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Impacts of modern molecular genetic techniques on conservation biology

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I am the family face;
Flesh perishes, I live on,
Projecting trait and trace
Through time to times anon,
And leaping from place to place
Over oblivion.

(Thomas Hardy, 'Heridity' in *Moments of Vision*, 1917.)

Introduction

Conservation biology strives to conserve biodiversity and biological processes in ecosystems, of which genetic variation is a key component. Genetic variation is the underlying foundation of higher levels of biodiversity (e.g. populations and species). Without genetic variation, populations could not evolve and adapt to future environmental changes. Because DNA (deoxyribonucleic acid) is fundamental to all biological systems, the practice of conservation often requires genetic studies. Beyond the measurement and conservation of genetic variation *per se*, the uses of molecular genetic techniques in conservation biology include:

1. identification of individuals, species, populations and conservation units;
2. detection of hybrid zones and admixed populations;
3. quantification of dispersal and gene flow;
4. estimation of current and historical population size;
5. assessment of parentage, relatedness, reproductive success, mating systems and social organization.

Molecular markers also assist forensic detection of illegally killed and trafficked plants and animals or their body parts. Finally, markers that are under selection (and thus influence fitness) can identify locally adapted populations that could have special value for conservation.

Two developments in molecular biology have had unprecedented significance for conservation biology: the PCR (polymerase chain reaction) process and the discovery of microsatellites. Since its development in 1985, PCR has transformed the life sciences, including

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conservation biology, due to the ease (and still declining cost) with which it generates millions of copies of any DNA fragment from minuscule quantities. The PCR technique has allowed the non-destructive study of living specimens and their long-dead ancestors. A surge of mitochondrial DNA (mtDNA) sequence studies on phylogeny, hybridization and gene flow among populations ensued, including some based on fragments of museum skins and specimens preserved in ethanol (Brown & Brown 1994). For example, ancient bones of the Laysan duck (*Anas laysanensis*) were identified by mtDNA analysis from lava tubes on the main Hawaiian islands, where they apparently had become extinct (Cooper et al. 1996). These data justified reintroduction and suggested that many island endemics may be relics of former cosmopolitan species (Wayne et al. 1999).

Microsatellites consist of a length of DNA in which sequences of one to four nucleotides are repeated many times (e.g. $[AC]_n$, where $n = 5$ to 50 repeats). The number of repeats defines an allele at a locus. Microsatellites are typically highly variable, often with > 10 alleles per locus in a population. They are widely dispersed in eukaryotic genomes and inherited in a Mendelian fashion. They can be amplified by PCR from only tiny amounts (one-to-several molecules) of DNA and thus can be salvaged from partially degraded DNA, such as in museum skins, dried faeces or fossil bones. Because of these features, microsatellites have become the most widely used molecular genetic marker. Numerous other PCR-based molecular markers and analysis systems exist, including SNPs (single nucleotide polymorphisms), and direct sequencing of PCR products (see Sunnucks (2000), Morin et al. (2004) and (Schlotterer 2004) for reviews).

Genetics is a key component of many aspects of conservation biology. From the design of reserves to the management of breeding programmes, molecular techniques are indispensable and are increasingly being used to address questions of conservation relevance. Molecular biology is undoubtedly the fastest evolving field of science. Conservation biologists can make use of these emerging techniques, which are rapidly transforming the field to one that is more molecular oriented. Conservation biology is an inexact science because new crises emerge every day and in most cases solutions are but extrapolations from related cases. Molecular biology is helping to change that trend by allowing conservation biologists to quickly scan a wide range of individual and population characteristics at a given site. Genetic data are most useful in conjunction with more traditional data, such as demographics, life history, distribution, etc. Rapid gain of detailed information on a population at risk may allow better understanding of the system at hand, and more sound recommendations for the decision makers.

Systematics and hybridization

Defining a species can be vital to its legal protection – for example, under the Convention on International Trade in Endangered Species (CITES) agreement, which regulates trade in endangered species, or the Endangered Species Act (ESA) in the USA. The ESA efforts to restore the red wolf, *Canis rufus*, to its native North American range began 25 years ago, founded on the belief that the red wolf was a distinct species. More recent genetic analyses from captive individuals and museum skins (Wayne & Jenks 1991; Roy et al. 1996), however, found no unique genetic characters in the red wolf and suggested a close genetic relationship to the coyote, *Canis latrans*. Reich et al. (1999) hypothesized that red wolves arose as a result of hybridization between grey wolves, *Canis lupus*, and coyotes during the past 2500 years, thus calling into question their conservation status under the ESA. Although this conclusion has been disputed (Wilson et al. 2000), the red wolf genetic studies have highlighted the issues of how to determine what constitutes a valid unit for conservation purposes and what conservation value should be afforded to hybrids.

What molecular tools are available for deducing the systematic status of an animal? One promising source of information about evolutionary relationships among species and populations is the circular, 16,000 base pair segment of DNA contained in mitochondria. The genes in mtDNA are well defined, and numerous universal primers, which target particular DNA segments in specific genes and operate effectively across a wide range of taxa, are available commercially. As each cell contains many more copies of mtDNA than nuclear DNA, mtDNA is easier to extract from minute, degraded samples. In most organisms, mtDNA is maternally inherited, so only one sequence copy can be extracted (as opposed to two for nuclear genes – one from each parental chromosome). Disadvantages of mtDNA include:

1. it represents the evolution only of maternal DNA and provides no direct information about genetic contributions of males;
2. it is inherited as a unit, so represents only a single marker and phylogenies based on it can be less robust that those based on nuclear DNA, for which it is typically possible to assay a large number of independent markers.

