical records. Alternatively, the level of genetic diversity is high because the two species are recovering rapidly from sealing, showing high population growth rate (Hofmeyr *et al*. 2006). This is in contrast to other species such as the Northern elephant seals (*M. angustirostris*), for which only a few haplotypes (or as few as 10 individuals) are believed to have survived (Hoelzel *et al*. 1993). The Marion Island study also confirmed previous postulations based on a small number of individuals from this island, namely that *A. gazella* have lower genetic variability and exhibit reduced population structure compared to *A. tropicalis* due to the more intensive exploitation of the former species (Wynen *et al*. 2000). Habitat preference is likely to have contributed to this as *A. gazella* prefer open vegetation areas that provide easier access to sealers, instead of the more inaccessible rocky areas and caves where *A. tropicalis* prefer to breed (Condy 1978). Theoretically, when the level of variation is low, it may lead to extinction of a species- the "central dogma of conservation genetics" (meaning that genetic

variability should be of primary concern because it is beneficial) (Lehman 1998, Pertoldi *et al*. 2007). An increase in genetic variability improves the probability of survival of a population (Frankham 2005). However, many species, in particular marine mammals, are doing well in terms of population growth and survival of pups, despite the recent, documented population reduction and environmental changes (inclusive of anthropogenic modifications and resource withdrawal) (Weber *et al*. 2004). *A. tropicalis* and *A. gazella* populations are recovering rapidly from the sealing bottleneck (Wynen *et al*. 2000). In contrast, populations that recover slowly often experience a decrease in fitness due to low heterozygosity (Miller and Hendrick 2001).

In terms of population structure, using mtDNA only, data from different islands were used to construct phylogenetic trees. For the *A. tropicalis* analyses, individuals from Marion and Gough islands, Iles Crozet, and Ile Amsterdam were included, with sequences from Iles Crozet and Ile Amsterdam being limited to just five sequences that are presently available on the Genbank database. The *A. tropicalis* tree revealed three divergent lineages indicating historical/evolutionary differentiation for this species as a whole. All lineages comprised individuals from Marion and Gough islands and that indicates migration between the two islands, but there was only one haplotype shared between these two islands. These observed between-island links were facilitated by the availability of a large number of sequences from both islands, whereas the estimates of migration and geneflow between the other islands may have been underestimated due to the limited number of sequences available from Iles Crozet and Ile Amsterdam. High structure (Φ_{ST}; 0.320) was seen between the Marion and Gough populations and the species at the two islands were moderately genetically different (F_{ST}; 0.168). For *A. gazella*, the phylogenetic tree inferred with data from two islands (Marion and Bouvetøya) revealed that individuals from these two islands share common ancestry based on the high levels of bootstrap support for the lineages containing individuals from these islands. Recent migration between the two islands was indicated by the high number of shared haplotypes, with Bouvetøya being the source of immigrants to Marion Island (Wynen *et al*. 2000) because *A. gazella* survived in remnant populations at Bouvetøya following sealing. This suggests that there is no difference between the populations at the two islands, which was confirmed by an F_{ST} of 0.062. Being more intensively harvested, *A. gazella* revealed lower levels of structure (Φ_{ST}; 0.082) between the two islands.

Hybridization can be underestimated if species are only classified as hybrids in the field without assessing them genetically. Gene exchange can occur between two species without any distinct morphological intermediates being observed, due to selection operating against species with intermediate morphology (Chiba 1998). Both markers (mtDNA and microsatellite DNA) were used to estimate hybridization between both species (*A. tropicalis* and *A. gazella*) at Marion Island. With the mtDNA marker, it was found that hybridization was low at Marion Island with only one hybrid out of 134 individuals classified phenotypically as *A. gazella* being shown genotypically to have *A. tropicalis* maternal ancestry. This corresponds to just 0.75 %, but is likely to be an underestimate as the samples in this study were primarily collected from sites where the two species do not co-occur, and a hybrid would only be indicated where there was conflict between the phenotype and maternal genotype, due to the strict maternal inheritance of mtDNA. Microsatellites confirmed that the same hybrid individual was indeed a hybrid, with most of the nuclear genetic material being contributed by *A. gazella* and less by *A. tropicalis*, indicative of this individual most likely arising from a cross between an *A. gazella* male and a hybrid female. As more samples were typed with the microsatellite panel (146 individuals versus 134 for the mtDNA component), this corresponds to an even lower level of introgression, namely 0.68 %.

The impacts of hybridization differ from case to case, and in some instances, sufficient introgression occurs to change entire taxa (Dowling and Secor 1997, Lehman *et al*. 1991, Roy *et al*. 1994, Wayne and Jenks 1991). This is clearly not of concern at Marion Island. Despite hybridization being possible, there is strong assortative mating at Marion Island due to the fact that there are more allopatric sites than sympatric sites. In addition, there are differences in ecological aspects such as habitat preferences, breeding strategies including lactating strategies, seasonal cycles and vocalizations (calls). All of these factors may explain the notably lower levels of hybridization recorded at Marion Island (Hofmeyr *et al*. 2006), compared to that reported for other islands where the two species co-occur (Lancaster *et al*. 2006, Kingston and Gwilliam 2007). Furthermore, low levels of introgression and hybrids together with the genetic differentiation (0.097, $P<0.001$), behavioural and phenotypic differences between the two species suggests that hybridization will most likely remain rare at this sub-Antarctic island, at least at those sites that were included in this initial genetic evaluation.

FUTURE PROSPECTS

This study has made a valuable contribution in providing the first genetic assessment of the level of hybridization between fur seals at Marion Island. The sampling at localities where the two fur seal species co-occur on the island would give a better idea of the true level of hybridization and may yield higher estimates. Further studies on the viability and fitness levels of the first generation hybrids would provide a platform for understanding the implications of hybridization between the two species. The current study sampled pups only. Sampling subadult and adult fur seals may also assist in improving hybridization estimates because it is easier to identify the hybrids based on physical appearance. This is not easily achieved with the pups because they are very difficult to distinguish. In addition, the use of a larger panel of microsatellites that is standardized across all sub-Antarctic islands where *Arctocephalus* species co-occur is needed in order to obtain better estimates of hybridization.

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