QUANTITATIVE METHODS IN CONSERVATION BIOLOGY

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Role of Genetics in Conservation Biology

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Introduction

Many species are represented solely by populations that are highly fragmented, isolated, or captive and therefore lack the number of individuals considered necessary for healthy sustainable populations. These species run an increased risk of local and global extinction due to environmental, demographic, and genetic stochasticity (Shaffer 1981) and may benefit from special management attention. Two major goals of conservation genetics are to minimize loss of genetic variation in managed populations and define the taxonomic units (i.e., rare populations, subspecies, species) we ought to conserve. In this chapter, I outline the rationale for managing genetic diversity, introduce molecular tools available to assess genetic variation and taxonomic relationships, and provide examples illustrating applications of population genetics to conservation biology. This chapter is not an exhaustive review. The discussion applies to many captive and natural threatened populations ranging from insects to primates. The apparent bias among the examples toward mammals and birds is not a value statement but simply a reflection of the general interest in conservation biology toward higher vertebrates as well as my own background in mammalian biology.

Concept of Genetic Diversity

The genetic composition of a population can be described by the alleles present at different loci for a representative sample of individuals within a population. Genetic diversity can be quantified by the number and distribution of alleles within and between individuals and populations. Diploid organisms have two alleles per locus. Individuals are considered homozygous at this locus if both alleles are the same and heterozygous if they are different from one another. Genetic variation can be described at a single locus or multiple loci by using several methods (Lande and Barrowclough 1987; Lacy et al. 1995; Hamrick and Godt 1996). For example, a polymorphic locus is defined as having several alleles with the population frequency of the most common allele being smaller than 0.99. The proportion of polymorphic loci, in turn, is a measure of the number of variable loci among all sampled loci within a population. Genetic variation may also be described by quantitative variation in traits derived from the actions (and interactions) of many genes, termed *quantitative genetic variation.* The most frequently used measure of populationwide genetic diversity is the amount of heterozygosity. Individual heterozygosity describes the observed proportion of heterozygous loci in an individual (Mitton and Pierce 1980), and average heterozygosity reflects the proportion of heterozygous individuals in a population measured across several loci (Hartl and Clark 1989). The theoretical predictions based on the Hardy Weinberg Principle provide the framework within which to evaluate the amount and distribution of genetic variation documented in natural populations. Deviations from the expected genotype frequencies within a population, for example, can be indicative of past bouts of strong selection or inbreeding.

To evaluate how loss of genetic diversity affects population survival, it is crucial to distinguish the significance of different types of genetic variation. For example, single-

locus diversity is measured by individual heterozygosity, whereas diversity associated with polygenic quantitative traits is measured by phenotypic variation. Empirical studies suggest that most phenotypic changes in a population are the result of small alterations at numerous loci rather than a consequence of major mutations at a single locus (Lande 1981; Lande and Barrowclough 1987; Lande 1995). Consequently, the adaptive potential of a population may depend more on variation of quantitative traits determined by multiple loci than on single-locus polymorphisms (Lynch 1996).

Lande and Barrowclough (1987) contrast the adaptive importance of these types of genetic variation and discuss their maintenance in the context of neutral and stabilizing selection. They suggest that a population of several hundred individuals is necessary to maintain amounts of quantitative genetic variation necessary for evolution. Similarly, Lynch (1996) emphasizes that quantitative genetics focuses on the evolutionary properties of morphological and behavioral traits and therefore can provide insights into the effects of small population size on fitness and ultimately extinction risks. He goes further than Lande and Barrowclough (1987) in his suggestion that maintenance of the adaptive genetic variation of populations requires more than 10,000 reproductive individuals and that current conservation policies leave most endangered species at risk of losing genetic integrity.

A general prediction from population genetics theory is that existing heterozygosity erodes by 50% within 1.39*Ne* generations, where *Ne* is the effective size of a randomly mating population (Wright 1931; Hartl and Clark 1989; see definition below). Small populations run a greater risk of becoming genetically depauperate and, therefore, are at the center of attention of conservation genetics. In the following sections, I discuss the merits of genetic diversity, tools to assess genetic variation and phylogenetic uniqueness, factors affecting genetic diversity, and the question of what to preserve.

Merits of Genetic Diversity

Genetic Diversity and Adaptation

Long-term survival of a species depends on its adaptation to current and future biotic and abiotic aspects of its environment. Natural selection results in the survival and propagation of those individuals that are best adapted to prevailing conditions. In a genetically diverse population, individual fitnesses vary, and differential survival affects gene frequencies within and between populations. On changes in the environment, natural selection may favor different genotypes and hence alter the distribution of gene frequencies. However, a population depauperate of genetic variation may not carry any individuals that are genetically preadapted to the new environment and hence runs an increased risk of extinction. To maximize the probability of long-term survival of species, especially in changing environments, conservation geneticists seek to maintain high genetic diversity, although this variation may be represented by neutral single locus variation or by variation in fitness-related quantitative traits.

Genetic Diversity and Inbreeding Depression

Inbreeding (nonrandom mating with respect to relatedness) skews genotype frequencies within populations toward increased frequencies of homozygotes. Inbreeding in combination with selection against homozygotes can reduce the reproductive performance of naturally outbreeding populations (Wright 1977; Falconer and Mackay 1996) and

therefore may decrease long-term survival (Ralls et al. 1988; Thornhill 1993; Frankham 1995c, 1998). In general, inbred offspring are expected to be less fit than offspring produced by random matings (Ralls et al. 1979, 1988; Thornhill 1993; Falconer and Mackay 1996). This reduction of fitness with inbreeding (inbreeding depression) is manifested by reduced growth rate, fertility, fecundity, survival, developmental stability, or changed mating behavior among inbred offspring (Lerner 1954; Wright 1977; Ralls and Ballou 1982; Miller et al. 1993; Keller et al. 1994; Rave et al. 1994; Falconer and Mackay 1996). Likely mechanisms for inbreeding depression have been debated for decades (Charlesworth and Charlesworth 1987; Shields 1993; Thornhill 1993), and the two competing explanations (among others such as partial and associative overdominance), the overdominance and dominance hypotheses, are compatible with many theoretical and empirical results. Proponents of overdominance argue that heterozygous genotypes are on average fitter than homozygotes and as inbreeding reduces heterozygote frequencies, it depresses population performance (Mitton 1993). The dominance hypothesis contends that inbreeding depression is due to the increased expression of recessive deleterious alleles resulting from an increase in the number of homozygous loci.

