THE USE OF AFLP MARKERS FOR ESTIMATING

RELATEDNESS AND INBREEDING

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Ph.D. dissertation
This dissertation is my own work and contains nothing which is the outcome of work done in collaboration with others, except as specified in the text and Acknowledgements. No part of this dissertation has been or is currently being submitted for a degree or diploma or any other qualification at any other university.
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SUMMARY

Inbreeding depression has been demonstrated in many species and is an important theme in conservation genetics. A detailed analysis of factors affecting fitness in captive Sumatran tigers was conducted and this revealed that although maternal inbreeding, calculated from the pedigree, negatively affected the fitness of litters, in this species the influence of maternal identity was much greater, with some females being consistently better than others at rearing litters. These results are different to those of a previous study that carried out a less rigorous analysis and reported evidence for the purging of deleterious alleles in the Sumatran tiger.

Although individuals’ inbreeding coefficients and the relatedness coefficients between individuals can be calculated from pedigrees, such pedigrees are not available in most natural populations. Where pedigrees are absent, molecular markers such as microsatellite loci have been used to estimate individuals’ levels of inbreeding and relatedness between individuals. In this thesis, a novel method for estimating levels of inbreeding using amplified fragment length polymorphisms (AFLPs) is described. These AFLP-based estimates of inbreeding correlate strongly with known inbreeding coefficients in captive-bred population of old-field mice (Peromyscus polionotus). At low levels of inbreeding in this species, for similar amounts of genotyping effort, the AFLP measure appears to give better estimates of inbreeding compared to estimates of inbreeding obtained from microsatellite markers. In contrast, for similar genotyping effort, microsatellite loci appear to give better estimates of pairwise relatedness compared to AFLP markers.

AFLP and microsatellite markers were used to detect heterozygosity-fitness correlations (HFCs) in four different species. In the house sparrow (Passer domesticus) and the common mallard (Anas
platyrhinos) HFCs were detected using AFLP markers but not with microsatellites, while the reverse was true in the California sea lion (Zalophus californianus). Both AFLP and microsatellite markers detected the same HFC in old-field mice (Peromyscus polionotus). These are the first cases where HFCs have been uncovered using AFLP markers.

AFLP markers were also used to examine phylogenetic relationships within the pinnipeds. There was broad agreement between the AFLP phylogeny and existing phylogenies based on mitochondrial DNA. While some relationships could be determined between Phocid species, the phylogenetic signal was not strong enough to establish relationships between Otariid species. This study shows the utility of AFLP markers at discerning the relatedness between species that have diverged over a time scale of 1-25 million years ago.

The findings reported in this thesis demonstrate that AFLPs are useful markers for revealing both short and longer-term patterns of evolution. Their use in estimating individuals’ levels of inbreeding, relatedness between individuals and phylogenetic reconstruction show their versatility, and illustrate that they are an important tool in conservation genetics as well as other areas of biology.
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ABBREVIATIONS

AFLP  amplified fragment length polymorphism
bp    base pairs
Ci    Ciuries
df    degrees of freedom
dATP  deoxy-adenosine triphosphate
dCTP  deoxy-cytidine triphosphate
dTTP  deoxy-thymidine triphosphate
dGTP  deoxy-guanosine triphosphate
DNA   deoxyribonucleic acid
EDTA  ethylenediamine tetra acetic acid
g    grams
GLM   general linear model
HCl   hydrochloric acid
HFC   heterozygosity-fitness correlation
HWE   Hardy-Weinberg equilibrium
IR    internal relatedness
KCl   potassium chloride
l    litre
m    milli
mtDNA mitochondrial DNA
M    molar
MgCl\(_2\) magnesium chloride
n    nano
p    pico
PCR   polymerise chain reaction
rpm   revolutions per minute
s    seconds
SDS   sodium dodecyl sulphate
Tris  2-amino-2-(hydroxymethyl) propane 1:3 diol
TBE   0.79 M Tris, 0.79 M boric acid, 1 mM EDTA
TE    10 mM Tris, 1 mM EDTA
TEMED \(N,N,N',N'\)-tetramethylethylenediamine
U    units
VB    Visual Basic
w/v  weight per volume
W    Watts
\(^\circ\)C degrees Celsius
\(\mu\) micro
%    percent
CHAPTER 1

GENERAL INTRODUCTION

1.1 Conservation genetics

The past century has seen rapid increases in human population and in the impact of human activities on the environment. This has lead to dramatic habitat loss, degradation and fragmentation resulting in many species of organisms becoming extinct or coming to the verge of extinction (IUCN 1996a; IUCN 1996b; WCMC 1992). The conservation of an endangered species depends mainly on direct protection of the species in question and preservation of its habitat. Nonetheless, since the 1980s, conservation genetics, or the application of genetics to the preservation of species, has received increasing attention (Frankel and Soule 1981; Frankham 1995a; Frankham et al. 2002; Ralls and Ballou 1986; Schonewald-Cox et al. 1983).

Human-associated factors such as habitat destruction, hunting and competition from introduced exotic species are the driving force behind the majority of recent extinctions. In addition to these factors, species are also vulnerable to stochastic factors that may cause extinctions even when the species is protected and there appears to be adequate habitat. These stochastic factors can be extrinsic and include biotic elements such as the influence of predators, competitors and pathogens (Roelke-Parker et al. 1996), or abiotic elements such as floods, fires and droughts (Raup 1991). Other stochastic factors such as demographic and genetic stochasticity (Frankham et al. 2002) are intrinsic to species. Demographic stochasticity arises from chance fluctuations in birth rates, death rates and sex ratios (Avise and Nelson 1989). Genetic stochasticity includes inbreeding depression resulting from unavoidable matings between relatives, loss of genetic
variation and the accumulation of deleterious alleles. The effects of genetic stochasticity depend on the effective population size. As stochastic factors are more important in small populations, they can exacerbate a situation once human factors have reduced a species’ population size (Gilpin and Soulé 1986). Therefore, in small captive populations, where extrinsic factors that may cause extinctions are usually minimised, demographic and genetic stochasticity play important roles.

There has been much discussion and controversy over the contribution of genetic factors to protecting endangered species. Some authors have suggested a limited role of genetic factors (Caro and Laurensen 1994; Lande 1988), while others have supported a greater role (Frankham and Ralls 1998; Hedrick 2001; O’Brien et al. 1983). There is now a growing body of evidence demonstrating that the fates of small populations are linked to genetic changes. Inbreeding has been shown to affect fitness in captive animals of conservation interest (Ralls and Ballou 1983), and Crnokrak and Roff (1999) reviewed a number of studies demonstrating inbreeding depression in wild populations. Other studies have found that genetically impoverished endangered populations often do not show signs of recovery until crossed with individuals from other populations (Land and Lacy 2000; Westemeier et al. 1998). In addition, wild populations exhibiting lower genetic diversity have been found to be at greater risk of extinction (Saccheri et al. 1998). Such studies underline the importance of genetic factors in the protection and recovery of endangered species.

Frankham et al. (2002) has identified 11 major areas in conservation genetics: i) inbreeding depression, ii) loss of genetic diversity, iii) population fragmentation and reduced gene flow, iv) accumulation and loss (purging) of deleterious mutations, v) genetic adaptation to captivity, vi) taxonomic uncertainties, vii) outbreeding depression, viii) defining management units within species, ix) forensic species identification, x) genetic drift and xi) understanding species biology.
A range of molecular markers and techniques has been used to address conservation problems in these areas.

1.2 Molecular markers in conservation genetics

Since the 1950s a variety of molecular markers have become available and have been applied to many areas in biology such as population genetics, forensics, paternity testing and gene mapping. However, it is only since the 1980s that these molecular markers have become widely used in the field of conservation biology.

The first true genetic markers to be used were allozymes, otherwise known as protein isozymes. These are enzymes, and variation in their amino acid sequences is detected through the resulting differences in electrophoretic mobility. Allozymes were widely used to assay genetic variation in populations (Lesica et al. 1988; O'Brien et al. 1983) and to study population differentiation (Barrowclough and Gutierrez 1990; Daugherty et al. 1990). However, they suffer from a number of problems such as possible non-neutrality and low allelic variation (O'Brien et al. 1983). In addition, as synonymous codon substitutions cannot be detected, allozymes do not reveal much of the underlying DNA sequence variation. These limitations mean that allozyme loci contain relatively low levels of information and any variation may be indicative of selection rather than neutral processes. DNA-based markers have largely superseded allozymes.

The first of these DNA-based markers was restriction fragment length polymorphisms (RFLPs). These are generated by digesting DNA with a suite of restriction enzymes that cleave DNA at particular sequences resulting in fragments of DNA that are detected by gel electrophoresis followed by hybridisation to particular probes. Minisatellites analysis, or DNA fingerprinting, is based on this principle of digesting genomic DNA, but the probe targets parts of the DNA
containing highly polymorphic minisatellite sequences (Jeffreys et al. 1985). Minisatellites are short DNA sequence motifs, 10-60 bp in length, which are repeated many times in tandem. Owing to their high variability, minisatellites were popular for detecting genetic variation (Brock and White 1992; O’Brien 1994) and for paternity testing (Ely and Ferrell 1990). They were also used for uncovering population differentiation (Gilbert et al. 1990) and for determining the relatedness between wild individuals founding captive populations (Geyer et al. 1993; Haig et al. 1994; Haig et al. 1995). However, there are several drawbacks to the minisatellite procedure. First, it requires relatively large amounts of DNA, second, it has low reproducibility due to its technically challenging protocol and third, it suffers from the problem that alleles cannot be assigned to particular loci as the resulting banding patterns are complex. The use of minisatellites has therefore been largely supplanted by methods that utilise the polymerase chain reaction (PCR).

The invention of PCR (Saiki et al. 1988) allowed the targeted amplification of specific sections of genomes from small amounts of DNA. PCR is essentially an *in vitro* version of natural DNA replication except that only a small section of the DNA is replicated. The reaction mix typically contains template DNA, a thermostable DNA replicating enzyme, the four DNA nucleotide bases, a pair of oligonucleotide primers, magnesium ions and buffer. The primers, usually 18-25 bases long, are complementary to DNA sequences flanking the region to be amplified. The reaction is subjected to repeated denaturing, annealing and extension temperature phases. The DNA strands are first separated in the denaturing phase, then over the course of the annealing phase the primers attach to the DNA flanking the sequence of interest, and during the extension phase the sequence between the primers is replicated. Usually 25-40 cycles are performed allowing amplification of targeted sequences from small starting amounts of DNA.
The finest scale genetic information is obtained by sequencing particular regions of a genome that have been amplified by PCR, and most conservation genetics studies making use of sequence information use mitochondrial DNA (mtDNA) sequences. A number of useful features of mtDNA have resulted in its widespread usage. First, conserved primers have been developed that allow amplification of a number of regions of the mtDNA molecule in a wide range of species. Second, because most cells contain multiple copies of the mtDNA molecule, mtDNA sequences can often be obtained from very small amounts of tissue containing degraded DNA. Third, the high mutation rate of mtDNA compared to that of most nuclear genes means that mtDNA sequences often contain high levels of informative variation. The main uses of mtDNA sequences in conservation genetics have been to establish population structuring (Barrowclough et al. 1999; Bowen et al. 1995) and to resolve taxonomies (Laerm et al. 1982; Xu and Árnason 1996). Other uses have included establishing interspecific hybridisation (Gottelli et al. 1994; Land and Lacy 2000), identification of species from hair and faecal samples (Foran et al. 1997; Paxinos et al. 1997) and the detection of illegal hunting and collecting (Dizon et al. 2000; Holden 2000).

Within a population, the majority of nucleotides of a particular sequence of DNA will be invariant and DNA sequencing will produce much redundant information. Therefore, an alternative to sequencing is to target single nucleotide positions in the genome that are known to be polymorphic. These single nucleotide polymorphisms (SNPs) are growing in popularity, but are costly and time consuming to develop. In addition, the biallelic nature of SNPs means that the information content of each SNP is low. Thus, the utility of SNPs in conservation genetics has yet to be established.

Microsatellites, or simple sequence repeats, were the first PCR-based markers to become widely used. Similar to minisatellites, microsatellites are short sections of DNA where a simple motif, generally 1-5 bp long, such as (AC)\textsubscript{\textalpha}, (AAT)\textsubscript{\textalpha} or (GATA)\textsubscript{\textalpha}, is repeated in tandem up to \(\sim\)60 times.
Microsatellite markers are highly polymorphic, abundant and fairly evenly distributed throughout eukaryotic genomes. They are also co-dominant markers as heterozygotes can be discriminated from homozygotes because alleles at a particular microsatellite locus vary in the number of tandem repeats and can be differentiated on the basis of the resulting differences in sequence length. Such characteristics mean that since they were first isolated and amplified in humans in 1989 (Litt and Luty 1989; Tautz 1989; Weber and May 1989), microsatellite loci have become the molecular markers of choice in many biological fields including conservation genetics.

Microsatellites have been used in a variety of studies to show population structuring (Forbes and Hogg 1999; Nesje et al. 2000), establish paternities (Houlden et al. 1997; Marshall et al. 1999), assay genetic diversity (Brown and Houlden 1999; Mundy et al. 1997) and to identify species and individuals from small amounts of non-invasively collected tissue (Ernest et al. 2000; Sloane et al. 2000).

Amplification of microsatellites, SNPs, mtDNA and other specific regions of a genome require prior knowledge of the sequences flanking the locus of interest so that the necessary primers can be designed. Although conserved primers have been developed for amplifying mtDNA sequences, primers are unavailable for nuclear loci in the vast majority of species. Random amplified polymorphic DNA (RAPDs) (Williams et al. 1990) and amplified fragment length polymorphisms (AFLPs) (Vos et al. 1995) were developed as PCR-based nuclear markers that could be amplified in any species without the requirement for prior sequence information. Both RAPDs and AFLPs are multilocus systems making use of primers that simultaneously bind to many different parts of a genome resulting in the amplification of many loci at the same time.

The RAPD system uses short non-specific primers that are 8-12 bases in length. The shortness of these primers means that they are complementary to a number of random locations of any genome. These random locations are simultaneously amplified by PCR and the amplified DNA
fragments separated by electrophoresis. A maximum of two alleles exist for each locus amplified using this system, and due to the dominant nature of the loci, it is not possible to distinguish a heterozygote from a homozygote. As a result, the per locus information content of RAPD markers is low. A more serious drawback of RAPDs is that the low stringency PCR required in the procedure results in a high genotyping error rate and in lower genotyping reproducibility compared to single locus markers. Such limitations mean that RAPD markers have found limited use in conservation genetics (Kozol et al. 1994; Robichaux et al. 1997).

Compared to the RAPD system, the AFLP system is a PCR-based multilocus system that provides a relatively reliable method of amplifying of hundreds of genetic markers distributed randomly throughout the genome. AFLP markers are restriction fragments that are amplified by PCR following the addition of adapters to the fragments (Vos et al. 1995). Samples to be genotyped for AFLP markers have to undergo three stages of processing. These stages are depicted in Figure 1.1 and are as follows. 1) Total digestion of purified genomic DNA with two restriction enzymes, a two base and a four base cutter, resulting in the production of short fragments of DNA with “sticky” ends. 2) Ligation of adaptors with complementary sticky ends to these DNA fragments so that the fragments are flanked by known DNA sequences. 3) Amplification by high stringency PCR of a small subset of these fragments using primers that bind to the adaptor sequence but which extend 1-3 bases beyond the restriction cut site into the unknown part of the DNA fragments. To reduce artefacts, when large genomes are examined, this protocol is modified such that there are two amplification stages: a pre-amplification using primers with a 1 bp extension beyond the cut site, followed by a more selective amplification using primers with a 3 bp extension. During the selective PCR, the fragments are labelled by the incorporation of radioisotopes or fluorescent dyes. The labelled amplified fragments are usually resolved by electrophoresis through acrylamide gels and scored either manually or automatically.
Figure 1.1  Diagrammatic representation of the stages required to generate AFLP markers.

a) Digestion with two restriction enzymes. b) Ligation of adapters to the digested DNA. c) Selective amplification of some of the fragments of DNA using selective amplification primers. For large genomes a pre-amplification phase precedes the selective amplification and is designed to reduce artefacts. Diagram adapted from Mueller and Wolfenbarger (1999).
A pair of AFLP primers will amplify a large number of AFLP loci. The majority of AFLP loci are dominant markers and are scored as either present or absent. As the present allele is dominant to the absent allele, it is not possible to distinguish heterozygotes from homozygotes. These features mean that, as in the case of RAPD markers, the per locus information content of AFLP markers is lower than that of co-dominant markers like microsatellites. This lower information content means that to address a particular problem such as parentage assignment, many more AFLP markers are needed to provide the same resolution as a few co-dominant markers (Campbell et al. 2003).

Not all of the AFLP markers amplified using a pair of AFLP primers will be polymorphic. Polymorphic AFLP loci can arise in several ways. First, if a mutation occurs at one of the restriction enzyme cut sites, the restriction enzyme will no longer cut the DNA at this position and individuals with the mutation will lack a band at the locus in question and will instead possess the absent allele. Second, if a mutation occurs at one or more of the selective amplification primer binding sites of a particular locus, the locus may not amplify. Third, an insertion or deletion within one the AFLP fragments will cause different sized products to amplify at the locus in question.

The main advantage of AFLP markers is that they can be amplified in any species without any prior DNA sequence knowledge. As the same protocol is used regardless of species, there is no requirement for optimisation. This is in contrast to microsatellite and SNP loci where in general new loci have to be isolated de novo for any new species being studied, and PCR conditions optimised for each locus. Thus, AFLP markers have been used to study a wide variety of taxa, from bacteria (Janssen et al. 1997; Siemer et al. 2004) and fungi (Gonzalez et al. 1998; Rosendahl and Taylor 1997), to diverse groups of animals and plants (Ajmone-Marsan et al. 2001; Beardsley et al. 2003; Krauss 1999; Questiau et al. 1999).
AFLP markers are generated by PCR, therefore they can be amplified from less than 50 ng of DNA. Only small amounts of tissue are required and even very small organisms can be genotyped. Rosendahl and Taylor (1997) successfully amplified AFLP markers from single fungal spores. Another advantage is that by using different AFLP primer combinations, large numbers of AFLP markers can be generated. Since many of these markers will be polymorphic, even minor genetic differences between organisms can be detected.

Owing to their greater reliability, AFLP markers have largely taken over from RAPDs as a PCR-based multilocus genotyping method. The high stringency of the PCR results in reliable and reproducible banding patterns for AFLP markers. Jones et al. (1997) reported an error rate of only 0.6 % per AFLP band in a study that compared AFLP genotypes generated by eight different laboratories from the same samples. Other studies have reported overall error rates to be less that 2 % per AFLP band, a rate that includes mispriming and scoring errors (Arens et al. 1998; Janssen et al. 1997; Questiau et al. 1999). A 2 % error rate, though much lower than the error rate with RAPD markers, is about an order of magnitude higher than genotyping errors when using microsatellite loci. This higher error rate is seen as a problem by some researchers.

Though mainly used as markers in plant systems, AFLP loci are increasingly being used in animal and other taxa to answer a wide variety of biological questions. Owing to their high variability, AFLP markers have been used successfully to infer phylogenetic relationships between closely related species (Bakkeren et al. 2000; Sullivan et al. 2004). As substantial amounts of AFLP variation also exist below the species level, they have also been used extensively in population and conservation genetics studies to infer population structuring, genetic diversity, gene flow and hybridisation (Arens et al. 1998; Beismann et al. 1997; Gaiotto et al. 1997; Rosendahl and Taylor 1997; Semblat et al. 1998). With sufficient numbers of markers, the variability of AFLP loci allows parentage assignment and individual identification (Krauss 1999; Questiau et al. 1999). The
ability to produce large numbers of markers scattered throughout the genome without prior sequence knowledge has also resulted in AFLP markers being used for constructing linkage maps and for identifying quantitative trait loci. (Jin et al. 1998; Lu et al. 1998; Otsen et al. 1996). However, the use of AFLP markers for estimating levels of inbreeding in individuals and relatedness between individuals has not been fully explored.

1.3 Relatedness and inbreeding

Inbreeding occurs when offspring are produced from the mating of individuals who are related by descent. In general, such inbred individuals show reduced levels of fitness compared to the mean fitness of the population, a phenomenon known as inbreeding depression. Two genetic mechanisms have been proposed as the cause of inbreeding depression. First, inbreeding will result in the expression of genetic load due to deleterious recessive alleles throughout the genome becoming homozygous (the dominance hypothesis: Charlesworth and Charlesworth 1987; Crow 1952). Second, if heterozygotes at particular loci have a higher fitness compared to both types of homozygote, the increase in homozygosity throughout the genome will result in the reduction in any such heterozygote advantage (the overdominance hypothesis: Crow 1948; Crow 1952; Mitton 1993). Most studies suggest that deleterious recessive alleles account for a large proportion of the inbreeding depression observed (Johnston and Schoen 1995; Lande 1994; Simmons and Crow 1977).

As inbreeding reduces fitness, inbreeding depression is one of the major themes in conservation genetics (Frankham et al. 2002). Inbreeding is likely to be a bigger problem in species with small populations where incestuous mating may be unavoidable, and in plants species capable of self-fertilisation. The reduction in fitness caused by inbreeding can further endanger an already vulnerable small population, making it more likely to go extinct. Such small populations are
unavoidable in most captive breeding programs and a large number of studies have documented
the incidence of inbreeding depression in captivity in a variety of organisms of conservation
interest (Ralls and Ballou 1982a; Ralls and Ballou 1982b; Ralls et al. 1979). Careful management
of such populations is required to minimise inbreeding and the resulting reduction in genetic
variability. Compared to the situation in laboratory and zoo environments, the effects of
inbreeding on fitness are expected to be greater in the wild where environmental conditions are
in general harsher and more variable (Ralls et al. 1988). Although inbreeding depression has also
been reported in wild populations (Crnokrak and Roff 1999), owing to the difficulty of making
the necessary observation required to calculate individuals’ levels of inbreeding, good examples of
inbreeding depression in the wild are scarce.

The relatedness between pairs of individuals is important in many areas of biology. In
quantitative genetics, where the inheritance of quantitative traits such as height and body mass is
studied, the resemblance between relatives in the trait of interest depends directly on the
probability that the individuals have genes that are identical by descent. Therefore, knowledge of
the relatedness between individuals is essential and forms the basis for the estimation of the
 genetic components of variance in traits, such as additive and dominance genetic variance
(Falconer and Mackay 1996; Lynch and Walsh 1998). Research on eusocial and cooperative
breeding species that makes use of Hamilton’s theory of kin selection (Hamilton 1964) rely on
relatedness coefficients between the altruists and the recipients of the altruism in order to
 estimate the costs and benefits of altruistic behaviour (Emlen and Wrege 1988; Trivers and Hare
1976).

In conservation genetics, knowledge of the relatedness between individuals is particularly
important in captive breeding programs that seek to reduce incestuous matings in order to
minimise inbreeding and the loss of genetic variation (Frankham et al. 2002; Montgomery et al.
Breeding programs are usually started on the assumption that the wild founders initiating the captive population are unrelated. This is likely to be true if the species has a large population size and if the wild founders are a random sample from this population. However, endangered species brought into captivity often have small population sizes, and as a result the founders may be related to one another (Geyer et al. 1993; Haig et al. 1994). Relatedness is also important in strategies that aim to recover small populations by introducing individuals from other populations. If the strategy is to maintain the genetic identity of the population, the introduced individuals should be closely related to the recipient population. On the other hand, if the strategy is to maximise outbreeding, individuals that are distantly related to the recipient population will be required. Thus, relatedness measures are important in a number of areas of conservation genetics.

An individual’s level of inbreeding is given by its inbreeding coefficient. The inbreeding coefficient is the probability that two alleles at a particular locus in the individual are identical by descent (Wright 1922). Thus, offspring from self-fertilisation have an inbreeding coefficient of 0.5 and offspring from parent-offspring or full-sib matings have an inbreeding coefficients of 0.25. The level of relatedness between two individuals is given by their coefficient of relatedness, which is the probability that a gene at a particular locus in one individual is identical by descent to a gene in the other individual (Wright 1922). Therefore, the coefficient of relatedness between monozygotic twins or clones is 1, between parents and their offspring or between full-sibs the relatedness is 0.5, and for second and third order relationships the relatedness is 0.25 and 0.125 respectively.

In systems where pedigrees are available, the coefficients of relatedness between any pair of individuals, and individuals’ inbreeding coefficients can be readily calculated using a number of different methods such as path analysis and the additive matrix method (Ballou 1995). If lineages
can be traced back far enough, any two individuals in a population can be seen to have descended from a common ancestor. Therefore, it is necessary to define inbreeding relative to some “base” population in which individuals are assumed to be unrelated to one another. When inbreeding coefficients and relatedness values are calculated from pedigrees, the founders at the base of a pedigree are usually assumed to be unrelated to one another.

Although complete pedigrees are sometimes available for organisms bred in captivity (Christie 1999; Lacy et al. 1996), pedigrees are available for only a small handful of natural populations (Keller 1998; van Noordwijk and Scharloo 1981). There are many practical difficulties with obtaining accurate pedigree data for wild populations. Most wild populations are too large for all individuals to be observed, and some organisms are too long lived. It is also often difficult to assess paternity in many species from behavioural observations, as females may be multiply mated. It may even be difficult to assign maternity when organisms live in social groups. Polymorphic genetic markers like microsatellites are widely used to assess paternity in natural populations (Hoffman et al. 2003; Taylor et al. 1997), but accurate parentage assignment requires reliable genotyping and sampling of the majority of candidate males in the population. Where pedigrees are unknown or incomplete, genetic markers may be used to estimate levels of inbreeding and relatedness.

Related individuals are expected to have a greater genetic similarity compared to non-relatives. Therefore, molecular methods for estimating the relatedness between individuals attempt to gauge this similarity by comparing the genotypes of pairs of individuals at a number of polymorphic genetic loci. Minisatellite DNA fingerprinting profiles have been used to estimate the relatedness between two individuals by calculating a similarity index between their banding profiles. The similarity index is calculated as being two times the number of common fragments
in their profiles divided by the total number of fragments exhibited by both individuals (Lynch 1988; Lynch 1990).

Genotypes from co-dominant markers can be used to calculate more sophisticated measures of pairwise relatedness. The simplest measure does not take allele frequencies into account and is simply the proportion of allelic positions of the markers at which two individuals match (Blouin et al. 1996). A number of relatedness measures have been developed that incorporate allele frequency information (Li et al. 1993; Lynch and Ritland 1999; Queller and Goodnight 1989; Ritland 1996; Wang 2002). All these estimators are designed for estimating the probability of a pair of individuals being identical by descent. These estimators are essentially unbiased and produce estimates that are on average identical to their expected values.

Van de Casteele et al. (2001) and Wang (2002) have reviewed the performance of these relatedness estimators based on co-dominant markers. They found that in general there is no single estimator that outperforms the rest. The relative advantage of each estimator depends on a large number of factors such as the number of loci used, the allele frequency distributions and sample sizes. However, all the estimators show a large sampling variance. The main source of this variance is the variance in identity-in-state for alleles that are not identical-by-descent (Lynch and Ritland 1999). Using more informative loci with large numbers of alleles and uniform allele frequencies can reduce the sampling variance. However, selecting loci which such characteristics is usually not practical.

