Evolutionary units in wildlife: a case study of the bald eagle
by Catherine Raven

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biological Science
Montana State University
© Copyright by Catherine Raven (1999)

Abstract:
The evolutionary unit is a Darwinian concept acknowledging the subspecific process of evolution. Any species characterized by nonrandom mating and unequal selective pressure may evolve on a sub-specific, not a species level. Are bald eagles evolving as a species, or are subspecific groups on separate evolutionary paths? I present a model of evolutionary separation occurring among the bald eagles inhabiting the American southwest. The model is based on phenotype, behavior, ecology, and geography. Reproductive isolation can be further clarified by examining patterns in the chemical units of heredity. The most useful units are hyper-variable simple repeats. Simple repetitive DNA is believed to be selectively neutral and have a high mutation rate. Although the project was not funded for molecular work, I created a DNA library with bald eagle genomic DNA, designed probes to screen the library for di-nucleotide repeats, and screened about 300 clones, each containing 300-600 bases of recombinant DNA. I isolated and sequenced an interrupted (dCA)n simple repeat. The repeat is (CA)8 N10(CA)15. I designed primers for the flanking regions. The primers amplify alleles in bald eagles in a polymerase chain reaction using nucleotide incorporation. The primers do not bind unspecified sequences. Six alleles are visible on nondenaturing polyacrylamide when stained with SYBR green. Funding expired before a significant number of samples could be amplified. The most important contribution is that researchers who lack the aptitude and technical ability to design their own primers, but have time, money, and lab space, can do publishable work with very little additional time and money. The system can be used to compare genetic variability among populations, and follow the genetic effects of translocations. These markers can also be used to trace the reproductive success of the Texas male bald eagle which was the first non-Arizona hatched bird believed to have bred in Arizona in modern times. Additional funding will allow several more variable regions to be sequenced and protocols developed for amplifying DNA from both bald eagles and related Accipiters.
EVOLUTIONARY UNITS IN WILDLIFE:
A CASE STUDY OF THE
BALD EAGLE

by

Catherine Raven

A thesis submitted in partial fulfillment
of the requirements for the degree

of

Doctor of Philosophy

in

Biological Science

MONTANA STATE UNIVERSITY
Bozeman, Montana

April 1999
© COPYRIGHT
by
Catherine Raven
1999
All Rights Reserved
APPROVAL

of a thesis submitted by

Catherine Raven

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

4/30/99
Date

Chairperson, Graduate Committee

Approved for the Major Department

4/30/99
Date

Head, Biology Department

Approved for the College of Graduate Studies

4-2-99
Date

Graduate Dean
STATEMENT OF PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a doctoral degree at Montana State University-Bozeman, I agree that the Library shall make it available to borrowers under the rules of the Library. I further agree that copying of this thesis is allowable only for scholarly purposes, consistent with "fair use" as prescribed in the U.S. Copyright Law. Requests for extensive copying or reproduction of this thesis should be referred to University Microfilms International, 300 North Zeeb Road, Ann Arbor, Michigan 48106, to whom I have granted "the exclusive right to reproduce and distribute my dissertation in and from microform along with the non-exclusive right to reproduce and distribute my abstract in any format in whole or in part."

Signature  Cathleen Casey

Date  4/30/99
ACKNOWLEDGMENTS

While waiting for funding to continue Lance Craighead’s grizzly bear studies, I spent eight months as a graduate research assistant at a DOE-Battelle fish and wildlife genetics lab. My assignment was arranged by Dr Jerry Bromenshenk, University of Montana, Missoula. While at Battelle, I applied for a DOE Ph.D. student traineeship. I received the traineeship award which included a one-year stipend and $2,000 in supplies. At the time of the award, Dr. Jon Longmire, Human Genome Project, Los Alamos, New Mexico, agreed to be my DOE-scientist sponsor.

As a DOE graduate research trainee, I spent 5 months working as a visiting researcher at Los Alamos during which time I reapplied for, and was granted, a second and final year of the EPSCOR traineeship (1995-1996). I worked independently while at Los Alamos. Jon arranged for my receipt of eagle DNA samples from Kathy Hobson at the San Francisco Zoo, expanded my repertoire of molecular techniques, and provided a generous amount of lab and office space. Using my DOE supply grant, and contributions from Jon, I purified and sequenced plasmids, designed primers from the sequence data, and amplified bald eagle DNA. I designed all the primers described herein while visiting Los Alamos. The cost of synthesizing these primers was donated by the Human Genome Project.

For the remainder of this project, except for a leave of absence in the summer of 1997, the summer and fall of 1998, and half of the 1999 spring semester, I was supported by the MSU Biology Department. My dissertation committee consisted of Drs. Ernie Vyse, major professor; Matt Lavin and Martin Teintze, reading committee members; Lance Craighead (who also read the entire dissertation), Dave Cameron, Andy Hansen; and Robert Nowierski. This was a very active and helpful committee, and the dissertation has been improved by their comments.