Distinction of these hypotheses is not merely of academic value but could have implications for conservation management, especially for small inbred populations. If inbreeding depression is a consequence of the reduction of heterozygous individuals, mating among relatives will result in a population with less fit individuals, irrespective of its mating history. By contrast, inbreeding should no longer result in depression of individual fitness if recessive deleterious alleles underlie inbreeding depression and can be purged from populations with regular inbreeding (Lande 1988; Hedrick 1994; Fu et al. 1998). Byers and Waller (1999) reviewed evidence for the purging hypothesis among plant populations and concluded that “purging appears neither consistent nor effective enough to reliably reduce inbreeding depression in small and inbred populations.”

Lande (1995) suggested that inbreeding depression is the result of segregation of deleterious alleles and that there is little evidence to support the overdominance hypothesis. He concluded that gradual inbreeding will only reduce the risk of inbreeding depression due to lethal and sublethal mutations but will not succeed in purging mildly deleterious alleles (Hedrick 1994; Lande 1995). This prediction is consistent with Ballou’s (1995) results that a long history of inbreeding in 25 captive populations resulted in a slight reduction but not complete removal of inbreeding depression.

Experimental studies of inbreeding effects in wild populations are rare and have produced mixed results. Brewer and co-workers (1990) tested the prediction of the dominance hypothesis that small isolated populations of White-footed Mice with low genetic diversity would show less inbreeding depression than large central populations. The severity of fitness depression in inbred litters did not correlate with genetic diversity, and deleterious effects did not diminish through several generations of inbreeding (support for overdominance hypothesis). However, maternal care was only depressed in inbred mothers from genetically diverse populations (support for dominance hypothesis). Brewer and associates (1990) concluded that “overdominance of fitness traits probably contributed as much to the genetic load as did deleterious recessive alleles.”

Keane (1990a, b) bred wild-caught mice from a single population. Whereas significant inbreeding depression was documented for full-sibling and halfsibling matings, mating among cousins resulted in high reproductive success, and there was potential for outbreeding depression (fitness reduction due to matings among genetically distant

individuals). Jimenez and colleagues (1994) documented severe inbreeding depression in White-footed Mice when they reintroduced inbred offspring of wild-caught mice into natural habitat. Inbred mice showed continual weight loss and suffered higher mortality than noninbred mice after release. These deleterious effects of inbreeding were much more severe in the natural environment than in captivity. In a "natural experiment" involving a Song Sparrow population, outbred individuals had a higher probability of survival during severe population crashes. Keller and associates (1994) concluded that environmental and genetic effects on survival interact and that inbreeding depression among the Song Sparrow population was expressed when the population experienced environmental stress (i.e., severe winter weather). These studies reiterate a point made by Hedrick and Miller (1992) who caution that inbreeding depression may be greater in natural populations than in laboratory animals due to more severe environmental conditions.

Genetic Diversity at Selected Sites: The Case of the Major Histocompatability Complex

Proponents of the neutral theory argue that most genetic variability at the genomic or molecular level is selectively neutral and allele frequencies within populations are merely a function of mutation rate and the effective population size (Kimura 1968; Kimura and Ohta 1971). Neutralists nevertheless agree that deleterious alleles are eliminated through directional selection and that adaptive evolution is mediated through fitness differences among alleles (for further discussion, see Li 1997).

The selectionist-neutralist debate has spilled into the conservation biology arena in discussions about the kind of genetic variation that ought to be preserved in endangered taxa (Hughes 1991; Vrijenhoek and Leberg 1991; Vrijenhoek 1994; Avise 1995; Miller 1995; Lynch 1996). Unfortunately, the precise relationships between most fitness-related traits and genetic diversity at specific allozyme loci, DNA, or quantitative markers continue to be elusive. Nevertheless, a recent debate focused on conservation management for maximizing heterozygosity at fitness-related traits, such as the major histocompatibility complex (MHC) (Hughes 1991; Vrijenhoek and Leberg 1991; Hedrick and Miller 1994). MHC molecules play a key role in the immune response of mammals and birds (possibly all vertebrates) and have been linked to kin recognition based on individual odor profiles in mice (Yamazaki et al. 1979, 1983; Klein 1986; Egid and Brown 1989; Potts et al. 1991; Brown and Eklund 1994) and possibly humans (Wedekind et al. 1995). MHC genes encode cell-surface proteins that bind foreign molecules and aid in recognition and elimination of these potentially harmful antigens. Extensive allelic diversity at MHC loci has been documented for most populations studied (Klein 1986; Nei and Hughes 1991). Notable exceptions are the virtually monomorphic Syrian Hamster, mouse populations on North Sea islands, and the Cheetah, all of which probably lost overall genetic diversity due to severe population crashes or small population sizes for many generations (Streilein et al. 1984; McGuire et al. 1985; O'Brien et al. 1985). A variety of mechanisms for maintaining MHC polymorphism have been proposed in the past few decades (for reviews see Potts and Wakeland 1990; Nei and Hughes 1991; Alberts and Ober 1993; Klein et al. 1993; Parham and Ohta 1996). The major explanations include (1) maternal–fetal interactions (Clarke and Kirby 1966; Hedrick and Thomson 1988), (2) disassortative mating preference based on MHC genotypes (Yamazaki et al. 1976; Hedrick 1992a; Manning et al. 1992; Wedekind et al. 1995), (3) disease-based overdominance leading to an increased chance of survival in environments with infectious diseases (Doherty and Zinkernagel 1975;

Hughes and Nei 1988; Nei and Hughes 1991), and (4) disease-based frequency dependence based on the selective advantage of new mutant alleles (Snell 1968; Bodmer 1972).

The role of pathogen-mediated mechanisms in maintaining MHC variability is of particular importance to conservation biology. If current high levels of MHC variability are a consequence of disease-based overdominance selection in the past, then new pathogens may pose a significant threat to the future survival of populations with low MHC variability. Host-parasite coevolution as a selection mechanism has been supported by studies that have documented a relationship between MHC haplotypes and disease resistance such as Marek's disease and fowl cholera in chicken and malaria in humans (Briles et al. 1977; Lamont et al. 1987; O'Brien and Everman 1988; Hill et al. 1991). Slade (1992) attributed low MHC variability in Southern Elephant Seals to their evolution in a pathogen-free marine environment. Reduced genetic diversity at MHC loci has also been linked to disease susceptibility in Cheetahs (O'Brien et al. 1985).