A frustrating paradox is that nuclear genes have the potential to provide more robust phylogenies but have been used less commonly, in part because many require specific primers and cloning before sequencing is possible. Nuclear genes that have been used commonly in systematic studies include those associated with the male-inherited Y chromosome genes in mammals (e.g. Lundrigan et al. 2002; Makova & Li 2002) and the highly variable major histocompatibility complex (MHC) region (Holmes & Ellis, 1999). As an alternative to sequence data, phylogenetic reconstruction can be achieved from short interspersed nucleotide elements (SINEs; Shedlock & Okada, 2000). Short interspersed nucleotide elements are dispersed throughout eukaryotic genomes in great numbers. Because an insertion (i.e. a small DNA segment that was inserted into the sequence of a gene) is an essentially irreversible event, the sequence of the insertions can be traced through a lineage to infer common ancestry among taxa. Short interspersed nucleotide elements have been used to infer phylogeny of African mammals, primates and reptiles, among other taxa (e.g. Nikaido et al. 2003). The abundance of molecular data has promoted development of several new statistical methods for phylogenetic reconstruction that have been discussed elsewhere (Felsenstein 1981, 2003; Hendy 1993; Hillis et al. 1996; Larget & Simon 1999).

An example of the use of phylogenetic reconstruction in conservation is the taxonomic status of endangered subspecies of the leopard (*Panthera pardus*). The leopard has an extensive geographical distribution, and in many regions it is quite common. However, some subspecies are extremely rare (e.g. the Arabian, *P. p. nimer*, and the Amur, *P. p. orientalis*, subspecies). Uphyrkina et al. (2001) used phylogenetic reconstruction to determine whether these rare subspecies of leopard are genetically unique (Fig. 4.1). These results can be used as guidelines for management of this species. For example, a highly phylogenetically distinct subspecies might have high conservation value and merit separate management (e.g. without interbreeding with other subspecies, as can occur in zoos and reintroduction programmes).

Non-invasive sampling and population size estimation

It is difficult to monitor or evaluate the population status of many threatened and endangered species because they live at low densities, roam over large areas, inhabit regions that are difficult to work in or have an elusive life style. Furthermore, many of these species are large (e.g. marine mammals), dangerous (e.g. carnivores) or secretive (e.g. nocturnal marsupials), meaning that trapping individuals for the purpose of

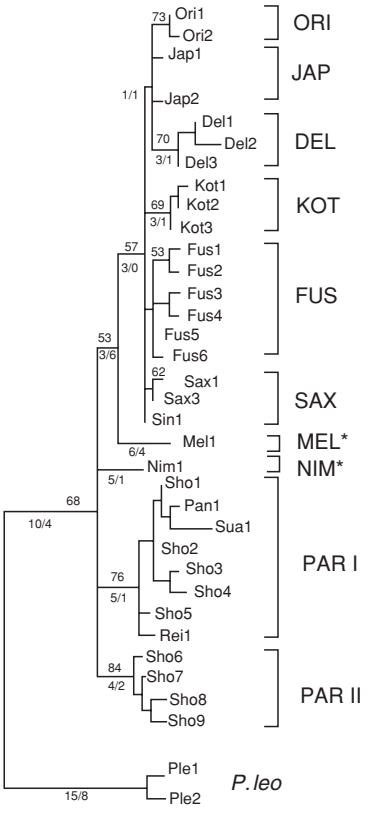

Fig. 4.1 Phylogenetic relationships among the leopard mtDNA haplotypes from combined NADH 5 (611 bp) and control region (CR, 116 bp) mtDNA. (Modified from Uphyrkina et al. 2001.) Lion (*Panthera leo*) samples were used as outgroup species. Maximum parsimony tree is shown. Numbers above branches represent bootstrap support (100 replicates); only those with > 50% are shown. Numbers below show number of steps/number of homoplasies. ORI stands for *Panthera pardus orientalis*, SAX for *P. p. saxicolor*, MEL for *P. p. melas*, KOT for *P. p. kotiya*, FUS for *P. p. fusca*, DEL for *P. p. delacouri*, JAP for *P. p. japonensis*, NIM for *P. p. nimer*, and PAR I and II for the two African clusters. Both ORI and NIM have a distinct position on the species phylogenetic tree, indicating their genetic uniqueness.

tagging is complex and expensive, even if the necessary permits can be secured. Scats, pellets, hair, feathers, egg shells, sloughed skin, urine and other body fluid secretions contain minute amounts of DNA that can be amplified by PCR. Hair was collected by hair-traps from black bears (Boersen et al. 2003) and from sleeping nests of chimpanzees (Morin et al. 1994), and a systematic survey for kit fox scats was carried out using trained dogs (Smith et al. 2001). Consequently, an array of molecular (Bellmain et al. 2004) and statistical (Valière et al. 2002) methods are being developed to monitor animal populations without the need to handle, or even observe, the subjects. For example, hair or scats collected from brown bears (*Ursus arctos*) (Bellmain & Taberlet 2004) and scats collected from coyotes (Kohn et al. 1999) have been used to estimate population size (abundance) and to track individual movements and home ranges.

A prerequisite for such techniques is that samples are correctly identified, often using species-specific DNA sequences. Sequences of any of the mtDNA genes (e.g. cytochrome *b*) are often sufficient to allow distinction between scats (or other material) from several species at a study

site (e.g. black bear (*Ursus americanus*) versus brown bear (*Ursus arctos*), or wolf versus red fox (*Vulpes vulpes*)). The DNA extracted from each scat (or other material) can be subjected to microsatellite analysis, which can identify different individuals based on their unique multilocus genotypes (their DNA 'fingerprint'). An interesting complication could arise if, for example, a brown bear eats a black bear and the black bear's DNA shows up in the faecal sample, or if one wolf urinates on another wolf's faeces; in these cases, individual identification will be difficult because DNA from more than one individual would be amplified.