Methods of calculating pairwise relatedness from the genotypes of dominant markers have been described by Lynch and Milligan (1994), Hardy (2003) and Madden et al. (in press). Lynch and Milligan (1994) and Madden et al. (in press) both assume that the loci are in Hardy-Weinberg equilibrium, while the measure described by Hardy (2003) requires knowledge of the departure...
from Hardy-Weinberg proportions. The estimator described by Madden et al. (in press) uses the relatedness estimator described in Queller and Goodnight (1989), modified for use with dominant markers.

In addition to their use in estimating pairwise relatedness, molecular markers can also be used to estimate individuals’ levels of inbreeding. Inbreeding results in the loss of genetic variability. Since a more inbred individual has lower genetic variability, compared to a non-inbred individual it has a higher probability of being homozygous at loci. Therefore, molecular methods attempt to estimate an individual’s level of inbreeding by measuring its heterozygosity at a number of genetic loci.

The simplest estimate of an individual’s level of inbreeding that can be obtained from the genotypes of co-dominant markers such as allozymes and microsatellites is its multilocus heterozygosity. This is the proportion of loci at which the individual is genotyped for which it is heterozygous. As genotyping is rarely complete, an improvement that avoids potential biases introduced by individuals being untyped at some loci is standardised heterozygosity (SH). SH is calculated as the ratio of an individual’s heterozygosity to the mean heterozygosity of those loci at which the individual was genotyped (Coltman et al. 1999). A further improvement is internal relatedness (IR). IR takes allele frequencies into account and is based on the relatedness measure described in Queller and Goodnight (1989), weighting the sharing of rare alleles more than common alleles (Amos et al. 2001).

Minisatellite DNA fingerprints have been used to estimate population heterozygosity. A more heterozygous population will show greater variability in the DNA fingerprint profiles. The most basic estimate of population heterozygosity has been to calculate average band sharing between all pairwise comparisons of individuals from the population (Yuhki and O'Brien 1990). A
measure described by Kuhnlein et al. (1990) improves on this by incorporating band frequencies, while Stephens et al. (1992) calculates expected heterozygosity by estimating allele frequencies based on the assumption that each band is dominant. Measures have also been developed for estimating population heterozygosity using genotypes from dominant markers such as RAPDs and AFLPs (Lynch and Milligan 1994). However, to my knowledge there have been no studies that have used minisatellite DNA fingerprints, or genotypes from AFLP or RAPD markers to estimate individual heterozygosity.

The majority of studies estimating relatedness and heterozygosity make use of genotypes from microsatellite loci. The popularity of these markers is due to their ease of amplification by PCR, their co-dominant nature and their typically high levels of allelic diversity at each locus. However, microsatellite loci have not been isolated in the vast majority of species. Some cross-species amplification of microsatellites is possible between closely related species (Menotti-Raymond and O’Brien 1995; Primmer et al. 1996), but high mutation rates in the sequences flanking the microsatellites mean that conserved primer sequences often cannot be designed. When cross-species amplification is attempted, such mutations in the primer binding regions may prevent the amplification of alleles, causing erroneous genotyping results. Therefore, in general new species-specific microsatellite loci have to be isolated when a species is studied for the first time, a process that is both time consuming and expensive (Armour et al. 1994; Ostrander et al. 1992; Paetkau 1999).

In contrast to microsatellite markers, AFLP markers can be amplified relatively easily from any species by applying the standard AFLP protocol (Vos et al. 1995). There is no requirement for any prior genetic information about the species in question and the AFLP procedure is relatively cheap. The high stringency PCR used in the AFLP protocol amplifies markers that are more reliable than RAPDs and minisatellites. Compared to microsatellite markers, the dominant and
biallelic nature of AFLP markers mean that the per marker information content is low, but this is offset by the fact that large numbers of AFLP markers can usually be easily generated.

In spite of these characteristics, AFLP markers have been used only occasionally for estimating relatedness between individuals, and the potential of AFLP markers for estimating levels of inbreeding in individuals is unknown. In this thesis I will explore the use of AFLP markers for estimating the relatedness between individuals, and individuals’ levels of inbreeding. The performance of AFLP markers at estimating these parameters will be compared with that of the more widely used microsatellite markers.

1.4 Thesis aims

i) Inbreeding has been shown to affect the fitness of animals reared in captivity. A detailed analysis of factors affecting fitness in a captive breeding program will be conducted. In addition to pedigree-based inbreeding coefficients, the influence of a number of other factors such as maternal experience and birth environment will be investigated.

ii) To investigate whether AFLP markers can be used to estimate levels of inbreeding and pairwise relatedness by comparing estimates based on AFLP markers with pedigree-based inbreeding coefficients and relatedness values.

iii) Microsatellite markers are usually used for estimating levels of inbreeding and relatedness. I will compare the utility of microsatellite and AFLP markers for estimating levels of inbreeding and pairwise relatedness.
iv) A number of studies have found correlations between heterozygosity estimated using microsatellite markers and fitness. I will examine whether heterozygosity estimated from AFLP markers can be used to detect such heterozygosity-fitness correlations.

v) As studies showing heterozygosity-fitness correlations usually use microsatellite markers, I will compare the performance of AFLP and microsatellite markers at detecting these correlations.

vi) AFLP markers have generally been used to establish relationships within species, or between closely related species. I will explore the role of AFLP markers in determining the relationships between species, both within and between different genera.
CHAPTER 2

FACTORS AFFECTING FITNESS IN THE CAPTIVE SUMATRAN TIGER (*PANTHERA TIGRIS SUMATRAE*)

2.1 Introduction

When individuals who are related by descent mate, their offspring generally have lower fitness compared to the mean fitness level of the population. This phenomenon is known as inbreeding depression (Frankham et al. 2002). Inbreeding depression may result from the loss of heterozygote advantage, from the expression of genetic load in the form of deleterious recessive alleles (Charlesworth and Charlesworth 1987; Crow 1952) or from a combination of these two mechanisms. Most studies suggest that the expression of genetic load is responsible for a large proportion of the observed inbreeding depression (Johnston and Schoen 1995; Lande 1994; Simmons and Crow 1977).

Even before the genetic basis of inheritance was known, inbreeding depression was a well recognised phenomenon. Darwin (1876) documented the effects of selfing and out crossing in 57 species of plants, reporting that inbred plants were on average shorter, weighed less, flowered later and produced fewer seeds compared to outbred plants. Since then there have been numerous studies demonstrating inbreeding depression in a wide range of laboratory and domestic species (Abplanalp 1990; Simmons and Crow 1977; Wright 1977). Analysing a variety of ungulates, primates and small mammals reared in zoos, Ralls and Ballou (Ralls and Ballou 1982a; Ralls and Ballou 1982b; Ralls et al. 1979) showed reduced survival of inbred offspring compared to outbred offspring, convincingly demonstrating inbreeding depression in species of conservation interest. Subsequently, there have been a large number of studies reporting the
effects of inbreeding in captive populations (de Bois et al. 1990; Hinz and Foose 1982; Kalinowski and Hedrick 2001; Ralls and Ballou 1986; Ralls et al. 1988; Sausman 1984).

Inbreeding and inbreeding depression are inevitable in small closed captive populations founded by a few wild individuals. As inbreeding is known to have a negative impact upon survival, the breeding program of a captive population usually seeks to minimise inbreeding while maximising the retention of the genetic diversity of the wild founders (Frankham et al. 2002). This is achieved by attempting to equalise founder representation in the population by choosing individuals with the lowest average relatedness to the population to be parents in the future generation, a process also referred to as minimising kinship (Ballou and Lacy 1995; Montgomery et al. 1997). Thus, decisions on who should breed and how many offspring should be produced are based on pedigree information rather than on individual reproductive performance or phenotype.

Another method that can be used to reduce inbreeding depression in captive populations is to purge the population of the deleterious alleles responsible for inbreeding depression. Deleterious alleles are removed by intentionally subjecting the population to inbreeding and then selecting healthy inbred individuals as breeders since they are less likely to carry deleterious recessive alleles. However, the effectiveness of purging will depend on how deleterious the alleles are. Purging is particularly effective at removing lethal recessives, but is less effective for alleles with small effects. Genetic drift in the small population may even fix such deleterious alleles with small effects and cause reduced fitness (Hedrick 1994).

Although purging has been documented in experimental populations (Frankham 1995b; Hedrick 1994), its value in managing captive populations of endangered animals remains controversial. Templeton and Read (1984; 1998) reported that purging was effective at reducing high levels of inbreeding depression in the Speke’s gazelle. As a result, the purging strategy was recommended
for use on other species suffering from severe inbreeding depression. However, re-analysis of the
Speke’s gazelle data using better statistical methodology revealed that purging had not actually
occurred and that the increased survival of inbred individuals was a consequence of improved
husbandry (Kalinowski et al. 2000; Willis and Weise 1997). Ballou (1997) looked for the effects of
inbreeding and purging on litter survival and litter size in 25 captive mammalian species. In
Ballou (1997), purging was estimated by means of an ancestral inbreeding coefficient calculated as the
cumulative proportion of an individual’s genome that had been previously exposed to
inbreeding. Purging was found to be statistically significant in only the Sumatran tiger. Of the 16
other species that showed inbreeding depression, 14 species showed small, non-significant signs
of purging. These purging effects were too weak to be of practical use in eliminating inbreeding
depression (Ballou 1997).

Although a number of studies have looked at the effects of inbreeding on the fitness of captive
animals, few of these studies have looked at the influence of factors other than inbreeding
coefficients on fitness levels. The measure of fitness most commonly used in such studies is
juvenile mortality, which is likely to be affected by a number of other factors in addition to
inbreeding. For example, females are expected to differ in their abilities to rear young and this
ability might be expected to improve with experience. Furthermore, zoos will differ in their
husbandry practices, veterinary care and in the general environment they provide for the animals.
Thus, the birth environment might be another important factor influencing juvenile mortality.
The influence of maternal factors and birth environment on fitness could potentially be greater
than the effects of inbreeding.

The Sumatran tiger is of particular interest since it was the only species analysed in Ballou (1997)
to show significant signs of purging. In this chapter I will carry out a detailed analysis of factors
affecting litter fitness in the captive Sumatran tiger population in order to establish the
importance of inbreeding and purging in this species. In these analyses, a large number of other potentially confounding factors such as maternal effects and zoo of birth will be investigated in addition to inbreeding coefficients and ancestral inbreeding coefficients calculated from the pedigree.

The Sumatran tiger, *Panthera tigris sumatrae*, is one of five recognised extant subspecies of tiger. In the wild the species is currently found in fragmented habitat on the island of Sumatra in Indonesia. The current population size in the wild is estimated to be about 400. The subspecies is categorised as being critically endangered in the IUCN Red List of Threatened Species (http://www.redlist.org). A managed captive breeding program was set up outside Indonesia in 1937. Over the years, 15 wild animals have contributed to this captive breeding program. Although initially started in Europe, this captive population is now managed as two separate groups, one based in North America and the other, a combined population, based in Europe and Australia. Animals are no longer transferred between the two groups. As of November 2000, the European population stood at 102 animals, while the North America and Australian populations consisted of 57 and 18 animals respectively (Christie 1999).

### 2.2 Materials and methods

All data about the captive Sumatran tiger population were provided by the studbook keeper, Sarah Christie, in the form of SPARKS (Single Population Analysis and Records Keeping System) database files (Christie 1999). Unlike many other breeding programs, detailed information has been kept about the Sumatran tiger captive breeding program since it was initiated. In addition to pedigree data, the SPARKS files also contain data regarding reproductive performance, survival and inter-zoo transfers of animals.
Data obtained from the SPARKS files were used to compile a database containing information about litters born within the captive breeding program between 1961 and 1999. Litters born prior to 1961 were not included in the database as these lacked adequate survival data. Any other litters for which information was imperfect were also excluded. As the effect of institutional identity on fitness was going to be investigated, only zoological institutions at which a minimum of five litters had been produced were included in the database. The final database consisted of 255 litters containing 574 individuals. These litters were produced by 67 different pairs of tigers at 21 zoological institutions.

Assuming that the 15 wild founders of the captive population were unrelated, the pedigree was used to calculate Wright’s inbreeding coefficient of each litter produced, as well as the inbreeding coefficients of the parents of each litter using the additive relationship matrix method described in Ballou (1995). Wright’s inbreeding coefficient is the probability that two alleles at a locus in an individual are identical by descent (Wright 1922). Pedigree information was also used to calculate the ancestral inbreeding coefficient of each litter (Ballou 1997). The ancestral inbreeding coefficient measures the exposure of an individual’s genome to inbreeding in previous generations and therefore is an estimate of how purged a genome is of deleterious alleles. The SPARKS files were also used to obtain the following information about each litter: proportion of the litter surviving to seven days (litter survival), litter size, zoo of birth, year of birth, maternal experience (parity), age of mother when the litter was born, and sex ratio of the litter.

The fates of individuals within a litter are not independent because they share the same maternal environment. Therefore, each litter was considered as an independent data point in the analyses. For each litter, general linear models (GLMs) were created examining two fitness components: litter survival and litter size. Litter survival indicates viability of the offspring and the mother’s ability to rear young. Litter size may be an indicator of foetal mortality and also reflect a female’s
fertility. Since most mortality occurs within the first week after birth (Figure 2.1), the proportion of a litter surviving to seven days of age was used. GLMs with binomial error structures were used to model both litter survival and litter size. Following the approach used in Ballou (1997), litter size was classified as either one or zero depending on whether it was larger or smaller than the average litter size of Sumatran tigers in captivity. Influence of the following factors on the two fitness measures was investigated: litter inbreeding coefficient, maternal inbreeding coefficient, paternal inbreeding coefficient, ancestral inbreeding coefficient, zoo of birth, year of birth, parity, litter sex ratio, mother’s identity, and age of mother. For litter survival, the effect of litter size was also considered.

Initially, to independently examine the influence that each factor has on litter survival and litter size, models were created using single factors as predictors. GLMs with multiple factors were then created to determine what combinations of factors best predicted litter survival and litter size. To do this, full models containing all the terms were first constructed. To compensate for overdispersion in the models, significance testing of each term was assessed using F-tests. Standard deletion-testing procedures (Crawley 2002) were used to drop terms from the full models that were not significant at p < 0.05. The effects of first order interactions between terms were investigated, but higher order interactions were not considered.
2.3 Results

Analyses of the litters revealed that the average size of litters produced in captivity by Sumatran tigers was $2.3 \pm 0.9$. The sex ratio at birth was not significantly different from 1:1 (255 females:282 males, sign test, $p = 0.13$). Neonatal mortality was high, with 27% of individuals dying within the first week (Figure 2.1). However, this neonatal mortality was unequally distributed amongst litters, with all individuals dying in 23% of litters and 60% of litters experiencing no mortality at all within the first week. Table 2.1 indicates that litter survival was highest when litter size at birth was two or three, and that survival was lower when the birth litter size was smaller or larger than two or three.


### Table 2.1

<table>
<thead>
<tr>
<th>No. of litter individuals surviving to 7 days</th>
<th>Litter size at birth</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>0</td>
<td>23</td>
</tr>
<tr>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td>2</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>–</td>
</tr>
<tr>
<td>5</td>
<td>–</td>
</tr>
</tbody>
</table>

The effect of litter size at birth on survival of individuals within the litters.

Italicised figures represent the number of litters in each category.

Analysis of the effects of single factors on litter survival and litter size appears to indicate a strong influence of both birth environment and maternal condition (Table 2.2). While the litter, paternal and ancestral inbreeding coefficients did not appear to affect either fitness measure, the zoo at which a litter was born, the mother’s identity and maternal inbreeding coefficient had strong effects on both litter survival and litter size. Other maternal factors, such as the mother’s parity and her age, also impacted on litter survival. GLMs created using multiple factors revealed a slightly different pattern with the mother’s identity and the litter sex ratio having significant effects on both litter survival and litter size (Table 2.3). The effect of mother’s identity was particularly strong. Litter survival and litter size were greater in litters with more females indicating male-biased foetal and neonatal mortality.
<table>
<thead>
<tr>
<th>Term</th>
<th>n</th>
<th>Litter survival</th>
<th>Litter size</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Coefficient</td>
<td>p</td>
</tr>
<tr>
<td>Litter inbreeding coefficient</td>
<td>255</td>
<td>-0.997</td>
<td>0.2</td>
</tr>
<tr>
<td>Maternal inbreeding coefficient</td>
<td>255</td>
<td>-3.79</td>
<td>3×10⁻⁵</td>
</tr>
<tr>
<td>Paternal inbreeding coefficient</td>
<td>255</td>
<td>-0.777</td>
<td>0.4</td>
</tr>
<tr>
<td>Ancestral inbreeding coefficient a</td>
<td>255</td>
<td>-4.77</td>
<td>0.07</td>
</tr>
<tr>
<td>Mother’s parity</td>
<td>255</td>
<td>0.147</td>
<td>0.0004</td>
</tr>
<tr>
<td>Litter size</td>
<td>255</td>
<td>0.366</td>
<td>0.002</td>
</tr>
<tr>
<td>Year of birth</td>
<td>255</td>
<td>-0.0330</td>
<td>0.004</td>
</tr>
<tr>
<td>Zoo</td>
<td>255</td>
<td>–</td>
<td>5×10⁻¹²</td>
</tr>
<tr>
<td>Age of mother at birth (days)</td>
<td>247 b</td>
<td>2.97×10⁻⁴</td>
<td>0.002</td>
</tr>
<tr>
<td>Litter sex ratio</td>
<td>232 b</td>
<td>-0.337</td>
<td>0.3</td>
</tr>
<tr>
<td>Mother’s identity</td>
<td>–</td>
<td>4×10⁻¹⁶</td>
<td>–</td>
</tr>
</tbody>
</table>

a This term is an interaction between the litters’ inbreeding and ancestral inbreeding coefficients as used in Ballou (1997) to represent the amount of purging a genome has been subjected to.

b Litter sex ratios and age of mother at birth were not available for all litters

### Table 2.2

Effects of single terms on litter survival and litter size in the Sumatran tiger.

Italicised numbers represent non-significant terms.

<table>
<thead>
<tr>
<th>Term</th>
<th>Estimate</th>
<th>% deviance explained</th>
<th>df</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) Litter survival</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mother’s identity</td>
<td>–</td>
<td>39.5</td>
<td>47</td>
<td>3.7</td>
<td>2×10⁻¹⁶</td>
</tr>
<tr>
<td>Litter sex ratio</td>
<td>-0.014</td>
<td>1.5</td>
<td>1</td>
<td>6.3</td>
<td>0.01</td>
</tr>
<tr>
<td>b) Litter size</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mother’s identity</td>
<td>–</td>
<td>26.2</td>
<td>47</td>
<td>1.8</td>
<td>0.0005</td>
</tr>
<tr>
<td>Litter sex ratio</td>
<td>-1.0</td>
<td>3.5</td>
<td>1</td>
<td>10.8</td>
<td>0.001</td>
</tr>
</tbody>
</table>

### Table 2.3

General linear models predicting a) litter survival, \(n = 232, 41.0\%\) of deviance explained, \(F = 3.66, p = 2×10⁻¹⁶\) and b) litter size \(n = 232, 29.6\%\) of deviance explained, \(F = 1.96, p = 0.0001\) in the Sumatran tiger. Only significant terms are shown. The percentage deviance explained refers to the proportion of the total deviance explained by each term in the model.
Of the 21 zoos considered in these GLMs, ten zoos held only a single litter-producing female. Thus, it is difficult to separate the effect of female identity on litter survival and litter size from the effect of zoo of birth. To throw further light on the relative importance of zoo of birth and maternal identity, a reduced dataset consisting of 180 litters born at zoos that held at least two litter-producing females was created from the initial dataset of 255 litters. Figure 2.2 indicates that there is appreciable variation between females in the average litter size and especially the average litter survival rate of litters produced by the females. In contrast, there appears to be less inter-zoo variation in average litter size and average litter survival rate of litters produced in zoos (Figure 2.3). GLMs of litter survival and litter size created using the reduced dataset show a very similar pattern (Table 2.4) to the GLMs created using the full dataset (Table 2.3), with mother’s identity appearing strongly influential. For both litter survival and litter size, models created with the reduced dataset using zoo of birth as an explanatory factor explained significantly less deviance compared to models with mother’s identity as an explanatory factor (ANOVA: litter survival, $F = 2.7, df = 29, p = 2 \times 10^{-6}$; litter size, $F = 1.8, df = 29, p = 0.004$). These lines of evidence suggest that maternal identity has a stronger influence on litter survival and litter size than the zoo of birth and that the strong effect of zoo of birth found in the single factor analysis (Table 2.2) is an artefact of the quality of females in a zoo’s possession, rather than a reflection of a zoo’s ability to rear tigers.
Figure 2.2  Variation in A) average proportion of litters surviving and B) average litter size between captive female Sumatran tigers. Figures above each column represent the number of litters produced by the female in question. The order of females is different in A and B.
Figure 2.3  Variation in A) average proportion of litters surviving and B) average litter size between zoos holding Sumatran tigers. Figures above each column represent the number of litter producing females held by the zoo in question. The order of zoos is different in A and B.
<table>
<thead>
<tr>
<th>Term</th>
<th>Estimate</th>
<th>% deviance explained</th>
<th>df</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) Litter survival</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mother’s identity</td>
<td>–</td>
<td>45.8</td>
<td>39</td>
<td>3.4</td>
<td>3×10⁻¹⁶</td>
</tr>
<tr>
<td>Sex ratio of litter</td>
<td>-1.75</td>
<td>4.1</td>
<td>1</td>
<td>12.0</td>
<td>0.0005</td>
</tr>
<tr>
<td>b) Litter size</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mother’s identity</td>
<td>–</td>
<td>28.6</td>
<td>40</td>
<td>1.8</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Table 2.4  General linear models predicting a) litter survival, \((n = 165, 49.9\% \text{ of deviance explained, } F = 3.64, p = 7×10⁻¹⁴)\) and b) litter size \((n = 180, 28.6\% \text{ of deviance explained, } F = 1.75, p = 0.002)\) in the Sumatran tiger. Models are based on litters produced in zoos with at least two females. Only significant terms are shown. The percentage deviance explained refers to the proportion of the total deviance explained by each term in the model.

2.4 Discussion

Analysis of Sumatran tiger litters born in captivity revealed that both litter survival and litter size are strongly influenced by the mother’s identity. Although the zoo at which a litter is born and the mother’s identity are to a certain extent confounded, evidence points to the mother’s identity being more important than the zoo of birth. Litter survival and litter size are also negatively correlated with the litter sex ratio indicating male biased foetal and neonatal mortality. There was some evidence for maternal inbreeding affecting litter survival, however, it was not possible to separate the effect of maternal inbreeding from that of maternal identity.

Ballou (1997) investigated the role of inbreeding in purging genomes of deleterious alleles in 25 mammalian captive breeding programs, including that of the Sumatran tiger. The captive Sumatran tiger population was the only species in the study in which significant purging was
reported. However, there are a number of problems with the analysis reported in Ballou (1997). First, the reported purging effects are weak ($p < 0.05$) and considering the number of tests carried out, could represent Type I errors. Second, despite the considerable overlap between my dataset of 255 litters and that used in Ballou (1997), it was not possible to replicate the results of Ballou (1997). The ancestral inbreeding model described in Ballou (1997) included the following terms: year of birth, litter inbreeding coefficient, maternal inbreeding coefficient, and an interaction between litter inbreeding and ancestral inbreeding. Using the same model, I failed to find any effect of ancestral inbreeding (or purging) on litter survival or litter size, and instead found a strong negative correlation between litter survival and maternal inbreeding. This lack of replicability indicates that the ancestral inbreeding model described in Ballou (1997) is not robust. Third, the models proposed in Ballou (1997) explain only 3.6% and 2.9% of the total deviance in the GLMs of litter survival and litter size and the models are highly overdispersed. This compares with 41% and 30% of the deviance explained by the models for litter survival and litter size described in this chapter. Large amounts of unexplained deviance coupled with overdispersion indicate weak models from which major explanatory factors, such as birth location, have been omitted. Fourth, in the Sumatran tiger, the ancestral inbreeding term used in Ballou (1997) correlates very strongly with both maternal and paternal inbreeding coefficients, $r = 0.79$ and 0.77 respectively. Therefore, the use of ancestral inbreeding simultaneously with maternal inbreeding as terms in a model is likely to create problems with model interpretation, especially as the effects from each term are expected to be in opposite directions. Thus, the evidence for the purging of deleterious alleles in the Sumatran tiger is weak.

The large amounts of deviance explained by the models describing litter survival and litter size that were developed in this chapter suggest that these models may be both more powerful and more appropriately formulated than the models described in Ballou (1997). A major limitation of the models described in this chapter is that it was not possible to satisfactorily separate the effect
of the zoo at which a litter is born from its mother’s identity. The analyses presented here indicate that the mother’s identity is more influential than the zoo of birth. A more statistically rigorous approach would entail carrying out a nested analysis with models containing both zoo of birth and mother’s identity as explanatory factors, the latter as a random factor nested within the former. Such an analysis is complex and requires of knowledge of mixed effects models, so only simple analyses that are easier to interpret have been presented in this chapter.

A number of studies have looked for inbreeding depression in captive populations and have generally found fitness to be affected by an individual’s inbreeding coefficient (de Bois et al. 1990; Hinz and Foose 1982; Ralls et al. 1988). However, most of these studies do not consider the possible impact of maternal inbreeding coefficient or other potentially important factors such as birth location and mother’s identity. Where the roles of both an individual’s own inbreeding and maternal inbreeding have been considered, maternal inbreeding has often been found to affect fitness (Lacy et al. 1996). In the Sumatran tiger, the fitness of litters does not appear to be affected by the litter’s inbreeding coefficient. However, maternal inbreeding is negatively correlated with both litter survival and litter size. As maternal identity and maternal inbreeding coefficient are confounded, it was impossible to investigate the effect of both these factors simultaneously. Therefore, it was not possible to establish how much of the effect of mother’s identity is due to the mother’s inbreeding coefficient, and how much is due to other factors such as fertility or body condition that may be unrelated to inbreeding. This study does however reveal the importance of maternal effects in determining litter fitness.

As the mother’s identity is important in determining litter fitness in the Sumatran tiger, it will be interesting to see whether any factors influencing a mother’s litter rearing capability can be identified. The mother’s inbreeding coefficient appears to have some effect on her litter rearing ability. Other factors that may have effects are her body condition and the environment. Another
potentially important factor is the extent to which a mother’s litter rearing ability is inherited from her own mother. If heritability of litter rearing ability is significant, it may be desirable to exclude matrilines with poor breeding success from the breeding program even if they include genetically important individuals. If the factors that affect a female’s litter rearing ability can be altered, improvements in breeding success are likely.

