population. Anyone that doubts the ability of humans to have devastating impacts on avian diversity need only be shown the clearly elucidated history of Pacific Island avifaunas following their contacts with colonizing humans (Steadman 1996). Anyone concerned with biodiversity must work to convince the world how important it is to decrease our own species' birth rate and resource use. Second, Hutto points out that although we focus on important research needs in this book, what we really need is the "widest possible variety of quality research." Moreover, he suggests that the limiting factor in avian conservation is not a shortage of quality data, but a lack of willingness to *use* it. Thus, important tasks for avian conservationists, outside of the collection of our data, are the general education of the public to be more sensitive to the perils of overpopulation and the determination of the types of data most likely to influence policy makers.

CHAPTER 3

Genetics and Avian Conservation

Robert C. Fleischer

Anthropogenic causes of avian endangerment and extinction may vary among taxa, geographic regions, and habitats (Dobson et al. 1997). While habitat destruction and fragmentation are clearly the most important factors, other agents, such as overhunting, pesticides, pollution, increased UV-B, global warming, salinization, and introduced predators, competitors, parasites, and diseases can also be significant, if not primary, causes of population decline for some bird species (Pulliam and Babbitt 1997). In the face of this variety of threats to biodiversity, genetic theory and technologies have been increasingly used to assist in the management of taxa in peril. Methods of conservation genetics have been applied in many case studies, especially during the past decade, and there have been many reviews that discuss these applications in detail (e.g., Soulé 1980, Lande and Barrowclough 1987, Hedrick and Miller 1992, Avise 1994, Moritz 1994a, O'Brien 1994, Frankham 1995a, Lynch 1996, Hedrick et al. 1996). Most conclude, of course, that genetic methods are not by themselves a panacea for the conservation problems that organisms face. However, most also conclude that such methods can provide useful information for biodiversity management.

In general, conservation genetic methods have not been applied to birds as much as to other vertebrate taxa, especially mammals (see volume with Haig and Avise 1996, or O'Brien 1994 for evidence of this). In addition, most previous studies in avian conservation genetics assessed genetic diversity within and among populations or species, and then recommended ways to conserve these levels and patterns (Haig and Avise 1996). There are alternative uses for genetic methods that have not been greatly explored. Here I take a functional approach to how these applications can be categorized and divide the primary uses into:

1. forensic applications (i.e., identification of individuals, populations, or species to solve "crimes against nature");
2. assessments of inbreeding and associated fitness depression;

3. assessments of current (along with past and future) levels of genetic variability within and among populations;
4. use of population genetic models with molecular data to estimate long-term gene flow rates, effective population sizes, and population fluctuations; and
5. identification of evolutionarily significant and management units (ESUs and MUs), hybrids, and determining if such units are "worth" saving.

These categories are not mutually exclusive: some applications involve conceptual bases and methodologies that overlap to some degree. In addition to those listed there are several more-novel ones. In particular, because of recent conceptual and technical advances in developmental genetics, immunogenetics, and applied genetic engineering, a number of applications may before long be possible that will assist management efforts in ways undreamed of before.

In this chapter I review the uses (and what I view as some potential misuses) of genetics for the conservation of avian diversity. I begin with a brief caveat, followed by categorization and description of the primary quantitative and molecular tools in conservation genetics. I then characterize and illustrate (with mostly avian examples) the five types of conservation applications noted above, and provide rationales for and problems with each approach. I conclude with some predictions concerning possible future uses for genetic theory and molecular genetic methods in avian conservation biology.

As in most applied fields of study, there are controversies within conservation genetics about how different methods should be applied, and about the relative merits of particular applications for management decisions (Hedrick et al. 1996). For example, one controversy revolves around whether inbreeding depression and genetic variation have important consequences for short- or long-term survival of a population or species. Another concerns how we should preserve levels and patterns of genetic variation—as they presently occur, at maximum variability, or how they occurred prior to human impacts? Or perhaps we should be concerned only with restoring or maintaining healthy species so that an ecosystem can function properly, regardless of the genetic makeup of its populations. These questions reflect the types of uncertainty that often exist for other aspects of conservation biology. In many cases we may be required to make management decisions based on conflicting or flawed theory or empirical databases, or when we cannot properly experiment and consider alternatives because of considerations of time, impact to endangered populations, or financial cost. These controversies may also reflect a tendency of us as competitive social organisms to take extreme positions partly out of concern for our standing in dominance hierarchies ("political" reasons). Lastly, as much as we biologists like to think we have all of the solutions, we must realize that most issues cannot be resolved entirely with biological or even other scientific information, but also often involve philosophical, legal, economic, or social concerns.

Methods of Conservation Genetics

Pedigree Analyses

The analysis of pedigrees is useful for the detection of inbreeding and its associated depression of fitness (Ralls, Ballou, and Templeton 1988), and for designing breeding strategies to avoid future losses of genetic variation or adaptation to captivity (Foose and Ballou 1988; Lacy 1989; Haig, Ballou, and Derrickson 1990). Such analyses are usually conducted with captive populations and involve maintaining records of parentage for all individuals in large databases called studbooks. Coefficients of inbreeding or coancestry (i.e., the probability that two alleles sampled from an individual are identical by descent; Ballou 1983) are calculated and then used to design breeding strategies that maintain particular levels or patterns of genetic variability (Foose and Ballou 1988; Lacy 1989; Hedrick and Miller 1992). Probabilities of loss of genetic variation also are often estimated via simulation experiments ("gene drops"; MacCluer et al. 1986). Alternatively, measures of fitness can be regressed onto these coefficients to assess inbreeding depression (Ralls, Ballou, and Templeton 1988).

Models of Genetic Structure

A second set of quantitative methods involves the use of demographic data within population genetics models to make predictive estimates of genetic variability and structure (e.g., for birds: Barrowclough 1980; Fleischer 1983; Rockwell and Barrowclough 1987). For example, Barrowclough (1980) used field-based estimates of bird dispersal and effective population sizes in equations of isolation-by-distance and stepping-stone models to estimate expected levels of among population genetic differentiation. Two concerns about this approach are that these models rely on accurate input values (that can often be very difficult to obtain without long-term field studies) and on assumptions that need to be met to varying degrees in order to have any predictive power.