This evidence and the apparent positive selection for a balanced MHC polymorphism led Hughes (1991) to believe that MHC diversity is paramount to the survival of small endangered populations. He then made the controversial argument that "all captive breeding programs for endangered vertebrate species should be designed with the preservation of MHC allelic diversity as their goal" (Hughes 1991). However, Vrijenhoek and Leberg (1991) cautioned that Hughes' (1991) management recommendations are based on assumptions that need experimental evaluation before their general validity is established. Selective breeding for maintenance of MHC diversity could, for example, increase loss of whole genomic diversity and hence bear the risk of inbreeding depression (Haig et al. 1990; Falconer and Mackay 1996). In addition, disease protection is not only a consequence of the adaptive immune response of organisms and hence the diversity of MHC molecules but involves numerous other genes associated with the humoral immune system (Janeway and Travers 1994). Vrijenhoek and Leberg (1991) recommended preservation of genetic diversity at the level of the whole genome instead of selected loci while intensifying efforts to monitor and understand the relationship between disease susceptibility and MHC variability.

Critics point out that several populations with low MHC variability are viable and healthy, such as northern European Beaver populations (Ellegren et al. 1993). However, the apparent well-being of a population with low genetic variability at either neutral or selected loci may be (1) the result of selection in the past and survivors represent the fittest genotypes; (2) because low variability is the result of drift or inbreeding in combination with selection, and although the population is healthy now, it lacks the potential for future adaptations; or (3) because there is actually no correlation between fitness and genetic variability. Similarly, a positive correlation between low fitness and low genetic diversity does not necessarily mean a causal relationship exists. Instead low reproductive success may be explained by nongenetic factors, such as predation, low densities, or disrupted behavior.

Tools to Assess Genetic Diversity and Phylogenetic Uniqueness

Molecular tools can contribute to conservation biology by revealing the genetic structure of the population, evolutionary history, and evolutionary potential of taxa (Ashley 1999). This information can serve to detect past, present, and possibly future population declines, besides establishing evolutionary uniqueness. Natural populations of threatened species

are managed to conserve genetic diversity to enhance individual fitness and maintain the evolutionary potential for future adaptations. Such genetic management generally requires information on demography (e.g., migration rates, population size). The amount and distribution of genetic variation within and between populations can provide relatively quick indirect estimates of migration, population subdivisions, and isolation, which can serve to define the appropriate scale for short- and long-term management (Moritz 1994b). Furthermore, characterization of the genetic make-up (i.e., the pedigree) of captive individuals can help to determine appropriate breeders to maximize offspring survival through inbreeding avoidance and genetic compatibility (Ryder 1986; Garner and Ryder 1992).

The proliferation of genetic markers in recent years has facilitated reliable estimates of some forms of genetic diversity and phylogenetic analysis at several taxonomic levels. The genetic variation detected by these molecular markers differs quantitatively and qualitatively as a result of the kind and number of genomic sites they assay. Protein electrophoresis uncovers putative genetic variation associated with protein coding regions, whereas DNA-DNA hybridization, restriction fragment length polymorphism (RFLP), and DNA sequencing analysis reveal differences at the DNA level for coding as well as noncoding regions. DNA sequencing data generally provide genetic distance estimates among taxa based on single locus comparisons whereas the other methods reveal variation at several loci. Resolution power of any molecular marker depends on the number of independent linkage groups (sites) it assays and the evolutionary rate of change associated with those groups. Animal and plant mitochondrial DNA (mtDNA), for example, usually is maternally inherited as one nonrecombining linkage group. Although the rate of sequence evolution varies along the mtDNA molecule, it is generally higher than the rate of single-copy nuclear DNA (scnDNA) (Moritz et al. 1987). The intramolecular variability of the rate of evolution makes mtDNA ideally suited to resolve taxonomic differences at various levels of divergence. In addition maternal inheritance uniquely qualifies mitochondrial markers for tracing of maternal genealogies. Lack of recombination, however, renders all mitochondrial genes part of one linkage group that are subjected to the same stochastic (e.g., random lineage extinction) and deterministic events (e.g., selective sweeps). Therefore they do not provide independent estimates of evolutionary change.

Choice of the appropriate marker for a particular task has to be based on the resolution power necessary to determine genetic differences and similarities among the individuals, populations, or other taxonomic units of interest. In addition, quality and quantity of the DNA source as well as to the required sample size have to be taken into consideration as these factors affect the applicability and cost-efficiency of a particular molecular tool. In general, rapidly evolving DNA (e.g., mini- and microsatellite DNA) generates genetic differences among individuals and populations that provide information on individual identity, paternity determination, and intraspecific genetic isolation among populations. Genetic markers with moderate evolutionary rates (e.g., allozymes, mtDNA control region) are used to resolve genetic distances at intermediate taxonomic levels. Resolution of deep branching patterns or divergence among distantly related taxa requires highly conserved regions (e.g., ribosomal RNA genes) within which evolutionary changes accumulate slowly (for reviews, see Moritz et al. 1987; Avise 1994; Simon et al. 1994; Avise et al. 1995). Following is a brief introduction to commonly used genetic tools and some examples of application in conservation biology (see Avise and Hamrick [1996] for an excellent recent collection of case studies in conservation genetics).

Protein Electrophoresis (Lewontin and Hubby 1966)

Electrochemical differences among proteins allow separation in either starch or acrylamide gels. The net charge, size, and shape of the protein determine the speed and direction of movement through a gel matrix in an electric field, and the position of each protein is visualized with specific histochemical stains. Homozygous and heterozygous genotypes are detected as single and double bands for monomeric proteins; polymeric proteins show more than two bands for heterozygotes. The quick and inexpensive resolution of genetic variation associated with unlinked loci and the Mendelian inheritance of allozyme polymorphisms made protein electrophoresis a good tool to assay heterozygosity within and between populations and estimate gene flow (Koehn and Eanes 1978; Selander and Whittam 1983; Philipp and Gross 1994).

Among vertebrates, however, the level of protein polymorphism is frequently too low to resolve specific genetic issues such as parentage. Molecular analysis of nuclear or mtDNA markers replaces protein electrophoresis in such cases (Gilbert et al. 1991; Packer et al. 1991; Sherwin et al. 1991; Martin et al. 1992a, b). There are numerous applications of this technique in conservation biology, including the identification of gene pools to guide conservation priorities, establishment of restocking programs, and evaluation of causes of decline in endangered species (e.g., Vrijenhoek et al. 1985; Quattro and Vrijenhoek 1989; Wayne et al. 1991; Petit et al. 1998).