From a smear of faeces or a pinch of hair follicles, the molecular detective can identify the sex (e.g. using sex-linked genes such as ZFX/ZFY, which are carried by either sex chromosome; Lucchini et al. 2002), reproductive status and parasite load of the subjects (Kohn & Wayne 1997; Fedriani & Kohn 2001). Further sleuthing can provide estimates of population abundance, based on a variation of the standard ecological practice of mark-recapture. In this case, however, the 'marks' are the naturally occurring DNA fingerprints of individuals, and recaptures are detections of a DNA finger print more than once among non-invasive samples (e.g. faecal samples). Abundance can be estimated as the asymptote of a curve plotting the cumulative number of unique genotypes (*y* axis) as a function of the number of new samples collected (Kohn et al. 1999; Banks et al. 2003). Failure to find new genotypes in additional samples suggests that most of the population has already been sampled.

Although non-invasive techniques are powerful, they (i) can be difficult to develop, (ii) require a pilot study to validate reliability, and (iii) usually require repeated genotyping of each locus on each sample to avoid genotyping errors (and thus cost more in time and money than when using fresh tissue samples). Low concentrations or partially degraded DNA can lead to genotyping errors during PCR amplification. For example, low DNA concentration in a sample occasionally causes an amplification of only one allele in a heterozygote (termed allelic drop out), an error that can yield a false homozygous genotype, leading to biased estimates, especially when a small number of individuals are involved (Taberlet et al. 1999). New DNA extraction protocols and software to detect and control for scoring and other errors are being developed. In coming years, those efforts, along with systematic use of rigorous laboratory and scoring protocols, automation of protocols, and error rate quantification and reporting (Broquet & Petit 2004) should help overcome many of these methodological problems.

Genetic diversity within populations

Why do species become extinct? This is one of the most debated questions in conservation biology (Caughley 1994). Deterministic forces, such as unrelenting harvest or incremental losses of habitat, obviously can place species at high risk. When populations become small, however, random events become relatively more important and may play a major role in many extinctions. For example, in a population of 1000, if males and females are equally likely, the sex ratio will seldom deviate far from 1:1, ensuring sufficient females to produce the next generation. In a population of 10, however, two or fewer will be females 5% of the time, just by chance. Large variations in birth rates, age structure and other demographic processes also occur by chance in small populations. For the same reason, small populations are prone to lose genetic diversity because the rate of genetic drift (random fluctuations in allele frequency) increases and alleles become extinct by chance faster than they are being generated by mutation. Loss of diversity constrains long-term evolution, because genetic variation is the raw material for natural selection to act upon. More diverse populations are better able to accommodate environmental variation and the outbreaks of disease.

On shorter time frames, loss of diversity reduces fitness primarily due to the expression of deleterious, recessive alleles. In large populations, selection keeps such alleles at a low frequency, so they usually occur in heterozygotes, where their deficiencies are masked by a copy of 'normal' alleles. In small populations, deleterious alleles can drift to high frequencies just by chance and become expressed in homozygotes, thus reducing fitness of the population through inbreeding depression. Populations that decline rapidly in size are said to suffer a genetic bottleneck, so termed because only alleles that make it through the bottleneck will survive in the population.

Which random processes, demographic or genetic, pose greater risks to small populations? This also has been one of the most hotly debated topics in conservation biology. For many years it was commonly believed that demographic stochasticity was more likely to cause extinctions (e.g. Lande 1993), the argument being that populations were likely to become extinct through random fluctuations in size before cumulative losses of genetic diversity became severe enough to seriously reduce fitness. However, recent studies demonstrate that genetic factors quite often play an important role in the extinction process (Spielman et al. 2004).

A number of empirical studies have found a correlation between reduced heterozygosity (and other measures of genetic diversity) and lowered individual fitness (Reed & Frankham 2003); more homozygous (inbred) individuals often have lower survival and fecundity. For example, the energetic cost of burrowing, a trait essential to survival in the pocket gopher (*Thomomys* spp.), was significantly lower in populations with higher genetic variability (Hildner & Soulé 2004). Many small populations of endangered species are restricted to isolated patches in the wild, or even housed in captivity as part of breeding programmes. Such populations have no immigration, a natural process that counteracts the fixation of deleterious alleles and loss of heterozygosity by importing novel alleles from other populations. Saccheri et al. (1996) dramatically illustrated the importance of this natural process in a butterfly metapopulation in Finland. Subpopulations with low levels of heterozygosity had a significantly higher subsequent probability of extinction (after controlling for environmental and demographic extinction risks). This was probably the result of inbreeding depression that affected larval survival, adult longevity and egg hatching rate. It appears that the other populations were rescued from this fate by receiving sufficient immigration, bringing novel alleles into the population.

Conservation biologists have drawn on fundamental principles of population genetics to develop the concept of **genetic rescue**, which occurs when immigrants make a positive contribution to fitness over and above the demographic effects of simply adding more individuals. This rescue effect is most likely to occur if the recipient population is small, isolated and suffering from inbreeding depression. Under these circumstances, genetically divergent immigrants can import new alleles into the population to counteract the tendency for erosion of genetic diversity and to mask deleterious alleles responsible for inbreeding depression. The Finnish butterfly laboratory study (Saccheri et al. 1996) illustrates how this process can function in the wild. Vila et al (2003) showed that a single breeding immigrant into a severely bottlenecked and geographically isolated Scandinavian population of grey wolf could recover genetic diversity.