Quantitative Genetics

A group of methods receiving increased attention in conservation genetics is that of the more traditional field of quantitative genetics (Falconer 1981). In fact, a growing cadre (e.g., Lande and Barrowclough 1987; Hamilton 1994; Lynch 1996) believes that such methods reveal variation that is of greater conservation importance than the putatively "neutral" variation revealed by molecular or other methods (but see Hughes 1991). They reason that traits that are most likely to affect fitness are polygenic or quantitative trait loci (QTLs) as opposed to single-locus traits. Effective mutation rates at QTLs are very high ($\sim 10^{-3}$ mutations/gamete/generation; Lande and Barrowclough 1987) in comparison to more typical coding loci ($\sim 10^{-5}$ to 10^{-7}; Nei 1987) but are similar to rates found

for variable number tandem repeat loci, VNTRs (e.g., Jeffreys et al. 1988; see Fleischer, Fuller, and Ledig 1995).

Quantitative genetic methods may be useful for conservation because they estimate genetic variability in potentially selected and important traits, like body size or clutch size. However, estimation of heritabilities (not to mention nonadditive genetic variances and covariances) requires that relatively large numbers of family groups be measured for such traits. This may not be a simple matter for some avian species, especially endangered ones that are sparsely distributed or for which nest disturbance could lead to abandonment. In addition, heritabilities could be biased if much extra-pair mating occurs, which is normal for many passerine and some nonpasserine birds (Fleischer 1996). Lastly, the work of James (1983) suggests that growth in different environments may impact heritabilities in ways that are not straightforward, although a recent review (Weigensberg and Roff 1996) revealed a high correlation between heritabilities estimated in captive animal studies with those measured in nature.

Biochemical Methods

The majority of genetic applications in avian conservation have used electrophoretic analyses of allozymes and other proteins (e.g., Haig and Oring 1988; Barrowclough and Gutiérrez 1990; Fleischer, Conant, and Morin 1991). Proteins can be assayed from soft tissues, blood, and feather pulp, and the latter two, less invasive approaches have obviously been favored in conservation applications. Unfortunately, blood and feather pulp yield fewer protein loci than soft tissues. When this drawback is combined with their relatively low mutation rate ($\mu = 10^{-5}$ to 10^{-7}) and variability (mean heterozygosity for birds is ~5.8 percent; Barrowclough, Johnson, and Zink 1985), allozymes generally appear less useful than other methods for assessment and comparison of genetic variability. The low proportion of variable loci makes it difficult to resolve differences in heterozygosity and assess population structure, especially in bottlenecked populations. Also, if a population has low allozyme variability, it is difficult to estimate the timing of a bottleneck, as it would take about $1/\mu$ generations (Nei, Maruyama, and Chakraborty 1975) for mutation to restore to equilibrium the neutral variation that had been lost. Some advantages of protein electrophoresis include its relatively simple extraction, electrophoresis, and staining protocols; the universality of its application; and its low cost compared to most molecular methods. See Hillis, Moritz, and Mable (1996) for a recent review of allozyme methods.

Molecular Genetic Methods

There has been a virtual explosion of new molecular genetic (nucleic acid) methods over the past decade, many of which have application to avian conservation biology. Some of the latest techniques are extremely powerful, and with

rapid advances in technology, many of the methods are becoming easier and, to some extent, less expensive than before. There is a tendency among researchers, however, to use newer methods even when some of the older, less expensive methods may produce adequate results.

The methods can be categorized by the type of analysis that is performed and the type of marker that is characterized. In figures 3.1 to 3.3 and below, I briefly summarize some of the common molecular methods. For detailed treatments of methods and explicit protocols, see Avise (1994); Hoelzel (1992); Hillis, Moritz, and Mable (1996); or Ferraris and Palumbi (1996). An older method, the analysis of Restriction Fragment Length Polymorphism (RFLP; figure 3.1) uses enzymes to cleave DNA at particular short sequences. A more recent and powerful method, the Polymerase Chain Reaction (PCR; figure 3.2A, 3.2B) copies, or amplifies, specific DNA sequences using a DNA replicating enzyme (polymerase) and short, synthesized DNA primers. PCR is very sensitive and can amplify sequences from very small starting quantities of DNA, including partly degraded DNA from feathers and avian museum specimens (Ellegren 1991a; Leeton, Christidis, and Westerman 1993; figure 3.2C), mammalian feces (e.g., Paxinos et al. 1997), and avian subfossil bones (Cooper et al. 1996). Its extreme sensitivity demands careful execution to avoid accidental contamination with foreign DNA. DNA sequencing (Figure 3.3A) determines exact nucleotide sequences of PCR amplified (or cloned) genes. There are many novel alternatives to DNA sequencing that also reveal variation (see Avise 1994; Ferraris and Palumbi 1996 for such methods).

Mitochondrial DNA

The only organellar genome identified in birds is mitochondrial DNA (mtDNA), which has proven to be one of the most useful (and used) marker systems (Moritz 1994a; Avise 1994). As in nearly all vertebrates, mtDNA has been shown to be maternally inherited, nonrecombining, and relatively rapidly evolving in birds. Thus, it is a very effective marker for detecting genealogical structure within and among avian populations and for reconstructing phylogenetic relationships among taxa (e.g., Avise 1994; Moore 1995). The primary method of assaying variation in mtDNA is PCR amplification of all or part of a particular gene, followed by direct sequencing. Different regions of the molecule appear to have different levels of selective constraints and thus exhibit different levels of variability (with the slowest-evolving regions being the ribosomal and transfer RNAs and the fastest usually being the noncoding control region). Because mtDNA is a single linkage group, any force (e.g., selection) that impacts one part of the molecule also impacts others (Degnan 1993; Rand 1996). In addition, copies of mtDNA genes transposed to the nuclear genome have been found in birds (e.g., Quinn 1992; Sorenson and Fleischer 1996), and these result in a variety of problems (as well as some opportunities).