Extending this type of analysis to other big cat species bred in captivity would enable investigation into whether the same maternal effects found in the Sumatran tiger are important in other species. Using a number of species bred in the same zoological institutions could also shed light on the role of birth environment on fitness. If the same zoos perform better over a number of different species, one could conclude that the effect is a result of differences in animal rearing practices between zoos. Detailed studbook records exist for a number of other big cat species such as the Amur leopard, Persian leopard, snow leopard, clouded leopard and the Asiatic lion. Although these breeding programs are smaller than that of the Sumatran tiger, it should be possible to incorporate these species into a large multi-species analysis. Such analyses will allow the identification of factors that can be changed to increase the breeding success of captive breeding programs. While some factors are likely to be species specific, factors that universally affect fitness regardless of the identity of the species may also be uncovered.

Laboratory studies have shown that inbreeding depression is typically more severe in stressful environments (Miller 1994). In captivity, environmental conditions are likely to be more stable and juvenile mortality reduced through veterinary intervention. Therefore, compared to the situation in captivity, inbreeding depression is expected to be more severe in the wild (Crnokrak and Roff 1999; Ralls et al. 1988). Despite this expectation, convincing studies demonstrating the effects of inbreeding in natural environments are scarce (Greenwood et al. 1978; Keller 1998; Kempenaers et al. 1996). This is primarily because detailed long-term studies over three to four
generations are required to gather the required pedigree data needed to calculate inbreeding coefficients. In the absence of good pedigree data, it may be possible to estimate an individual’s level of inbreeding using molecular markers (Balloux et al. in press; Hedrick et al. 2001; Slate and Pemberton 2002). The rest of this thesis will investigate the use of molecular methods for estimating levels of inbreeding in individuals, and levels of relatedness between individuals.
CHAPTER 3

MATERIALS AND METHODS

3.1 Samples

In this thesis, samples from a large number of species have been genotyped using AFLP markers. The samples used are summarised in Table 3.1 and represent samples obtained from a collaboration that I established with Dr R C Lacy (Chicago Zoological Society), and from various ongoing studies in the Molecular Ecology Laboratory, at the Department of Zoology, University of Cambridge.

3.2 Genotyping

3.2.1 DNA extraction

Genomic DNA was extracted from the various tissue samples using a protocol adapted from Walsh et al. (1991). A small amount of tissue was placed in a 1.6 ml tube together with 300 μl of extraction buffer (5% Chelex 100™ w/v, 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 1% SDS, 0.3 mg/ml Proteinase K). The tube was then incubated for 2-4 hours at 56 °C and periodically vortexed. After adding RNAase A to a concentration of 0.15 mg/ml, the extract was incubated overnight at 37 °C on a rotating wheel. The tube was then centrifuged at 13,000 rpm for 60 s, and 200 μl of the supernatant transferred to a fresh 1.6 ml tube containing 300 μl of 5% Chelex in TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA). The resulting DNA extractions were stored at -20 °C.
To check the quality and concentration of the resulting DNA extractions, 5 μl of the extracts were run out on a 0.7 % agarose gel. The DNA was stained by soaking the gel in a 0.5 μg/ml solution of ethidium bromide, and visualised using an ultraviolet trans-illuminator.
<table>
<thead>
<tr>
<th>Class</th>
<th>Order</th>
<th>Family</th>
<th>Genus and species</th>
<th>Common name</th>
<th>No. of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mammalia</td>
<td>Rodentia</td>
<td>Muridae</td>
<td>Peromyscus polionotus subgriseus</td>
<td>Old-field mouse</td>
<td>258</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>Peromyscus polionotus rhoadsi</td>
<td>&quot;</td>
<td>60</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>Peromyscus polionotus lenoccephalus</td>
<td>&quot;</td>
<td>61</td>
</tr>
<tr>
<td>Carnivora</td>
<td>Odobenidae</td>
<td>Odobenus</td>
<td>Odobenus rosmarus</td>
<td>Walrus</td>
<td>8</td>
</tr>
<tr>
<td>&quot;</td>
<td>Otariidae</td>
<td>Zalophus</td>
<td>Zalophus californianus</td>
<td>Californian sea lion</td>
<td>181</td>
</tr>
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<td>&quot;</td>
<td>&quot;</td>
<td>Lagenorhynchus jubatus</td>
<td>Stellar sea lion</td>
<td>5</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>Arctocephalus gazella</td>
<td>Antarctic fur seal</td>
<td>9</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>Callorhinus ursinus</td>
<td>Northern fur seal</td>
<td>5</td>
</tr>
<tr>
<td>&quot;</td>
<td>Phocidae</td>
<td>Erignathus</td>
<td>Erignathus barbatus</td>
<td>Bearded seal</td>
<td>4</td>
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<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>Phoca</td>
<td>Phoca bipecta</td>
<td>Ringed seal</td>
<td>3</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>Halichoerus</td>
<td>Halichoerus grypus</td>
<td>Grey seal</td>
<td>7</td>
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<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>Phoca</td>
<td>Phoca astalica</td>
<td>Caspian seal</td>
<td>4</td>
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<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>Phoca</td>
<td>Phoca groenlandica</td>
<td>Harp seal</td>
<td>13</td>
</tr>
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<td>&quot;</td>
<td>Phoca</td>
<td>Phoca vitulina</td>
<td>Hooded seal</td>
<td>5</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>Phoca</td>
<td>Phoca lalhga</td>
<td>Harbour seal</td>
<td>5</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>Halichoerus</td>
<td>Halichoerus grypus</td>
<td>Spotted seal</td>
<td>3</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>Phoca</td>
<td>Phoca groenlandica</td>
<td>&quot;</td>
<td>9</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>Leptonychotes</td>
<td>Leptonychotes weddelli</td>
<td>Southern elephant seal</td>
<td>5</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>Lobodon</td>
<td>Lobodon carcinophagus</td>
<td>Crabeanter seal</td>
<td>5</td>
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<td>&quot;</td>
<td>&quot;</td>
<td>Sus</td>
<td>Sus scrofa</td>
<td>Wild boar</td>
<td>20</td>
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<tr>
<td>Aves</td>
<td>Falconiformes</td>
<td>Accipitrdae</td>
<td>Aquila heliaca</td>
<td>Imperial eagle</td>
<td>40</td>
</tr>
<tr>
<td>&quot;</td>
<td>Anseriformes</td>
<td>Anatidae</td>
<td>Anas platyrhynchos</td>
<td>Common mallard</td>
<td>60</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>Mimusidae</td>
<td>Ramphocinclus brachyrhynus</td>
<td>White-breasted thrashers</td>
<td>10</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>Alaudidae</td>
<td>Alauda razae</td>
<td>Raso lark</td>
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</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>Passeridae</td>
<td>Passer domesticus</td>
<td>Sparrow</td>
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</tr>
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<td>Paridae</td>
<td>Parus</td>
<td>Parus major</td>
<td>Great tit</td>
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</tr>
<tr>
<td>&quot;</td>
<td>Galliformes</td>
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<td>Alectoris nova</td>
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</tr>
<tr>
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<td>Caprimulgiformes</td>
<td>Caprimulgida</td>
<td>Caprimulgus caudicatissimus</td>
<td>White-winged nightjar</td>
<td>38</td>
</tr>
<tr>
<td>&quot;</td>
<td>Coraciiformes</td>
<td>Meropidae</td>
<td>Merops apiaster</td>
<td>European bee-eater</td>
<td>8</td>
</tr>
</tbody>
</table>

**Table 3.1**  Samples genotyped in this study
3.2.2 PCR amplification of microsatellite markers

Microsatellite markers were only amplified in the *Peromyscus polionotus* samples. Fourteen loci were used to genotype the samples (Table 3.2). A further 19 loci (Table 3.3) were also tried, but were not used due to problems such as high null allele frequencies, difficulty in scoring, inconsistent amplification and non-amplification. Extracted DNA was diluted 50 times with low TE (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA) before being used as template in polymerase chain reactions (PCRs). The PCR conditions used for the amplification of microsatellite markers in *P. polionotus* were similar in all 14 of the loci used, varying only in the annealing temperature (Table 3.2). The annealing temperatures for the different loci were optimised using a gradient PCR machine.

PCR reactions were carried out in 10 µl reaction volumes containing 0.5 µl of diluted template DNA, 1× Thermalase buffer A (100 mM Tris-HCl pH 8.0, 50 mM KCl, 1.5 mM MgCl₂, 0.01 % Tween 20, 0.01 % gelatine, 0.01 % IGepal), 0.5 mM additional MgCl₂, 0.2 mM each of dATP, dTTP and dGTP, 0.04 mM dCTP, 400 nM of each primer, 0.25 U *Taq* polymerase, and 0.1 µCi [α³²P]-dCTP. The reactions were overlaid with 10 µl of mineral oil. The following PCR program was used: 3 minutes denaturing at 94 °C; followed by 35 cycles of 30 s at 94 °C, 30 s at the annealing temperature (Table 3.2), 25 s at 72 °C; ending with a 20 minute final elongation stage at 72 °C. Following PCR amplification, 10 µl of stop solution (50 % formamide, 10 mM EDTA, 1 mg/ml xylene cyanol, 1 mg/ml bromophenol blue) were added to each of the reactions. To resolve the PCR products, reactions were denatured by heating to 94 °C for 4 minutes, and 3.5 µl of each reaction loaded onto a 38×30 cm 6 % denaturing acrylamide gel (19:1 acrylamide:bis-acrylamide) in 1×TBE running buffer. The gels were run at 45-50 °C for 2-3 hours, after which they were transferred onto filter paper and dried. The PCR products were then visualised by autoradiography or by exposure to a sensitive phosphorimager plate.
In addition to microsatellite genotyping of *P. polionotus*, as part of my laboratory training I also isolated microsatellite markers in the European bee-eater (Dasmahapatra et al. 2004).

<table>
<thead>
<tr>
<th>Locus</th>
<th>Primer sequence (5'-3')</th>
<th>$T_a/ °C$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pml1</td>
<td>F: CATTCAAGACCTGGCTTTTT</td>
<td>56.5</td>
<td>(Chirhart et al. 2000)</td>
</tr>
<tr>
<td></td>
<td>R: TGGGTTTTCATCAGTGCTCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pml2$^b$</td>
<td>F: GTACAGGGATGAACATAGT</td>
<td>51.0</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>R: GAATAATTTCGCTGTGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pml3$^a$</td>
<td>F: GCCATTAGCTATGTGACAG</td>
<td>63.0</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>R: GCAGGTACCCAGAAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pml4</td>
<td>F: CATAAGGTGGTCGGGAATCA</td>
<td>63.0</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>R: CAGGAGGGAAATGACCAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pml6</td>
<td>F: CAGGCTGTAAGGGAGGACAC</td>
<td>63.0</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>R: ACTGGAGCAGAGGATT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pml7</td>
<td>F: GCCCTGTCACCCAGTGAAAT</td>
<td>50.5</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>R: TCCCATGGGTGACTTTTTTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pml8$^a$</td>
<td>F: AATGGCTCAGCCTCCTCTCC</td>
<td>51.0</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>R: GGCTGTCACCCCTGTCTTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pml10</td>
<td>F: CACGCTGACACACGAGACG</td>
<td>63.0</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>R: TCCCTAACACACTCACCCTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pml11</td>
<td>F: ACCCAGTGCTGAGATT</td>
<td>63.0</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>R: TCGGTGCTCCGCCACAGAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plgt58</td>
<td>F: GATCTTGTGAAACAGCCTCT</td>
<td>60.5</td>
<td>(Schmidt 1999)</td>
</tr>
<tr>
<td></td>
<td>R: TTGATGCGCTGGAGGACT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plgt62$^b$</td>
<td>F: AGAGCTAGCTAGAAATAG</td>
<td>60.0</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>R: GATTTACACTGCAACAGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plgt66</td>
<td>F: CTTGTGCCTCCACAGTGTGCA</td>
<td>63.0</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>R: GTGCTATCCAGATGGTAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Po3-68</td>
<td>F: GTAGTCTGAGAAACGAAAGG</td>
<td>62.0</td>
<td>(Prince et al. 2002)</td>
</tr>
<tr>
<td></td>
<td>R: TTTATTTGGCTGGACTGCCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Po697</td>
<td>F: TGAGCGTATCTCTCTCTCA</td>
<td>59.0</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>R: CCTGGAGCCTTTATCTGAA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Only amplified for the *P. polionotus* caught in 1990; $^b$ Only amplified for the *P. polionotus* caught in 1998; $T_a =$ annealing temperature

Table 3.2  Microsatellite primers used in *P. polionotus*. 
<table>
<thead>
<tr>
<th>Locus</th>
<th>Primer sequence (5'-3')</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pml5</td>
<td>F: CTGAGCCAAAAAGTGTCCTT</td>
<td>(Chirhart et al. 2000)</td>
</tr>
<tr>
<td></td>
<td>R: TGAAGACAGCCCCCTCCTC</td>
<td></td>
</tr>
<tr>
<td>Pml9</td>
<td>F: GATTCATACAAACATGC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: TGCTTTTCGTCAAGTTTTT</td>
<td></td>
</tr>
<tr>
<td>Pml12</td>
<td>F: GCCAGCCTGTATCTCTCAAC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: GCCAACATTTTTCTCAAGTG</td>
<td></td>
</tr>
<tr>
<td>Plgt15</td>
<td>F: GATCAAGTCTCCTATGTAG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: GACCTCCACAAATACACTGT</td>
<td>(Schmidt 1999)</td>
</tr>
<tr>
<td>Plgt16</td>
<td>F: GACAGACAGAGGTCACAG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: TCATAGTAACATATGCTCAG</td>
<td></td>
</tr>
<tr>
<td>Plgt22</td>
<td>F: GATCTCTAGTCTGACAACA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: ATTACTATTTTCTCATTAG</td>
<td></td>
</tr>
<tr>
<td>Plgt48</td>
<td>F: GCATACAGATTCGAATCTG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: TGACTGAAAGAGCCAGTCC</td>
<td></td>
</tr>
<tr>
<td>Plgt50</td>
<td>F: GCTCAGTGTTGGTCAAGGCAC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: GACIAAGTCCTGATATGTAG</td>
<td></td>
</tr>
<tr>
<td>Plgt56</td>
<td>F: GATCAAGTCAGCGC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: AGCTGATACACACC</td>
<td></td>
</tr>
<tr>
<td>Plgt67</td>
<td>F: GCACCTGCTGCACTGACAGA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: TCACTACAGGGTCGGCCTG</td>
<td></td>
</tr>
<tr>
<td>Plct5</td>
<td>F: CCTCTAGTGACTGAGTG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: CATATGTCATTTAGAAA</td>
<td></td>
</tr>
<tr>
<td>Plgata70</td>
<td>F: CTTGATGTCAGCTCAGCT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: TAATCTCTGTAGCTCATGT</td>
<td></td>
</tr>
<tr>
<td>Po40</td>
<td>F: CACTTGTTGGAGACGTTTGAG</td>
<td>(Prince et al. 2002)</td>
</tr>
<tr>
<td></td>
<td>R: GGAGCAGGTCCTACCTTC</td>
<td></td>
</tr>
<tr>
<td>Ppa1</td>
<td>F: ATGTGCACGTTGTTTGAGTTA</td>
<td>(Wooten et al. 1999)</td>
</tr>
<tr>
<td></td>
<td>R: ATTTCATCTGTCCTTCCTA</td>
<td></td>
</tr>
<tr>
<td>Ppa3</td>
<td>F: GGACAAGTTGGGCCTCCTTTAGT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: TGAAAATATCTTGGGTTACTT</td>
<td></td>
</tr>
<tr>
<td>Ppa12</td>
<td>F: TGCCAACATACAAAGAGACCTCT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: GGACAGAGAGGTCTCAAGGCCTGA</td>
<td></td>
</tr>
<tr>
<td>Ppa28</td>
<td>F: GCCATAAATGTCCAAAGCTCTCCTT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: AGTTACAGGAAAGGTCGCAAAGCTG</td>
<td></td>
</tr>
<tr>
<td>Ppa46</td>
<td>F: ATGCCACAGGGAGGCTCTCTAACAG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: TTTTACAAGAAGGAGCGCAACCATTTC</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.3  *Peromyscus* microsatellite primers that did not amplify successfully.
3.2.3  PCR amplification of AFLP markers

The protocol followed for the amplification of AFLP markers was similar to that used in Vos et al. (1995) and Ajmone-Marsan et al. (2001), and consisted of five stages: i) extraction and purification of the genomic DNA, ii) digestion of the DNA using two restriction enzymes, iii) ligation of adapters, iv) pre-amplification and v) selective amplification.

3.2.3.1  Extraction and purification of DNA

Following the extraction of genomic DNA as described previously, the DNA was quantified by running out 5 μl of extract on a 0.7 % agarose gel. Approximately 100-400 ng of DNA were then used per sample for the AFLP reactions. This DNA was further purified by a standard phenol-chloroform protocol (Sambrook et al. 1989). One volume of phenol-chloroform was added to the DNA in 1.6 ml tubes, mixed gently on a rotating wheel, and then centrifuged for 10 minutes at 13,000 rpm. The supernatant was removed from each tube and added to fresh tubes. If impurities were still present in the supernatant after phenol-chloroform treatment, a second phenol-chloroform stage was performed. Following the phenol-chloroform stage, an equal volume of chloroform was added to each sample, mixed gently on a rotating wheel and centrifuged for 10 minutes at 13,000 rpm. The supernatant was again removed and added to 0.5 ml tubes, and mixed with 0.5 volume of 8 M ammonium acetate. To precipitate the purified DNA, 2.5 volumes of ice-cold ethanol were added to the tubes and the tubes kept at –20 °C for at least 30 minutes. After centrifuging the tubes for 15 minutes at 13,000 rpm, all traces of the supernatant were carefully removed by aspiration, and the DNA pellet washed with 500 μl of ice-cold 70 % ethanol. The tubes were centrifuged at 13,000 rpm for 3 minutes and the ethanol completely removed by aspiration. DNA in the tubes was air dried for 20-30 minutes and then re-hydrated with 8.5 μl of sterile distilled water. Care was taken to ensure that all the DNA went
into solution by flicking the tubes such that any DNA adhering to the tube walls was also dissolved.

### 3.2.3.2 Digestion using restriction enzymes

The DNA was first digested with *Taq*I in a 10 μl reaction by adding 5 U of the enzyme and the relevant amount of digestion buffer to each tube of re-hydrated DNA. The tubes were incubated at 65 °C for 2 hours. Following this, 5 U of *Eco*RI and the appropriate amount of digestion buffer were added and the reaction volume made up to 20 μl. The tubes were then incubated at 37 °C for a further 2 hours.

### 3.2.3.3 Ligation of adapters

*Eco*RI adapters were made by adding equimolar amounts of the *Eco*RI top and bottom single strands (Table 3.4) to a tube. To anneal the single strands, the tubes were heated to 65 °C for 5 minutes, and then allowed to slowly cool to room temperature. *Taq*I adapters were made similarly in a separate tube. 5 pM of *Eco*RI and 50pM of *Taq*I adapters were ligated on to the digested DNA in a 50 μl reaction volume using 1 U of T4 DNA ligase. A larger amount of *Taq*I adapters is required because many more of the DNA fragments created by the restriction enzyme digests have been cut by *Taq*I than by *Eco*RI, as the former is a 4-base recognition enzyme and the latter a 6-base recognition enzyme. The ligation reaction was incubated at 37 °C for 3 hours, and then diluted 10 fold with low TE (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA).

### 3.2.3.4 Pre-amplification

5 μl of the diluted ligated DNA were added to a 50 μl PCR reaction mix containing 1× Thermalase buffer A, 0.2 mM each of dATP, dTTP, dCTP and dGTP, 1 U *Taq* polymerase and
50 ng each of the EcoRI-A and TaqI-C pre-amplification primers (Table 3.4). The reactions were overlaid with 10 µl mineral oil. The PCR program used was: 3 minutes denaturing at 94 °C; followed by 20 cycles of 30 s at 94 °C, 60 s at 50 °C, 60 s at 72 °C; ending with a 20 minute final elongation stage at 72 °C. Following the reaction the samples were diluted 10 fold with low TE.

3.2.3.5 Selective amplification and electrophoresis

2.5 µl of the diluted pre-amplification reaction were added to a 12.5 µl PCR reaction mix containing 1× Thermalase buffer A, 0.2 mM each of dATP, dTTP and dGTP, 0.04 mM dCTP, 1 U Taq polymerase, 0.1 µCi [α³²P]-dCTP, 30 ng and 5 ng respectively of a TaqI-CXX and an EcoRI-AXX selective amplification primer (Table 3.4). The reactions were overlaid with 10 µl mineral oil and amplified using the following PCR program: 3 minutes denaturing at 94 °C; followed by 12 cycles of 30 s at 94 °C, 60 s at 65 °C (reducing by 0.7 °C each cycle), 60 s at 72 °C; with another 23 cycles of 30 s at 94 °C, 60 s at 56 °C, 60 s at 72 °C; ending with a 20 minute final elongation stage at 72 °C.

Following PCR amplification, 10 µl of stop solution were added to each of the reactions. To resolve the AFLP loci, the reactions were denatured by heating to 94 °C for 4 minutes, and 3.5 µl of each reaction loaded onto a 38×50 cm 6 % denaturing acrylamide gel (19:1 acrylamide:bis-acrylamide) in 1×TBE running buffer. The gels were run at 45-50 °C for 5-6 hours, after which they were transferred onto filter paper and dried and the PCR products visualised by autoradiography.
### Table 3.4 Pre-amplification and selective amplification primers used for AFLP genotyping.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TaqI-adapter top strand</td>
<td>For making adapters</td>
</tr>
<tr>
<td>TaqI-adapter bottom strand</td>
<td></td>
</tr>
<tr>
<td>EcoRI-adapter top strand</td>
<td></td>
</tr>
<tr>
<td>EcoRI-adapter bottom strand</td>
<td></td>
</tr>
<tr>
<td>TaqI-C</td>
<td>Pre-amplification primer</td>
</tr>
<tr>
<td>EcoRI-A</td>
<td></td>
</tr>
<tr>
<td>TaqI-CAC</td>
<td>Selective amplification primer</td>
</tr>
<tr>
<td>TaqI-CAG</td>
<td></td>
</tr>
<tr>
<td>TaqI-CCA</td>
<td></td>
</tr>
<tr>
<td>EcoRI-ACA</td>
<td></td>
</tr>
<tr>
<td>EcoRI-AGC</td>
<td></td>
</tr>
<tr>
<td>EcoRI-ATG</td>
<td></td>
</tr>
<tr>
<td>EcoRI-AAC</td>
<td></td>
</tr>
</tbody>
</table>

#### 3.2.4 Scoring microsatellite markers

All microsatellite loci were scored by eye. Microsatellite loci amplified by PCR are characterised by a distinct banding pattern consisting of the main alleles together with fainter stutter bands.

Stutter bands are thought to result from the slippage of *Taq* polymerase during PCR (Hauge and Litt 1993), and in the case of dinucleotide tandem repeats, are usually at intervals of two base pairs above and/or below the actual alleles. Each locus has a different stutter pattern, several examples of which are shown in Figure 3.1. Stutter bands can aid the scoring of microsatellite alleles, especially when the locus in question has a high allelic diversity and a large variation in allele size. However, when the two alleles of a heterozygote differ by only a small number of bases, overlapping stutter bands from the two alleles can make accurate allele size estimation difficult. This problem is particularly pronounced when the two alleles of a heterozygote are only one repeat unit different in size. In such cases, careful examination of the particular stuttering pattern is required to distinguish heterozygotes from homozygotes.
The orientation of gels and autoradiographs were carefully noted at all times to eliminate left-right orientation problems. The loading of known positive standards on all gels also prevented such problems and reduced scoring errors. Genotypes from scored loci were entered into Excel spreadsheets and double-checked against the original autoradiographs to eliminate transcription errors.
Figure 3.1    Microsatellite banding patterns obtained from the amplification of a) Pml10, b) Plgt58 and c) Pml1 in *P. polionotus*. 
3.2.5 Scoring AFLP markers

AFLP loci were scored by eye. Each AFLP primer combination results in the amplification of a large number of loci (Figure 3.2). Every locus potentially has two alleles, the absent and the present allele. Many of the amplified loci are monomorphic and only the present allele is found amongst all genotyped individuals of the species in question. Some of the loci are polymorphic and not all individuals possess a band at the locus in question. Only polymorphic loci that could be unambiguously scored as present or absent were used in further analyses. These presence and absence genotypes were entered into Excel spreadsheets as ones and zeros respectively. To eliminate transcription errors, the entered genotypes were double-checked against the original autoradiographs.

Occasionally aberrant banding patterns were observed for some of the samples. These were easily identifiable, as they were in general characterised by the presence of only a few strongly amplifying bands which were atypical of the species. For the sample in question, similarly aberrant banding patterns would be present for all AFLP primer combinations. In the majority of cases, these aberrant banding patterns were found to be a result of the DNA used in the AFLP procedure being degraded. In other cases, they were probably a result of the digestion and/or ligation phases of the AFLP procedure not proceeding satisfactorily. Where the DNA quality was high, such failures were infrequent, affecting fewer than one in 40 samples. In these cases, the AFLP genotype could usually be obtained by repeating the AFLP procedure on the samples. Aberrant banding patterns were not included in the genotype data.
Figure 3.2  AFLP banding patterns obtained from the amplification of three different primer combinations in *P. polionotus* (a) *TaqI*-CAC + *EcoRI*-AGC, (b) *TaqI*-CAC + *EcoRI*-AAC and (c) *TaqI*-CCA + *EcoRI*-ACA. Some bands could not be scored easily and the black triangles indicate polymorphic loci that were scored unambiguously.
3.2.6 Microsatellite marker characteristics

3.2.6.1 Allele frequencies and heterozygosity

For each microsatellite locus, GENEPOP v3.3 (Raymond and Rousset 1995) was used to calculate the allele frequencies, observed heterozygosity and expected heterozygosity. Observed heterozygosity, $H_O$, was calculated as:

$$H_O = \frac{\sum p_{ij}}{n} \quad (Equation\ 3.1)$$

Expected heterozygosity, $H_E$, was calculated as:

$$H_E = 1 - \sum p_i^2 \quad (Equation\ 3.2)$$

($p_i$ = the frequency of the $i^{th}$ allele, $p_{ij}$ = the number of heterozygotes for alleles $i$ and $j$; and $n$ = the number of individuals)

Expected heterozygosity is the heterozygosity that would be obtained given the allele frequencies at a particular locus under conditions of Hardy-Weinberg equilibrium (HWE) (Hardy 1908). The main conditions are random mating, a large (infinite) population size, negligible migration, no or negligible mutation and selection acting on the locus under consideration. Discrepancies between the observed and expected heterozygosities were tested using GENEPOP. Any such deviation from HWE at specific loci may indicate a high null allele frequency, sex linked loci or linkage to other loci under selection. A locus with a high null allele frequency is characterised by an excess of homozygotes and non-amplification of some samples. Depending on whether the locus is located on the Y or the X chromosome, a sex-linked locus is either characterised by non-amplification in females or by an excess of homozygotes in males. Single loci that deviated significantly from HWE were not used in analyses. If departures from HWE are present in the majority of loci, this may indicate some other violation of the HWE conditions, such as the presence of population structuring, migration, or population bottlenecks.
### 3.2.6.2 Linkage disequilibrium

Two loci are said to be in linkage disequilibrium when the alleles at these loci are in non-random association with each other in a population. This may happen if the two loci are physically linked by being positioned close to one another on the same chromosome so that they do not segregate randomly during meiosis. Linkage disequilibrium may also arise as an artefact of admixture of subpopulations, the presence of kin, or founder effects. GENEPOP was used to estimate whether any linkage existed between all pairs of microsatellite loci.