DNA-DNA Hybridization

The complementary strands of the DNA duplex are connected by hydrogen bonds that are unstable at high temperatures and become single stranded when boiled. With cooling temperature, the complementary nucleotide sequences reanneal (i.e., become double stranded). In general, the greater the similarity or homology between DNA sequences, the more hydrogen bonds exist between matched nucleotides and the greater the thermal stability of the DNA duplex. To determine the homology of DNA sequences, single-stranded DNA of two individuals is allowed to anneal. DNA-DNA hybridization simultaneously samples genetic differences across most of the genome and hence provides valuable genetic distance estimates to resolve phylogenies, especially at intermediate taxonomic levels (Sibley and Ahlquist 1981). However, it does not provide qualitative data on character states crucial for many phylogenetic analyses. Few laboratories are set up for routine DNA-DNA hybridizations. This tool is rarely applied in conservation biology.

Restriction Fragment Length Polymorphism (RFLP)

Restriction enzymes cut double-stranded DNA at specific recognition sites that consist of short oligonucleotide sequences (typically four, five, and six base pairs). Restriction of DNA with one or several enzymes results in a range of fragment sizes that are electrophoretically separated in either agarose or polyacrylamide gels. Complex banding patterns can be detected when either all or selected fragments are visualized by means of a chemical stain (e.g., ethidium bromide) or radioactive DNA probes (i.e., short labeled DNA fragments that bind and hence mark complementary sequences). Furthermore, the development of the polymerase chain reaction (PCR) allows amplification of homologous locusspecific DNA fragments that can be subjected to restriction fragment analysis. PCR-based RFLP analysis has the advantages that very little tissue provides sufficient amounts of DNA and that large copy numbers of each fragment facilitate visualization of the banding patterns. As restriction sites are distributed throughout nuclear and cytoplasmic

DNA, RFLP analysis can be applied to a variety of DNA regions by using a number of DNA sources, as discussed below.

Single-Copy Nuclear DNA (scnDNA) (Quinn and White 1987; Karl and Avise 1993)

In contrast to repetitive DNA, single copy nuclear DNA (scnDNA) is represented only once or possibly a few times in a haploid genome. As scnDNA is found in both coding and noncoding regions, evolutionary rates of change associated with these sites vary considerably. Genetic variation is detected by hybridizing a locusspecific scnDNA probe to digested whole genomic DNA. Alternatively, scnDNA can be PCR-amplified at specific loci and digested with restriction enzymes, and electrophoretically separated fragments are visualized with ethidium bromide staining. As scnDNA polymorphisms are numerous and their Mendelian inheritance can be established through pedigree analysis, scnDNA provides a wealth of genetic markers to estimate genetic diversity. Karl and colleagues (1992) showed the usefulness of scnDNA in a study on the genetic population structure of the endangered Green Turtle. Previous mtDNA analysis had shown high nest site fidelity among females and suggested severe limitation of gene flow between breeding populations (Bowen et al. 1992). Analysis of scnDNA, however, revealed only a moderate degree of genetic substructure, suggesting moderate levels of male-mediated gene flow between rookeries (Karl et al. 1992).

Random Amplified Polymorphic DNA (RAPD) (Williams et al. 1990; Welsh and McClelland 1990)

Random amplified polymorphic DNA (RAPD) analysis uses primers of a randomized oligonucleotide sequence to amplify DNA from various anonymous sites. The detected polymorphism can be applied to estimate genetic distances among closely related species or recent hybrids and in some cases to perform paternity exclusions (Lewis and Snow 1992; Levitan and Grosberg 1993; Milligan and McMurray 1993; Avise 1994). However, RAPD fragments do not always amplify reliably or show Mendelian inheritance, which renders some polymorphisms unsuitable for population genetics and parentage analyses (Riedy et al. 1992).

Ribosomal RNA Genes (rRNA)

Ribosomal RNA (rRNA) plays a crucial role in protein assembly, and therefore most regions of the molecule are structurally and functionally highly constrained (Mindell and Honeycutt 1990; Hillis and Dixon 1991). Nuclear rRNA is considered middle-repetitive DNA and generally consists of repeat units that contain highly conserved coding regions and less-conserved noncoding spacer regions (Avise 1994). Probes for rRNA genes are readily available and can detect intraand interspecific restriction fragment length differences, which are either the consequence of heterogeneity in the length of noncoding DNA regions or the position of restriction sites. Several studies have found the hypervariability of the noncoded part of the nuclear rRNA gene families useful to differentiate populations and closely related species (Williams et al. 1985; Davis et al. 1990).

Major Histocompatability Complex (MHC) Genes (Klein 1986)

MHC genes code for cell surface proteins crucial to the immune response of animals as discussed above. The MHC is a family of tightly linked loci, many with scores of alleles, making it one of the most variable regions in the genome. Allelic variation associated with those regions can be detected through RFLP analysis using DNA probes (Klein 1986; Nei

and Hughes 1991). MHC variability has been used to study inbreeding and fitness, genealogies, and population histories. Specific MHC haplotypes are thought to be associated with kin recognition and mate choice in mice and humans and disease resistance in birds and humans (Briles et al. 1977; Tiwari and Terasaki 1985; Hedrick et al. 1991; Howard 1991; Hughes 1991; Potts et al. 1991; Vrijenhoek and Leberg 1991; Hedrick 1992a; Ellegren et al. 1993; Klein et al. 1993; Brown and Eklund 1994; Wedekind et al. 1995).

Minisatellite DNA (Jeffreys et al. 1985a, b)

Minisatellite DNA consists of highly conserved core areas of short oligonucleotide sequences (10–65 base pairs rich in GC nucleotides) that are strung together in long arrays. These arrays of repetitive DNA vary considerably in size within and between genomes due to differences in the number of tandem repeat units (core areas) across 10–25 loci. Minisatellite DNA is inherited in a Mendelian fashion. Consequently, RFLP analysis of minisatellite DNA reveals unique inherited banding patterns for each individual ("DNA fingerprints") ideally suited for assigning individual genetic profiles and parentage (Burke and Bruford 1987; Burke et al. 1989; Rabenold et al. 1990; Westneat 1990; Morin and Ryder 1991; Martin et al. 1992b).