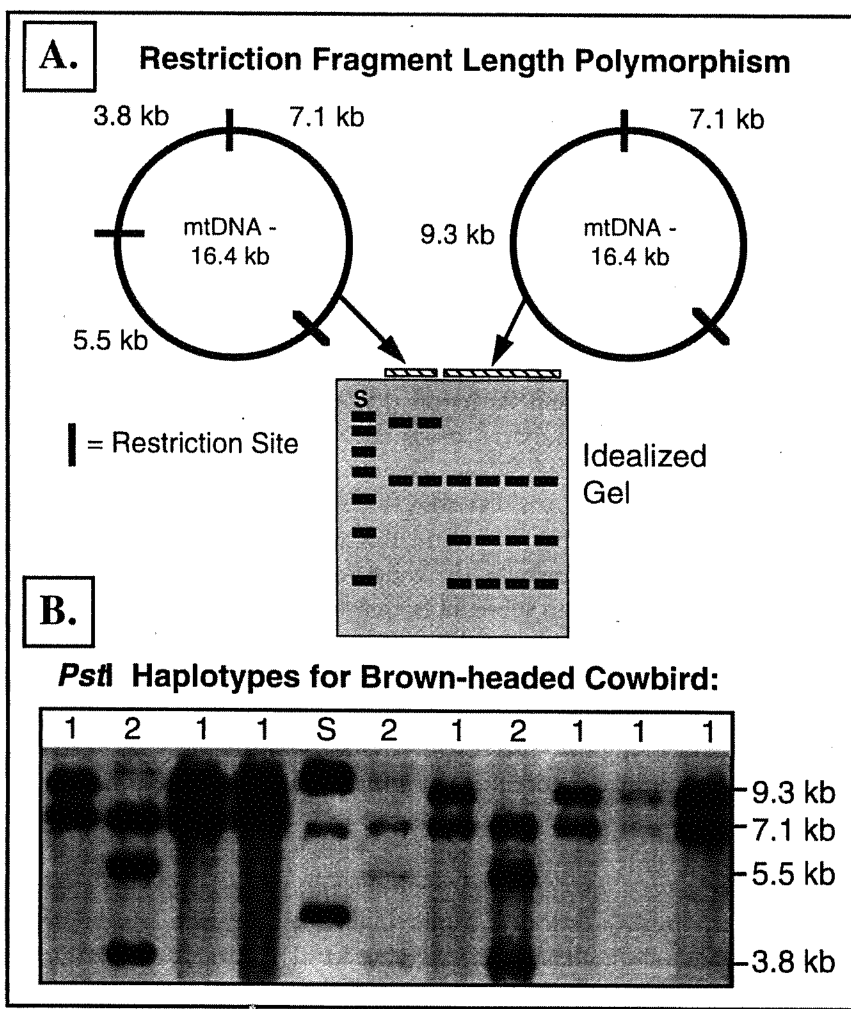

Figure 3.1. Restriction Fragment Length Polymorphism (RFLP) analyses. DNA is digested with bacterial enzymes called restriction endonucleases (RE). Each RE recognizes a particular sequence of DNA bases and cleaves the DNA at that point (e.g., the enzyme HaeIII cleaves at 5'GGCC'3). If mutation modifies the sequence, whether by substitution, insertion, or deletion, the RE will not cleave the DNA. By comparing the distributions of fragments on gels following digestion, one can usually determine the presence (or absence) of a restriction site and tally the minimum changes required for the pattern. Because most mutations involve substitutions, and usually only a single base change per site, models of restriction site evolution can be used to estimate the proportional change in nucleotide sequence (Nei 1987). (A) Schematic diagram of RFLP analysis. The molecule on the right has two recognition sites, and digestion by RE results in two fragments. The molecule on the left has three restriction sites, and digestion produces three fragments. Fragment sizes add up to (approximately) the same size, based on size standards run in adjacent lanes (S). (B) Example of an actual RFLP analysis of mtDNA in the Brown-headed Cowbird (*Molothrus ater*) using the RE PstI. The number above each lane indicates the haplotype (1 = 1 cut, 2 = 2 cuts). S = size marker. Sizes of the mtDNA fragments are noted in kilobases to the right of the gel.

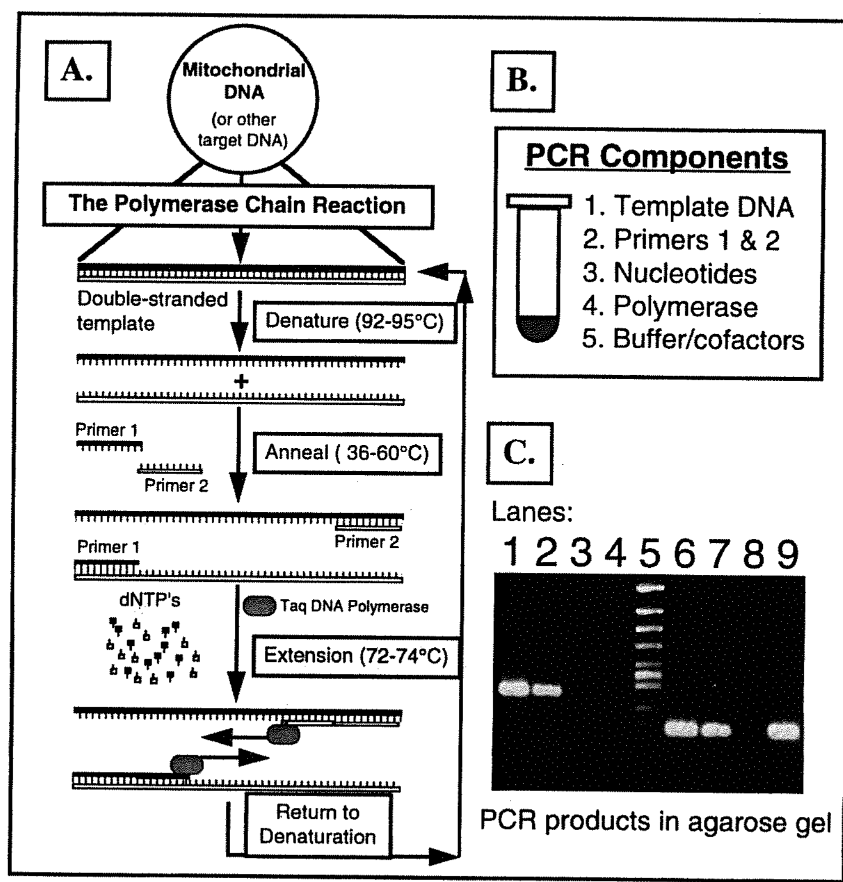

Figure 3.2. Polymerase Chain Reaction (PCR). PCR copies (amplifies) specific sequences of DNA using a DNA polymerase and synthetic primers. (A) Schematic of PCR showing the three major steps: *denaturation* of double-stranded template DNA to single-stranded by heating; *annealing* of complementary flanking primers (at temperatures that depend on the degree of match between primer and template and the proportion of G:C versus A:T bonds); and *extension* from the 3' end of the primer as the polymerase covalently binds deoxynucleotides from solution. The result is an accurately synthesized complementary strand of sequence. The process is repeated thirty or more times, each time as much as doubling the number of copies of the sequence. (B) Components of the PCR mix: "Template DNA" is from the organism of study, nucleotides = dNTPs. The polymerase (Taq) is thermally stable, obtained originally from bacteria capable of living in water near boiling temperature. (C) Examples of PCR products for the control region of mtDNA amplified from DNA isolated from museum specimens of Hawaiian Honeycreepers (Tarr et al., unpublished). A size marker is in lane 5. The lighter bands near the bottoms of lanes 2, 3, and 8 are PCR artifacts called primer dimers. Lanes 3 and 8 contain amplifications of extract controls (all components but template DNA) and serve to identify possible contamination. Amplification products of the correct size were obtained in lanes 1, 2, 6, 7, and 9. Amplified products can be run in a gel, visualized by one of a variety of methods, cleaned of primers and nucleotides, and then cloned into plasmid or viral vectors in bacteria or directly analyzed for variability by RFLP, DNA sequencing, or other methods.