### 3.2.7 AFLP marker characteristics

#### 3.2.7.1 Allele frequency estimation

AFLP markers are dominant loci with two alleles, the present allele (1) and the absent allele (0). An individual that lacks a band at a particular locus is homozygous for the absent allele (the 0,0 genotype), while an individual with a band at that locus is either homozygous for the present allele (the 1,1 genotype) or is heterozygous (the 0,1 genotype). Thus, at a particular locus, \( i \), the frequencies of the absent allele \( p_i(0) \) and the present allele \( p_i(1) \) are given by:

\[
\begin{align*}
p_i(0) &= \sqrt{1 - \frac{\sum i_n}{n}} \\
p_i(1) &= 1 - p_i(0)
\end{align*}
\]

(Equation 3.3) (Equation 3.4)

\( i_n \) is 1 or 0 depending on whether the \( n^{\text{th}} \) individual possesses or lacks a band at locus \( i \).

The accuracy of the estimates of \( p_i(0) \) and \( p_i(1) \) depend on two assumptions. First, it is assumed that AFLP markers are dominant loci inherited in a Mendelian fashion. Second, it is assumed that the individuals used to make the allele frequency estimates are unrelated to one another.

Verification of the first assumption would require many parent-offspring pairs to be genotyped, something that was not possible in this study. However, previous studies using AFLP markers
(Liu et al. 1998; Maughan et al. 1996) have found this assumption to be valid in most cases. The second assumption is likely to be valid in the majority of species as population sizes are usually large enough such that individuals sampled at random from it are unlikely to be closely related to one another.

### 3.2.7.2 Testing for linkage among AFLP loci

The AFLP genotypes at each locus were encoded as 1 or 0 depending on whether individuals possessed or lacked a band at the locus in question. Possible linkage between the AFLP loci was checked by calculating the correlation coefficient between the genotypes of all pairwise combinations of loci. The correlation coefficient can vary from -1 to 1. If the correlation coefficient between two loci \( i \) and \( j \) is -1, then the presence of a band at locus \( i \) corresponds to the absence of a band at locus \( j \) and vice versa. A correlation of 1 indicates consistent co-migration, or linkage between the two loci in question.

### 3.2.8 Calculating heterozygosity and IR using microsatellites

Microsatellite genotypes were used to calculate multilocus heterozygosity and internal relatedness (IR). Multilocus heterozygosity was calculated simply as the total number of loci at which a particular individual is heterozygous, divided by the number of loci at which it was genotyped. An individual’s IR was calculated from its microsatellite genotypes using Equation 3.5, as described in Amos et al. (2001).

\[
IR = \frac{2H - \sum f_i}{2N - \sum f_i} \quad \text{(Equation 3.5)}
\]

\((H = \text{the number of loci that are homozygous}, \ N = \text{the number of loci and} \ f_i = \text{the frequency of the} \ i\text{th allele contained in the genotype})\)
3.2.9 Calculating pairwise relatedness

Queller and Goodnight (1989) described a method for calculating unbiased estimates of pairwise relatedness between individuals based on co-dominant genetic markers such as allozyme and microsatellite markers. The general formula for calculating the pairwise relatedness, $r_{AB}$, of individual $A$ to individual $B$ is given by Equation 3.6.

$$r_{AB} = \frac{\sum_k \sum_a (p_B - \bar{p})}{\sum_k \sum_a (p_A - \bar{p})}$$

(Equation 3.6)

($p_B$ = frequency of the allele that occupies the $a$th allelic position of the $k$th locus in individual $B$,
$p_A$ = frequency of the allele that occupies the $a$th allelic position of the $k$th locus in individual $A$ and \( \bar{p} \) = population frequency of the $a$th allele in the $k$th locus)

Making use of Equation 3.6, the software Relatedness 4.2 (http://es.rice.edu/projects/Bios321/relatedness.html) was used to calculate pairwise relatedness between individuals from their microsatellite genotypes. Madden et al. (in press) used formulae derived from Equation 3.6 to calculate pairwise relatedness from AFLP genotypes (Table 3.5). By summing across all AFLP loci, these equations were used to calculate the pairwise relatedness between individuals.
### Table 3.5

Formulae used to calculate relatedness values between two individuals, A and B at each AFLP locus. Adapted from Madden et al. (in press). $p_i$ = present allele frequency at the $i$th locus, $q_i = 1 - p_i$ = absent allele frequency at the $i$th locus, $P_{i01}$ = probability that an individual with a band at the at the $i$th locus is heterozygous and $P_{i11}$ = probability that an individual with a band at the at the $i$th locus is homozygous.

<table>
<thead>
<tr>
<th>Band presence in individual A</th>
<th>Band presence in individual B</th>
<th>Relatedness</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>$\frac{4 - 4q_i}{4 - 4q_i} = 1$</td>
</tr>
<tr>
<td>0</td>
<td>1</td>
<td>$\frac{P_{i01}(1 - 2q_i) - 2P_{i11}}{P_{i01}(2 - 2q_i) + 2P_{i11}}$</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>$\frac{P_{i11}^2 (4 - 4p_i) + 2P_{i01}P_{i11} (1 - 2p_i)}{P_{i01}^2 (4 - 4p_i) + 2P_{i01}P_{i11} (2 - 2p_i)}$</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

### 3.3 Data analysis and statistical testing

Unless otherwise mentioned, all statistical analysis was carried out using R 1.6.2 (http://www.r-project.org/). Significance testing was carried out at the $\alpha = 0.05$ level. Programs used for simulations and data analysis were written in Microsoft Visual Basic 6.3.
CHAPTER 4

EVALUATION OF AFLP AND MICROSATELLITE MARKER FOR
ESTIMATING INBREEDING AND RELATEDNESS

4.1 Introduction

A large number of studies have been published that purport to show inbreeding depression. Captive and laboratory studies most convincingly demonstrate the effects of inbreeding depression (Ballou 1997; Charlesworth and Charlesworth 1987; Lacy et al. 1996; Pray and Goodnight 1995; Ralls et al. 1988). In such studies, matings between individuals are arranged, and knowledge of the pedigree allows the calculation of inbreeding coefficients. Wright’s inbreeding coefficient, \( f \) (Wright 1922), is the probability of two homologous genes in an individual being identical by descent. The ability to arrange matings in the laboratory means that individuals with a wide range of inbreeding coefficients can be generated from pairings between unrelated, closely related or distantly related parents. In such situations the effects of inbreeding are more easily observed compared to situations in which only a narrow range of inbreeding coefficients are available. In captivity, reduced fitness as a result of inbreeding has been found in many traits such as neonatal survival (Ballou 1997; Ralls et al. 1988), adult weight (Pray and Goodnight 1995) and reproductive success (Lacy et al. 1996).

In comparison to these captive and laboratory studies, there are very few examples that provide compelling evidence for inbreeding depression in wild populations. This is primarily due to the fact that in order to estimate an individual’s inbreeding coefficient, knowledge of its pedigree for three to four generations is required. These accurate long-term observations are available for only
a handful of populations in a few species such as the Mandarte Island song sparrow (Keller 1998), great tit (Greenwood et al. 1978) and blue tit (Kempenaers et al. 1996). In general, these are small populations where incestuous matings are a consequence of limited numbers of potential mates and result in offspring with a range of inbreeding coefficients.

While pedigree-based measures are seen to be the best possible estimate of an individual’s level of inbreeding, large population sizes together with the requirement for long-term observations mean that pedigree data are not available for the vast majority of systems. In the absence of detailed pedigree information, researchers have attempted to use genetic markers to estimate how inbred wild individuals are. Individuals are genotyped using genetic markers and average heterozygosity at these markers is taken to be an estimate of the individuals’ level of inbreeding.

For a genetic marker to be suitable for estimating individual heterozygosity, it should be selectively neutral. Allozymes were originally used for measuring heterozygosity. However, as allozymes are functional proteins, they are not selectively neutral (Thelen and Allendorf 2001). Different alleles, or combinations of alleles, of a particular allozyme may affect an individual’s fitness, making allozymes far from ideal markers for measuring individual heterozygosity. Since their discovery in 1989 (Litt and Luty 1989; Tautz 1989; Weber and May 1989), microsatellite markers have largely superseded allozymes. Microsatellite markers are thought to be selectively neutral, although they may be linked to other loci that are under selection. They are widespread throughout most genomes and characteristically have high variability. These features make microsatellite markers particularly suitable for measuring individual heterozygosity.

Genotypes from genetic markers such as microsatellites can be used to assess individual heterozygosity by a number of methods. Multilocus heterozygosity can be calculated as simply the proportion of typed loci for which an individual is heterozygous. Derived from this is
standardised heterozygosity (SH), which is the ratio of the heterozygosity of an individual to the mean heterozygosity of those loci at which the individual is typed (Coltman et al. 1999). SH avoids any biases being introduced by an individual not being genotyped at any particular locus. A further improvement is internal relatedness (IR), where the individual heterozygosity score is weighted by the frequency of the alleles in each genotype such that the sharing of rare alleles is weighted more than the sharing of common alleles (Amos et al. 2001). In practice, IR and SH are very closely correlated (Amos et al. 2001; Valsechhi et al. 2004), but IR tends to reveal slightly stronger heterozygosity-fitness effects than SH (Acevedo-Whitehouse et al. 2003; Amos et al. 2001).

A growing number of studies have used heterozygosity measures based on microsatellites to show heterozygosity-fitness correlations (HFCs). These studies have found significant correlations between heterozygosity measures calculated from 5-15 microsatellite markers and a wide variety of fitness traits in a range of systems: neonatal survival in red deer and harbour seals (Coltman et al. 1998; Coulson et al. 1999), parasite load in Soay sheep (Coltman et al. 1999), disease resistance in California sea lions (Acevedo-Whitehouse et al. 2003) and male reproductive success in Antarctic fur seals (Hoffman et al. in press).

Two hypotheses have been put forward to explain how observed correlations between microsatellite heterozygosity and fitness can arise. First, variation at microsatellite loci may reflect genome-wide heterozygosity at unlinked genes exhibiting overdominance or segregating for deleterious recessive alleles. This has been referred to as the general effect hypothesis and is the basis for inbreeding depression (David 1998; Hansson and Westerberg 2002). Second, one or more of the microsatellite markers used could be in linkage disequilibrium with genes under balancing selection. Such single locus heterosis is also known as the local effect hypothesis (Hansson and Westerberg 2002; Hansson et al. 2004).
The relative importance of the general and local effect hypotheses at explaining HFCs is currently under debate. Most studies showing HFCs are based on a small number of microsatellite loci (typically 5-15) and an accurate estimate of genome-wide heterozygosity might not be expected from sampling such a small fraction of the genome. This is especially so in systems consisting of large homogenous populations where the vast majority of individuals are expected to have levels of inbreeding that are zero, or close to zero (Balloux et al. in press).

Small population sizes and strong population structuring are circumstances that can give rise to conditions under which incestuous matings are not uncommon (Hoffman et al. in press; Keller 1998). There are a small number of studies of island populations where inbreeding coefficients are known in wild populations. Among these are populations of red deer and Soay sheep, both of which have polygynous mating systems, the former with a stable population of 1175-1724 and the latter with a fluctuating population of 694-1826 (Marshall et al. 2002), a population of great tits (population size ~100, van Noordwijk and Scharloo 1981), and a population of ground finches (population size ~1060, Gibbs and Grant 1989). These studies suggest that while inbreeding coefficients greater than 0.125 are rare in wild populations, comprising merely 0.5-2.7 % of the populations, non-zero inbreeding coefficients less than 0.125 are not uncommon, making up 3.2-19 % of the populations. In such populations, the question is whether or not a small number of microsatellite markers can detect inbreeding coefficients of 0.125.

Although microsatellite loci are currently widely used for detecting HFCs, there are some disadvantages associated with their use. The major drawback is that microsatellite loci have not been cloned for the majority of species. Some cross-species amplification of microsatellite loci is possible between closely related species (Menotti-Raymond and O’Brien 1995; Primmer et al. 1996). However, microsatellites have not been cloned for many species and there are particularly large gaps in certain groups such as plants and invertebrates. Therefore, in general, the study of a
new species requires the development of new species-specific microsatellite loci, a process that is
time consuming.

An alternative to using microsatellite loci for detecting HFCs is the use of molecular markers that
can be applied to any species. Multilocus DNA fingerprinting (Jeffreys et al. 1985) is a technique
that can be used in any species and has been used in the past to assess population heterozygosity
(Kuhnlein et al. 1990; Stephens et al. 1992). The DNA fingerprint profile of an individual
consists of a series of bands representing alleles at different loci and a more heterozygous
individual will have a larger number of bands. However, the technique of DNA fingerprinting is
not straightforward and there are problems with reliability and with comparisons between
samples and gels. Random amplified polymorphic DNA (RAPD, Williams et al. 1990) and
amplified fragment length polymorphisms (AFLP, Vos et al. 1995) succeeded DNA
fingerprinting as multilocus markers which can be amplified by PCR in any species. Of the two,
AFLPs show far higher levels of reliability and reproducibility.

AFLP markers can be used to estimate individual heterozygosity by simply counting the number
of bands an individual possesses. AFLP markers are dominant loci, with each locus having only
two alleles: the absent allele (0), and the present allele (1). For each locus, an individual either has
a band, the present state, or does not have a band, the absent state. In the absent state an
individual is homozygous for the absent allele (the 0,0 genotype). However, in the present state,
the individual is either homozygous for the present state (the 1,1 genotype), or is heterozygous
(the 1,0 genotype). An individual’s heterozygosity can therefore be estimated by counting the
number of polymorphic loci at which it has a band. The more bands an individual has, the more
heterozygous, and therefore less inbred, it is likely to be.
In this chapter I estimate the heterozygosity of over 180 wild and captive-bred old-field mice, *Peromyscus polionotus*, using AFLP markers and microsatellite loci. To test the validity of these estimators, they are compared with pedigree-based inbreeding coefficients. This system is also used to compare AFLP and microsatellite-based estimators of relatedness.

### 4.2 Materials and methods

#### 4.2.1 The *Peromyscus polionotus* system

Wild *Peromyscus polionotus subgriseus* were trapped in 1998. A total of 36 wild old-field mice were collected and used to found laboratory stocks at Brookfield Zoo (Brookfield, Illinois). Twenty-six of these wild mice were randomly selected to produce the experimental stocks. Over 10 generations, mice were paired to produce offspring with a range of inbreeding coefficients and the resulting pedigree was recorded. The breeding design was not regular and pairings were arranged to mimic the complex pedigree that might develop in a small closed natural population. In each generation, four classes of pairings were made: a) between mice with low inbreeding coefficients ($f < 0.1$) to produce non-inbred offspring, b) between non-inbred but related mice to produce inbred offspring, c) between inbred but unrelated mice to produce non-inbred offspring, d) between inbred and related mice to produce highly inbred offspring. Some pairings were between full-sibs or half-sibs, but never between littermates. The crosses were carried out at Brookfield Zoo by R. C. Lacy and G. Alaks. Assuming unrelated founders, the resulting progeny had inbreeding coefficients in the range of 0 to 0.453.

#### 4.2.2 Microsatellite and AFLP genotyping

All 36 of the original wild mice and 145 randomly chosen captive-bred mice with inbreeding coefficients in the range of 0 to 0.453 were genotyped at 12 microsatellite loci (Table 3.2) and with eight AFLP primer combinations (*Taq*I-CAC with *Eco*RI-ACA, *Taq*I-CCA with *Eco*RI-ACA, etc.)
\[ f_{AFLP} = \frac{\sum S_i}{n} \]  
(Equation 4.1)

\((S_i = 0 \text{ when a band is absent and } S_i = 1 \text{ when a band is present at each of the } n \text{ polymorphic loci}).\)

Inbreeding was also estimated using the microsatellite loci by calculating microsatellite heterozygosity, \(f_{\text{micro}}\) for all individuals. Microsatellite heterozygosity was calculated as the total number of loci at which a particular individual is heterozygous, divided by the number of loci at which it was genotyped. Other estimators of inbreeding such as internal relatedness (Amos et al.
2001) were not used as they require the knowledge of allele frequencies, and these varied widely from generation to generation within the *P. polionotus* pedigree.

### 4.2.4 Estimating relatedness

Using the known pedigree, and assuming unrelated wild founders, a pairwise relatedness matrix for both wild and captive mice was calculated representing the probability of sharing genes that are identical by descent, $r_{\text{ped}}$. Pairwise relatedness matrices were also estimated for the wild and captive-bred mice from the genetic data in three ways:

1) Using AFLP genotypes, $r_{\text{AFLP1}}$ was calculated as the number of identical states, either band present or absent, between a pair of individuals (0 or 1 per locus) divided by the number of loci used.

2) Using AFLP genotypes, $r_{\text{AFLP2}}$ was calculated between pairs of individuals as the correlation coefficient between the presence-absence scores of their AFLP genotypes based on the 94 polymorphic loci.

3) Using microsatellite genotypes, $r_{\text{micro}}$ was calculated as the total number of identical alleles between a pair of individuals (0, 1 or 2 per locus) divided by twice the number of loci used (Blouin et al. 1996; Ellegren 1999).

When allele frequencies are known, microsatellite and AFLP markers can be used to calculate more sensitive measures of relatedness such as the method described in Queller and Goodnight (1989). This measure gives greater weight to the sharing of rare alleles compared to the sharing of common alleles. AFLP and microsatellite allele frequencies in the wild population could be
estimated from the genotypes of the wild mice. Therefore, for the wild mice, in addition to the previous three measures, the AFLP genotypes were used to calculate $r_{\text{AFLPQG}}$ (Madden et al. in press) and the microsatellites genotypes were used to calculate $r_{\mu\text{GQG}}$ (Queller and Goodnight 1989).

### 4.2.5 Analysis of inbreeding and relatedness estimates

To assess the reliability of both types of genetic markers at estimating levels of inbreeding, microsatellite heterozygosity ($f_{\text{sat}}$) and AFLP band count ($f_{\text{AFLP}}$) were regressed against the pedigree-based inbreeding coefficients, $f_i$ individually, and then together in a multiple regression for all genotyped mice. Linear regressions between $f_{\text{sat}}$ and $f_{\text{AFLP}}$ were also carried out to assess the relationship between these estimators of inbreeding, using all individuals, and using just the wild mice.

Inbreeding coefficients greater than 0.125 are rare in wild populations, while non-zero inbreeding coefficients up to 0.125 are often not uncommon. To examine the utility of the genetic estimators, $f_{\text{sat}}$ and $f_{\text{AFLP}}$, at estimating inbreeding at the levels of inbreeding found in natural populations, the genotyped mice were split into low and high inbreeding groups. The low inbreeding group consisted of individuals with zero to moderate inbreeding levels, $f \leq 0.125$ ($n = 104$), and the high inbreeding group of individuals with high inbreeding levels, $f > 0.125$ ($n = 77$). The regressions of $f$ against $f_{\text{sat}}$ and $f_{\text{AFLP}}$ were repeated on these two groups.

To compare the AFLP and microsatellite-based estimates of relatedness with known pedigree-based relatedness, correlation coefficients were calculated between the relatedness matrices based on genotypes ($r_{\text{AFLP1}}$, $r_{\text{AFLP2}}$ and $r_{\mu\text{GQG}}$) and the pedigree-based relatedness matrix, $r_{\text{ped}}$, for all genotyped mice. To test the significance of the correlations, Mantel tests were carried out with
100,000 rounds of randomisation using the software \( zt \) (Bonnet and Peer 2002). In order to gauge how similar the genetic estimators of relatedness are, pairwise correlations between the relatedness matrices \( r_{AFLP1} \), \( r_{AFLP2} \) and \( r_{\text{sat}} \) were calculated. Again the significance of the correlations were tested with Mantel tests.

The wild mice were assumed to be unrelated to one another. However, to test whether detectable levels of relatedness exist within the wild mice, the correlation coefficient between the relatedness matrices \( r_{AFLP} \) and \( r_{\text{sat}} \) was calculated and its significance tested with a Mantel test.

### 4.2.6 Allele dropping simulations

Both the pedigree of the captive-bred mice and the genotypes of the wild founders at the base of the pedigree are known, therefore, it is possible to generate potential genotypes of the captive-bred mice by simulating the inheritance of known founder alleles through the pedigree.

Simulation of different scenarios allow comparisons to be made with the actual genotypes in order to check the inheritance of the markers. Such simulations can also indicate how the identity of alleles by state rather than by descent can affect the relationships between inbreeding coefficients and heterozygosity. Separate simulations were carried out for microsatellites and AFLPs.

Three situations were modelled for the microsatellite allele dropping. 1) Founders with actual genotypes, with wild mice being assigned their true genotypes. 2) Founders with random alleles, where the genotypes at each of the 12 microsatellite loci were randomly allocated to each founder at the beginning of each simulation round based on the known allele frequencies at the loci. 3) Founders with unique alleles, where 72 different alleles were assigned to the 36 wild mice at each of the 12 microsatellite loci such that no founder shared any alleles with any other founder.
Situation 1) was simulated in order to establish whether the observed relationship between \( f \) and \( f_{\mu_{sat}} \) and \( r_{ped} \) with \( r_{\mu_{sat}} \) obtained from genotyping match what would be expected given the pedigree and the founder genotypes. Differences in the results between situations 1) and 2) will provide an indication as to whether or not the actual genotypes of the wild mice are a random selection based on the allele frequencies. As the founding mice in situations 1) and 2) have some alleles in common with each other, alleles in their descendants may be identical by state or by descent. In contrast, as all the founders in situation 3) have unique alleles, none of the alleles in their descendants will be identical by state. Therefore, a comparison between the results of situations 1) and 3) will indicate the extent to which identity by state affects the strength of the relationships of \( f \) with \( f_{\mu_{sat}} \) and \( r_{ped} \) with \( r_{\mu_{sat}} \).

To simulate the Mendelian inheritance of alleles, in all three situations alleles were randomly chosen from the founders and passed down through the pedigree at each of the 12 loci. At the end of each simulation round, average heterozygosity of each of the 181 study mice was calculated from the simulated genotypes to obtain simulated \( f_{\mu_{sat}} \) values. In addition, the simulated genotypes were also used to calculate the pairwise relatedness matrix, \( r_{\mu_{sat}} \) for the 181 study mice. 1000 simulation rounds were carried out resulting in an array of simulated \( f_{\mu_{sat}} \) and \( r_{\mu_{sat}} \) values from which the variance in the simulated \( f_{\mu_{sat}} \) and \( r_{\mu_{sat}} \) could be estimated.

AFLP allele dropping was based on the 94 polymorphic loci that were genotyped. The simulations were carried out assuming the markers were dominant and had two alleles: the present allele (1) and the absent allele (0). Using the genotypes of the 36 wild mice, the frequencies of the absent and present alleles at each of the 94 loci was estimated assuming that the two alleles at each locus were in Hardy-Weinberg equilibrium. Knowledge of these allele
frequencies allowed estimation of the probability, \( p_i \), that a wild founder with a band at a particular locus, \( i \), was genotype (0,1) rather than (1,1).

For the AFLP allele dropping, two situations were modelled. 1) Founders with actual genotypes. At the beginning of each allele dropping simulation round, the presence-absence genotypes of each wild mouse, together with \( p_i \) at the 94 loci was used to assign founder genotypes. The absence genotype always had the (0,0) allele combination, while the ambiguous presence genotype was assigned the genotypes (1,0) or (1,1) with probability \( p_i \) and 1 - \( p_i \) respectively.

2) Founders with random alleles. At the beginning of each simulation round, alleles were randomly allocated to each wild mouse based on the estimates of the absent and present allele frequencies at each of the 94 AFLP loci. Differences in the results between these two situations may provide an indication as to whether or not the actual genotypes of the wild mice are a random selection based on the allele frequencies.

For both situations, alleles were passed down the pedigree by Mendelian inheritance. At the end of each simulation round, the simulated AFLP band count, \( f_{AFLP} \), and the pairwise related matrix, \( r_{AFLP} \), were calculated for each of the 181 study mice. This process was repeated over 1000 rounds, resulting in an array of simulated \( f_{AFLP} \) and \( r_{AFLP} \). Since the relatedness measures \( r_{AFLP1} \) and \( r_{AFLP2} \) were found to be very similar, only \( r_{AFLP1} \) was calculated from the allele dropping simulations.

Linear regressions of pedigree-based inbreeding coefficients, \( f_i \), were carried out separately against simulated \( f_{AFLP} \) and \( f_{\mu_{sat}} \) obtained from each of the 1000 rounds of allele dropping to obtain \( r^2 \) values. This allowed the calculation of the average value and the standard deviation of the \( r^2 \) for comparison with the same regressions based on the actual genetic data of the inbred mice.

Pedigree-based relatedness, \( r_{ped} \), was correlated separately against simulated \( r_{AFLP1} \) and \( r_{\mu_{sat}} \) from
each round of simulation. From these, the mean and the standard deviation of the correlation coefficient was calculated for comparison with the correlation coefficients of $r_{ped}$ against $r_{AFLP1}$ and $r_{\mu_{sat}}$ based on the actual genotypes of the inbred mice.

4.2.7 Locus dropping simulations - the effect of locus number

Reducing the number of AFLP and microsatellite markers used in estimating levels of inbreeding and relatedness will decrease the power of these estimators. To investigate how the relationships of pedigree-based inbreeding coefficient, $f$, with $f_{AFLP}$ and $f_{\mu_{sat}}$ and those of pedigree-based relatedness, $r_{ped}$ with $r_{AFLP1}$ and $r_{\mu_{sat}}$ are affected by the number of loci used in these estimators of inbreeding and relatedness, separate locus dropping simulations were conducted for AFLP and microsatellites loci.

In the locus dropping simulations, $n$ loci were chosen at random from the available 12 microsatellite and 94 AFLP loci. From the genotypes at these $n$ loci, $f_{AFLP}$, $f_{\mu_{sat}}$, and the pairwise relatedness matrices $r_{AFLP1}$ and $r_{\mu_{sat}}$ were calculated for all the 181 study mice. For each value of $n$, the process was repeated 100 times with different combinations of the $n$ loci so that an estimate of the variance in $f_{AFLP}$, $f_{\mu_{sat}}$, $r_{AFLP1}$ and $r_{\mu_{sat}}$ could be obtained. Where fewer than 100 combinations of $n$ loci existed, all possible combinations were used. For the microsatellites, $n$ was increased from 2 to 11, and for the AFLPs from 2 to 92, in steps of one.