Genetic variation associated with minisatellite DNA has been revealed by means of multilocus and single-locus DNA fingerprinting (Bruford et al. 1992). Multilocus DNA fingerprinting simultaneously assays genetic variation associated with numerous minisatellite loci through hybridization with a labeled minisatellite probe (Loew and Fleischer 1996). This relatively quick assay of multilocus variation is one of the major advantages of minisatellite DNA fingerprinting over microsatellite DNA, especially when the study population is suspected to be inbred. The detected polymorphism, can provide a snapshot of genetic variability within populations and, in some cases, relative estimates of genetic similarity between populations (Gilbert et al. 1990; Reeve et al. 1990). Useful applications in conservation biology have been developed by Fleischer and co-workers (1994) on the Palila, an endangered Hawaiian Honeycreeper, and by Fleischer and associates (1995) on endangered Clapper Rails, leading to recommendations for translocations between populations.

The complex banding pattern of multilocus DNA fingerprinting makes it less suitable for population genetics studies for determining exact degree of relatedness beyond full sibs (Lynch 1988, 1990, 1991; Burke et al. 1991; Jin and Chakraborty 1993, 1994). Coancestry coefficients are generally correlated with bandsharing coefficients (Lynch 1988, 1990; Kuhnlein et al. 1990; Reeve et al. 1990; Rave et al. 1994) and have been used to provide estimates of relatedness (Burke et al. 1991; Piper and Rabenold 1992).

Examples of applications of DNA fingerprinting are provided by Rave and colleagues (1994) for captive Hawaiian Geese, Brock and White (1992) for the Puerto Rican Parrot, Ashworth and Parkin (1992) for Rothchild's Mynah, and Haig and co-workers (1994) who reconstructed the pedigree of all living Guam Rails, a species that is extinct in the wild.

Single-locus fingerprinting resolves polymorphisms associated with different hypervariable loci sequentially and therefore addresses most technical, statistical, and theoretical difficulties associated with multilocus minisatellite DNA fingerprinting. Under very stringent conditions, a single-locus minisatellite probe detects variation only at a specific hypervariable minisatellite locus, consequently providing allelic diversity (= number of fragments per locus) and allele frequencies (= frequency of individual fragments within

the population). Sequential application of several probes provides levels of polymorphism comparable with those from multilocus DNA fingerprinting, while also providing estimates of heterozygosity (Jeffreys et al. 1990, 1991; Burke et al. 1991; Hanotte et al. 1992; Scribner et al. 1994). However, the development of single-locus probes is timeconsuming and more expensive than multilocus probes, and hence quick screening with multilocus probes may be the best first step.

Microsatellite DNA (Tautz 1989; Weber and May 1989)

Microsatellites are tandem repeats of di-, tri-, or tetranucleotides that are a result of mutation length changes and are found in many vertebrate species. As individual microsatellite arrays are relatively short, they can be amplified by using PCR primers designed to anneal to the conserved flanking regions of each microsatellite "locus." Subsequently, locus-specific microsatellite variation, or simple sequence length polymorphism can be visualized through electrophoresis of the marked PCR fragments (Burke et al. 1991; Schl¨otterer et al. 1991; Bruford et al. 1992).

Like minisatellite DNA, microsatellites are inherited in Mendelian fashion, and copy numbers of the repeat unit vary greatly within individuals at different loci and among individuals of a population at the same locus. The level of variability differs among microsatellite regions, offering an opportunity to select microsatellite loci that provide the appropriate level of resolution for a study. Microsatellite DNA fingerprinting is ideally suited for parentage analysis, as well as providing allele frequencies essential for estimates of gene flow and genetic diversity. In addition, this PCR-based method allows analysis of minute amounts of DNA (e.g., from hair or fecal samples), providing unrivaled opportunities to address questions of paternity and population genetics in wild animals (e.g., in wild chimpanzees) (Morin and Woodruff 1992). Thus far, microsatellite DNA fingerprinting has been less recommended for quick genetic assays of endangered or threatened species, as suitable microsatellite primers are still not readily available and are labor intensive in development (Edwards et al. 1991; Moore et al. 1991; Rico et al. 1994). However, the probability of detecting variable microsatellite loci and hence their usefulness for conservation genetics has recently increased significantly through the application of microsatellite-enriched genomic libraries (Armour et al. 1994; Fleischer and Loew 1996).

Mitochondrial DNA (Avise et al. 1987; Moritz et al. 1987; Harrison 1989; Wolstenholme 1992; Simon et al. 1994)

Animal mtDNA is a circular molecule 15–20 kilo bases in length and contains about 37 genes (coding for 22 mitochondrial transfer RNA genes [tRNA], two rRNAs, and 13 proteins) and a control region associated with the molecule's replication and transcription. mtDNA is generally maternally inherited and evolves relatively quickly due to the apparent lack of repair mechanisms for mutations during replication (Wilson et al. 1985; Avise et al. 1987; Martin et al. 1992a). Analysis of mtDNA restriction fragments detects sufficient polymorphism to resolve genetic distances among conspecific populations and closely related species (Tarr and Fleischer 1993; Avise 1994).

O'Brien and associates (1990) combined mtDNA analysis with data on protein polymorphism to inform management decisions for the Florida Panther. Menotti- Raymond and O'Brien (1993) used them to evaluate the history, status, and management of African Cheetahs. Taberlet and colleagues (1995) used DNA sequencing and RFLP analysis of mtDNA to delineate the contact zones of two divergent lineages of Scandinavian Brown Bears, to define conservation units, and to guide possible translocation efforts.

DNA Sequencing (Maxam and Gilbert 1977, 1980; Sanger et al. 1977)

DNA sequencing provides the greatest resolution of genetic divergence by actually determining the identity and sequence of all bases within a target region (typically 500 base pairs long). Consequently, all genetic differences between samples are detected, instead of only those that result in restriction site changes. The most commonly used manual sequencing method is called Sanger dideoxy sequencing. It is based on in vitro synthesis of radioactively labeled singlestranded DNA that is interrupted at either an A, T, C, or G nucleotide. DNA pieces ending in different nucleotides are run in separate lanes, and their positions are made visible as individual bands through autoradiography. Each band represents a particular nucleotide, and consecutive bands are separated by one base pair, consequently the band pattern reveals the DNA sequence of a molecule. More recently, automated DNA sequencing with fluorescent dye labels has started to replace the more cumbersome manual sequence analysis. Nuclear and mtDNA can serve as templates for DNA sequence comparisons among individuals and taxa, provided sufficient amounts of purified homologous DNA and suitable sequencing primers are available. The source DNAs most commonly used for DNA sequence analysis are discussed below.