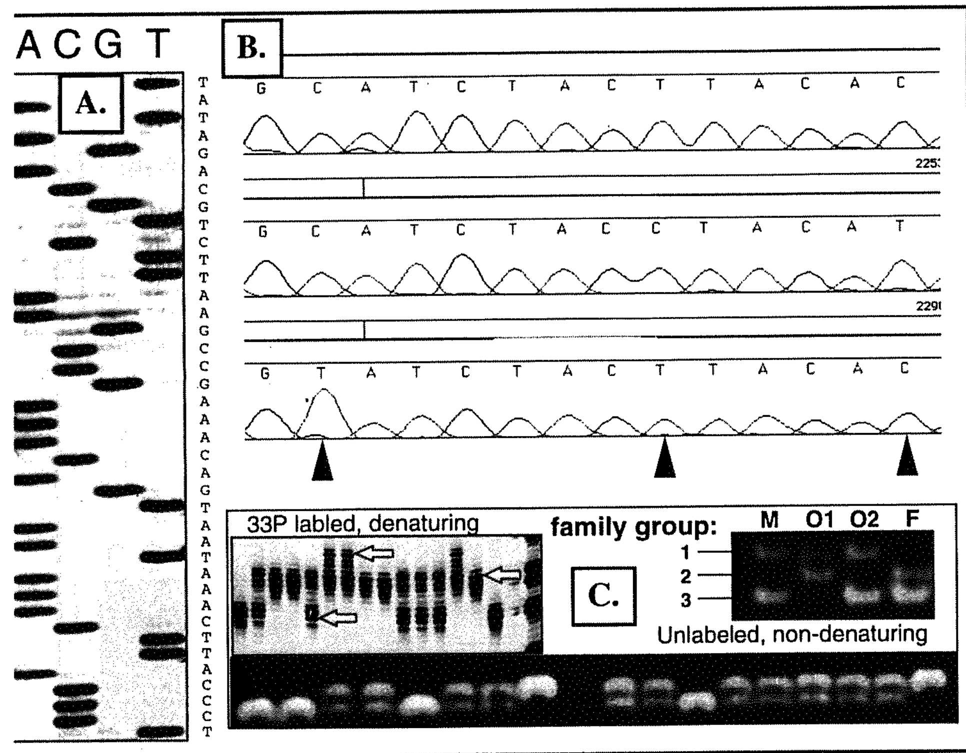

Figure 3.3. DNA Sequencing and Microsatellite VNTR analysis. DNA sequencing involves reactions similar to PCR: complementary strand synthesis from a primer annealed to a template DNA. However, DNA sequencing involves primers or free deoxynucleotides that are covalently labeled (radioactively in "manual" sequencing and with fluorescent dyes in "automated" sequencing) and other nucleotides that lack a hydroxyl group needed for strand synthesis (i.e., dideoxynucleotides). The dideoxynucleotides "randomly" terminate sequence extension, thus leaving a nested series of labeled, single-stranded products in size increments of a single base pair. (A) Manual DNA sequence. Extension reactions for each base (A, C, G, and T) are completed in separate tubes, and the denatured (single-stranded) products are loaded into adjacent lanes of a denaturing polyacrylamide gel. The sequence is read up the ladder as shown to its right. (B) Automated sequence. Cytochrome b mtDNA sequence of three Corvus species. In automated sequencing, each dideoxynucleotide (A, C, G, or T) is labeled with a different color of fluorescing dye. The sequencing reactions are completed in one tube and loaded into a single lane of a denaturing gel. The labeled, synthesized strands are run past a scanning laser at the bottom of the gel. The laser-excited dye fluoresces, and each color and its intensity are recorded into a computer and displayed as continuous spectrum in a "chromatogram." The bases are called according to the colors of the peaks (σ marks variable sites). (C) Microsatellites PCR amplified from drepanidine DNA. Upper left shows microsatellites PCR amplifed with a ^{33}P end-labeled primer followed by electrophoresis in a denaturing polyacrylamide gel. Note that "slippage" bands (a polymerase artifact) make it difficult to score the genotypes consisting of three alleles (marked with dots). Example below and on the right are of unlabeled amplifications of microsatellites followed by electrophoresis on nondenaturing gels. No slippage is evident, and genotypes and alleles are easy to score. The family group shows microsatellites for a male (M), female (F), and two offspring (O1 and O2) and matches Mendelian expectation for inheritance of variants.

Nuclear Genes

These are diverse in their rates of evolution and usefulness in conservation applications. The most used are the variable number of tandem repeat loci, random genomic markers, protein coding regions (exons), noncoding or spacer regions (introns), and immune system multigene families such as the major histocompatibility complex (MHC).

One of the best molecular marker systems for conservation genetics developed to date is the variable number of tandem repeat loci (see Fleischer 1996 for literature summarized here), which include minisatellite (7–25 bp repeat length) and microsatellite (1–6 bp repeat length) sequences in a tandem repetitive array or "core." These loci are highly variable, primarily because of their high mutation rates (10^{-2}–10^{-4} mutations per gene per generation). The mode of mutation also differs from other types of DNA markers. Mutation generally involves replication slippage or unequal sister chromatid exchange, rather than point substitutions, and results in a loss or gain in the number of repeats within a repeat array. Thus, array size varies, causing variation in the size (and thus position) of a fragment or product in a gel (figure 3.3C).

Multilocus DNA fingerprinting involves nearly "universal" minisatellite and microsatellite probes in an RFLP analysis. Multilocus probes can assay for as many as 15–25 highly variable loci on a single gel with one probe, and filters can be reprobed with several independent probes. Some problems with the method are that a large amount of DNA (>2 μg per individual) is required (not so great a problem for birds because of their nucleated erythrocytes), specific loci and alleles cannot usually be determined, and individuals can usually not be reliably compared among gels (Burke et al. 1991; Fleischer 1996). Some minisatellite probes take advantage of single-copy sequences that flank minisatellite repeat arrays and thus reveal variation at only one locus (Hanotte et al. 1991).