Separate linear regressions of $f$ against $f_{AFLP}$ and $f_{\mu_{sat}}$ were carried out for the 100 different locus combinations at each value of $n$. This allowed the calculation of the mean and the standard deviation of $r^2$ for each value of $n$. To investigate the effect of varying locus number on the accuracy of inbreeding estimates at high and low levels of inbreeding, the mice were also divided.
into two groups with \( f \leq 0.125 \) and \( f > 0.125 \). The regressions of \( f \) against \( f_{AFLP} \) and \( f_{\mu sat} \) were repeated for both these groups for the 100 locus combinations at each value of \( n \).

To investigate how reducing the number of AFLP and microsatellite loci affects relatedness estimates, correlation coefficients of \( r_{ped} \) with \( r_{AFLP} \) and \( r_{\mu sat} \) were calculated for each of the 100 locus combinations at each selected locus number. This allowed the calculation of the mean and the standard deviation of the correlation coefficient for the range of locus numbers investigated.

## 4.3 Results

### 4.3.1 Analysis of inbreeding and relatedness estimates

The mean pedigree-based inbreeding coefficient, \( f \), of the 181 *P. polionotus* was 0.151, and the variance in \( f \) was 0.023. Strong correlations exist between \( f \) and estimates of inbreeding based both on AFLP markers and microsatellite loci (\( f_{AFLP} \): \( r^2 = 0.32 \), \( n = 181 \), \( p < 2 \times 10^{-16} \); \( f_{\mu sat} \): \( r^2 = 0.39 \), \( n = 181 \), \( p < 2 \times 10^{-16} \); Figure 4.1 and Table 4.1). In the multiple regression of \( f \) of all mice against \( f_{AFLP} \) and \( f_{\mu sat} \), 53\% of the variance in \( f \) is explained (\( r^2 = 0.53 \), \( n = 181 \), \( p = 1 \times 10^{-11} \) and < \( 2 \times 10^{-16} \) respectively). As \( f_{AFLP} \) and \( f_{\mu sat} \) are correlated with one another it was not possible to establish the proportion of the variance explained by each estimator in the multiple regression.

Partitioning the mice into zero to moderate levels of inbreeding (\( f \leq 0.125 \)), and high levels of inbreeding (\( f > 0.125 \)), reveals that both \( f_{AFLP} \) and \( f_{\mu sat} \) are better estimators at lower levels of inbreeding (Figure 4.2 and Table 4.1). At lower levels of inbreeding, \( f_{AFLP} \) is a better estimator (\( r^2 = 0.15 \), \( n = 104 \), \( p = 5 \times 10^{-5} \)), explaining more than twice the variance in \( f \) compared to \( f_{\mu sat} \) (\( r^2 = 0.06 \), \( n = 104 \), \( p = 0.01 \)). At higher levels of inbreeding, although there is still a weak relationship between \( f_{\mu sat} \) and \( f \) (\( r^2 = 0.05 \), \( n = 77 \), \( p = 0.05 \)), no significant relationship exists between \( f_{AFLP} \) and \( f \) (\( r^2 = 0.02 \), \( n = 77 \), \( p = 0.2 \)).
When the inbreeding estimators for both wild and captive-bred mice are compared with one another, \( f_{AFLP} \) correlates significantly with \( f_{\mu sat} \) \((r^2 = 0.11, n = 181, p = 7 \times 10^{-6})\). This indicates that these estimators carry significant amounts of common information. There is however no such relationship between the estimators when only the wild mice were used in the analysis \((r^2 = 0.00, n = 36, p = 0.97)\).

<table>
<thead>
<tr>
<th></th>
<th>( f(0-0.453) )</th>
<th>( f \leq 0.125 )</th>
<th>( f &gt; 0.125 )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( n = 181 )</td>
<td>( n = 104 )</td>
<td>( n = 77 )</td>
</tr>
<tr>
<td>( r^2 )</td>
<td>( p )</td>
<td>( r^2 )</td>
<td>( p )</td>
</tr>
<tr>
<td>( f_{AFLP} )</td>
<td>0.32</td>
<td>0.15</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>( 2 \times 10^{-16} )</td>
<td>( 5 \times 10^{-3} )</td>
<td>( 0.2 )</td>
</tr>
<tr>
<td>( f_{\mu sat} )</td>
<td>0.39</td>
<td>0.06</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>( 2 \times 10^{-16} )</td>
<td>( 0.01 )</td>
<td>( 0.05 )</td>
</tr>
</tbody>
</table>

Table 4.1  Single factor linear regressions of pedigree-based inbreeding coefficients, \( f_i \), against the two genetic estimators of inbreeding based on AFLP markers, \( f_{AFLP} \), and microsatellite markers, \( f_{\mu sat} \).

AFLP and microsatellite-based estimators of relatedness \( (r_{AFLP}, r_{AFLP2} \) and \( r_{\mu sat}) \) correlate significantly with pedigree-based relatedness, \( r_{ped} \) (correlation coefficients of 0.391, 0.396 and 0.550 respectively, \( n = 181, p = 0.00001 \), Figure 4.3). Table 4.2 shows the correlation coefficients of all pairwise comparisons between the relatedness matrices \( r_{AFLP}, r_{AFLP2}, r_{\mu sat}, r_{AFLPQG} \) and \( r_{\mu satQG} \).

The two AFLP-based estimators, \( r_{AFLP1} \) and \( r_{AFLP2} \) are very similar to one another \((r = 0.993)\). With the wild mice it was possible to calculate relatedness measures that make use of allele frequencies, \( r_{AFLPQG} \) and \( r_{\mu satQG} \) (Madden et al. in press; Queller and Goodnight 1989). \( r_{AFLPQG} \) correlates strongly with \( r_{AFLP1} \) and \( r_{AFLP2} \) \((r = 0.932 \) and \( r = 0.946 \) respectively), as does \( r_{\mu satQG} \) with \( r_{\mu sat} \) \((r=0.957)\).
Table 4.2  Correlation coefficients between pairwise relatedness matrices based on wild and captive-bred mice (n = 181). The correlation coefficients between relatedness matrices for only the wild mice (n = 36) are italicised. \( r\text{AFLP}G \) and \( r\text{µsat}G \) were not calculated for the captive-bred mice.

The pairwise relatedness matrices \( r\text{AFLP}1 \) and \( r\text{µsat} \) for the 36 wild mice are significantly correlated with each other (\( r = 0.199, p = 0.00004 \)). The relatedness matrices \( r\text{AFLP}1 \) and \( r\text{µsat} \) estimate the relatedness between the wild mice using different types of markers. As they are estimating the same parameter, a significant correlation between the two relatedness matrices indicates that there is an overlap of information between the two estimators, which probably arises from there being detectable levels of relatedness between the wild caught mice.
Figure 4.1  Linear regressions of pedigree-based inbreeding coefficients against the genetic estimators of inbreeding based on A) 94 AFLP markers, $f_{AFLP}$ and B) 12 microsatellite markers, $f_{\mu sat}$ for 181 wild and captive-bred mice. $p < 2 \times 10^{-16}$. 
Figure 4.2  Linear regressions of pedigree-based inbreeding coefficients against the genetic estimators of inbreeding based on A) 94 AFLP markers, $f_{AFLP}$ and B) 12 microsatellite markers, $f_{micro}$ for 181 wild and captive-bred mice. The mice are partitioned into two groups: zero to moderate inbreeding ($f \leq 0.125$, filled circles) and high inbreeding ($f > 0.125$, open circles).
Figure 4.3 Pedigree-based pairwise relatedness between 181 wild and captive-bred mice, \( r_{\text{ped}} \), plotted against relatedness estimates based on A) 94 AFLP markers, \( r_{\text{AFLP}} \), B) 12 microsatellite markers, \( r_{\text{sat}} \).
4.3.2 Allele and locus dropping simulations

Results of the allele dropping simulations are summarised in Table 4.3 and Table 4.4 together with the corresponding results from the actual genotyping. The genotyping results fall within two standard deviations of the results obtained from the allele dropping simulation using actual alleles (Table 4.3 and Table 4.4) and so are not significantly different from them. The results of the allele dropping based on founders with their actual alleles and random alleles are very also similar. Allele dropping simulations based on the microsatellite data show that the relationships between \( f_{\mu} \) and \( f \), and that between \( r_{\mu} \) and \( r_{ped} \) are strongly affected by the amount of allele sharing between the founders of the population. When the founders all carry unique alleles, the \( r^2 \) for the linear regressions of \( \mu_{\text{sat}} \) with \( f \) is 0.72 ± 0.04, compared to 0.46 ± 0.06 obtained when the founders carry their actual genotypes. Similarly, the correlation between \( r_{\mu} \) and \( r_{ped} \) is 0.904 ± 0.004 when the founders do not share any alleles, compared to 0.57 ± 0.02 when the actual founder genotypes are used in the simulations.

Figure 4.4 and Figure 4.5 illustrate how the strength of the relationship between pedigree-based inbreeding coefficient and the genetic estimators of inbreeding, \( f_{\text{AFLP}} \) and \( f_{\text{sat}} \), are affected by the number of loci used in the estimations. When all levels of inbreeding are considered (Figure 4.4), for equivalent amounts of genotyping effort, microsatellite loci appear to be better estimators than AFLP loci, giving higher mean \( r^2 \) values for the relationship between inbreeding coefficient and the genetic estimators. However, when low levels of inbreeding are considered (\( f \leq 0.125 \)), Figure 4.5 indicates that AFLP loci are better than microsatellite loci at estimating inbreeding coefficients, giving higher mean \( r^2 \) values. The effect of locus number on the relatedness estimators \( r_{\text{AFLP}} \) and \( r_{\mu} \), is shown in Figure 4.6. A small number of microsatellite loci are capable of conveying more information about relatedness compared to AFLP loci, with three to four microsatellite loci being approximately equivalent to 90 AFLP loci in this respect.
### Table 4.3

Summary of the $r^2$ values obtained from linear regressions of pedigree-based inbreeding coefficients, $f$, against the genetic estimators of inbreeding, $f_{AFLP}$ and $f_{sat}$, calculated from i) actual genotypes and ii) genotypes obtained from allele dropping simulations. Results shown are $\pm$ one standard deviation.

<table>
<thead>
<tr>
<th></th>
<th>Simulated genotypes</th>
<th>Actual genotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>$f_{AFLP}$: actual alleles</td>
<td>0.20 ± 0.06</td>
<td>0.32*</td>
</tr>
<tr>
<td>$f_{AFLP}$: random alleles</td>
<td>0.23 ± 0.09</td>
<td>-</td>
</tr>
<tr>
<td>$f_{sat}$: actual alleles</td>
<td>0.46 ± 0.06</td>
<td>0.39*</td>
</tr>
<tr>
<td>$f_{sat}$: random alleles</td>
<td>0.48 ± 0.07</td>
<td>-</td>
</tr>
<tr>
<td>$f_{sat}$: unique alleles</td>
<td>0.72 ± 0.04</td>
<td>-</td>
</tr>
</tbody>
</table>

* $p < 2 \times 10^{-16}$

### Table 4.4

Summary of the correlation coefficients of the pedigree-based pairwise relatedness matrix, $r_{ped}$, against pairwise relatedness matrices based on AFLP and microsatellite markers: $r_{AFLP}$ and $r_{sat}$, calculated from i) actual genotypes and ii) genotypes obtained from allele dropping simulations. Results shown are $\pm$ one standard deviation.

<table>
<thead>
<tr>
<th></th>
<th>Correlation coefficient with $r_{ped}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Simulated genotypes</td>
</tr>
<tr>
<td>$r_{AFLP}$: actual alleles</td>
<td>0.44 ± 0.03</td>
</tr>
<tr>
<td>$r_{AFLP}$: random alleles</td>
<td>0.46 ± 0.04</td>
</tr>
<tr>
<td>$r_{sat}$: actual alleles</td>
<td>0.57 ± 0.02</td>
</tr>
<tr>
<td>$r_{sat}$: random alleles</td>
<td>0.51 ± 0.03</td>
</tr>
<tr>
<td>$r_{sat}$: unique alleles</td>
<td>0.904 ± 0.004</td>
</tr>
</tbody>
</table>

* $p = 0.00001$
**Figure 4.4** $r^2$ values for the relationship between pedigree-based inbreeding coefficients and genetic estimators of inbreeding based on A) 94 AFLP markers, $f_{AFLP}$, and B) 12 microsatellite markers, $f_{\mu}$. Using different numbers of loci for all levels of inbreeding, $r^2$ values were obtained from separate linear regressions of inbreeding coefficients against $f_{AFLP}$ and $f_{\mu}$. Each point is an average based on 100 unique sets of loci and the vertical lines indicate the standard deviation.
Inbreeding coefficients 0 – 0.125

**Figure 4.5** $r^2$ values for the relationship between pedigree-based inbreeding coefficients and genetic estimators of inbreeding based on A) 94 AFLP markers, $f_{AFLP}$ and B) 12 microsatellite markers, $f_{\mu sat}$, using different numbers of loci at low levels of inbreeding. $r^2$ values were obtained from separate linear regressions of inbreeding coefficients against $f_{AFLP}$ and $f_{\mu sat}$. Each point is an average based on 100 unique sets of loci and the vertical lines indicate the standard deviation.
Figure 4.6  Relationship between pedigree-based pairwise relatedness, $r_{ped}$, and genetic estimators of relatedness based on A) 94 AFLP markers, $r_{AFLP}$, and B) 12 microsatellite markers, $r_{micro}$, using different numbers of loci. The strength of the relationship is given by the correlation coefficient between the pairwise relatedness matrices $r_{AFLP}$ and $r_{micro}$ and $r_{ped}$. Each point is an average based on 100 unique sets of loci and the vertical lines indicate the standard deviation.
4.4 Discussion

In this chapter I have described a novel method for estimating levels of inbreeding using AFLP markers. The utility of this method at estimating inbreeding was evaluated and compared with that of microsatellite heterozygosity by genotyping wild and captive-bred *Peromyscus polionotus* at 94 AFLP and 12 microsatellite loci. A comparison between pedigree-based inbreeding coefficients and inbreeding estimates based separately on the AFLP and microsatellite genotypes showed that, for the numbers of loci used, both the AFLP and microsatellite-based measures carry similar amounts of information about inbreeding when a wide range of inbreeding coefficients are investigated. At lower levels of inbreeding, the AFLP-based estimator correlated more strongly with known inbreeding coefficients. However, for the numbers of loci used, relatedness measures based on microsatellite loci correlate more strongly with pedigree-based pairwise relatedness compared to relatedness measures based on AFLP loci.

Not many studies have investigated the relationship between microsatellite heterozygosity and inbreeding coefficients. Slate et al. (2004) has summarised the results of several studies in which pedigree-based inbreeding coefficients have been correlated against microsatellite heterozygosity. In two of the seven populations examined in Slate et al. (2004) there were strong correlations between inbreeding coefficients and heterozygosity (large ground finch, *Geospiza magnirostris*: $r^2 = 0.29$, Grant et al. 2001; wolves; *Canis lupus*: $r^2 = 0.52$, Hedrick et al. 2001) and in a third population with a low mean inbreeding coefficient there was a significant but weak correlation between the two parameters (medium ground finch, *Geospiza fortis*: $r^2 = 0.04$, Markert et al. 2004). In the four other populations examined, correlations between inbreeding coefficients and heterozygosity were either very weak or non-existent. However, in these four populations there was evidence of inaccuracies in the pedigrees. Such inaccuracies result in the incorrect calculation
of inbreeding coefficients, therefore, the correlations coefficients between inbreeding coefficients and heterozygosity observed in these four populations should be viewed with caution.

My study represents another example of a population in which a strong correlation exists between inbreeding coefficients and microsatellite heterozygosity. In contrast to many of the populations reviewed in Slate et al. (2004), the inbreeding coefficients of the *P. polionotus* used in the experiment are likely to be accurate as the mice were reared in a laboratory and errors in the pedigree will be low or absent. The wolf and large ground finch studies, in which strong correlations between inbreeding coefficients and microsatellite heterozygosity were observed, were based on relatively small populations of 30 and 76 individuals respectively, founded by a only a few individuals (Grant et al. 2001; Hedrick et al. 2001). In my study, a larger number of individuals were used, specifically 181 *P. polionotus* consisting of 36 wild individuals and 145 individuals from a captive-bred population founded by 29 of the wild mice. For the *P. polionotus* population, both the microsatellite and AFLP estimates of heterozygosity perform similarly, correlating strongly with pedigree-based inbreeding coefficients.

### 4.4.1 Detecting inbreeding in wild populations

The strong correlations between known inbreeding coefficients and estimators of inbreeding found in this study are based on animals with a wide range of inbreeding coefficients. However, a wide range of inbreeding coefficients is unlikely to be found in most wild populations. Population simulations reported in Balloux et al. (in press) indicate that in large homogenous populations of 1000 individuals, incestuous matings are not common enough to result in levels of inbreeding that can be detected by 200 microsatellite loci, let alone the small numbers usually employed in studies estimating inbreeding. This leads to the question of whether or not AFLP or microsatellite loci can detect inbreeding in wild populations.
A comparison between the AFLP and microsatellite-based estimators of inbreeding for the inbred mice showed a highly significant correlation between the two estimators ($r^2 = 0.11$). This indicates that when a wide range of inbreeding coefficients is considered, both estimators carry detectable amounts of common information about inbreeding. However, no significant correlation was found when the AFLP and microsatellite-based estimators of inbreeding for the 36 wild mice were compared. This lack of correlation suggests that the levels of inbreeding in the wild mice are too low to be detected by the numbers of markers employed in this study. However, the amount of overlap between these two estimators of inbreeding is not large ($r^2 = 0.11$) and a significant correlation may not be detectable in a small sample size of only 36 individuals. The fact that the pairwise relatedness estimates for the 36 wild mice based on AFLP and microsatellite markers do correlate significantly with each other indicates that there are detectable levels of relatedness between the wild mice. These levels of relatedness should in theory lead to the offspring from pairings of the wild mice having levels of inbreeding that can be measured using molecular markers.

The expectation that levels of inbreeding in the wild cannot be detected using large numbers of microsatellite loci is based on the assumption that all wild populations are large and homogenous. However, small population sizes are not uncommon, especially for large predators, for island populations and in fragmented habitats. In addition, most populations are not homogenous but instead have some level of population structuring, making incestuous matings more common. Overlapping generations, high reproductive skew and philopatry may also give rise to circumstances in which close inbreeding can occur naturally (Hoffman et al. 2003). In many plant species, in the absence of opportunities for cross-fertilisation, self-fertilisation is known to occur frequently, resulting in close inbreeding (Lande and Schemske 1985; Stebbins 1950). Thus, the combination of small population sizes with particular population structures and reproductive
strategies may give rise to circumstances under which levels of inbreeding in wild populations may be high enough to be detected using molecular markers.

As inbreeding coefficients greater than 0.125 are rare in wild populations, the relationship between heterozygosity and inbreeding coefficients was examined in all *P. polionotus* with $f < 0.125$. With the numbers of loci used, both AFLP and microsatellite-based estimators of inbreeding perform better at lower levels of inbreeding compared to at higher levels of inbreeding ($f > 0.125$). A possible reason for this pattern is that at higher levels of inbreeding a large fraction of the loci will be homozygous, saturating the information carrying capacity of both genetic estimators such that a larger number of loci would be required to achieve a better estimation. The AFLP band count is particularly prone to this saturation effect as each locus has only two alleles.

In the *P. polionotus* system, at low inbreeding coefficients the AFLP estimator appears to outperform the microsatellite estimator. Extrapolations based on Figure 4.5 suggest that at these low levels of inbreeding, 90 AFLP markers give the same resolution as 25 to 30 microsatellite loci. The amount of experimental effort required to genotype *P. polionotus* individuals at 90 AFLP markers was approximately equivalent to genotyping the individuals at 12 microsatellite loci. As high levels of inbreeding are expected to be rare in wild populations, this result suggests that for species with similar levels of AFLP polymorphism to *P. polionotus*, AFLPs would appear to be more suitable markers than microsatellites for estimating inbreeding in wild populations.

### 4.4.2 Advantages and drawbacks of the AFLP band count

The major advantage of the AFLP band count method over microsatellite heterozygosity for estimating inbreeding is that AFLP loci can be amplified with ease from any species without the need for the time consuming cloning and optimisation required when isolating new
microsatellites (Armour et al. 1994; Vos et al. 1995). In general, large numbers of polymorphic AFLP loci can be amplified by using different AFLP primer combinations. However, different species carry varying amounts of AFLP polymorphism. Species like the imperial eagle (Aquila heliaca) and the Raso lark (Alauda razae) that have small populations sizes or that have suffered population bottlenecks exhibit reduced AFLP variability. This reduced variability tends to be present in all AFLP primer combinations for the species in question. Although a larger number of primer combinations can be used to generate more polymorphic AFLP markers, in such species it would lead to a large genotyping effort.

Several recent studies that have used microsatellite loci to show heterozygosity-fitness correlations (HFCs) have reported marker specific effects (Foerster et al. 2003; Hansson et al. 2004; Merila et al. 2003) with a few microsatellite loci contributing excessively to the observed HFC. Such results could be due to these specific loci being in linkage disequilibrium with other genes under balancing selection. A typical study estimating inbreeding uses only 5-10 microsatellite loci. If such a single linked locus is present but not detected, it would lead to the erroneous conclusion of inbreeding depression when the result is actually a result of selection acting on a single locus. The larger number of loci used in the AFLP-based measure means that this estimator will not be strongly affected if a few of the markers are linked to loci that are under selection. This is because the large number of other unlinked markers will dilute the influence of any such linked markers. Therefore, any HFCs detected using a large number of AFLP markers are less likely to be a result of single locus heterosis and more likely to be an indication of inbreeding depression.

AFLP markers can be amplified with a high degree of reliability and reproducibility, especially compared to other multilocus markers such as RAPDs or minisatellites. AFLP genotyping error rates are usually < 2 % per AFLP band (Arens et al. 1998; Jones et al. 1997; Questiau et al. 1999).
This error rate is about an order of magnitude higher than error rates using microsatellite loci. Although an error rate of ~ 2% is seen as a problem by some workers, it is probably low enough not to have a major impact on most applications of AFLP markers.

### 4.4.3 Improving the estimators of inbreeding

Although strong correlations have been found between pedigree-based inbreeding coefficients and inbreeding estimators based on AFLP and microsatellite markers, there is substantial unexplained variation in the estimators that is not due to inbreeding. In the case of the microsatellite-based estimator, the results of the allele dropping simulations indicate that the sharing of alleles between the founders of the population is responsible for much of this unaccounted variation. When no alleles are shared between the founders, the \( r^2 \) between pedigree inbreeding and the microsatellite estimate of inbreeding is 0.72 ± 0.04, compared to an \( r^2 \) of 0.46 ± 0.06 when the founders’ actual genotypes are used in the allele dropping simulations. The reduction in \( r^2 \) is a result of alleles being shared between the founders at the different microsatellite loci, such that an individual derived from these founders may be homozygous at a locus for a particular allele, but the two alleles may not be identical by descent. Thus, the accuracy of microsatellite-based estimates of inbreeding will depend strongly on the number of alleles and the allele frequencies at the loci used in the estimation, more polymorphic loci in general being better.

As each AFLP locus has only two alleles, the problem of alleles at a locus being identical, but not identical by descent is particularly acute for the AFLP-based estimator of inbreeding. Ambiguity as to whether an individual with a band at a particular locus is heterozygous or homozygous adds to the unexplained variation present in the AFLP-based estimator. If large numbers of AFLP loci
are available, selecting loci with a low present allele frequency should reduce this source of noise, as there will be a high probability that individuals with bands at such loci are heterozygous.

Incorporating allele frequency information into the estimators can improve both estimators of inbreeding. With microsatellite loci, internal relatedness (IR, Amos et al. 2001) can be calculated. The AFLP band count approach simply adds the number of polymorphic AFLP bands an individual possesses, and ignores all information regarding allele frequencies. AFLP markers are dominant markers and it is not possible to distinguish between heterozygotes and homozygotes. However, if allele frequencies at the AFLP loci are available, they can be used to calculate the probability that an individual with a band at a particular locus is heterozygous. If a particular AFLP locus has a low present allele frequency, an individual with a band at that locus has a high probability of being heterozygous, and vice versa. The AFLP band count can therefore be weighted with these probabilities to provide a better estimate of inbreeding.

Weighted AFLP band counts and IR were not used in the analyses presented in this chapter as the allele frequencies at the loci in the captive population changed from one generation to the next. In practice, the correlations between IR and heterozygosity (Amos et al. 2001), and that between weighted AFLP band count and AFLP band count are high ($r > 0.95$). Consequently, a strong heterozygosity-fitness correlation should be significant regardless of whether they are based on weighted or un-weighted heterozygosity measures.

Increasing the number of loci used in the estimations is the obvious way of improving the accuracy of both the estimators of inbreeding. The locus dropping simulations indicate that substantial improvements are possible by increasing the number of loci used (Figure 4.4 and Figure 4.5). However, the simulations also indicate diminishing returns, meaning that the improvement is not proportional to the extra genotyping effort required.
4.4.4 Estimating relatedness using AFLP and microsatellite markers

Comparisons between pedigree-based pairwise relatedness and pairwise relatedness values based on AFLP and microsatellite markers show that the microsatellite-based estimator performs better. In the *P. polionotus* system, three to four microsatellite loci carry the same amount of information about relatedness as 90 AFLP markers. The higher per locus information carrying content of the microsatellite loci is a result of each locus in *P. polionotus* having on average ten allelic states, compared to only two alleles per locus for each AFLP marker. Therefore, for a given amount of genotyping effort, microsatellite loci appear to be better at estimating the relatedness between two individuals compared to AFLP markers.

Notably, when the pairwise relatedness estimates based on microsatellite and AFLP markers of the wild mice are compared, the estimators correlate significantly with each other. Such a correlation indicates the presence of common information about relatedness between the estimators and implies that the levels of relatedness amongst the wild mice are high enough to be detected using the numbers of loci employed in this study. This suggests that random pairings of these wild mice should result in some offspring with levels of inbreeding high enough to be detected with the numbers of molecular markers used in this study.

4.4.5 Allele dropping simulations

In the allele dropping simulations, the founders’ alleles were passed down the pedigree to generate simulated genotypes of the captive-bred mice. Results in Table 4.3 and Table 4.4 show that regressions and correlations based on actual genotypes and genotypes simulated by allele dropping do not differ significantly from each other. This concordance between actual genotypes and simulated genotypes lends further support to the validity of the estimators of inbreeding and relatedness evaluated in this chapter. However, it is evident that the agreement is closer for the
microsatellite loci than for AFLP loci. Microsatellite alleles are known to be co-dominant and inherited in a strict Mendelian fashion, therefore close agreement between simulated and actual genotypes is expected. The lack of close concordance for the AFLP loci is probably a result of two assumptions that have been made in the allele dropping simulations. First, it is assumed in the simulations that all AFLP loci are dominant. This affects the estimation of allele frequencies, which in turn influences the assignment of founder genotypes used in the simulations. Second, it is assumed that all the loci are inherited in a strict Mendelian manner. Studies have shown that these assumptions are likely to be correct for most loci (Liu et al. 1998; Maughan et al. 1996). Confirming the validity of these assumptions in the *P. polionotus* system would require genotyping a large numbers of known parent-offspring groups, something that was not possible in this study.