Mitochondrial DNA Control Region

This noncoding region contains sequences that control replication and transcription of the mtDNA molecule and is called D-loop region in vertebrates and A+Trich region in insects (Fauron and Wolstenholme 1980; Aquadro and Greenberg 1983). It is the only mtDNA region that consistently contains noncoding DNA in many taxa, and although evolutionary rates vary within the control region, it is generally a reliable source for hypervariable mtDNA. This high level of variability has made the control region suitable for sequence comparisons within and between populations and closely related species (Wilson et al. 1985; Harrison 1989; Thomas et al. 1990; Tarr 1995; Morales et al. 1997). For example, Morin and co-workers (1992) used D-loop sequence to identify chimpanzee subspecies 14. Role of Genetics in Conservation Biology 239 and thus recognize hybrids in captive populations and identify the origin of illegally traded animals.

Mitochondrial Protein Coding Genes

DNA coding for proteins is based on a triplet code for amino acids. Several triplets code for the same amino acids. The first and second codon positions are highly conserved, whereas the third position is less constrained in terms of nucleotide changes due to the degenerate nature of the amino acid code. Consequently, most base pair substitutions in the third position do not change amino acid transcription and are considered “silent.” Base pair substitutions that result in amino acid replacements generally occur at a lower rate than silent substitutions due to structural and functional constraints. Cytochrome b and ATPase 6 have been used extensively in vertebrate studies to resolve relationships at close and intermediate taxonomic levels (Kocher et al. 1989; Meyer et al. 1990). For example, Bowen and colleagues (1992, 1993) have used cytochrome b sequence data to determine the phylogeny of all known marine turtles, essential information in choosing which taxonomic groups to conserve and in designing recovery plans.

Mitochondrial transfer RNA genes (tRNA) and mitochondrial ribosomal RNA

(rRNA) evolve more slowly than protein coding genes (Wolstenholme and Clary 1985), indicating greater structural and functional constraints (Simon et al. 1994). Although their slow evolutionary rates render them generally unsuitable to answer typical conservation

genetics questions by themselves, they are occasionally combined with other molecular markers to resolve phylogenies (Mindell and Honeycutt 1990; Hillis and Dixon 1991; Hoelzel et al. 1993; Kretzmann et al. 1997).

Nuclear DNA Introns (Palumbi and Baker 1994)

Nuclear introns have been introduced as a suitable template for DNA sequencing analysis to complement mtDNA data. Universal PCR primers that anneal to exons of highly conserved nuclear genes are used to amplify across nuclear introns.

Noncoding introns generally exhibit high rates of evolutionary change, and sequence analysis can reveal high levels of diversity at these nuclear DNA sites.

Unlike mtDNA, nuclear DNA reflects the biparental contribution to population structure at several independently segregating sites, and like mtDNA sequencing, the resolution power of nuclear introns sequences is high. Consequently, nuclear introns are expected to find their greatest application in resolving genetic population structure and taxonomic relationships. Using actin intron alleles, Palumbi and Baker (1994) corroborated large-scale movements of Humpback Whales based on mtDNA analysis. They revealed that sequence analysis of mtDNA and nuclear introns predicted different amounts of gene flow between Hawaiian and Californian Humpback Whales, a result consistent with female philopatry and malebiased migration between populations.

In general, nuclear and mtDNA evolve and are transmitted differently, and because mtDNA is haploid and maternally inherited, its effective population size is four times smaller than that of nuclear markers. Consequently, small populations may show genetic diversity at nuclear DNA loci but have no mtDNA variation (Avise 1994, 1995). Consequently, ambiguities in phylogenetic relationships and the genetic structure of natural populations are best resolved by using a combination of nuclear and mtDNA markers (Moritz 1994b; Avise 1995). The value of this approach has been demonstrated in studies revealing hybridization in the history of the endangered Red Wolf (Wayne and Jenks 1991; Roy et al. 1994) and the Florida Panther (O'Brien et al. 1990), as well as in determining the phylogeographic history and infraspecific taxonomy of Leopards (Miththapala et al. 1995).

"Ups and Downs" of Genetic Diversity *Generating Diversity*

The amount of genetic diversity present at any point in time in an individual or population is the result of opposing forces that have affected allele frequencies in the past. For example, mutation events, recombination, and immigration are important evolutionary forces that introduce additional alleles and polymorphic sites into a population, whereas selection and inbreeding, as well as random genetic drift, generally homogenize gene pools (for further details, see Hartl and Clark 1989; Loeschcke et al. 1994).

Only mutations can generate novel alleles in entirely monomorphic populations. However, mutations occur rarely and are often deleterious or neutral and therefore cannot be relied on as a major source of genetic variation for short-term genetic management. Immigration offers mixing of gene pools and rapid infusion of new genes into genetically homogeneous populations. The fragmentation of natural habitats, however, decreases natural rates of migration and dispersal and results in population subdivision and eventual isolation.

Conservation biologists can increase gene flow by either encouraging successful dispersal among subpopulations through dispersal corridors or by translocating new genetically distinct breeders to isolated populations. In addition, wild populations can be supplemented with captive-bred individuals through reintroduction programs (for further discussions, see Gibbs 1991; Olney et al. 1994). Numerous threatened or endangered populations have benefited from these management strategies, and consequently, dispersal corridors (e.g., natural areas associated with rivers) and translocation have become an integral part of reserve design (Beier 1993; Dunning et al. 1995; Madsen et al. 1996; Beier and Noss 1998).

Nevertheless, there is a cost to helping gene flow along through human-made corridors or translocations (Simberloff and Cox 1987). Although such population management tools allow the influx of new genetic material into isolated populations, they might also create new problems, such as facilitation of the spread of pathogens and parasites and hybridization (Woodford and Rossiter 1994; Cunningham 1996; Stockwell et al. 1996). Green and Rothstein (1998), for example, reported that translocations of the endangered Black-faced Impalas to private farms have increased the threat of hybridization with resident Common Impalas in Namibia. They agreed with Robinson and associates (1991) that population manipulations (translocations, introductions) should only be carried out after careful genetic, taxonomic, and ecological considerations.