PCR allowed the development of highly variable single-locus genetic markers (Tautz 1989). This method usually involves *microsatellites* as opposed to minisatellites. Microsatellite probes (e.g., CAn, CACn) are initially used to screen random genomic or microsatellite-enriched libraries of a species of interest in order to locate clones containing microsatellite sequences (Fleischer 1996). Regions flanking the repeat region are sequenced, and synthetic oligonucleotide primers are designed so that they will PCR amplify across the microsatellite to produce small products (<300 bp) that can be resolved on a polyacrylamide gel. The products can be sized exactly in multiples of the repeat length, and are highly variable, sometimes with more than 10–20 alleles and heterozygosities above 80–90 percent (figure 3.3C).

Microsatellites are less common (perhaps by as much as an order of magnitude) and variable in birds than in other vertebrates (Ellegren 1992; Hanotte et al. 1994; Fleischer 1996; Glenn 1997). Microsatellites can be difficult and time-consuming to develop, and primers are not universal and may reveal variable and/or interpretable patterns only with related species (Hanotte et al. 1994). In addition,

problems, such as nonamplifying or null alleles (e.g., Pemberton et al. 1995), slippage or other artifacts, and uncertainty about the modes of evolution (e.g., Valdes, Slatkin, and Friemer 1993), can confound their interpretation and analysis. Nonetheless, amplifiable microsatellites are the most powerful markers in avian conservation genetics and have a wide range of applications (e.g., estimation of parentage, dispersal, genetic variation and structure, subspecies systematics, and genome mapping).

Some studies use markers that are, in a sense, randomly sampled from the genome (e.g., in birds: Haig, Rhymer, and Heckel 1994; Nusser et al. 1996). Anonymous single-copy markers can be specifically amplified by PCR from sequences identified from random clones (Avise 1994, 72), but the easiest markers to develop and use, though not necessarily the most trouble free, are Randomly Amplified Polymorphic DNAs (RAPDs; Grossberg, Levitan, and Cameron 1996). In this method, one or two random sequence primers usually ≥ 10 bp in length are used in a PCR reaction. In regions of the genome where the primers can anneal in opposite orientations while flanking a small (<2,000 bp) sequence, amplification of a product will occur. Sometimes amplification will not occur if priming site sequences are polymorphic. Thus, RAPD fragments usually show complete genetic dominance (i.e., presence of a fragment indicates homozygote dominant or heterozygote, while absence indicates homozygous recessive). Fragment profiles often vary among individuals (but see Nusser et al. 1996) and, with certain assumptions (Lynch and Milligan 1994), can provide useful markers for estimation of population heterozygosity and structure. They are perhaps most useful for development of simple markers to assess gene flow, hybridization among differentiated taxa, or population of origin (e.g., Haig et al. 1997).

Other potentially important single-copy markers are introns, exons (coding sequences), and pseudogenes (exons no longer translated to protein and thought to be free from selective constraints). Variation in neutral markers such as introns and pseudogenes presumably reflects processes (μ, m and N_e) that occur in the absence of selection (assuming no strong linkage to selected loci). Some feel that coding or regulatory regions may be preferable for conservation studies because they code for traits possibly related to fitness. Variation in one such group of genes, the MHC (highly polymorphic proteins that recognize foreign antigens), is putatively maintained by balancing selection caused perhaps by differential resistance to infectious disease (Hughes 1991), but the perils of relying on only one gene system for conservation management, especially one as difficult to characterize in birds as the MHC (Edwards, Grahn, and Potts 1995; Jarvi et al. 1995), have been noted (e.g., Miller and Hedrick 1991; Haig, Ballou, and Derrickson 1990).

Applications in Avian Conservation

Forensics

There has been a virtual explosion in the use of molecular genetic methods in human forensics over the past ten years, and these same methods have been introduced to wildlife conservation biology with only a minor lag period. In most cases some crime has been committed and the molecular data are used as evidence in a trial. The primary use of such methods is to identify or exclude at a variety of levels (i.e., that of the individual, relative, population, or species). Most such applications involve identification of particular wildlife species using mitochondrial DNA (e.g., Paxinos et al. 1997) or of individuals using VNTRs. Another important type of "forensic" method involves the diagnosis and identification of parasites or diseases in tissue samples (e.g., *Plasmodium* in the blood of Hawaiian birds at different elevations; Feldman, Freed, and Cann 1995).

Relatedness, Inbreeding, and Inbreeding Depression

Inbreeding, as defined as the mating between close relatives ($r > 0.125$, sometimes called consanguineous mating), is relatively rare in birds and mammals in nature (Ralls, Harvey, and Lyles 1986), although the preference to mate with relatives has been found in some captive bird studies (e.g., Bateson 1982). Interestingly, in the one case (the Splendid Wren, *Malurus splendens*) in which Ralls et al. note a very high rate of inbreeding in birds in nature (19.4 percent of pairings based on observed pedigree), the species has turned out to have a high rate of extra-pair fertilization that effectively cancels out the putatively high degree of inbreeding (Brooker et al. 1990). This is an example of molecular data invalidating an incorrect claim of high inbreeding without inbreeding depression in a wild population. There are also a number of examples in which calibrations of molecular data with relatedness of individuals (e.g., Rave et al. 1994; Haig and Avise 1996) have been applied to determine inbreeding levels.

The usually negative fitness consequences of inbreeding (known as inbreeding depression) have long been known by people who conduct captive breeding of livestock, laboratory animals, and zoo animals (Falconer 1981; Ralls, Ballou, and Templeton 1988; Lacy, Petric, and Warneke 1993). Inbreeding effects can be even more pronounced in studies in which outbred animals are brought in from wild populations and bred in captivity (Lynch 1977; Lacy, Petric, and Warneke 1993). There is also considerable variability in the incidence and impact of inbreeding depression among different taxa in captivity (Ralls, Ballou, and Templeton 1988; Frankham 1995a,b; Lacy, Alaks, and Walsh 1996), and some of this variability may be related to the degree of prior inbreeding and thus the "purging" of deleterious recessive alleles (but see Frankham 1995a). Molecular markers such as VNTRs have also been used in studies of captive birds to show relationships between the genetic similarity of pairmates and their reproductive success (e.g., in parrots and Hawaiian geese: Brock and White 1992; Rave et al. submitted).