### 4.5 Conclusions

I have described a novel method for estimating levels of inbreeding using AFLP markers and demonstrated that it correlates strongly with known inbreeding coefficients. For similar amounts of genotyping effort, the new measure appears to give better estimates at low inbreeding coefficients compared to microsatellite markers. The technique is easily applicable to any species without the need for time consuming cloning, primer development and optimisation. But for species with low levels of AFLP polymorphisms, a large genotyping effort may be required. In contrast, for similar genotyping effort, microsatellite loci appear to give better estimates of pairwise relatedness compared to AFLP markers. AFLP markers are currently widely used in studying plant populations, and their use in studying animal population genetics is gradually increasing. The method described here for estimating levels of inbreeding using AFLP markers should be applicable to the large amounts of AFLP genetic data that already exists.
CHAPTER 5

MEASURING HETEROZYGOSITY-FITNESS CORRELATIONS USING AFLP AND MICROSATELLITE MARKERS

5.1 Introduction

Many recent studies have reported correlations between heterozygosity at neutral genetic markers and a wide variety of fitness traits such as disease susceptibility (Acevedo-Whitehouse et al. 2003), juvenile survival (Coltman et al. 1998; Coulson et al. 1999), parasite resistance (Coltman et al. 1999) and male reproductive success (Hoffman et al. in press). In general, these studies have measured heterozygosity using 5-15 microsatellite loci, and have concluded that the observed heterozygosity-fitness correlations (HFCs) are a sign of inbreeding depression. However, many question whether heterozygosity at such a small number of loci can accurately represent genome-wide heterozygosity (Balloux et al. in press; Chakraborty 1981; Hansson et al. 2001; Slate and Pemberton 2002).

The ubiquity of such HFCs, and the range of traits affected, mean that they are of great evolutionary importance. Selection acts upon differences in fitness between individuals, and the presence of HFCs indicates genetic components upon which selection can in principle act. Any genetic factor affecting fitness will also be important in determining the viability of small populations. Therefore, HFCs are of relevance to conservation biology. As a result, there has been an increasing number of studies investigating HFCs (Acevedo-Whitehouse et al. 2003; Coltman et al. 1998; Coltman et al. 1999; Coulson et al. 1999; Coulson et al. 1998; Hansson et al. 2004; Markert et al. 2004).
HFCs can arise in two ways (Hansson and Westerberg 2002). First, heterozygosity at the microsatellite markers may reflect genome-wide heterozygosity. When the population carries deleterious recessive alleles at many different loci, individuals with high homozygosity will be relatively unfit compared to more heterozygous individuals. This is the cause of inbreeding depression, since unusually homozygous individuals most commonly arise from incestuous matings. Second, one or more of the microsatellite markers used could be linked to loci under balancing selection, such that the microsatellite markers themselves show signs of this selection, an effect referred to as single locus heterosis, or overdominance. While the first mechanism is a result of two individuals related throughout their genomes producing offspring, the second is a result of individuals that are similar at particular loci producing offspring. As the two mechanisms that may cause an HFC are very different, it is important to discriminate between them.

Distinguishing between whether any particular HFC is a result of inbreeding depression or single locus heterosis is not easy. If an HFC is a result of inbreeding depression, it is a genome-wide effect, and it is expected that the relationship between heterozygosity and fitness should be similar for each marker used. If, on the other hand, the HFC is due to single locus heterosis, the relationship should be present for only one or a few of the loci. Therefore, a typical analysis would involve performing separate statistical tests using each locus in turn to check whether the HFC is caused by a few, or many of the markers used. Some recent studies reporting HFCs show that the effect is due to one or two loci (Hansson et al. 2001; Merila et al. 2003), but others have shown results suggesting a genome-wide effect (Hoffman et al. in press; K. Acevedo-Whitehouse pers. comm.). Therefore, the relative importance of inbreeding depression and single locus heterosis remains unclear (Hansson and Westerberg 2002), and appears to be dependent on the system being studied.
Current studies reporting HFCs tend to use microsatellite markers to estimate heterozygosity, although a few have used allozymes for this purpose (Borrell et al. 2004; Thelen and Allendorf 2001). In the previous chapter I described the use of AFLP markers for estimating heterozygosity. Comparisons with pedigree-based inbreeding coefficients revealed that both microsatellite and AFLP markers can be used to estimate inbreeding when levels of inbreeding are high. There was also evidence indicating that for similar amounts of genotyping effort, AFLP markers may be more sensitive at measuring inbreeding at low levels of inbreeding compared to microsatellite markers. However, the usefulness of AFLP markers at measuring HFCs has not yet been investigated, and the relative ability of AFLP and microsatellite markers to detect HFCs is also unknown.

In this chapter, I investigate the utility of AFLP markers for measuring correlations between heterozygosity and fitness in four different species: common mallards, house sparrows, California sea lions, and old-field mice. Tissue samples and fitness data were available from studies conducted on these species, allowing direct comparisons to be made between microsatellite and AFLP markers for measuring HFCs. These analyses will also allow further investigation into the roles of genome-wide heterozygosity and single locus heterosis in explaining HFCs.

### 5.1.1 Study species

Each of the four species in which HFCs are investigated in this chapter is described briefly below.

#### 5.1.1.1 Common mallard

Female mallards (*Anas platyrhynchos*) form monogamous pair bonds and appear to exhibit strong mate choice based on male traits (Cunningham 2003; Cunningham and Russell 2000).
Cunningham and Russell (2000) conducted a study looking at factors affecting egg investment by female mallards, and found that females invest more by producing heavier eggs when paired with a high-ranking male than if paired with a low-ranked male.

As male mallards do not provide parental care, it is thought that females increase their offspring’s fitness by choosing mates based on a male’s genetic quality (Andersson 1994). However, there are few studies showing a direct link between male genetic quality and maternal investment in reproduction. Here I use the experimental system described in Cunningham and Russell (2000) to investigate whether maternal investment in mallard clutches is affected by either maternal or paternal genetic quality measured in terms of heterozygosity estimated using both microsatellite and AFLP markers.

5.1.1.2 House sparrow

The house sparrow, *Passer domesticus*, is a sexually dichromatic passerine. Compared to females, males have more conspicuous plumage and a throat patch of black feathers under the bill. This throat patch, also known as a badge or bib, varies greatly in size between individuals and is known to act as a badge of status, with its size correlating with male dominance (Liker and Barta 2001; McGraw et al. 2003; Møller 1987a; Møller 1987b; Møller 1988; Veiga 1993). However, the role of male badge size in mate choice by females is uncertain, with some studies suggesting that females prefer males with large badges (Møller 1987b; Møller 1988; Møller 1990), while in others, females prefer males with small badges (Griffith et al. 1999b), or show no preference (Cordero et al. 1999; Kimball 1996).

Møller (1987a) suggested that the honesty of the badge signal is controlled by aggressive interactions between flock members. Testosterone levels appears to be associated with the control of badge size in individuals, with higher testosterone levels being associated with larger
badge sizes. High levels of testosterone are in turn known to suppress the immune system (Buchanan et al. 2003; Evans et al. 2000). Honesty of the signal is therefore maintained as only high quality males can afford to compromise their immune systems with the high testosterone levels required to produce a large badge.

From its roles in male-male interactions and its potential influence on female mate choice, it is clear that male badge size in sparrows is a fitness trait under sexual selection. Models of sexual selection usually assume that variation in a sexually selected trait that is costly to maintain is determined largely by genetic, rather than environmental factors (Andersson 1994). However, in a cross-fostering study, Griffith et al. (1999a) found that badge size of male sparrows correlated with the badge size of foster fathers and not with that of genetic fathers, supporting the idea that badge size is influenced by environmental factors.

The results from Griffith et al. (1999a) do not rule out the possibility of genetic factors acting in conjunction with environmental factors to influence badge size, or of badge size being influenced to varying degrees by environmental and genetic factors depending on the conditions or the population studied. In this study I further investigate whether there are any genetic factors influencing badge size in house sparrows by examining the effect of heterozygosity, measured using both microsatellite and AFLP markers, on badge size.

### 5.1.1.3 California sea lion

California sea lions (*Zalophus californianus*) range from southern Mexico up to British Columbia. The population is growing steadily, and is at present estimated to be around 200,000. The species has a polygynous mating system and during the breeding season males defend territories containing a number of females. These sea lions are known to suffer from a range of diseases and injuries, and rescued animals are frequently treated at rehabilitation centres.
Acevedo-Whitehouse et al. (2003) investigated the relationship between microsatellite heterozygosity and disease susceptibility in California sea lions. Genotypes from 11 microsatellite loci were used to calculate the IR of California sea lions suffering from a variety of diseases. Animals were grouped into six categories: trauma, carcinoma, bacterial infection, helminth infection, algal intoxication, and non-specific infection. On average, sea lions suffering from diseases were found to have higher IR values, and were therefore less heterozygous, compared to the trauma individuals which suffered from injuries but had no disease. The strong HFC detected in this system using microsatellite markers is thought to be a consequence of inbreeding (Acevedo-Whitehouse et al. 2003). Therefore, it represents an ideal system in which the utility of AFLP markers at detecting HFCs can be investigated. I use the California sea lion system described in Acevedo-Whitehouse et al. (2003) to examine whether or not disease susceptibility in these sea lions is affected by heterozygosity estimated using AFLP markers.

### 5.1.1.4 Old-field mouse

The old-field mouse, *Peromyscus polionotus*, is a small nocturnal mouse found in the southeast of the United States of America. Genetic evidence indicates that these mice are monogamous. Pairs mate for life and raise a number of litters, with both parents providing parental care (Foltz 1981). Several subspecies of old-field mice have been described and have been found to possess different levels of genetic diversity. However, laboratory experiments have shown that variation in the amount of inbreeding depression between the subspecies is not linked to their differing levels of genetic diversity in the wild (Brewer et al. 1990).

Further studies using different subspecies of laboratory-bred old-field mice have found that a large number of fitness traits are affected by inbreeding (Lacy et al. 1996; Lacy and Ballou 1998). In spite of the differences between the subspecies in population size and genetic variation, there
were no subspecies differences in susceptibility to inbreeding depression. All variation in inbreeding depression between subspecies could be attributed to random founder effects (Lacy et al. 1996).

In the previous chapter, both microsatellite and AFLP markers were shown to be capable of estimating levels of inbreeding in old-field mice. There were also indications that AFLP markers may be better than microsatellite markers at estimating low levels of inbreeding. As inbreeding has been found to affect fitness in laboratory-bred old-field mice, they are an interesting species in which to examine whether or not inbreeding effects can be detected in wild mice. In this chapter I investigate the extent to which the fitness of litters produced by three subspecies of wild *P. polionotus* are affected by litter and parental heterozygosity estimated using microsatellite and AFLP markers.

5.2 Materials and methods

5.2.1 General methods

AFLP genotyping and scoring was carried out according to protocols described previously. AFLP heterozygosity was estimated from the AFLP genotypes by calculating AFLP band counts and weighted AFLP band counts of individuals. An individual’s AFLP band count was calculated using Equation 4.1. An individual’s weighted AFLP band count was calculated according to Equation 5.1 in which the AFLP band count is weighted with the probability, \( \rho \) (Equation 5.2), that an individual with a band at the \( i \)th locus is heterozygous.
Weighted AFLP band count = $\frac{\sum_{i=1}^{n} p_i S_i}{n}$  \hspace{1cm} (Equation 5.1)

$p_i = \frac{2p(0)_i p(1)_i}{2p(0)_i p(1)_i + p(1)_i^2}$  \hspace{1cm} (Equation 5.2)

($S_i = 0$ when a band is absent and $S_i = 1$ when a band is present at each of the $n$ polymorphic loci; $p(0)_i =$ absent allele frequency, and $p(1)_i = 1 - p(0)_i =$ present allele frequency, at the $i$th locus)

Microsatellite genotypes were used to calculate internal relatedness, IR, a measure of heterozygosity in which the heterozygosity score is weighted by the frequency of the alleles in each genotype (Amos et al. 2001). Relatedness between pairs of individuals were calculated from microsatellite and AFLP genotypes using the methods described in Queller and Goodnight (1989) and Madden et al. (in press).

To check for any overlap of information between the microsatellite and AFLP measures of heterozygosity, for each of the four species, correlation coefficients were calculated between IR and the two AFLP measures, AFLP band count and weighted AFLP band count.

### 5.2.2 Common mallard

Four measures of maternal investment were available for each clutch of eggs produced by a mallard: clutch size, average weight of eggs, total weight of eggs, and intra-clutch variation in egg weight (Cunningham and Russell 2000). Intra-clutch variation in egg weight was measured as the standard deviation of egg weights divided by the average egg weight. Blood samples were collected from 60 mallards, including all parent pairs used in the experiment described in Cunningham and Russell (2000). Following the extraction of genomic DNA, the mallards were
genotyped and scored at 10 microsatellite loci by P. Johnson, W. Lee and W. Amos. The following microsatellite loci were used: Du05, Du08, Du13, Du15, Du19, Du30, Du50 (unpublished loci, R. Paton), APH03, APH07, APH09 (Maak et al. 2000). Microsatellite genotypes were used to calculate the IR of each individual. The individuals were also genotyped using eight AFLP primer combinations (Table 5.1), from which 111 polymorphic AFLP loci were scored. AFLP band counts and weighted AFLP band counts were calculated from the AFLP genotypes. Heterozygosity of the clutches produced could be estimated from the genetic similarity of the parents as more closely related parents produce less heterozygous offspring. Relatedness between parent pairs was calculated from the microsatellite and AFLP genotypes (Madden et al. in press; Queller and Goodnight 1989).

To investigate the influence of parental relatedness and parental heterozygosity on maternal investment, linear regressions were performed on each of the four available measures of maternal investment using the following eight predictors: maternal IR, maternal AFLP band count, maternal weighted AFLP band count, paternal IR, paternal AFLP band count, paternal weighted AFLP band count, microsatellite-based parental relatedness, and AFLP-based parental relatedness. To examine whether the effect of paternal AFLP band count on intra-clutch variation in egg weight is a result of heterozygosity at a few AFLP loci, or due to contributions from a large number of loci, a single locus analysis was carried out. The presence-absence genotypes at the 111 scored loci were coded as 1 or 0 respectively. At each locus, t-tests were conducted to test whether the fathers’ genotypes (0 or 1) influenced intra-clutch variation in egg weight. Loci found to be significantly affecting intra-clutch variation in egg weight at the $\alpha < 0.05$ level were removed, and AFLP band count and weighted AFLP band count recalculated based on the remaining loci. To check whether or not paternal heterozygosity based on the remaining loci still influenced maternal investment, intra-clutch variation in egg weight...
was regressed against the recalculated paternal AFLP band count and AFLP weighted band count.

<table>
<thead>
<tr>
<th>AFLP primer combination</th>
<th>California sea lion</th>
<th>Common mallard</th>
<th>House sparrow</th>
<th>Old-field mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcoRI-ACA, TaqI-CAC</td>
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<td>✓</td>
<td>✓</td>
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<td>EcoRI-AAC, TaqI-CAC</td>
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<td>✓</td>
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</tr>
<tr>
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<tr>
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</tr>
<tr>
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<td>✓</td>
<td>✓</td>
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</tr>
</tbody>
</table>

Table 5.1 AFLP primer combinations used to genotype each species described in this chapter.

5.2.3 House sparrows

Blood samples from 22 male house sparrows were provided by M. Evans. Badge size of each male was measured twice (Evans et al. 2000) and averaged. The sparrows were genotyped and scored at eight microsatellite loci by P. Johnson and W. Amos. The following microsatellite loci were used: Pdoµ1, Pdoµ2, Pdoµ3 (Neumann and Wetton 1996), Pdoµ5, Pdoµ6 (Griffith et al. 1999c), Pdoµ8, Pdoµ10 (Genbank Accession numbers: AF354422 and AF354424). The IR of each individual was calculated from the genotypes at these loci. Using eight AFLP primer combinations (Table 5.1), the individuals were also genotyped at 72 polymorphic AFLP loci. These genotypes were then used to calculate AFLP band counts and weighted AFLP band counts.
In order to investigate the effect of heterozygosity on badge size, IR, AFLP band count and weighted AFLP band count were separately regressed against average badge area. A single locus analysis, identical to that described for the mallard, was carried out to examine whether the effect of AFLP band count on badge area is a result of heterozygosity at a few AFLP loci, or due to contributions from a large number of loci. At each of the 72 loci, t-tests were carried out to test whether an individual’s genotype influenced its badge size. Any locus found to be significant at \( \alpha < 0.05 \) was removed, and AFLP band count and weighted AFLP band count recalculated. The influence of the remaining loci was assessed by regressing badge size against the recalculated paternal band count and weighted band count.

### 5.2.4 California sea lion

Of the original 371 individuals used in Acevedo-Whitehouse et al. (2003), 169 were genotyped using AFLP primers. These consisted of the following numbers of sea lions in each of five categories: 11 carcinoma, 33 bacterial infection, 34 algal toxin, 56 helminth infection and 35 trauma. They represent all the trauma and carcinoma cases from the original study, barring samples that failed to amplify, sea lions with a wide range of gut parasite diversity, and approximately equal numbers of individuals suffering from algal intoxication and bacterial infection. The sea lions were genotyped using 12 different AFLP primer combinations (Table 5.1). Only 32 polymorphic AFLP markers could be scored. This is a substantially lower level of polymorphism compared to the other species. From the scored markers, AFLP band count and weighted AFLP band count were calculated for each individual. K. Acevedo-Whitehouse provided the genotypes of these 169 sea lions at 11 microsatellite loci. t-tests were carried to examine whether sea lions suffering from diseases had significantly different IR, AFLP band count and weighted AFLP band count compared to individuals suffering from trauma, but no disease. Analyses of variance (ANOVA) were also conducted to test whether IR, AFLP band count and weighted AFLP band count varied significantly between the five disease categories.
In order to examine whether the patterns of disease susceptibility with microsatellite heterozygosity reported in Acevedo-Whitehouse et al. (2003) could be attributed to sea lions suffering from each disease category originating from distinct populations, I used the AFLP genotypes to construct phylogenetic trees. There is little consensus regarding the most effective and appropriate method(s) of phylogenetic analysis using AFLP characters. Although distance and Dollo parsimony methods are most widely used (Buntjer et al. 2002; Kingston and Rosel 2004), other studies have used Wagner parsimony (Hollingsworth and Ennos 2004; Sullivan et al. 2004). This issue is discussed further in Chapter 6. I used distance and Dollo parsimony methods to analyse the AFLP data in PHYLIP (Felsenstein 1989).

For the distance analyses, the AFLP data was converted into a pairwise Jaccard distance matrix (Kingston and Rosel 2004) using Equation 5.3.

\[
J_{xy} = 1 - \left( \frac{a}{a + b + c} \right) 
\]  
(Equation 5.3)

\(a\) is the number of polymorphic bands shared by individuals \(x\) and \(y\), \(b\) is the number of bands present in \(x\) but absent in \(y\), and \(c\) is the number of bands present in \(y\) but absent in \(x\).

Pairwise distances were used to produce neighbour-joining trees using the NEIGHBOUR program in PHYLIP. Dollo parsimony analysis was carried out using the DOLLOP program in PHYLIP. To assess support for the tree nodes, 1000 bootstrap pseudoreplicates were performed for distance and Dollo parsimony, and consensus trees were calculated using SEQBOOT and CONSENSE.

5.2.5 Old-field mouse

Three subspecies of *Peromyscus polionotus*, *P. p. subgriseus*, *P. p. rhoadsi* and *P. p. leucocephalus*, representing central, peninsular and insular populations respectively, were collected from the wild.
in 1990 (Lacy et al. 1996). These wild mice were paired and used to found breeding stocks of mice that were subsequently used in inbreeding experiments (Lacy et al. 1996; Lacy and Ballou 1998). In general, each female was only paired with a single male, and vice versa. Three aspects of fitness were measured in the litters produced by the wild-caught mice: litter size, average mass of weaned pups in a litter, and proportion of litters surviving from birth to weaning (Lacy et al. 1996).

The wild-caught *P. polionotus* were genotyped at 13 microsatellite loci (Table 3.2). Using separate allele frequencies for each subspecies, the genotypes were used to calculate the IR of each individual in all subspecies. The samples were also genotyped using eight AFLP primer combinations (Table 5.1), yielding 143 polymorphic markers. AFLP genotypes were used to calculate AFLP band counts and weighted AFLP band counts. As allele frequencies for the AFLP markers varied considerably between subspecies, separate weights were calculated for each subspecies. Parental relatedness of litters, an estimate of litter heterozygosity, was calculated based on the microsatellite and AFLP genotypes separately (Madden et al. in press; Queller and Goodnight 1989).

To investigate the influence of parental relatedness and parental heterozygosity on litter size, average pup mass, and litter survival, general linear models (GLMs) were produced using the combined data from all subspecies of *P. polionotus*. The following predictors were used: maternal IR, maternal weighted AFLP band count, paternal IR, paternal weighted AFLP band count, microsatellite-based parental relatedness, AFLP-based parental relatedness, litter size, mother’s parity and subspecies. Litter size and average pup mass were approximately normally distributed and were modelled using normal error structures. GLMs with a binomial error structure were used to model the proportion of the litter that survived to weaning. Initially, full models containing all the terms were constructed. To compensate for overdispersion in the models,
significance testing of each term was assessed using F-tests. Standard deletion-testing procedures (Crawley 2002) were used to drop terms from the full models that were not significant at p < 0.05. The effects of first order interactions between terms were also investigated, but higher order interactions were not considered.

5.3 Results

5.3.1 Common mallard

IR did not correlate significantly with either AFLP band count or with weighted AFLP band count ($r^2 = 0.01$, $p = 0.4$, $n = 60$; $r^2 = 0.02$, $p = 0.3$, $n = 60$). None of the maternal heterozygosity, paternal heterozygosity or parental relatedness measures had any significant influence on clutch size, average weight of eggs or total weight of eggs. However, paternal AFLP band count and weighted AFLP band count were found to correlate negatively with intra-clutch variation in egg weight ($r^2 = 0.21$, $p = 0.008$, $n = 32$; $r^2 = 0.25$, $p = 0.004$, $n = 32$; Figure 5.1).

The investigation into the effect of individual AFLP loci on intra-clutch variation in egg weight could be carried out using only 91 of the 111 AFLP markers, as 20 of the markers were not polymorphic in the males that fathered clutches. Of the 91 t-tests using single AFLP loci, four had p-values less than 0.05. Three loci were significant at $p = 0.02$ and one locus at $p = 0.007$. However, after sequential Bonferroni correction, none of these are significant. Of the 91 t-tests, 55 had negative coefficients, a marginally significant majority ($p = 0.06$, sign test). After removal of the four loci that were significant before Bonferroni correction in the single locus analysis, intra-clutch variation in egg weight was still significantly correlated with paternal AFLP band count and weighted AFLP band count ($r^2 = 0.14$, $p = 0.03$, $n = 32$; $r^2 = 0.21$, $p = 0.008$, $n = 32$).
Figure 5.1  Influence of A) father’s AFLP band count ($r^2 = 0.21, n = 32, p = 0.008$) and B) father’s weighted AFLP band count ($r^2 = 0.25, n = 32, p = 0.004$) on intra-clutch variation in egg weight in mallards.
5.3.2 House sparrow

IR did not correlate significantly with either AFLP band count or weighted AFLP band count ($r^2 = 0.11$, $p = 0.14$, $n = 22$; $r^2 = 0.09$, $p = 0.18$, $n = 22$). Badge area was found to correlate positively with an individual’s AFLP band count and weighted AFLP band count ($r^2 = 0.26$, $p = 0.01$, $n = 22$; $r^2 = 0.29$, $p = 0.01$, $n = 22$), but not with IR ($r^2 = 0.03$, $p = 0.5$, $n = 22$; Figure 5.2).

Five of the 72 t-tests used in the single locus analysis had p-values less than 0.05. Three of the AFLP loci were significant at $p = 0.02$, and one locus each at $p = 0.01$ and $p = 0.008$. These are not significant after sequential Bonferroni correction. Badge size remained significantly associated with AFLP band count and weighted AFLP band count ($r^2 = 0.17$, $p = 0.05$, $n = 22$; $r^2 = 0.19$, $p = 0.04$, $n = 22$) even after exclusion of these five loci, though as expected, the resulting relationships were weaker. Of the 72 t-tests in the single locus analysis, 46 had positive coefficients, significantly more that would be expected by chance ($p = 0.02$, sign test).

5.3.3 California sea lion

There was no relationship between IR and either AFLP band count or weighted AFLP band count ($r^2 = 0.004$, $p = 0.4$, $n = 169$; $r^2 = 0.007$, $p = 0.3$, $n = 169$). Mean IR of diseased sea lions was significantly different from the control group of sea lions with trauma ($t$ test: $t = 4.69$, $p = 6 \times 10^{-6}$). However, there were no significant differences between diseased and trauma sea lions for AFLP band count and weighted AFLP band count ($t$ test: $t = 0.48$, $p = 0.6$; $t = 0.32$, $p = 0.8$). Mean IR varied significantly between the five disease categories (ANOVA: $F_{4,168} = 9.90$, $p = 3 \times 10^{-5}$), but there was no significant variation in mean AFLP band count or mean weighted band count between the disease categories (ANOVA: $F_{4,168} = 1.55$, $p = 0.19$; $F_{4,168} = 1.48$, $p = 0.21$). These relationships are depicted in Figure 5.3. Neither the parsimony nor the distance
analyses based on the AFLP markers showed the existence of any population structure within the sampled sea lions.

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)

**Figure 5.2** Influence of A) AFLP band count ($r^2 = 0.26, n = 22, p = 0.01$), B) weighted AFLP band count ($r^2 = 0.29, n = 22, p = 0.01$) and C) internal relatedness on badge size in male house sparrows ($r^2 = 0.03, n = 22, p = 0.5$).
Figure 5.3  Internal relatedness and A) AFLP band count, B) weighted AFLP band count in California sea lions in the four disease (C = carcinoma, H = helminth infection, Ba = bacterial infection, Al = algal toxin) and the control (T = trauma) categories. Values are means ± 1 s.e.
5.3.4 Old-field mouse

There were no significant correlations between IR and either AFLP band count or weighted AFLP band count for any of the subspecies of *P. polionotus*. GLMs were carried out to investigate how parental heterozygosity and parental relatedness affect fitness in *P. polionotus*. Data from all the subspecies were pooled to look for any patterns common to all the subspecies. The results are summarised in Table 5.2. Litter size was not found to be affected by any of the factors used in the models.

Litter survival appears to be positively correlated with father’s IR, and negatively correlated with parental relatedness measured using microsatellites. There was also some evidence of subspecies differences in litter survival. However, as the model is overdispersed (dispersion factor = 1.3), and explains only 5.0% of the total deviance, these results should be interpreted with caution. In addition, the residuals are not normally distributed, with large negative residuals associated with a number of the data points. For these reasons, no analyses were carried out to investigate whether or not the effect of father’s IR on litter survival is a result of single loci.