Animal breeders have long practiced a form of genetic rescue by periodically injecting 'new blood' into their broodlines. Direct interventions to effect genetic rescue of natural populations of conservation interest is an exciting new development with some apparent successes. For example, by the 1980s numerous developmental and reproductive abnormalities indicated that the endangered Florida panther, *Felis concolor coryi*, was suffering from inbreeding depression. Population genetic models indicated that a brief episode of high gene flow (using animals from Texas), followed by subsequent gener-

ations of low gene flow, could genetically restore the population by reducing the frequency of deleterious alleles without substantially reducing the frequency of alleles responsible for local adaptation (Hedrick 1995). Although long-term results will not be known for several generations, preliminary data suggest that this strategy may be working (Hedrick 2001).

However, such interventions are risky with no guarantee of success. In fact, it is quite possible that genetic rescue attempts could reduce fitness rather than increase it. Just as matings between genetically similar individuals can lead to inbreeding depression, interbreeding of genetically divergent individuals can lead to outbreeding depression, either through dilution of locally adapted genes or disruption of gene complexes that function effectively together (Lynch 1991). Furthermore, a host of behavioural, ecological and demographic factors (e.g. unintentional importation of exotic diseases; McCallum & Dobson 2002) can influence the consequences of human manipulated migration. Therefore, although the concept of genetic rescue may seem elegantly simple and empirical examples document its potential benefits, developing testable models to predict when genetic rescue may seem likely to succeed (and fail) is a major challenge for the future (Tallmon et al. 2004). Rescue is most likely to occur (without outbreeding depression) when gene flow is being restored into inbred populations that only **recently** became small and isolated such that little time existed for adaptive differentiation to develop.

Gene flow among populations

Not only is nature patchy, but habitat fragmentation is accelerating as roads, agriculture, logging and other developments divide continuous habitats into isolated patches, disrupting immigration as well as reducing population sizes (Hanski & Gaggiotti 2004). In many species, local populations are connected by dispersal into larger metapopulations, and these connectivities can be essential to the long-term persistence of the metapopulation as a whole, for both demographic and genetic reasons. Estimating the rate and pattern of migration among patches is thus vitally important for the conservation biologist.

Genetic markers are well suited to the study of gene flow, or movement of genes among populations, because they integrate information about migration or isolation over evolutionary time frames. Genetic markers thus can provide information not only about contemporary migration, but also historical patterns of connectivity. For example, the African wild dog, *Lycacn pictus*, is among the most endangered canid species. Girman et al. (2001) showed that although populations cluster into two genetic units (eastern and southern), the admixture zone spans much of the current geographical range of the wild dog. The authors concluded that the Selous population in Tanzania is an appropriate source of individuals for reintroduction into Masai Mara and Serengeti, where wild dogs declined precipitously in recent years. This example illustrates that population genetic analysis is not a theoretical exercise but an important tool for developing translocation plans, long-term management programmes and reserve design (Palumbi 2003).

Genetic analysis of population structure commonly comprises three main stages:

1. identification and enumeration of populations;
2. analysis of relationships among populations;
3. evaluation of patterns of differentiation as a function of geographical distance.

The first step, determining how many populations exist, is a necessary precursor to many subsequent types of analyses. In some cases, candidate populations are easy to infer from the discontinuous geographical distribution of individuals, and standard statistical methods can be used to test the null hypothesis that all

samples belong to a single random mating population. In other cases, distributions may be continuous or overlapping, making it difficult to collect meaningful samples for statistical tests. In this situation, clustering methods (Pritchard et al. 2000; Manel et al. 2005) can be used to estimate the number of gene pools present in a mixed sample and assign individuals to specific gene pools. Pritchard et al. (2000) used this approach to show that at least three populations of the endangered Taita thrush, *Turdus helleri*, occur in Kenya. This method can be powerful if strong genetic differences exist among populations, but its general applicability is still being evaluated.

Once populations are identified, it is important to examine their genetic relationships to gain insights into patterns of migration. The first step is typically calculation of a genetic distance between pairs of populations. A commonly used measure is the fixation index (F_{st}), which measures the fraction of the total variation in allele frequency that is found between populations. The F_{st} is inversely related to the number of migrants (N_m) per generation between the populations of interest. Allele or haplotype frequencies are used to calculate F_{st} or related genetic distances, some specific to microsatellites. A matrix of pairwise genetic distances can be visualized as a tree network connecting all populations or as a two- or three-dimensional plot (e.g. fig. 1 in Girman et al. 2001). Analysis of molecular variance (AMOVA; Excoffier et al. 1992) is a procedure that allows the overall genetic variance to be partitioned into components of interest, such as geographical subdivisions or temporal replicates. In the Australian green turtle (*Chelonia mydas*), AMOVA was used to show that about 99% of the genetic variation in microsatellite loci was contained within rookeries. In contrast, only 22.5% of the genetic variation in mtDNA haplotypes occurred within rookeries, whereas 77.5% was partitioned among regions and none among rookeries within regions (FitzSimmons et al. 1997). The combined genetic and tagging evidence allowed the authors to conclude that the observed genetic subdivision is due to migration of turtles from the south Great Barrier Reef through the courtship area of the north Great Barrier Reef population.