There are few data that directly reveal inbreeding depression in wild populations (Lacy, Petric, and Warneke 1993). One recent exception is from a study of song sparrows (Keller et al. 1994) in which outbred individuals had greater survivorship during a natural bottleneck than inbred ones. A rather ingenious study by Jiminez et al. (1994) demonstrated that inbred field mice (*Peromyscus polionotus*) survived less well following release than inbred lines remaining in captivity and all outbred controls. The general prediction is that inbreeding depression should be more severe in nature (Frankham 1995b), where conditions are generally more stressful than in captive environments (in which food, medical care, and mates are provided more or less *ad libitum*)) and where inbreeding may generally be rare because of intrinsic inbreeding avoidance mechanisms (Blouin and Blouin 1988), and thus little or no purging of deleterious alleles occurs.

Can inbreeding impact fitness to the extent that it causes the extinction of populations? Contrasting theoretical results have been obtained from Lande (1988) on the one hand and Mills and Smouse (1994) and Frankham (1995b) on the other; however, recent studies by Lande (1994) and Lynch, Conery, and Burger (1995) have indicated that "mutational meltdowns" are likely to have a very strong impact on fitness and may have greater impacts on population survival than demographic or even environmental stochasticity. There are few direct empirical assessments of this question, and none that I know of in birds. Frankham's study (1995b) of inbred strains of *Drosophila* and mice provides the clearest direct demonstration that inbreeding can directly affect rates of extinction of lines, but there are a huge number of indirect studies in the agricultural and laboratory genetics literatures that strongly suggest such a relationship. In particular, because the theory is so complex and contentious, this is one area of research where additional direct empirical tests are of critical importance (Frankham 1995a).

Genetic Variability and Structure

Genetic variation is thought to be important to the probability of population extinction at both proximate and ultimate levels (Soulé 1980). In the former, variability is thought to be important because of mechanisms that increase individual fitness, such as heterozygote advantage or frequency-dependent selection (e.g., in disease resistance genes). In the latter, standing genetic variation is important because it allows a population to respond more rapidly to selection than if mutation is the only source of new variability; i.e., it is required for adaptation to occur. Lastly, in order for higher levels of evolutionary change to have an opportunity to occur (i.e., cladogenesis) the structuring of genetic variation should also be conserved.

The evidence for the importance of heterozygosity to proximate fitness is mixed, with a few studies showing a strong, clear relationship, and perhaps most showing little or no direct impact (see Mitton 1994 for review). In addition, a recent meta-analysis of the available data relating fitness to heterozygosity revealed only weak support for such a relationship (Britten 1996).

Particular criticisms have been raised (Lande 1988; Caughley 1994) and countered (Lande 1994; Lynch, Conery, and Burger 1995; O'Brien 1994; Hedrick et al. 1996) about theory concerned with the genetic impacts within the "small population paradigm": that is, do small or endangered populations have reduced genetic variation, and does this reduction significantly impact short- or long-term survival of populations? There are clearly cases in which apparently healthy avian populations have extremely low allozyme variability (e.g., see review of allozyme studies in Haig and Avise 1996). Given the low mutation rate in allozymes, it might not be surprising that these populations could harbor significant amounts of quantitative genetic variation for fitness in spite of low allozyme variability. Perhaps better markers to reflect polygenic variation may be hypermutable VNTRs (Fleischer, Fuller, and Ledig 1995; Hedrick et al. 1996), which have mutation rates nearer to those of quantitative trait loci. However, several studies have revealed high similarity among individuals and thus low variability for VNTRs (e.g., Blue Duck [*Hymenolaimus malacorhynchos*], Triggs et al. 1992; Nene [*Branta sandvicensis*], Rave et al. 1994; Light-footed Clapper Rail [*Rallus longirostris*], Fleischer, Fuller, and Ledig 1995; Black Robin [*Petroica traversi*], Ardern and Lambert 1997), and yet these populations are currently surviving if not thriving. One should note that there is often an unrealistic expectation that when populations get very small (bottlenecks to the tens or hundreds of individuals), they will go extinct. We need only to look at the current population sizes and fitnesses of species for which small founder populations were introduced, such as House Sparrows (*Passer domesticus*) or Red-whiskered Bulbuls (*Pycnonotus jocosus*) in Hawaii (Long 1981), to see that this is not the case. In fact, reduced population size and reduced genetic variation only imply a greater probability of reduced fitness and population survival, rather than guaranteeing it (Hedrick and Miller 1992; Frankham 1995a).

Estimation of Demographic Variables

Because the levels and patterns of variability in neutral genes (i.e., those not subject to selection) are determined by the population processes of mutation (μ), migration (m), and genetic drift, the analysis of molecular genetic or quantitative trait variation can provide "indirect" estimates of demographic parameters such as m, and N_e and their direction of change (Slatkin and Maddison 1989; Tajima 1989; Hudson 1990; Donnelly and Tavaré 1995; Wakeley and Hey 1997; but see Moritz 1994a and Rand 1996 for caveats). Models can be directly analytical or can use simulations to obtain parameter estimates (e.g., Neigel, Ball, and Avise 1991; Slatkin and Barton 1989). Coalescent, or genealogical, methods (Donnelly and Tavaré 1995) are particularly powerful in that they take advantage of all of the information in a set of molecular data (i.e., both the frequency of an allele

and its divergence from other alleles), and they can provide historical information on population change and migration largely independent of mutation rates. Such models combined with better methods of tree construction are revolutionizing population genetics and have considerable application in conservation.

Applying these models invariably requires assumptions, some of which need to be met more stringently than others. One assumption of most models is that populations have reached genetic equilibrium (with regard to mutation, drift, and migration). Assuming equilibrium conditions can be problematic for populations that fluctuate greatly, are recently founded, or that have been recently impacted by humans (like many endangered ones). A second assumption of such models is the mode of mutation acting on the markers under study (e.g., infinite alleles, stepwise mutation; Nei 1987).

A third assumption for which violation can confound estimates of demographic variables is neutrality of alternative variants (Rand 1996). For example, gene diversity can be calculated from pairwise sequence divergence (π) or from the number of variable (segregating) sites under a stochastic model (θ)(Nei 1987). These should be equivalent (and equal to $4N_e\mu$) under equilibrium conditions, but a value of π significantly less than θ may indicate either a recent population decline or an episode of directional selection (Tajima 1989). Determining the liable alternative may be resolved by comparing π and θ for more than one unlinked marker (e.g., Tajima 1989; Rand 1996): it would be very unlikely for unlinked loci to show the same signature of selection, but all loci should reveal the impact of a population bottleneck. Anomalous genes may be under selection and can be excluded from estimating demographic parameters.

A variety of models have been used to assess effective population sizes and gene flow rates in birds. For example, Fleischer, Fuller, and Ledig (1995) used minisatellite data and the analytical models of Lynch (1991) to estimate N_e and the effective number of migrants (N_em) among endangered populations and subspecies of the Clapper Rail in California. A coalescent approach was used with an mtDNA control region sequence by Edwards (1993) to estimate and provide evidence for long-distance gene flow among populations of the Grey-crowned Babbler (*Pomatostomus temporalis*).