A strong negative association was found between average pup mass and litter size. Maternal heterozygosity measured using AFLP markers positively correlated with pup mass. There was also a negative interaction between mother’s weighted AFLP band count and mother’s IR, indicating that mothers with both a high AFLP band count and a low IR produce heavier pups, and *vice versa*. More experienced mothers also produced significantly heavier pups.

To investigate whether the effects of mother’s IR and weighted AFLP band count on average pup mass are a result of heterozygosity at a few loci, or due to contributions from a large number of loci, separate single locus analyses were carried for the microsatellite and the AFLP loci. At each of the 13 microsatellites, the mother’s genotype was recorded as one or zero depending on
whether she was heterozygous or homozygous. Similarly, at each of the 143 AFLP markers the mother’s genotype was recorded as one or zero depending on whether or not she possessed a band at the locus in question. The final model for pup mass contains the following terms: litter size, parity, mother’s weighted AFLP band count, mother’s IR, and mother’s weighted AFLP band count × mother’s IR (Table 5.2a). To make it easier to interpret the results, the roles of single microsatellite loci were investigated using simpler models containing the following terms: litter size, parity and mother’s heterozygosity (as a categorical variable). The contributions of single AFLP loci were investigated using a model containing the following terms: litter size, parity and mother’s band presence or absence (as a categorical variable).

<table>
<thead>
<tr>
<th>Term</th>
<th>Estimate</th>
<th>% deviance explained</th>
<th>df</th>
<th>F</th>
<th>p</th>
</tr>
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<tr>
<td><strong>a) Average pup mass</strong></td>
<td></td>
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<tr>
<td>Litter size</td>
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<td>19.0</td>
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<tr>
<td>Parity</td>
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<td>14.4</td>
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<td>7.9</td>
<td>0.002</td>
</tr>
<tr>
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<td>4.7</td>
<td>1</td>
<td>6.1</td>
<td>0.013</td>
</tr>
<tr>
<td>Mother’s weighted band count × mother’s IR</td>
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<td>7.9</td>
<td>1</td>
<td>10.2</td>
<td>0.002</td>
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</tr>
<tr>
<td>Father’s IR</td>
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<td>2.2</td>
<td>1</td>
<td>11.7</td>
<td>0.006</td>
</tr>
<tr>
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<td>1</td>
<td>7.1</td>
<td>0.04</td>
</tr>
<tr>
<td>Subspecies</td>
<td>—</td>
<td>1.4</td>
<td>3</td>
<td>3.7</td>
<td>0.03</td>
</tr>
</tbody>
</table>

**Table 5.2** General linear models of fitness in the old-field mice: a) average pup mass in a litter (n = 77, total deviance = 100.8, total deviance explained = 44.8 %, F = 11.5, p = 4×10⁻⁴) and b) litter survival (n = 378, total deviance = 528.5, total explained deviance = 5.0 %, F = 6.55, p = 3×10⁻⁵). No factors significantly affected litter size. Data from all subspecies have been combined in these analyses. Only significant terms are shown. The percentage deviance explained refers to the proportion of the total deviance explained by each term in the model.
In the single locus analyses using microsatellite loci, of the 13 loci, four associated significantly with pup mass with the following p-values: 0.0001, 0.002, 0.01 and 0.04. Eleven of the 13 loci had positive coefficients (p = 0.02, sign test). These results indicate that maternal heterozygosity at a majority of the microsatellite loci contributes towards the association between maternal IR and pup mass. The single locus analysis for the AFLP markers was carried out for only 96 of the 143 AFLP markers, as 47 of the markers were not polymorphic in the 16 females that contributed litters in the models of pup mass. Of these 96 markers, half had positive coefficients in the single locus analyses. The results of the single locus analyses should be interpreted with some caution as they were carried out on simpler models rather than on the more complex full model with an interaction between mother’s IR and mother’s weighted AFLP band count.

5.4 Discussion

5.4.1 Common mallard

Intra-clutch variation in mallard egg weight correlated negatively with paternal AFLP band count. Results indicate that this association is not likely to be due to heterozygosity at any single locus. No other paternal or maternal measures of heterozygosity, or of parental relatedness were found to affect the four measures of maternal investment: clutch size, average egg weight, total clutch weight, and intra-clutch variation in egg weight.

While testing for HFCs in this study, 32 statistical tests have been carried out, raising the possibility that the one significant result obtained could be a Type I error. This result, the correlation of intra-clutch variation in egg weight with paternal AFLP heterozygosity, is significant at the p = 0.004 level. Strict usage of a sequential Bonferroni correction would mean the acceptance of this result only if p < 0.0016. However, many of the 32 statistical tests are non-independent, making a meaningful sequential Bonferroni correction difficult. AFLP band
count is very similar to weighted AFLP band count, and average egg weight is derived from total clutch weight and clutch size. Therefore, a conservative estimate of the number of independent tests is 18. With Bonferroni correction, this makes $p = 0.0028$ the critical level of significance.

The correlation of intra-clutch variation in egg weight with paternal AFLP heterozygosity lies just outside this level, and the rest of the discussion is based on the assumption that this correlation is not a Type I error.

Although it is difficult to rule out the possibility of single locus heterosis, there are several indicators that the correlation between intra-clutch variation in egg weight and AFLP band count is an effect of variation in genome-wide heterozygosity. First, the single locus analysis reveals that heterozygosity at only four of the 91 AFLP markers is associated significantly with intra-clutch variation in egg weight. However, these associations are weak, and none remain significant after sequential Bonferroni correction. Second, after removal of these four loci, AFLP heterozygosity remains significantly associated with intra-clutch variation in egg weight demonstrating that the other AFLP loci also contribute to the observed HFC. Third, in more than half of the 91 single locus regressions, AFLP heterozygosity was negatively associated with intra-clutch variation in egg weight indicating an effect spread over many loci.

Variation in egg weight within a clutch is likely to be under female control (Eberhard 1996). Regardless of whether the negative correlation between AFLP band count and intra-clutch variation in egg weight is a result of genome-wide heterozygosity or single locus heterosis, a male with a low AFLP band count is presumed to be a low quality male. Therefore, my results indicate that when paired to a male with low genetic quality, female mallards lay eggs with a wider range of weights than if paired with a higher quality male. A female mallard may be hedging her bets, and attempting to increase offspring survival by laying a mixture of small and large eggs when
paired with a low quality male, rather than by laying lots of small eggs or a few large eggs. However, further research would be required to validate such an explanation.

### 5.4.2 House sparrow

Badge size in male sparrows correlated positively with an individual’s AFLP band count. Further analyses indicate that this result is not likely to be due to heterozygosity at any single locus, and that AFLP band count is measuring genome-wide heterozygosity. There was no effect of an individual’s IR on its badge size.

A number of facts point towards the correlation between badge size and AFLP band count being a result of genome-wide heterozygosity and not a consequence of single locus heterosis. First, the single locus analysis revealed that heterozygosity at five of the 72 AFLP markers is weakly associated with badge size, but these associations are not significant after sequential Bonferroni correction. Second, even after the removal of these five loci, AFLP band count remained significantly associated with badge size. Third, heterozygosity at 46 of the 72 single locus t-tests were positively associated with badge size, more than would be expected by chance alone, indicating an effect spread over many loci. The lack of any correlation between IR and badge size is not inconsistent with an effect of genome-wide heterozygosity on badge size. At the low levels of inbreeding that are likely to be encountered in wild populations, 72 AFLP markers should better at estimating genome-wide heterozygosity compared to eight microsatellite loci.

Badge size in male sparrows is known to correlate with male dominance and studies suggest that badge size is an honest signal (Buchanan et al. 2003; Evans et al. 2000; Møller 1987a). The positive correlation between AFLP heterozygosity and badge size supports theories of sexual selection and signalling in which variation in costly sexually selected traits is maintained by genetic factors (Andersson 1994). AFLP heterozygosity appears to be measuring genome-wide
heterozygosity in sparrows. A male with a low AFLP band count is presumed to be inbred, and finds it difficult to maintain a costly signal such as badge size. If on the other hand the association between AFLP heterozygosity and badge size is due to linkage of some AFLP markers to a few loci under selection, it is possible that these loci under selection are directly involved in maintaining badge size. Alternatively, they could be loci that affect the fitness of male sparrows in other ways such as disease resistance and have an indirect effect on badge size.

Whether the association between heterozygosity and badge size is a result of inbreeding or single locus heterosis, this study provides evidence for a genetic factor influencing a sexually selected trait.

In a cross-fostering study, Griffith et al. (1999a) found that badge size of male sparrows correlated with the badge size of foster fathers and not with that of genetic fathers. This result supports the idea that badge size is influenced by environmental factors, such as the ability of the parents to provide for their young, and not by genetic factors. The reported environmental effect is of similar magnitude ($r^2 = 0.38$) to the effect of heterozygosity on badge size ($r^2 = 0.29$) that I find in this study. The results in Griffith et al. (1999a) do not exclude the possibility of environmental and genetic factors acting in conjunction, especially as the genetic factor in question, heterozygosity, is not heritable. Thus, an outbred male can produce inbred offspring if related to the female he is paired with.

The sparrows studied in Griffith et al. (1999a) come from a relatively small island population of about 100 breeding adults. In such a small population, incestuous matings resulting in inbred offspring are likely to be frequent. It is therefore possible that inbreeding effects might be easier to observe in this wild population. Blood samples were collected from the individuals used in Griffith et al. (1999a). Genotyping these sparrows using AFLP markers will enable the combined
contributions of both genetic and environmental factors on badge size to be investigated simultaneously.

This study was based on a small number of sparrows from a single population. The observed effect of AFLP heterozygosity on badge size could vary depending on the population history of the sparrows studied. Therefore, to investigate the generality of the observed effect of AFLP heterozygosity on badge size, it will be interesting to conduct the study on larger samples sizes of sparrows collected from different populations, for example, mainland populations, island populations and bottlenecked populations.

5.4.3 California sea lion

California sea lions suffering from disease had lower microsatellite heterozygosity than sea lions with trauma, but no disease (Acevedo-Whitehouse et al. 2003). Microsatellite heterozygosity also varied significantly between the five different disease classes. In contrast, there were no such patterns with AFLP heterozygosity. Diseased and trauma individuals did not differ in their AFLP band counts, and there were no differences in AFLP band count between disease categories.

Examination of Figure 5.3 reveals that the four disease categories show a trend suggesting that disease categories, like carcinoma, which contain seal lions with high IR, also tend to have a low AFLP band count. In comparison, categories like bacterial and helminth infection have lower IR and higher AFLP band counts. The trauma class breaks this trend. Although the trauma category is a good group against which to compare the diseased sea lions, it is not necessarily a perfect control group. Sea lions with trauma consisted of animals injured by boats, gun shots or net trappings and they may not represent a random sample of sea lions.
Disease susceptibility in the sea lions appears to be affected in different ways by IR and AFLP band count. If both genetic measures are estimating levels of inbreeding in the sea lions, the opposing results obtained are incompatible with one another. However, the lack of concordance between the results obtained from the two types of markers could be a result of one, or both of the markers failing to measure levels of inbreeding. There is the possibility that the significant results obtained with microsatellite markers are a consequence of heterozygosity at one of a few microsatellite loci affecting disease susceptibility.

AFLP genotyping of the sea lions did not reveal a large number of polymorphic markers. Using 12 primer combinations, only 32 polymorphic markers could be scored reliably. In contrast, two to three times as many polymorphic markers were obtained in the other species described in this chapter. The small number of markers used in the estimation of AFLP heterozygosity means that the AFLP band count will carry limited information about inbreeding. Therefore, it is possible that the lack of any relationship between disease susceptibility and AFLP band count is due to AFLP heterozygosity based on only 32 markers not providing a sufficiently accurate estimate of genome-wide heterozygosity.

The relationship between microsatellite heterozygosity and disease resistance could either be an indication of inbreeding depression, or a result of single locus heterosis. If the relationship is due to inbreeding depression, heterozygosity at all the microsatellite loci would contribute towards the relationship. On the other hand, if the relationship is due to single locus heterosis, disease susceptibility would be associated with heterozygosity at only one or a few of the microsatellite loci. Except for carcinoma, susceptibility to the different diseases has not been found to depend on heterozygosity at particular microsatellite loci. Heterozygosity at two microsatellite loci was found to be associated with carcinoma. Although these associations are fairly strong, they are not significant after Bonferroni correction (Acevedo-Whitehouse, pers. comms.). These results
suggest that the observed relationship between disease susceptibility and microsatellite heterozygosity is a manifestation of inbreeding depression.

An alternative explanation for the observed differences in IR between the different disease groups is that these differences reflect population structuring rather than susceptibility to disease. In this scenario, the different populations vary in their incidence of the different diseases, and due to limited gene flow between the populations, also in their levels of heterozygosity. Phylogenetic analysis using the AFLP markers did not reveal any population structuring amongst the sea lions. Although, the power of this analysis was limited by being based on only 32 AFLP markers, very strong population structuring would be required to cause the observed differences in IR between the disease groups and even a small number of AFLP markers might be expected to capture such population structuring. Analysis of the microsatellite genotypes using DADSHARE (http://www.zoo.cam.ac.uk/zoostaff/amos/DadShare.doc) also did not reveal any population structuring associated with the disease groups (Acevedo-Whitehouse, pers. comms). Therefore, the possibility of population structuring within the sea lion population causing the observed relationship between disease class and IR seems unlikely.

5.4.4 Old-field mouse

The effects of parental heterozygosity and parental relatedness on three measures of fitness, litter survival, average pup mass, and litter size, were examined in three subspecies of old-field mice. Over all subspecies, average pup mass was negatively correlated with maternal heterozygosity based on AFLP and microsatellite markers, and positively correlated with litter size. Litter survival was positively associated with father's IR and negatively associated with parental relatedness measured using microsatellites. None of the factors investigated had significant effects on litter size.
There is good evidence to suggest that the effect of maternal heterozygosity on pup mass is a result of genome-wide heterozygosity. The strongest support comes from the fact that maternal heterozygosity estimates based on independent types of molecular markers affect pup mass as evidenced by the significant negative effect of the interaction between mother’s IR and mother’s weighted AFLP band count on pup mass. In addition to this, the results of the single locus analyses reveal that heterozygosity at a significant majority of microsatellite markers correlates positively with pup mass. Therefore, it is likely that this HFC is a sign of inbreeding depression.

The study described here is based on the first generation of litters produced by the same wild mice that were used to produce the inbred lines used in Lacy et al. (1996). In a series of experiments designed to examine variation in inbreeding depression between different replicate stocks for each of three subspecies of *P. polionotus*, Lacy et al. (1996) investigated the effect of litter and maternal inbreeding on a variety of fitness traits, including the three measures of fitness I have used. A comparison can be made between factors affecting fitness in litters produced by wild mice, and litters produced by inbred laboratory mice. For the purposes of this comparison, the mother’s inbreeding coefficient is equivalent to mother’s IR or mother’s AFLP band count, and the litter’s inbreeding coefficient is equivalent to parental relatedness based on either microsatellites or AFLP loci.

Lacy et al. (1996) found that litter survival was negatively correlated with the litter’s inbreeding coefficient in two of the three subspecies. A similar pattern is found in my analysis of litter survival, with parental relatedness negatively correlating with the survival of litters produced by wild mice (Table 5.2). This indicates that the relatedness between the founders of the laboratory population may provide additional information about inbreeding on top of that contained within pedigree-based inbreeding coefficients. While pedigrees provide information about inbreeding
coefficients, genetic estimators of inbreeding may give information about single locus effects in
addition to level of inbreeding.

Results in Lacy et al. (1996) show that in two of the three subspecies of mice, pup mass was
negatively associated with the litter’s inbreeding coefficient, while mother’s inbreeding coefficient
also correlated with pup mass in two subspecies, but in opposite directions. Unlike these results, I
find that the mass of pups is affected by maternal experience and maternal heterozygosity
estimated by both microsatellite and AFLP markers. Pup mass is not seen to be affected by the
parental relatedness of the litters. The effect of mother’s parity on pup mass makes intuitive
sense, as a more experienced mother might be expected to be better at rearing pups. The average
mass of pups in a litter at weaning will also depend on the ability of the mother to provide her
pups with nourishment both before and after birth. Compared to a less heterozygous female, a
more heterozygous female is likely to be fitter and have better body condition, making her better
able to nourish her pups.

The differences between the factors affecting pup mass in my analysis and in Lacy et al. (1996)
could be due to the analyses in Lacy et al. (1996) being based on litters produced by captive
inbred mice, and my analysis being based on litters produced by wild mice. However, my analysis
shows that litter size has a major effect on pup mass, explaining 15 % of the deviance. Litter size
was not included in the regression model for pup mass in Lacy et al. (1996). Leaving out a major
explanatory factor from a regression is likely to produce results that are difficult to interpret.
Therefore, it is possible that the ambiguity about the effects of maternal inbreeding on pup mass
found in Lacy et al. (1996) is a Type II error.
5.4.5 General discussion

No significant correlations were found between IR and AFLP band count in any of the four species investigated in this chapter. Although both IR and AFLP band count are estimating an individual’s level of inbreeding, the accuracy of both estimates is low. In Chapter 4, an $r^2$ of only 0.11 was obtained from regressing the microsatellite heterozygosity of 181 $P. polionotus$ with inbreeding coefficients ranging from 0 to 0.45 against their AFLP band counts. As the species studied in this chapter come from wild populations, the sampled individuals are likely to have zero or very low levels of inbreeding. When very low levels of inbreeding are being considered, it unlikely that a correlation between IR and AFLP band count could be detected with the small sample sizes used in the four species examined here.

Analysis of the sparrow and mallard data has revealed cases where HFCs have been detected using AFLP band count, but not with IR. In both cases the indications are that AFLP band count is estimating genome-wide heterozygosity and that the observed HFCs are the effects of inbreeding depression rather than single locus heterosis. These cases are the first reported HFCs detected using AFLP markers and they provide further evidence in support of the results in Chapter 4 which suggested that a large number of AFLP markers ($> 70$) might be better at estimating low levels of inbreeding compared to a small number ($8 – 12$) of microsatellite markers.

In contrast to the sparrow and mallard cases, the California sea lion represents a study in which a strong HFC had already been detected using IR, but was not apparent with AFLP band count. In this case, IR appears to be estimating genome-wide heterozygosity. The lack of a relationship of fitness with AFLP heterozygosity is probably due to only a small number of polymorphic markers being available for the AFLP band count in this species. This highlights a potential problem with using AFLP markers for measuring HFCs. Some species have low levels of AFLP
polymorphisms, and although this can be overcome by using many different AFLP primer combinations, the genotyping effort required will be large.

HFCs can be detected using both microsatellite and AFLP based measures. If, as in the case of the pup mass in the old-field mice, the same HFC is detected using both types of markers, it is probable that the HFC in question is a sign of inbreeding depression. On the other hand, if different HFCs are revealed using the two types of markers, it is possible that one, or both of the markers are revealing HFCs that are due to single locus heterosis. Irrespective of what marker is used to detect HFCs, it is important that appropriate analyses are undertaken to establish whether the observed HFC is likely to be a result of single locus heterosis or inbreeding depression.
CHAPTER 6

USING AFLP MARKERS TO INFERENCE PHYLOGENETIC RELATIONSHIPS BETWEEN PINNIPED SPECIES

6.1 Introduction

Results in previous chapters indicate that amplified fragment length polymorphisms can be used to estimate the relatedness between individuals from the same species. In this chapter I will investigate the role of AFLP markers at revealing longer-term patterns of evolution by using them to examine the relatedness between individuals from different species, genera and families.

6.1.1 Phylogenetic analysis using AFLP markers

AFLP markers are widely used in reconstructing phylogenies in plant and fungal species (Baayen et al. 2000; Bakkeren et al. 2000; Caicedo et al. 1999; Hodkinson et al. 2000; Kardolus et al. 1998; Labra et al. 1999). These markers are increasingly being used in animal systems where sequence based studies have been unable to resolve phylogenetic relationships (Albertson et al. 1999; Allender et al. 2003; Buntjer et al. 2002; Giannesi et al. 2001; Parsons and Shaw 2001; Seehausen et al. 2003). However, there is little agreement about the most appropriate and effective methods for analysing AFLP characters. No analytical methods have been developed specifically for phylogenetic analysis using AFLP genotypes, and studies use existing methods that have been developed for other kinds of data.

Most studies generating AFLP markers do so using a protocol that utilises two restriction enzymes, a 6-base and a 4-base recognition enzyme, together with two selective amplification primers (Ajmone-Marsan et al. 1997; Vos et al. 1995). When these procedures are followed, the
amplification of each AFLP fragment requires 16 bases to be of a particular sequence: ten bases for the two restriction enzymes and 3 bases for each selective primer (Figure 1.1). In effect, the presence of an AFLP band is acting like the existence of a 16-base restriction site (Felsenstein 2004). Therefore, the parsimony and distance methods used for analysing restriction site data are probably applicable to the analysis of AFLP characters.

A problem with analysing AFLP characters and restriction site data is the asymmetry in the probabilities of gaining and losing bands. If a particular sequence is only one substitution away from the 16 bases required for AFLP amplification then, given that a substitution occurs, only one in 48 possible substitutions will result in the band being amplified. On the other hand, if the band already exists, a substitution at any of the 16 bases will cause the band to be lost. Therefore, losing a particular band is much more likely than gaining the band.

The most common parsimony methods applied to AFLP data are ones based on Dollo and Wagner parsimony. Dollo parsimony (Farris 1977; Quesne 1974) is based on “Dollo’s Law” (Dollo 1893) according to which a complex character once attained cannot be attained in that form again. Thus, once a complex character is lost, it cannot re-evolve except in a different form. Dollo parsimony can be applied to data where the gain of a character is improbable but its loss not as rare. In Wagner parsimony (Kluge and Farris 1969) there is no asymmetry regarding changes in the state of a character, making character losses and gains equally probable. Due to the asymmetry in character loss and gain, DeBry and Slade (1985) suggested that Dollo parsimony is more appropriate for analysing restriction site data than Wagner parsimony. In contrast, Albert et al. (1992) found that the constraints of Dollo parsimony are too strong, and that Wagner parsimony with an unknown ancestral state comes closer to approximating restriction site changes. Although a proper quantitative evaluation has not been carried out, because AFLP markers can be treated as large restriction sites with strong asymmetries of
change, it is expected that Dollo rather than Wagner parsimony would be better for analysing AFLP data (Felsenstein 2004).

Most studies computing distances from AFLP data either use Jaccard distances (Buntjer et al. 2002; Jaccard 1908; Kingston and Rosel 2004) or the restriction fragment distance described by Nei and Li (1979) (Sullivan et al. 2004). As it is easier to lose an AFLP band at a particular locus than it is to gain the same band, the alleles responsible for band presence at a locus are likely to be homologous. However, the absent state at a particular locus can arise from a number of different mutations that prevent amplification of the band in question. Therefore, a locus may have a several different absent alleles that not homologous, but it is not possible to tell these alleles apart. The longer the period of evolutionary time over which comparisons are being made, the higher the probability that the absent alleles are not homologous as more time would have elapsed over which different mutations could have generated different absent alleles. The calculation of Jaccard distances does not assume homology between absent alleles and therefore is the more conservative distance measure for use with AFLP data.

AFLP markers usually show high levels of intraspecific polymorphism and for this reason have usually been used to establish phylogenetic relationships either within a species (Giannesi et al. 2001; Ivors et al. 2004; Riberon et al. 2004), or between closely related species (Carisio et al. 2004; Kingston and Rosel 2004; Semerikov et al. 2003). At these scales, they have often performed better than other nuclear or mitochondrial markers (Després et al. 2003; Sullivan et al. 2004; Xu and Sun 2001). However, the utility of AFLP markers for examining the phylogenetic relationships between more distantly related species and genera has not been properly examined. Pinnipeds provide an excellent test case for AFLP-based phylogenies because they comprise species with a wide range of species divergence times of 1-25 million years (MY).
6.1.2 Pinniped phylogenetic relationships

There are 33 extant pinniped species from three families: Otariidae, Phocidae and Odobenidae. Fourteen sea lion and fur seal species make up the otariids and among other characters they all have external ear flaps or pinnae and can turn their hind flippers forward and use them for walking. The phocids are also known as earless seals or true seals. Made up of two subfamilies, the Phocinae and the Monachinae, the Phocid family has 18 extant member species and is characterized by the lack of visible pinnae, and their inability to turn their hind flippers forward for supporting the body, resulting in a humping/crawling locomotion on land. The walrus is the only surviving representative of a once diverse assemblage of species comprising the Odobenidae. Pinniped fossils dating back 25-27 MYA (million years ago) (Berta et al. 1989) have been found, and suggest that the Phocid lineage diverged 15 MYA, but molecular data place the extant Phocid species diversification as occurring 2-6 MYA (Árnason et al. 1995). The Otariid lineage is dated from 6-12 MYA (Berta 2002), with the line leading to extant sea lion and *Arctocephalus* fur seal species diverging ~6 MYA (Miyazaki et al. 1994).

A large number of studies have been conducted using both morphological and molecular data to shed light on the evolutionary origin of the pinnipeds (Árnason 1974; Árnason and Widegren 1986; Berta 2002; Berta and Wyss 1994; Lento et al. 1995; Ray 1976; Sarich 1969; Slade et al. 1994). Traditionally, the pinnipeds were thought to have evolved from two separate carnivore lineages, with the phocids being related to the mustelids, and the ursids giving rise to the otariids and odobenids (Figure 6.1A) (McLaren 1960; Wozencraft 1989). The current view, supported by both morphological and molecular data, strongly endorses a monophyletic origin for the pinnipeds (Figure 6.1B) (Árnason et al. 1995; Lento et al. 1995; Wyss 1988).
Figure 6.1  Phylogenetic trees describing the major arguments in the debate on pinniped evolutionary origins. A and B depict monophyletic and diphyletic origins for the pinnipeds. Given a monophyletic origin for the pinnipeds, the placement of the Odobenids within the group is ambiguous: C and D.
Given a monophyletic origin for the pinnipeds, there is still disagreement about the relationships between the three families comprising the extant pinnipeds. Whether or not they support pinniped monophyly, studies using flipper and post-cranial characters suggest a sister taxon relationship between the Odobenidae and the Phocidae (Figure 6.1C) (Berta et al. 1989; Wyss 1988). On the other hand, molecular data and studies using different morphological characters suggest a relationship between the Odobenidae and Otariidae (Figure 6.1D) (Árnason et al. 1995; Lento et al. 1995).

Analyses based on morphological and molecular data also disagree about relationships within the Otariidae. The division of the otariids into two subfamilies, sea lions and fur seals, although widely recognized in the literature (Reynolds et al. 1999; Riedman 1990), is not supported either by molecular data (Wynen et al. 2001) or by more recent fossil and morphological evidence (Bininda-Emonds et al. 1999). Only a few morphological characters separate the two subfamilies, the presence or absence of underfur and the presence of five or six upper canines, and these may not be sufficient to justify the division. Analysing mtDNA for all extant otariids, Wynen et al. (2001) found the northern fur seal, genus *Callorhinus*, to be basal to the rest of the otariids. However, they failed to find evidence either supporting or refuting the monophyly of either the sea lions or the major fur seal genus, *Arctocephalus*. Interspecific and intergeneric hybrids between otariids have been reported (Goldsworthy et al. 1999; Rice 1998) and may be partly responsible for the reduced genetic differentiation between some otariid species.