Another topic of interest is the role of geographical distance in shaping the observed genetic structure. Understanding the relationship between geographical and genetic distance is important for any conservation plan. When this association is high, geographical distance can be a meaningful barrier to dispersal and care should be taken to conserve populations that are close enough together to permit sufficient genetic exchange. If an association between geographical and genetic distance is not found, it may indicate that few barriers to dispersal exist even at large spatial scales, but it could also mean that the populations are isolated and historical factors have shaped the present-day structure.

How are individuals associated with populations? Typically, we assign individuals based on the collection site. However, this approach risks misclassifying migrant individuals. Applying an assignment test (Paetkau et al. 1995) – a powerful statistical tool that 'assigns' each individual to the most likely population of origin based on its multilocus genotype – has the potential to provide information for a broad range of questions of conservation relevance (Manel et al. 2005). For example, assignment tests and related analyses have been used to document male-biased dispersal in the whitefooted mouse, *Peromyscus leucopus* (Mossman & Wasser 1999); to show that treating wolverines (*Gulo gulo*) from Montana as a single population is not a sound conservation strategy, even though they have high apparent dispersal capability (Cegelski et al. 2003); to highlight risks of fragmentation due to overharvest of the kelp *Laminaria digitata* in the English Channel (because gene flow from adjacent, continuous strands is generally more important than distant transport by currents; Billot et al. 2003); and to evaluate introgression of coyote genes into the red wolf (Miller et al. 2003). A precursor of assignment tests known as genetic stock

identification (Pella & Milner 1987; Brown et al. 1999) has been used for many years to help manage mixed-stock fisheries of Pacific salmon and other commercial species to avoid unsupportable harvest of depressed wild populations. For example, real-time (24-h turnaround) genetic analysis of samples from a Chinook salmon (*Oncorhynchus tshawytscha*) fishery in the Lower Columbia River has helped managers determine when endangered populations from the upper Columbia and Snake River basins enter the fishery, at which point the fishery can be closed (Shaklee et al. 1999).

Effective population size

The effective population size (N_e) is one of the most important parameters in conservation genetics because it influences the rate of loss of genetic variation, the rate of inbreeding (mating between relatives) and the efficiency of selection in eliminating deleterious alleles and maintaining adaptive ones. A rough approximation of N_e is the number of breeding individuals in a population that leave offspring that survive to reproductive age. The effective population size is defined more technically as the size of the ideal population that loses genetic variation at the same rate as the population being studied. In an 'ideal' population, population size is constant, sex ratio is equal and variation in reproductive success among individuals is random. All of these provisions are typically violated in real populations, with the result that $N_e/N < 0.5$, and sometimes a great deal less (Frankham 1995).

Several recent studies of marine species have estimated N_e to be three to six orders of magnitude lower than N. For example, Hauser et al. (2002) used variation at seven microsatellite loci to estimate N_e in the New Zealand snapper, *Pagrus auratus*, using two independent molecular-based methods. Scale samples were collected beginning in 1950 around the time a commercial fishery started to harvest the Tasman Bay population. Genetic variation (allelic richness and heterozygosity) was much lower in 1998 than in the samples from 1950, a result that would not be expected in large populations. Allele frequency changes over this period were also typical of those found in small populations. The effective size estimates consistent with these observed genetic changes were 46 and 176, respectively, for the heterozygosity loss and temporal change methods. In contrast, the census size was estimated in the mid-1980s to be 3.3 million fish. Hedgecock (1994) proposed a hypothesis to explain this phenomenon in marine species with very high fecundity and very high mortality of eggs and larvae: most families produce no offspring that survive to reproduce, and the next generation is derived from progeny of a very few families that are 'sweepstakes' winners in the reproductive lottery. This hypothesis and the empirical estimates of tiny N_e/N ratios remain controversial, but they demonstrate that even large populations can be at risk of losing genetic variation, and that monitoring of genetic variation and N_e can be useful, even when the census size is large.

The effective population size, N_e, can be calculated from demographic data, such as lifetime variance in reproductive success, but these data are difficult to obtain for most species. Furthermore, demographic methods often overestimate N_e because they do not include all factors causing N_e to be less than N. For these reasons, methods for estimating and monitoring N_e based on molecular markers were developed and have made an important contribution to conservation (reviewed by Schwartz et al. 1999).

Genetic bottlenecks

A population bottleneck, or rapid reduction in N_e, generates characteristic genetic signatures that can be detected with realistic samples (e.g. c.30 individuals scored for 10–20 molecular markers). One signature is a deficit of rare alleles (frequency < 0.10), which develops in

small, declining populations. In a large stable population, most alleles occur at low frequency (Fig. 4.2; Luikart et al. 1998). During a bottleneck, rare alleles are lost first, leading to an apparent excess of alleles at moderate frequency. Populations in which a large fraction of alleles are at intermediate frequencies thus are likely to have recently experienced a bottleneck. Another signature, detectable using microsatellite data, depends on the ratio of the number of alleles to the range in allele sizes (Garza & Williamson 2001). During a bottleneck, the number of alleles declines faster than the range, leading to a low ratio. Yet another signature is an excess of heterozygosity (i.e. Hardy Weinberg expected H_e) compared with the theoretical equilibrium gene diversity expected for a large, stable population (Luikart & Cornuet 1998). All these kinds of information (allele length, allele frequencies and heterozygosity excess) are used in the Bayesian approach for detecting bottlenecks developed by Beaumont (1999). Thus the Beaumont approach should, in theory, be the most powerful; however, its performance and reliability has not been thoroughly evaluated.

The signature of a bottleneck event is an alarm call for those who monitor populations of an endangered species. For example, if a strong bottleneck is detected, it would be prudent to initiate monitoring of the genetic and demographic status of the population – and perhaps take action such as translocations (as in the example of the Florida panther, above). In extreme cases, such as the African cheetah (*Acinonyx jubatus*), the effects of an apparently ancient bottleneck event (approximately 10,000 years ago) are still observed today in the form of very low genetic variability on a continental scale (Menotti-Raymond & O'Brien 1993).