Evolutionarily Significant Units, Hybridization, and Phylogenetic Triage

Sometimes decisions have to be made about what constitutes a unit to be conserved. In essence, this results in two problems: how to define a unit for evolutionary or management significance, and how to determine whether such a unit is "worth" the investment of resources required to recover or conserve it.

Several definitions of "evolutionarily significant unit" have been proposed since the term was hesitantly coined by Ryder (1986). In general, an ESU is a population or group of populations that has had an independent evolutionary trajectory through time, which should be evidenced by some degree of genetic differentiation from other such populations. The keys to designating ESUs are in the analytical methods used to hierarchically order individuals into such units and in how much differentiation should exist among units.

A number of approaches to making ESU determinations have been developed (e.g., Ryder 1986; Barrowclough 1992; Vogler and DeSalle 1994; Moritz 1994b). The most stringent essentially applies the phylogenetic species concept (Vogler and DeSalle 1994): that is, an ESU is "delimited by characters that diagnose clusters of individuals to the exclusion of other such clusters." Thus, any fixed, alternative difference between taxa makes them "diagnosable" as a distinct unit. This cladistic approach is the most straightforward and perhaps logical, but it is not without difficulties. For example, under this definition a "single nucleotide change in a DNA sequence" defines an ESU. From models of genetic drift we know that small populations can diverge and fix variants quite rapidly (the number of generations required to fix a new mutant is directly proportional to the effective population size, i.e., $4N_e$). Thus if populations become fragmented to small N_e by recent human impacts, some alternate genetic variants may "fix" in a very short period of time. We would then be delineating an "unnatural" ESU, one based on an evolutionarily unimportant and anthropogenically originated event (e.g., Fleischer, Tarr, and Pratt 1994).

Moritz (1994b) defined ESUs as requiring reciprocal monophyly of taxa in an mtDNA phylogenetic tree along with evidence of genetic differentiation at nuclear loci. This, like Vogler and DeSalle's method, uses phylogenetic methods to avoid the question of how much divergence is enough? But should we avoid that question? Some differences that define ESUs could be trivial, either because they do not truly reveal prehistoric patterns of diversity and levels of gene flow, or because they would not result in any biological problems for the taxa should they be managed as a single unit (e.g., Zink and Kale 1995). One way to address the how much question is to assay highly variable markers in nonfragmented populations of the same species or closely related members of the same genus (e.g., Tarr and Fleischer 1995). Divergence between putative ESUs may not be important if it is significantly less, for the same markers, than variability within the relative. Examination of genetic markers from museum or subfossil material may also reveal that genetic change has resulted from recent, anthropogenically induced, population change. Moritz (1994b) also defined management units as "populations with significant divergence of allele frequencies . . . regardless of the phylogenetic distinctiveness" and suggested that they be considered equivalent to "stocks" in wildlife biology.

Another problem with ESU and MU approaches, especially for species that view the world as a fine-grained entity (like most birds), are the rationales for an ESU approach and its management implications. These include:

1. Mixing of ESUs or MUs could result in reduced fitness of the hybrids (outbreeding depression) because of local adaptation and/or co-adapted gene

complexes. There is little evidence for either phenomenon in avian or other vertebrate species (Hedrick and Miller 1992; Ballou 1995). For example, both the survival of many bird species introduced to new ranges and environments (Long 1981) and the relatively high levels of gene flow found in most avian taxa (e.g., Barrowclough 1980; Rockwell and Barrowclough 1987; Fleischer and Rothstein 1988) argue against local adaptation. Even hybridization between fairly divergent populations or subspecies rarely appears to result in significant outbreeding depression, both in the few bird species assessed (e.g., Rhymer and Simberloff 1996) and in a sizeable sample of subspecies or population crosses of mammals in captivity (Ballou 1995; Frankham 1995a). These factors may be more problematic for plants, invertebrates, and lower vertebrates, which appear to have greater potential for local adaptation.

2. Several small populations can maintain more variability than a single large one with the same number of individuals because drift in small populations will fix alternative alleles (Varvio, Chakraborty, and Nei 1986; Nei and Takahata 1993). However, if fitness is impacted by small size and low heterozygosity in the subpopulations, this mode of management may be detrimental.
3. Behavioral isolating mechanisms alone may be insufficient to maintain distinct species. The high level of hybridization and introgression among fairly differentiated avian taxa (e.g., summarized in Rhymer and Simberloff 1996) suggests that most avian subspecific taxa may not have behaviors different enough to cause problems with reproductive isolation.
4. Preservation of "natural" patterns of variation and processes of evolution may have far-reaching consequences. Perhaps a "philosophical" rationale—but do patterns we find today reflect structure after human impacts? What if preservation of ESUs impacts the probability of survival of one or both ESUs (Ryder 1986)? On the other hand, what if ESUs are weakly defined and protected under the endangered species act principally to protect vulnerable habitats? We must be careful not to abuse this approach until the ESA changes to allow it, as trivial designations, or ones that can be easily challenged, may make us appear biased or fickle in the eyes of management agencies, congress, and the public.

Another problem for defining conservation units and survival of avian endangered species is hybridization (Rhymer and Simberloff 1996). How should hybrid individuals or populations be managed, especially if only one of the hybridizing taxa is endangered (e.g., O'Brien and Mayr 1991)? Each case may require independent evaluation as to the level of introgression reached. Habitat changes and species introductions have greatly modified ranges, resulting in greater opportunity for hybridization. Molecular genetic methods are proving useful for measuring the extent of hybridization (Rhymer and Simberloff 1996).

Phylogenetic information can also help in allocating effort and resources to endangered species recovery. This approach is sometime called phylogenetic triage after the method of culling hopeless cases from wartime casualties (Barrowclough 1992), and decisions depend on how phylogenetically unique a taxon is relative to other surviving taxa. Several quantitative measures of uniqueness have been devised and applied (e.g., Vane-Wright et al. 1991; Krajewski 1994). Avian examples of triage ask whether more resources should go into preserving barely differentiated, but endangered, populations of a common species—e.g., the Common Amakihi (*Hemignathus virens*) on Molokai (Tarr and Fleischer 1993); Mississippi populations of Sandhill Crane (*Grus canadensis*) (Krajewski 1994); and San Clemente Island Loggerhead Shrikes (*Lanius ludovicianus mearnsi*) (Mundy, Winchell, and Woodruff 1997)—or into preservation of phylogenetically (not to mention morphologically and ecologically) unique species on the brink of extinction (e.g., the California Condor [*Gymnogyps californianus*], Poouli [*Melamprosops phaesoma*], and Spix Macaw [*Cyanopsitta spixii*]). Clearly other factors enter into triage decisions, including, how likely is recovery of the taxon, how attractive or important it is to the public, and how might preserving a species result in umbrella protection for other organisms.