Artificial insemination and embryo transfer are alternative methods of introducing genes into a population. Inseminating females or implanting them with embryos circumvents the problem of mate choice and does not depend on the establishment and mating success of immigrant males. These methods are, however, highly intrusive, cumbersome, and expensive and hence are only practical for captive populations and only recommended in cases in which species survival depends on imminent increase in genetic diversity. Furthermore, reproductive technology of endangered species is a relatively new and still experimental field. Although the techniques have proved useful in livestock management, much more basic research on the reproductive biology of rare species is needed to guarantee safe application in genetic management efforts (see Ballou and Cooper [1992] and Moore et al. [1992] for discussion of reproductive technology and conservation genetics).

In general, increased habitat fragmentation has subdivided many populations to the point at which individual subpopulations would become extinct unless they are managed as part of the larger highly structured population. The extreme complexity of modeling stochastic and deterministic events in such structured populations has hampered efforts to assess genetic and demographic effects simultaneously (Burgman et al. 1993; Ballou et al. 1995; Ratner et al. 1997).

However, population viability models are becoming increasingly more sophisticated and valuable in risk assessment of endangered populations (Boyce 1992; Lacy 1993; Kenny et al. 1995; Mills et al. 1996).

Losing Diversity

Inbreeding can be due either to the mating of related individuals (as in selfing or assortative mating) or it may be because, in finite populations, there is a chance for two identical genes to be sampled together. This chance increases when the size of the population decreases. Consequently, levels of homozygosity in inbreeding populations are higher than predicted under random mating (Falconer and Mackay 1996). (Templeton and

Read [1994] discuss different measures of inbreeding and their relationship with genetic diversity.)

Natural selection is a significant evolutionary force that can maintain or erode genetic diversity, depending on the relationship between genotypes and fitness. Directional selection, for example, favors extreme phenotypes and their underlying genotypes, and the entire array of intermediate phenotypes are selected against and eventually lost in the population. Consequently, strong selection for particular traits homogenizes the gene pool, ultimately reducing the adaptive potential of a population. This relationship is of concern in captive propagation programs that breed animals for future reintroduction to the wild; any inadvertent selection to improve management in captivity may reduce survival chances back in the wild. In general, the interactions between phenotypes, genotypes, and fitness are far too complex to predict reliably, and conservation biologists generally refrain from managing captive populations based on specific beneficial traits; instead, they try to maintain overall genetic diversity on which natural selection can act.

Maintaining Genetic Diversity and Effective Population Size

Ideal populations are defined as infinite populations, consisting of sexually reproducing diploid organisms that mate at random and have nonoverlapping generations and whose allele frequencies are not affected by migration, mutation, or selection (Wright 1931). The probability of loss of an allele in ideal populations is equal to its allele frequency.

As real populations almost always violate the assumptions of an ideal population, Sewall Wright (1931) introduced the concept of the effective population size (*Ne*) to evaluate the evolutionary potential of populations that deviate from the ideal. The effective population number is most commonly estimated by relating the variance in allelic frequency (the "variance effective size") or the rate of inbreeding ("inbreeding effective size") of the real to the ideal population (Wright 1931; Crow and Denniston 1988). Accordingly, the effective population size of a real population equals the size of an ideal population that has the same amount of variance in allele frequencies or the same amount of inbreeding as the actual population (Wright 1931, 1938; Crow and Kimura 1970).

Maximizing the inbreeding effective size maintains heterozygosity within local populations, whereas high variance effective size decreases loss of genetic diversity across local populations and significantly affects allelic diversity (Crow and Kimura 1970; Gliddon and Goudet 1994; Ballou and Lacy 1995; Hedrick et al. 1995). In general, *Ne* is smaller than the actual population size, and loss of genetic variation increases with decreasing effective population size. Numerous factors, such as unequal sex ratio and fluctuating population size, can significantly affect effective population size (for detailed discussion, see Falconer and Mackay 1996). For example, calculations of effective population size are usually based on discrete generation models, which are not applicable to many real populations.

A realistic diploid model with overlapping generations, however, is very complex as it attempts to estimate allele frequency changes per unit time for males and females that might differ in their age-specific birth and death rates (Felsenstein 1971; Lande and Barrowclough 1987). The effective population size per unit time is maximized if the generation lengths of males and females are equal, and it decreases with shorter maturation time. In other words, species that experience delayed sexual maturation and have long generation times (e.g., large-bodied mammals) have a higher probability of retaining genetic diversity for any given period of time (see Hedrick 1992b for review).

These species will, however, lose genetic variation at the same rate as short-lived animals following a bottleneck if time is measured in generations.

It is crucial to realize that high genetic diversity in long-lived species (e.g., One-horned Rhinoceros; Dinerstein and McCracken 1990) does not necessarily mean that previous population crashes did not affect them, but instead it may mean that they have not reproduced much since then. Hence, we should realize the opportunity to maintain the genetic diversity stored through such "walking gene banks" by maximizing their effective population size for future generations. To determine effective population size when several factors are of importance, Chepko-Sade and colleagues (1987) suggested sequential calculation of *Ne* for all variables by using an iterative process. They used this approach as a first approximation to reach more realistic estimates of the evolutionary potential (*Ne*) for a variety of well-studied natural populations (e.g., wild horses, Black Bears, Dwarf Mongoose), but its theoretical validity has not been tested. Numerous alternative approaches to estimate *Ne* have been developed to address incomplete data sets and effects of multiple variables (Frankham 1995a; Rockwell and Barrowclough 1995). In addition to demographic approaches, change in various genetic measures (e.g., allozyme heterozygosity, pedigree inbreeding) has been used to estimate effective population size (Avise et al. 1988; Tomlinson et al. 1991; Briscoe et al. 1992). Frankham (1995b) argued that genetic versus demographic methods show comparable results, provided the same variables were used to determine short-term estimates of *Ne.* In general, he concluded that population fluctuations, variance in family size, and unequal sex ratio affect the ratio of effective to actual population size most significantly and that the effective size for most wild population is disconcertingly small ($Ne/N = 0.10–0.11$).