Detecting selection and local adaptation

Most studies in conservation genetics have used markers assumed to be neutral (i.e. not associ-

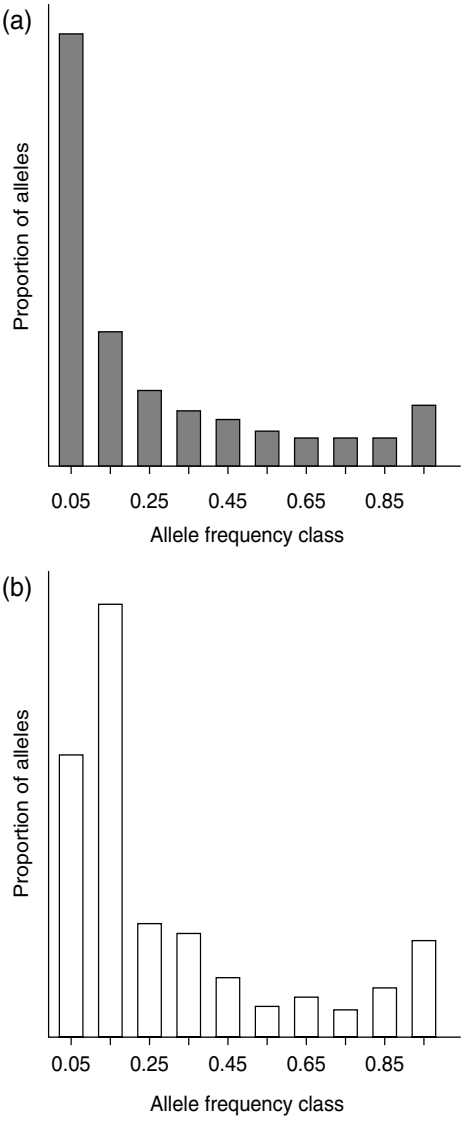

Fig. 4.2 Genetic signature of a population bottleneck: a mode shift in the distribution of allele frequencies. Large stable populations (i.e. populations near muta tion drift equilibrium) have a large proportion of alleles at low frequency (a). Why? Because new mutations are rare (occurring as a single copy), and new alleles usually fluctuate at low frequency until they are lost via random genetic drift. However, a bottleneck causes rapid loss of rare alleles and generates a deficit of alleles at low fre quency (frequency < 0.10) (b). This shifts the mode of the distribution from the low frequency class (0.0 0.1) to an intermediate frequency class (e.g. 0.1 0.2). Bottle necks can be thought of as strong sampling events where rare alleles are lost.

ated with fitness), largely because population genetics theory and models are best developed for neutral alleles. For example, methods for estimating levels of gene flow, effective population size, bottlenecks, mating system characteristics (F_{is}) and some methods for inferring phylogenies, all assume that markers are neutral. Applications not requiring neutral markers include parentage and relatedness estimation.

Although in general the assumption of neutrality for molecular markers may be reasonable, with the increasing number of markers in a typical data set, it is likely that some will be under selection. In addition, more and more studies are using markers located in genes (e.g. SNPs, Morin et al. 2004), making selection effects or 'signatures' more likely. Markers in genes (e.g. introns) are more likely to be affected by selection than most markers that are seldom near genes and thus unlikely to be under selection directly or through linkage to a selected gene. Fortunately, new statistical tests now make it feasible to identify loci under selection. Two important uses are (i) excluding selected loci for applications in which neutrality must be assumed, and (ii) using selected loci to help identify locally adapted populations with special value or concern for conservation.

Loci under selection should be excluded from inferences about population demography and evolutionary history, because selection can bias inferences – even if only one out of many markers is under selection (Landry et al. 2002; Luikart et al. 2003; Storz et al. 2003). For example, Allendorf & Seeb (2000) studied 36 markers from four populations of salmon, and found one locus with extremely high F_{st} (0.71) relative to the other loci: mean F_{st} with and without the outlier locus was 0.20 and 0.09, respectively (Fig. 4.3). In this example, one strong outlier locus more than doubled the estimation of population differentiation. The locus was probably under selection because such a high F_{st} is extremely unlikely by chance alone, at a neutral locus. Fortunately, several computer programs are now freely available to allow tests for outliers and to help differentiate between selected and neutral loci.

Once markers under selection are identified, they might be used in conservation to help design translocation programmes. For example, if two populations are candidates as sources for translocation into a small or declining population, the source with the least genetic differentiation at selected loci (fewest F_{st} outliers), relative to the declining population, might be used preferentially. This is true especially if the source with few F_{st} outliers also has the most similar environment or habitat compared with that of the recipient population. These views expand further the concept of 'genetic rescue', and in the future may become guidelines in translocation programmes.

Molecular markers, if confirmed as adaptive, also may be used to prioritize or rank populations for conservation importance. For example, a population containing a high proportion of adaptive and unique alleles might be of higher conservation value than another population with fewer such alleles. Adaptive markers (and other adaptive characters) could be integrated along with neutral markers (and other non-genetic data) when prioritizing populations for conservation (Fig. 4.4). Unfortunately, prioritizing preservation of one population based on a sample of adaptive genes could actually reduce diversity across the rest of a species' gene pool. This could jeopardize the adaptive potential of a species to future environmental changes (Luikart et al. 2003). For example, if we prioritize conservation of one population based on a few divergent adaptive genes unique to that population, we might lose adaptive genes in other populations that would improve the species persistence in future environments. Further difficulties arise in predicting which genes will be adaptive in future environments. Thus, although the use of adaptive gene markers for prioritizing populations is desirable, it can be difficult and risky to apply effectively. More research is needed to assess the usefulness of adaptive markers in conservation.