The Future?

With fears about incorrect prognostication thrown aside, I attempt below to discuss advances in theory and technology that may, in the future, be applied to particular cases in avian conservation biology.

Better Models and Faster Molecular Methods

As noted above, population genetics is undergoing a revolution as new theories and analytical methods are being applied to large databases derived from faster molecular techniques. In particular, coalescent models and simulations that estimate age of most-recent common ancestor, effective number of migrants, and effective population sizes are able to maximize information from DNA sequence data (Slatkin and Maddison 1989; Donnelly and Tavaré 1995; Wakeley and Hey 1997). Population viability models also need improvement, with impacts of low genetic variability and inbreeding on extinction probabilities more accurately incorporated (Lande 1994; Lynch, Conery, and Burger 1995; Frankham 1995b).

Molecular methods of analysis have become incredibly rapid and efficient during the past ten years (primarily with the advent of PCR), and there is no doubt that the methods will become even faster during the next ten years. In fact, technologies have already changed to the point that many complex protocols are automated via robotics, and "digitally" assayed human genetic markers (e.g., DNA coated "chips"; Schena et al. 1996) are in development. We may soon be

able to electronically "type" thousands of DNA variants per individual in very short periods (approaching the tricorder of *Star Trek* fame!).

Another recent technological advance with potential application to conservation of birds is the analysis of "ancient DNA." For example, mtDNA sequences from crane museum specimens were used to directly assess loss of variability associated with a population bottleneck (Glenn 1997), and DNA sequences from subfossil bones have been used to confirm that Laysan Ducks (*Anas laysanensis*) recently existed on islands on which they are now extinct (Cooper et al. 1996). Lastly, hypervariable markers such as VNTRs appear very useful in assays of the mutational effects of environmental pollutants (e.g., in Herring Gulls, *Larus argentatus*; Yauk and Quinn 1996).

Genetic Engineering and Immunogenetics

For a relatively small number of cases there may be genetically based problems that impact individual fitness and population survival. In these cases, advances in gene therapy and genetic engineering may prove useful. Although their routine use is probably more than five or ten years away, I speculate here about how such methods may eventually be applied.

Some cases may involve genetic disease. VNTR and RAPD markers are proving extremely valuable for gene mapping and they are also useful, along with other approaches, for gene identification (e.g., Georges et al. 1993; Ferraris and Palumbi 1996; Ghosh and Collins 1996). One avian disease that may have a genetic basis is hemochromatosis (iron storage disease), evidenced by the circulation of excess iron in the blood and in the liver and most commonly observed in frugivorous species in captivity (Kincaid and Stoskopf 1987). If a simple genetic basis for absorption of iron can be isolated, it may be possible to clone it and conduct gene therapies to correct the problem. Molecular "fixes" perhaps should initially be engineered into somatic cells, so they can be easily reversed if problems later arise.

Another future application of genetic engineering methods is to modify genetic systems that confer resistance to infectious disease. Drepanidines (Hawaiian Honeycreepers) have been greatly impacted by introduced disease, along with other factors (van Riper et al. 1986). Currently, only twenty-four (of fewer than fifty) species exist, and of these only eight are not endangered. Infectious disease is considered to be a major limiting factor in drepanidine distribution, especially the introduced mosquito-transmitted disease, malaria, caused by *Plasmodium relictum*. Many honeycreeper species do not occur, or occur in very small numbers, at lower elevations where mosquito density is high. Most species at higher elevations appear to lack natural resistance to malaria (van Riper et al. 1986; Atkinson et al. 1995), and mosquitoes may be gradually moving upslope. Common Amakihi occur at low and high elevations on several islands, and half or more survive malarial infection in controlled challenge experiments (including presumably "unchallenged" individuals from high elevations; Atkinson et al. 1995). Such resistance appears to be genetically based, and Jarvi, Fleischer, and Atkinson (unpublished) are attempting to identify this basis by searching for linkage disequilibrium between malaria resistance and variants of: (1) MHC proteins (which do appear to provide some resistance to infectious disease in chickens and to malaria in humans [Hill 1996]); and (2) microsatellite markers (Ghosh and Collins 1996). If we can locate the genetic complexes that confer resistance in the Common Amakihi, it may be possible in the future to develop gene therapies or even gene transfers to susceptible, endangered drepanidines. Another fanciful application is to engineer genes for rejection of parasitic eggs (if these exist in a simple, clonable form) from rejector species (e.g., Eastern Warbling Vireos, *Vireo g. gilvus*) to closely related acceptor species impacted by brood parasitism (e.g., Western Warbling Vireos, *V. g. swainsoni*; Sealy 1996).

The recently described method of cloning sheep from adult cell lines (Wilmut et al. 1997) may also present some opportunities for conservation biology. For example, founders of a captive population that are senescent or ill and will not breed in captivity could be cloned and maintained for future breeding into the population. An individual that shows resistance to an epidemic disease could be cloned and the clones used to "swamp" the population with disease-resistant genes. In this light, it may have been remarkably prescient of zoo geneticists to initiate cell lines from different endangered species and store them in "frozen zoos" since the late 1970s (Benirschke, Lasley, and Ryder 1980).

The biotechnology applications described above should enhance conservation efforts for only a small number of endangered species. They will also genetically alter the species, if only by a small amount. Numerous questions have been raised about the ethics and safety of genetic manipulations of humans and their agricultural products. Such issues have not yet been considered intensively for the prospect of genetic engineering in wildlife that are then released into nature, but I imagine this will change in the near future.

Acknowledgments

I would like to thank Cheryl Tarr, Sabine Loew, Jon Ballou, Kathy Ralls, Matt Hamilton, Judith Rhymer, Susan Jarvi, Carl McIntosh, Bob Zink, and Dina Fonseca for enlightening discussions on many of the topics included in this chapter, and Cheryl Tarr, Carl McIntosh, and Pedro Cordero for examples of their lab work for display in figures. Sue Haig and John Marzluff provided feedback on an earlier draft. The Smithson Society, Friends of the National Zoo, National Biological Service, National Geographic Society, and National Science Foundation funded original research illustrated in this chapter.

Avian Conservation

Research and Management

Edited by John M. Marzluff
and Rex Sallabanks

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