In conclusion, loss of genetic variation in endangered or rare species can be minimized through breeding programs that aim to maximize effective population sizes and reduce inbreeding. Outbreeding is generally considered an appropriate measure to maintain or generate genetic diversity; however, individuals should not be outbred indiscriminately. If most genetic variation is found in a number of different geographic regions, regular genetic exchange between those populations would actually reduce overall genetic diversity and homogenize rather than diversify the species' gene pool (= genetic cost of dispersal corridors and translocation). Furthermore, populations that experience varying ecological conditions and have been isolated from each other for an extended period of time might show important local adaptations. Under such conditions, gene flow would enhance genetic variation within populations but might also result in reduced fitness of the less well-adapted outbred offspring (outbreeding depression) (Shields 1982; Templeton et al. 1986; Ballou 1995).

In addition, long-term historical divisions within species may be the sign of ongoing adaptive radiation, and individual populations might have to be treated as separate evolutionary entities. Consequently, recommendations for the genetic management of endangered species should be based on an assessment of the present genetic structure and take the population biology and breeding history of individuals into consideration wherever possible (Vrijenhoek et al. 1985; Vrijenhoek 1994; Ballou et al. 1995; Avise and Hamrick 1996).

Who Is to Embark on the Ark?

To maintain biodiversity and to identify those taxonomic units worthy of our protection, we need clearly defined criteria to determine conservation units. Since the 1973 Endangered

Species Act mandated the protection of species, determination of species status has necessarily become of great importance to conservation management (U.S. House of Representatives 1973).

The species concept, however, has been at the center of an ongoing debate among evolutionary biologists, and numerous criteria, such as reproductive isolation and ancestral relationships, have been applied to define a species (O'Brien and Mayr 1991; Crozier 1992; Geist 1992; Rojas 1992). For example, Mayr's (1963, 1969) biological species concept defines species as freely interbreeding populations that are reproductively isolated. Criteria of interbreeding ability, however, are of limited use for clarifying taxonomic relationships of discontinuous populations because reproductive barriers among allopatric populations are difficult to prove (Cracraft 1983; McKitrick and Zink 1988). Similarly, distinguishing species on the basis of morphology suffers, in part, from the fact that many morphological traits are considerably affected by environmental conditions (Geist 1987). Therefore phenotypic differences between populations might reflect temporary local adaptations rather than independent evolutionary histories.

By contrast, phylogenetic analysis based on neutral genetic markers can contribute additional measures of the genetic distinctiveness of taxa and may be more reflective of their evolutionary history (Avise et al. 1987; Hillis 1987; Dizon et al. 1992; Moritz 1994b; Wayne et al. 1994; Avise and Hamrick 1996; but see Cronin 1993). In general, a variety of genetic markers in combination with morphometric analysis is preferable to establish the amount of reproductive isolation and phylogenetic uniqueness of a particular taxonomic group. Accordingly, Avise and Ball (1990) suggest that a suite of phylogenetically concordant characteristics should be used to define a taxonomic group as a population of individuals that can be united by one or more derived traits.

Rojas (1992) has pointed out that conservation based on the above typological approaches to species is problematic for several reasons. If nature reserves are designed to ensure survival of a representative sample of "types" or species, unresolved species status will have a major impact on the number of preserved species and ultimately the level of biodiversity. She points out that "the numbers, however, are unlikely to be the same if we are considering biological species, cladistic species, or evolutionary species" (Rojas 1992). In addition, she notes that conserving species as types ignores the importance of preserving geographic variation within species.

Such infraspecific variation, however, is potentially crucial for the long-term survival of a species. Conserving species as evolutionary units addresses the importance of variation within species to preserve the evolutionary potential of the protected organisms and ultimately minimize the extinction probability across their range (Vrijenhoek 1989; Rojas 1992). Although reserve management based on evolutionary units is less subject to "bean counting" of rare types, clear delimiting of taxonomic groups is still essential to avoid inadvertent hybridization and outbreeding depression.

Amendments to the Endangered Species Act (ESA) extended protection of species to include distinct population segments that interbreed when mature. This broader definition of species protection allowed flexibility to preserve species in only part of their range without resolving their taxonomic status (Pennock and Dimmick 1997). As ESA provided no clear definition for "distinct population segments," criteria for identifying fragile populations were highly varied, including uniqueness based on geographic isolation and morphological and genetic differences (Pennock and Dimmick 1997).

The concept of evolutionary significant units (ESU) was developed, in part, to provide biological guidelines for determining conservation units below the species level (Ryder 1986; Vane-Wright et al. 1991; Waples 1991). This attempt to apply the concept of ESUs to identify "distinct population segments" for conservation has resulted in ongoing debates that focus on the biological merits (Dizon et al. 1992; Moritz 1994a; Vogler and DeSalle 1994; Waples 1998) and the fulfillment of the ESA mission (Rohlf 1994; Pennock and Dimmick 1997; Waples 1998). Rojas's (1992) conclusion that "considering the species problem more critically may result in recognizing the limitations of the taxonomic information used; it may also contribute to the refinement of the concepts and methods" still holds, and we continue to benefit from the interaction between systematists and conservation biologists to determine conservation units.

Conclusions

Which species are most at risk of extinction may depend on many interactive aspects of their biology, such as their ecology (e.g., specialists), mating or migratory behavior (e.g., threatened breeding or wintering grounds), demography (e.g., low birth rate), and population genetics (e.g., small effective population size). Populations that are dwindling in size are generally marked by high mortality or low birth rates and eventually may show changes in gene frequencies due to increased population subdivisions and reduced gene flow. Hence, ecologists make use of alarming demographic trends such as low growth rates and conservation geneticists focus on changes in genetic variation (e.g., loss of heterozygosity) to screen for possible threatened populations. Population viability analyses that estimate extinction risks of populations due to both demographic and genetic stochasticity provide powerful monitoring tools that will help to guide the allocation of scarce resources to taxa most likely in need of management (Burgman et al. 1988; Lacy 1993; Nunney and Campbell 1993; Ballou et al. 1995).

Failure to detect signs of increased extinction risk based on the demographic and genetic records of populations should not be interpreted as a guarantee of survival of a taxon, especially not for healthy populations with a limited range or species of a specialized ecological niche that might become endangered by a single catastrophic event. Hence listing taxa as endangered should be based on demographic and genetic indicators of decline, while taking into consideration vulnerabilities of the species due to its specific biology, as well as abiotic factors such as the local political situation (Avise 1994; Ballou et al. 1995; Avise and Hamrick 1996). In their reevaluation of the IUCN threatened species categories, Mace and Lande (1991) applied a similar logic in their development of quantitative criteria to determine endangerment.

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