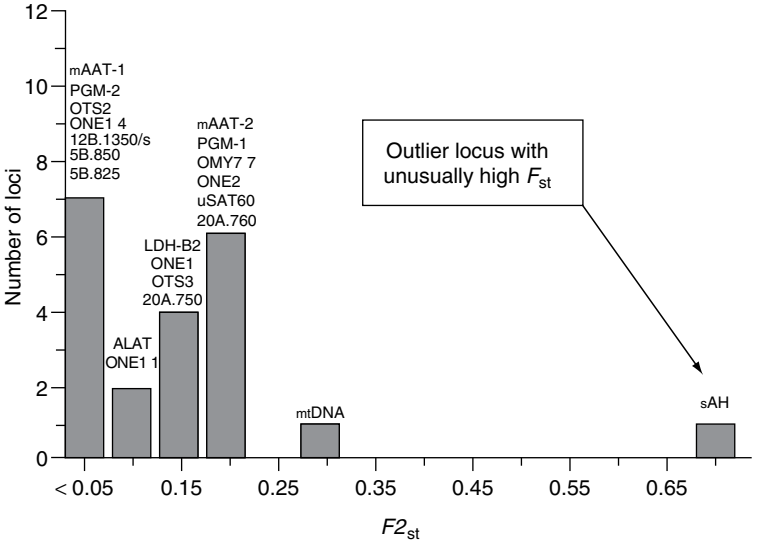

Fig. 4.3 Distribution of F_{st} (differentiation among populations) for 21 molecular marker loci (including mtDNA, microsatellites and allozymes and RAPDs (random amplified polymorphic DNA)) from sockeye salmon, *Oncorhynchus nerka*, populations from Cook Inlet, Alaska. (Modified from Allendorf & Seeb 2000.) $F2_{st}$ differs from the classic 'F_{st}' in that all minor alleles (at low frequency) are pooled to make only two allele classes, before computing F_{st}. This allows a less biased comparison between multi allelic microsatellites and allozymes, which are mostly bi allelic. Note that mtDNA is expected to have a relatively high F_{st} because the mtDNA effective population size (N_e) is small and thus drift is strong. N_e is small because only females (half the population) transmit mtDNA, and because mtDNA is haploid (half the number of chromosomes com pared with nuclear genes). This makes the mtDNA effective population size only one quarter the effective size of the nuclear genes (assuming a 50:50 sex ratio).

A powerful tool for evaluating selection in specific genes is the relative frequency of DNA substitutions at sites that do and do not result in changes in the amino acid sequence of proteins. Because of redundancies in the genetic code, some mutations are synonymous (S, no change in amino acid sequence, hence are considered neutral), while others are non-synonymous (N, result in a change). As most mutations are deleterious, N mutations are generally much rarer because they are quickly eliminated. In an analysis of DNA sequence data for the transferrin gene (important in binding and sequestering iron), Ford (2001) found very high N/S ratios in salmon but not other vertebrates – indicating strong, positive selection for transferrin variation in Pacific salmon. The positively selected sites occur primarily on the outside of the molecule in regions subject to binding by bacterial proteins. One possible explanation for this result is an 'arms race' for access to iron between pathogenic bacteria (for which iron is often a limiting nutrient) and the host salmon, which must continually change the structure of transferrin to keep ahead of bacterial mutations.

Populations often differ in many phenotypic and life-history traits. How does one decide under what circumstances these differences are important to conserve, and if so, which traits are the most valuable? Has a particular trait evolved many times within the species (hence it might be regenerated again in the future if lost) or only once? Joint analysis of genetic and life-history data can provide a powerful means to help set conservation priorities. Waples et al. (2004) examined chinook

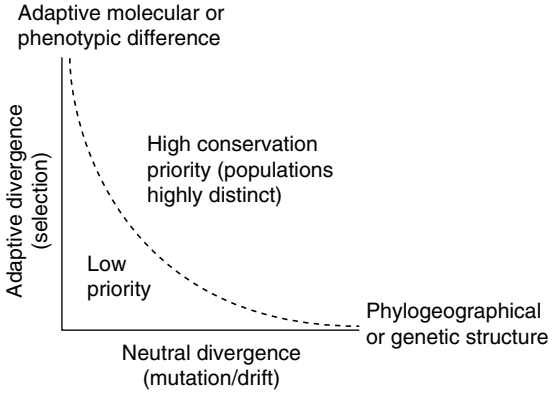

Fig. 4.4 Adaptive markers could be treated separately from (but integrated with) neutral markers when prioritizing populations for conservation. Populations with the highest diversity and uniqueness for both adaptive and neutral markers would receive highest priority for conservation. Other non genetic information (life history, morphology, environment) should also be integrated when ever possible for prioritizing or identifying populations for conservation. (From Luikart et al. 2003.)