The division of the 18 extant phocid species into the two subfamilies, Phocinae and Monachinae, is supported by both morphological and molecular data (Árnason et al. 1995). Two karyotypes are found within the phocids, $2n = 32$ and $2n = 34$. The $2n = 34$ karyotype is thought to be ancestral, and is found in all Monachinae, as well as two of the Phocinae, the bearded (*Erignathus barbatus*) and hooded seals (*Cystophora cristata*). The rest of the Phocinae have the $2n = 32$
karyotype (Árnason 1977). Árnason et al. (1995) used mtDNA data to examine the relationships between the phocids and failed to find strong support for relationships between species within the Phocinae and the Monachinae.

Extant species of pinnipeds are thought to have diverged over a wide time scale of 1-25 MY (Árnason et al. 1995; Berta 2002). I will examine the utility of AFLP markers as a tool for determining phylogenetic relationships between these pinniped species. Patterns emerging from an AFLP phylogeny of pinnipeds will be compared against existing phylogenies based on mtDNA (Árnason et al. 1995; Wynen et al. 2001). As AFLP markers evolve quickly, they may shed further light on relationships between recently diverged otariid and phocid species.
6.2 Materials and methods

6.2.1 Samples and genotyping

Eighteen pinniped species comprising 4 otariids, 13 phocids, and the walrus (Table 6.1) were used in the analyses presented in this chapter. A total of 104 individuals from these species were genotyped using eight AFLP primer combinations (TagI-CAG with EcoRI-ACA, TagI-CAC with EcoRI-ATG, TagI-CCA with EcoRI-ACA, TagI-CTG with EcoRI-ACA, TagI-CAC with EcoRI-ACA, TagI-CAC with EcoRI-AGC, TagI-CAC with EcoRI-AAC, TagI-CGA with EcoRI-ACA).

To reduce the amount of computation time required during the tree building procedures, a maximum of five individuals were randomly chosen from each species for the subsequent analyses. As fewer than five individuals were genotyped in four of the species, a total of 84 individuals were used in the phylogenetic analyses (Table 6.1). The AFLP genotypes of the 84 individuals from the 18 pinniped species were scored at 218 AFLP markers that were variable either within or between species. Bands that were difficult to size, or that did not amplify reliably, were not scored. Most of the scored bands were between 100-300 bp in length. It was assumed that AFLP bands that were the same size across species represented homologous markers (Parsons and Shaw 2001).
Table 6.1 Pinniped species genotyped and used in the phylogenetic analyses.

6.2.2 Phylogenetic analysis

The final character matrix used as input for the phylogenetic analyses consisted of binary characters representing the presence and absence genotypes of 84 individuals from 18 pinniped species at 218 AFLP markers. Both distance and parsimony methods were used to create unrooted trees. As the various pinniped species may have diverged up to 25 million years ago, Jaccard distances were used in the distance analyses to avoid the problem of possible non-homology between absent alleles. All analyses were carried out using programs in PHYLIP (Felsenstein 1989).
A matrix of pairwise Jaccard distances, $J_{xy}$, between all individuals was created from the binary character matrix using Equation 6.1:

$$J_{xy} = 1 - \left( \frac{a}{a + b + c} \right)$$

(Equation 6.1)

(a is the number of polymorphic bands shared by individuals $x$ and $y$, $b$ is the number of bands present in $x$ but absent in $y$, and $c$ is the number of bands present in $y$ but absent in $x$). From this distance matrix, the neighbour-joining (NJ) method (Saitou and Nei 1987) was used to construct minimum evolution trees using NEIGHBOR (Felsenstein 1989). Both Dollo and Wagner parsimony methods were used to construct minimum evolution trees using the DOLLOP and PARS programs in PHYLIP (Felsenstein 1989). To assess support for the trees, 1000 bootstrap pseudoreplicates were performed for the distance method. Only 200 pseudoreplicates were performed for the Dollo and Wagner parsimony methods due to the much greater computational time required for these algorithms (Felsenstein 1985). For the bootstraps, starting trees for each replicate were obtained by randomising the input order of the species.

### 6.3 Results

Three trees were constructed to examine pinniped relationships: a distance-based NJ tree (Figure 6.2), a Dollo parsimony tree (Figure 6.3) and a Wagner parsimony tree (Figure 6.4). Except for within the Otariidae, all three trees exhibit identical branching patterns, indicating that the phylogeny is robust to the assumptions made in constructing them. Bootstrap support for the various branching events do vary depending on the assumptions made in building the trees. Bootstrap support for branching events is similar in both the Dollo and Wagner parsimony trees, but is in general lower when compared with the distance-based tree.
Figure 6.2 Unrooted neighbour-joining tree for pinnipeds derived from Jaccard distances. Bootstrap values at nodes at or above the 50 % level are indicated.

(CSL, California sea lion; SSL, Stellar sea lion; NFS, northern fur seal; AFS, Antarctic fur seal; W, Walrus; CRB, crabeater seal; RSS, Ross seal; LEO, leopard seal; WED, Weddell seal; ES, southern elephant seal; BRD, bearded seal; HD, hooded seal; HRP, harp seal; RNG, ringed seal; SPT, spotted seal; HBR, harbour seal; GRE, grey seal; CSP, Caspian seal)
Figure 6.3  Unrooted Dollo parsimony tree for pinnipeds. Bootstrap values at nodes at or above the 50 % level are indicated.

(CSL, California sea lion; SSL, Stellar sea lion; NFS, northern fur seal; AFS, Antarctic fur seal; W, Walrus; CRB, crabeater seal; RSS, Ross seal; LEO, leopard seal; WED, Weddell seal; ES, southern elephant seal; BRD, bearded seal; HD, hooded seal; HRP, harp seal; RNG, ringed seal; SPT, spotted seal; HBR, harbour seal; GRE, grey seal; CSP, Caspian seal)
Figure 6.4  Unrooted Wagner parsimony tree for pinnipeds. Bootstrap values at nodes at or above the 50 % level are indicated.

(CSL, California sea lion; SSL, Stellar sea lion; NFS, northern fur seal; AFS, Antarctic fur seal; W, Walrus; CRB, crabeater seal; RSS, Ross seal; LEO, leopard seal; WED, Weddell seal; ES, southern elephant seal; BRD, bearded seal; HD, hooded seal; HRP, harp seal; RNG, ringed seal; SPT, spotted seal; HBR, harbour seal; GRE, grey seal; CSP, Caspian seal)
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Table 6.2 Pairwise Jaccard distances between representatives of the 18 pinniped species used in this study. Shaded regions contain distances within the Otariid, Phocine and the Monachine groups.
All three trees (Figure 6.2, Figure 6.3 and Figure 6.4) show a clear division of the pinnipeds into
three clades representing the three pinniped families. The lack of an appropriate outgroup makes
it difficult to draw conclusions about the relationships between these three families, however,
some inferences can be made based on the average Jaccard distances between the families. There
are three possible groupings: (Odobenidae, (Phocidae, Otariidae); (Phocidae, (Odobenidae,
Otariidae); (Otariidae, (Phocidae, Odobenidae). Based on values in Table 6.2, the average
pairwise distance between the phocids and the otariids is 0.957, and the distance between the
phocids and the odobenid is 0.929. In comparison, the average distance between the otariids and
the odobenid is only 0.874. This suggests that the (Phocidae, (Odobenidae, Otariidae) grouping is
the most likely arrangement, and is consistent with phylogenies based on mtDNA (Árnason et al.
1995; Lento et al. 1995). Therefore, although morphological analyses do not agree on the
grouping of the three pinniped families, all of the molecular evidence to date supports the
grouping of the odobenids with the otariids.

Although the trees based on AFLP markers strongly support monophyly of the otariids, the
relationships between the four species used in the analyses are uncertain. The Stellar sea lion is
basal to the other species in the group in both the parsimony methods as well as the distance
method. However, the placement of the other three species is not consistent across methods.
Bootstrap support is low for all branching patterns within the Otariidae.

Parsimony and distance trees divide the phocids into two clades corresponding to the subfamilies
Phocinae and Monachinae. There is good support for this division in the distance analysis, but
the support is poorer in the parsimony analyses, especially using Dollo parsimony. Within the
Monachinae, the southern elephant seal is basal to the four Antarctic seal species. Relationships
between these Antarctic seal species are difficult to resolve, but there is some support for a clade
containing the leopard and Weddell seals (Figure 6.2). Within the Phocinae, the hooded and
bearded seals are basal to the rest of the species. Phylogenetic relationships between the more recently radiated *Phoca* species complex are more difficult to resolve. Both distance and parsimony methods place the harp seal at the base of the *Phoca* complex. Although the grey seal has traditionally been assigned its own genus, *Halichoerus*, the phylogenetic analyses based on AFLP markers groups the grey seal with the Caspian seal within the *Phoca* genus (Figure 6.2), a finding supported by mtDNA data (Árnason et al. 1995). Within the *Phoca* grouping, AFLP markers fail to distinguish strongly between the spotted and the harbour seals. The average Jaccard distance between these two species is 0.043. This is similar to the average distance of 0.031 between individual harbour seals. Other *Phoca* relationships cannot be resolved using the AFLP markers.

Multiple individuals were genotyped for each of the 18 pinniped species used in the AFLP analyses. Using Jaccard distances, there was 100 % bootstrap support for 14 of the species groups, and 99 % bootstrap support for a further two species groups. AFLP-based distances were unable to distinguish clearly between the spotted and harbour seal. These results indicate the utility of genetic distances based on AFLP markers for species recognition purposes within the pinnipeds.

6.4 Discussion

Phylogenies based on AFLP markers were generated to investigate the relationships between 18 pinniped species comprising 13 phocids, 4 otariids and 1 odobenid. The distance-based NJ tree and both parsimony trees showed similar branching patterns and clearly distinguished between the three pinniped families. Results suggest that the Odobenidae and the Otariidae are sister taxa. Relationships between otariid species could not be resolved. Within the phocids, two clades corresponding to the Phocinae and Monachinae were evident. Within the Monachinae the
southern elephant seal emerged basal to a monophyletic clade of Antarctic seals, while the bearded and hooded seals were basal to the recently radiated group of *Phoca* species.

Both parsimony methods employed for analysing the pinniped relationships resulted in trees that in general had lower bootstrap support for branching events compared to the Jaccard distance-based NJ tree. Although Dollo and Wagner parsimony methods are widely used to analyse AFLP data, it has been argued that neither the evolutionary constraints of Dollo parsimony, nor the free reversibility allowed in Wagner parsimony are appropriate for the analysis of binary characters generated by RAPD or AFLP genotyping (Backeljau et al. 1995). The lower bootstrap support for the parsimony trees may be a reflection of the inadequacies of these parsimony models in dealing with AFLP data. Another problem with AFLP characters is that while the present alleles at a locus are unlikely to have arisen independently, the absent state at a particular locus may result from different mutations, especially over long periods of evolutionary time. The parsimony methods assume that the absent alleles at a particular locus are homologous, an assumption that is not necessarily accurate and may contribute to the lowered confidence in the branching events. On the other hand, the calculation of Jaccard distances used in the NJ tree is based on the sharing of present bands, and does not count the sharing of absent bands. Therefore, in this analysis, the branching patterns and the associated bootstrap supports of the distance-based NJ tree are thought to be more accurate compared to those of either parsimony tree.

No species from outside the pinnipeds were included in the analyses to act as outgroup species. The Jaccard distance between two species is measure of the number of homologous AFLP bands shared by them (Equation 6.1). When two species do not share any bands, the Jaccard distance between the two is 1. Within the pinnipeds the maximum distance between two species exceeded 0.99 (Table 6.2) indicating that these species share very few AFLP bands. The distance between potential outgroup candidates from within the ursids or mustellids and any pinniped species
would be greater than this maximum distance. Therefore, it is unlikely that any homologous AFLP markers would be found between the pinnipeds and such outgroup species making the inclusion of an outgroup uninformative. The lack of such an outgroup does make it difficult to draw conclusions about the relationships between the three pinniped families (Figures 6.1C and 6.1D). However, a comparison of average Jaccard distances between the families suggests the grouping (Phocidae, (Odobenidae, Otariidae), a result that is consistent with those from other molecular studies. The absence of outgroup species also makes it impossible to make inferences regarding the monophyly or diphly of the pinnipeds (Figures 6.1A and 6.1B). However, studies based on both molecular and morphological characters now agree on the monophyly of the pinnipeds and this is no longer a very contentious issue.

Phylogenies based on morphological characters divide the otariids into two groups: sea lions and fur seals. However, this division is based on only a few characters. Both the AFLP data presented here and mtDNA data presented in Wynen et al. (2001) fail to support such a division. Wynen et al. (2001) presented a mtDNA phylogeny using all Otariid species and found strong support for the basal relationship of the northern fur seal to the rest of the group, but was unable to clarify relations between the other fur seal and sea lion species. With the AFLP data, although the parsimony and distance methods place the Stellar sea lion at the base of the group, there is no evidence for the monophyly of either the fur seals or the sea lions. Support for the placement of the Stellar sea lion and not the northern fur seal at the base of the Otariidae is weak, consequently the results of the AFLP analysis should not be viewed as being inconsistent with that of Wynen et al. (2001).
Figure 6.5 Neighbour joining tree summarizing Phocid relationships presented in Figure 6.2.

Within the pinnipeds, two clades corresponding to the subfamilies Phocinae and Monachinae are evident from the AFLP phylogenies. In agreement with the mtDNA phylogeny in Árnason et al. (1995), the bearded and hooded seals are at the base of the phocine clade (Figure 6.5) but the bootstrap support for these relationships is stronger using AFLP markers compared to using mtDNA sequences. These relationships support the findings of karyotypic studies where all Monachinae have a $2n = 34$ karyotype, while a $2n = 32$ karyotype is found in all Phocinae except
for the bearded and hooded seals, which have a $2n = 34$ karyotype (Árnason 1977) suggesting that the $2n = 34$ karyotype is the ancestral state.

The Monachinae consist of four Antarctic seal species, the southern and northern elephant seals and the monk seals. The AFLP phylogeny shows that the four Antarctic seal species form a monophyletic group and that the southern elephant seal is basal to this group. Monophyly of the Antarctic species suggests that diversification in the Southern Hemisphere occurred following a single invasion, a finding that is concordant with the evidence from mtDNA (Árnason et al. 1995). Analysis of mtDNA data also suggests that the Monachinae are a paraphyletic group with the monk seals basal to all phocids and not just the monachines (Árnason et al. 1995). However, no monk seals were included in the AFLP analyses, thus this relationship cannot be corroborated.

Although relationships between the four Antarctic monachine species are difficult to resolve, there is some support for a clade containing the leopard and Weddell seals. Morphologically the leopard and Weddell seals are highly divergent. In contrast, the genetic distance between these two species is one of the smallest between pinniped species (Table 6.2). This discrepancy between morphological and molecular distances shows how the rate of morphological evolution can accelerate compared to that of molecular evolution when rapid diversification occurs to fill new ecological niches. Such differences in rates of evolution can cause incongruities between phylogenetic trees based on morphological characters and those based on molecular characters.

AFLP, mtDNA and karyotype data agree on the placement of the hooded and bearded seals at the base of the Phocinae, however, phylogenetic relationships between the recently radiated Phoca species complex have been difficult to resolve. The Phoca species form a monophyletic group that includes the grey seal (genus Halichoerus) with the harp seal at its base. A number of dispersal
hypotheses have been put forward to explain the current biogeographic patterns for phocine seals. One hypothesised dispersal involves an initial migration from the Paratethys Sea, an area now occupied by the Black, Caspian and Aral Seas, into the Arctic Basin followed by an eastward migration that gave rise to the extant *Phoca* species (Repenning et al. 1979). Another dispersal hypothesis argues for a North Atlantic origin for all phocines with cyclical glaciation events causing speciation in refugia (Davies 1958; Deméré et al. 2003). Under the first hypothesis the landlocked Caspian seal remained in the Caspian Sea as an isolated remnant of the original Paratethys Sea species and should therefore appear near the base of the phocine clade. Under the second hypothesis the Caspian seal is derived from an ancestral *Phoca* species that entered the Paratethys from the Arctic Basin during the Pleistocene by means of the Volga River drainage and later became land locked. Under such a scenario the Caspian Seal would be placed within the *Phoca* species complex. Lending support to the second hypothesis is the fact that in the AFLP phylogeny the Caspian Seal is positioned within the *Phoca* group and appears have diverged relatively recently from the grey seal. The basal positions of phocine taxa such as *Erignathus* and *Cystophora* that appear to have originated in the Arctic during the Pleistocene (Deméré et al. 2003) provide additional support for the North Atlantic origin for all phocines.

Although 18 of the 33 extant pinniped species were included in the AFLP phylogeny, the coverage of species is not uniform. In particular, the otariids are underrepresented, with only four of the 14 extant species being included in the analyses. Within the phocids, the northern and southern seal species are well covered in the analyses. However, no monk seal species were present in the analyses. The monk seals are important species as they are representatives of an early diverging group of phocids. Traditionally they were thought to be basal to the monachines, but recent work suggests that they are basal to all phocines [Árnason, 1995 #145]. Any future work should attempt more comprehensive species coverage by including monk seal and further otariid species.
The pinniped relationships obtained here using AFLP markers agree closely with previous phylogenies based on mtDNA. In some cases, improved resolution of relationships was obtained using AFLP data. The concordance between phylogenies obtained from two independent types of molecular markers indicates the robustness of the detected relationships. This study demonstrates the utility of AFLP markers at discerning the longer-term patterns of evolution between species that have diverged over a time scale of 1-25 million years.

In addition to the AFLP-based phylogenies presented in this thesis, pinniped phylogenies using karyotype data and mtDNA sequences have also been published. Unpublished data from Y-chromosome loci also exists (M. Kinnear pers. comm.) and work is currently in progress looking at microsatellite length differences between pinniped species (E. J. Vowles pers. comm.). While AFLP and microsatellite markers are inherited from both parents in a Mendelian manner, mtDNA is inherited through the female line and Y-chromosome loci through the male line. These independent sets of markers evolve at different rates and should provide optimal resolution at different time scales. Few species groups have data from such a diverse range of markers and this wealth of data that should be brought together to generate a definitive molecular phylogeny of the pinnipeds.
CHAPTER 7

CONCLUDING REMARKS

Although some authors have expressed scepticism about the importance of inbreeding in conservation (Caro and Laurensen 1994; Caughley 1994), evidence documenting the importance of inbreeding depression has accumulated, making inbreeding depression a major theme in conservation genetics. In addition to accounts of inbreeding depression in laboratory populations of *Drosophila* and mice (Bowman and Falconer 1960; Miller and Hedrick 1993), inbreeding has been found to affect fitness in species of conservation interest held in zoos (Ralls et al. 1988; Ralls et al. 1979). There are also a few convincing studies demonstrating inbreeding depression in wild populations (Keller and Waller 2002), and evidence that inbreeding increases the risk of extinction in wild populations (Saccheri et al. 1998).

In this thesis, I first looked at the importance of pedigree-based inbreeding coefficients and other factors in determining fitness in a captive population of Sumatran tigers. A detailed analysis revealed that although maternal inbreeding negatively affected the fitness of litters, in this species the influence of maternal identity was much greater, with some females being consistently better at rearing litters than others. These conclusions are contrary to those reached in a previous analysis of this species that only examined the effects of a few factors. The study highlights the importance of including major explanatory factors in regression models and the risk of reaching erroneous conclusions when they are excluded.

In order to investigate the impact of inbreeding on fitness, knowledge of individuals’ levels of inbreeding is required. In the case of the Sumatran tiger, detailed pedigree information was available that allowed the calculation of individuals’ inbreeding coefficients. While pedigrees may
be available for some laboratory and zoo populations, they are not available for most wild populations. Therefore, I investigated the use of molecular markers for estimating individuals’ levels of inbreeding.

Most studies use heterozygosity measured at 5-15 microsatellite markers as an estimator of inbreeding (Acevedo-Whitehouse et al. 2003; Hansson et al. 2001; Hoffman et al. in press). The main drawback of using microsatellites is that these markers have not been isolated in the majority of species. Therefore, a useful alternative to microsatellites would be a marker that can be amplified in any species. As AFLP markers are reliable PCR-based markers that can be amplified in any species without the need to optimise conditions, I investigated whether individual heterozygosity estimates based on AFLP markers reflect levels of inbreeding. In a laboratory population of *P. polionotus*, both AFLP and microsatellite heterozygosity were found to strongly correlate with pedigree-based inbreeding coefficients, demonstrating that both types of markers carry information about an individual's level of inbreeding. In this species, for similar amounts of genotyping effort, AFLP-based heterozygosity appeared to give better estimates of inbreeding at low levels of inbreeding compared to microsatellite-based heterozygosity.

As heterozygosity at genetic markers is expected to correlate with an individual’s level of inbreeding, many researchers have investigated correlations between heterozygosity and fitness as a means of assessing inbreeding depression (Coltman et al. 1998; Coulson et al. 1999; Hansson 2004). I investigated whether heterozygosity estimated using AFLP markers could be used to detect such heterozygosity-fitness correlations (HFCs) in four species. In two of the species, HFCs were detected using AFLP but not microsatellite markers, in one species HFCs were detected using microsatellite but not AFLP markers, and in another species HFCs were detected with both types of markers.
A number of studies have taken the presence of an HFC in a population to be an indication of inbreeding depression (Acevedo-Whitehouse et al. 2003; Coltman et al. 1999; Slate et al. 2000). However, this will only be true if heterozygosity at the markers used in the studies is equivalent to an individual’s level of inbreeding. An alternative explanation for the presence of an HFC is that one or a few of the markers used to measure heterozygosity are in linkage disequilibrium with genes under balancing selection, resulting in single locus heterosis. Under this alternative explanation, an HFC does not indicate the presence of inbreeding depression.

As studies applying microsatellite markers for detecting HFCs usually employ only 5-15 markers, if one of these markers were linked to a gene under balancing selection, but analyses to detect this linkage are not carried out, an erroneous conclusion of inbreeding depression would be reached. Therefore, when an HFC is detected, it is important that appropriate analyses are undertaken to establish which of the two alternatives is most likely to explain the HFC in question. In order to establish whether heterozygosity at a few markers causes the HFC, or whether the effect is spread over the majority of the loci used, such analyses usually involve looking at the contribution of heterozygosity at individual markers to the HFC.

Since the AFLP estimate of individual heterozygosity is usually based on a large number of markers, this measure of heterozygosity is less likely to be affected if a few of the markers are linked to genes under balancing selection. This is because the presence of a large number of other unlinked markers will weaken the effect of any linked markers. Thus, it is expected that an HFC detected using a large number of AFLP markers is more likely to be indicative of inbreeding depression than single locus heterosis.
While correlations between pedigree-based inbreeding coefficients and fitness measures are a sign of inbreeding depression, an HFC may either indicate inbreeding depression, the existence of single locus heterosis, or a combination of the two. It will therefore be interesting to compare the effects of both pedigree-based inbreeding coefficients and heterozygosity at molecular markers on fitness. In populations where fitness correlates with both inbreeding coefficient and heterozygosity, it may be possible to determine how much of the HFC is due to inbreeding depression and how much is a result of single locus heterosis. The *P. polionotus* subspecies used in Lacy (1996) would be ideal populations for such a study as the mice have good pedigree records and fitness information, and because tissue samples are available. Other potential populations that fulfil the criteria would be well-managed zoo populations such as the Sumatran tiger (Christie 1999), California condor (Geyer et al. 1993) or the Puerto Rican parrot (Brock and White 1992). In such studies it would be important to include other potentially significant factors that may affect fitness such as zoo environment and maternal identity, in addition to inbreeding coefficients and heterozygosity.

As AFLP markers can be amplified from any species, these markers could potentially become widely used for detecting HFCs. AFLP markers are currently used extensively to study plant populations and are increasingly being used in animal systems. Therefore, much genotype and fitness data already exists for examining HFCs. However, any post hoc analysis for HFCs must be carried out cautiously to guard against the possibilities for Type I errors.

It is not expected that AFLP markers will replace microsatellites in research into HFCs. Once a suite of polymorphic microsatellite markers has been developed in a species it is easy to genotype large numbers of samples at these markers rapidly and reliably. In a number of species, such as the imperial eagle (*Aquila heliaca*) and the Raso lark (*Alauda razae*), only low levels of AFLP polymorphism have been detected. Generating a large enough number of polymorphic AFLP
markers in such species would entail using many AFLP primer combinations, leading to a considerably increased genotyping effort. In such species it may be worthwhile making an initial time and financial investment to develop species-specific polymorphic microsatellite markers.

As different species show varying levels of AFLP polymorphism or diversity, it would be interesting to investigate factors that may influence the amount of AFLP diversity present in a species. Having genotyped a large number of species using AFLP markers, it is evident that while some species such as the house sparrow, common mallard and the old-field mouse exhibit high levels of diversity, other species such as the Raso lark and the imperial eagle have very low levels of diversity. There seems to be a correlation with population size, and species with large population sizes appear on average to harbour higher levels of AFLP diversity. This pattern was evident even within a species, with the island subspecies of old-field mice having substantially lower levels of diversity compared to the mainland subspecies. Past population history is also likely to play an important role in determining the current AFLP diversity of a species. Species with a large current population size may have a low AFLP diversity as a result of past population bottlenecks. In this respect the pinnipeds could be an interesting group to concentrate on, as a number of pinniped species, such as the northern elephant seal, were hunted down to very low numbers.

The California sea lion presents an interesting case as this species has low levels of AFLP diversity, but has high levels of microsatellite diversity. Such a discrepancy may arise from the fact that microsatellite markers evolve faster than AFLP markers. This higher rate of evolution means that following a historical population bottleneck, diversity at microsatellite markers is expected to be regenerated faster than diversity at AFLP markers. In order for microsatellite diversity to have regenerated, such a bottleneck would probably have occurred long enough ago to have left its signature on the AFLP: microsatellite diversity ratio in sister taxa. If the differences
between AFLP and microsatellite diversity is a result of a historical bottleneck, examination of AFLP:microsatellite diversity ratios in other otariid species and in pinniped species outside the Otariidae could help trace when such an event occurred. Therefore, discrepancies between microsatellite and AFLP diversity in species may carry valuable information regarding past population history and is an area where interesting future work could be carried out.

I have investigated the use of AFLP markers for estimating individuals’ levels of inbreeding and relatedness between individuals. In addition, AFLPs have also been found to be useful markers for establishing longer-term evolutionary patterns as shown by their use in generating a phylogeny of pinniped species. Their role in phylogenetic reconstruction means that AFLP markers should also be valuable tools in areas of conservation genetics such as species identification, resolution of taxonomic uncertainties and the definition of management units within species.


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