salmon (*O. tshawytscha*) populations from California to British Columbia and mapped life-history variation on a tree depicting genetic relationships. They focused on differences in run timing (the season adults enter fresh water to begin their spawning migration), which commonly is used by managers to define management and conservation units. In coastal basins and the Lower Columbia River, populations with different run timing co-occur in many distinct genetic lineages, providing strong evidence for repeated, parallel evolution of run-timing differences. In these regions, genetic differences between populations from the same river basin but having different run-timing are typically small enough that they can be explained by fairly recent divergence (within about 100 years) or, more likely, low levels of ongoing gene flow. A very different pattern, however, was found in the interior Columbia and Snake River Basins. In this region east of the Cascade Mountains, all spring-run populations are strongly differentiated from all fall-run populations, to the extent that they are behaving largely as separate biological species even where they overlap in distribution. Furthermore, the interior spring-run populations have a unique suite of tightly correlated life-history traits that perhaps has evolved only once within the species. The results helped to identify evolutionarily significant units (ESUs; Waples 1991) of Chinook salmon – groups of populations that collectively represent major components of genetic diversity of the species as a whole and which are believed to be on largely independent evolutionary trajectories. In coastal areas and the lower Columbia River, spring- and fall-run populations from the same geographical area are part of the same ESU, but in the interior of Columbia spring- and fall-run populations are in separate ESUs. Over half the ESUs of Chinook salmon are now protected as threatened or endangered 'species' under the Endangered Species Act of the USA.

Forensic genetics and conservation

Recent molecular techniques allow forensic scientists to extract DNA from tiny remains at a crime scene and relate it to an offender. Conservation biologists have used related methodology to trace the source of whale meat sold at the Japanese markets (Cipriano & Palumbi 1999) and to monitor illegal ivory trade (Comstock et al. 2003). The power of forensic science is especially important in the marine environment and in remote wilderness areas where poaching of threatened species is otherwise difficult to detect (Avise 1998). In future years, molecular approaches for species and population identifications may become standard procedure with law enforcement agencies. Mitochondrial genes are useful for species identification using large databases available online (e.g. NCBI Genbank; DNA Surveillance, Ross et al. 2003). Microsatellites and other highly variable markers can be used to identify the source population

of poached specimens by comparison with a reference database using the assignment test (Manel et al. 2002).

Assignment tests and microsatellite genotyping were used to detect fraud in a fishing tournament in Finland. A fisherman claimed to have caught an excessively large salmon (*Salmo salar*) in the local Saimaa Lake. Officials doubted that the salmon was of local origin and had a genetics laboratory genotype nine micosatellite loci in the fish as well as in samples from Saimaa Lake and nearby fishing areas. The alleles in the 'winning' fish were so uncommon in the Saimaa Lake sample that its multilocus genotype was extremely unlikely (probability $<1/10{,}000$) to have originated in the lake (Primmer et al. 2000); instead, its genotype was much more likely in other populations that were subjected to fishing. When confronted with this information, the fisherman confessed to purchasing the big fish at a distant fish market.

The future

Non-invasive and forensic techniques will become standard tools for the conservation biologist in coming years. Forensic and biodiversity inventorying studies could benefit from emerging ambitious projects to 'barcode' (i.e. to sequence a single mtDNA gene) all species on the planet (e.g. Hebert et al. 2004). It will be important, however, to couple these emerging molecular techniques with more traditional morphological analyses of vouched specimens to confidently match genotypes with actual species. Such information and mobile PCR or genotyping machines could allow rapid (on site) identification of species from tiny tissue samples. Combined with the availability of GPS technology, much of the information that required years of tedious fieldwork will be obtained via the Internet and at the laboratory bench.

Rapid identification of genes expressed in a variety of organisms has been achieved by the systematic sequencing of cDNA libraries. Specific transcripts, generally known as expressed sequence tags (ESTs), are prepared from different tissues or developmental stages of a single organism. The ESTs can be used to construct catalogues of tissue-specific or stage-specific genes. Such libraries constructed for endangered or keystone species may help monitor environment-related stress and developmental disorders in these populations. Similarly, single nucleotide polymorphisms (SNPs) recognized for key genes (coding and non-coding regions of the genome) in a target species can predict, for example, the resistance of a population to specific diseases (i.e. having or lacking gene-mediated resistance) and the need for vaccination. For example, Liu & Lamont (2003) scanned a chicken population for susceptibility to *Salmonella* using key SNPs. We anticipate that similar applications would be developed rapidly for conservation purposes.

Another exciting tool is micro-array technology (Gibson 2002; Pfunder et al. 2004), which opens up new perspectives for biodiversity monitoring. A single DNA micro-array contains many thousands of genetically based characteristics (cDNA or oligonucleotides) on one microscopic glass slide (termed 'genome chip'). This technology promises to monitor the whole genome on a single chip so that the researcher can have a better picture of the interactions among thousands of genes simultaneously. A 'Mammalia Chip', for example, could include redundant diagnostic markers to unambiguously identify all European mammal species (Pfunder et al. 2004). Such application could serve as a forensic tool for poaching control or for scanning scats and hair samples (Davison et al. 2002). Micro-arrays were designed originally to measure gene expression (e.g. production of mRNA), but now also exist for measuring DNA sequence variation (e.g. genotyping hundreds of loci simultaneously). A chip designed for a specific endangered species can detect expression changes in multiple genes. Understanding adaptive phenotypic variations in a species is most important for conservation purposes because

these expression changes are intimately connected to fitness. Scanning by micro-array analysis a portion of a population could reveal whether individuals are behaviourally or environmentally stressed and the reasons causing it, the sex and reproductive state of individuals, parasite load, ability of individuals to accommodate various selection pressures, etc. (Gibson 2002). Combining non-invasive methodology with micro-array technology is a powerful tool that in the future could provide a complete profile of a population from a single sampling trip or as a means to monitor populations over a long period in unprecedented detail.

The first rule of intelligent tinkering is to keep the parts
(Aldo Leopold, *Round River*, Oxford University Press, New York, 1993, p. 146.)

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