Eagle samples were offered by Al Harmata. I also solicited, and received, samples, and with extensive documentation from Drs. Bill Bowerman, Grainger Hunt, and Dave Best. Cheyenne Mountain Zoo donated griffon vulture blood. All samples were received under U.S. Fish and Wildlife permit number 823439 issued to the author. I refused samples from people who wanted to sell them. The US Fish and Wildlife Service Forensic Labs in Ashland, Oregon, refused to donate samples, choosing instead to wait until my primers were published so they could write grants and use my primers to generate their own funding.
TABLE OF CONTENTS

<p>| CHAPTER 1  | INTRODUCTION TO THE DISSERTATION | .......................................................... 1 |
| References Cited | .......................................................... 4 |
| CHAPTER 2  | EVOLUTION IN THE BALD EAGLE | .......................................................... 7 |
| Introduction | Bald Eagles: Background | .......................................................... 7 |
| Evolution: Background | .......................................................... 8 |
| A Model of Evolution in the Bald Eagle | .......................................................... 10 |
| Materials | .......................................................... 12 |
| Methods | Cloning the Bald Eagle Library | .......................................................... 13 |
| Planning Strategy for the Oligonucleotide Probe | .......................................................... 15 |
| Screening the Library | .......................................................... 17 |
| Visualizing the Microsatellites | .......................................................... 18 |
| Results | Frequency of Microsatellites | .......................................................... 21 |
| Regions Associated with Microsatellites | .......................................................... 24 |
| Use of Microsatellites with Related Raptors | .......................................................... 25 |
| Discussion | .......................................................... 28 |
| References Cited | .......................................................... 31 |
| CHAPTER 3  | INTEGRATING SCIENCE AND MANAGEMENT: EVOLUTION AND THE ENDANGERED SPECIES ACT | .......................................................... 36 |
| Introduction | .......................................................... 36 |
| Methods | .......................................................... 43 |
| Results | .......................................................... 45 |
| Discussion | .......................................................... 51 |
| Recommendations | .......................................................... 54 |
| References Cited | .......................................................... 55 |
| CHAPTER 4  | EVOLUTIONARY UNITS AND CONSERVATION BIOLOGY: ANALYZING THE BALD EAGLE PARADIGM | .......................................................... 59 |
| Introduction | Evolution and Ecology of Sea Eagles | .......................................................... 59 |</p>
<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Repeat regions cloned from bald eagle genomic DNA</td>
<td>20</td>
</tr>
<tr>
<td>2. Distribution of alleles for 21 individuals at locus 6</td>
<td>22</td>
</tr>
<tr>
<td>3. Distribution of alleles at locus 6</td>
<td>23</td>
</tr>
<tr>
<td>4. Non-random sequences associated with bald eagle microsatellites</td>
<td>26</td>
</tr>
<tr>
<td>5. Taxon involved in actions from 1995 through 1999</td>
<td>48</td>
</tr>
<tr>
<td>6. Reproductive isolation within distinct population segments</td>
<td>49</td>
</tr>
<tr>
<td>7. Reproductive isolation within DPS listings</td>
<td>51</td>
</tr>
<tr>
<td>8. Species recorded with their Evolutionary Units</td>
<td>54</td>
</tr>
<tr>
<td>9. Characteristics of <em>Haliaeetus</em> species</td>
<td>60</td>
</tr>
<tr>
<td>10. Survey of Nei's distances</td>
<td>75</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Sequenced microsatellite regions that were not primed</td>
<td>24</td>
</tr>
<tr>
<td>2. Principal Components of 1990 isozyme frequencies</td>
<td>73</td>
</tr>
<tr>
<td>3. First two principal components of 1985 allozyme frequencies</td>
<td>74</td>
</tr>
</tbody>
</table>
ABSTRACT

The evolutionary unit is a Darwinian concept acknowledging the sub-specific process of evolution. Any species characterized by nonrandom mating and unequal selective pressure may evolve on a sub-specific, not a species level. Are bald eagles evolving as a species, or are subspecific groups on separate evolutionary paths? I present a model of evolutionary separation occurring among the bald eagles inhabiting the American southwest. The model is based on phenotype, behavior, ecology, and geography. Reproductive isolation can be further clarified by examining patterns in the chemical units of heredity. The most useful units are hyper-variable simple repeats. Simple repetitive DNA is believed to be selectively neutral and have a high mutation rate. Although the project was not funded for molecular work, I created a DNA library with bald eagle genomic DNA, designed probes to screen the library for di-nucleotide repeats, and screened about 300 clones, each containing 300-600 bases of recombinant DNA. I isolated and sequenced an interrupted (dCA)n simple repeat. The repeat is (CA)_n N_{10}(CA)_{15}. I designed primers for the flanking regions. The primers amplify alleles in bald eagles in a polymerase chain reaction using nucleotide incorporation. The primers do not bind unspecified sequences. Six alleles are visible on nondenaturing polyacrylamide when stained with SYBR green. Funding expired before a significant number of samples could be amplified. The most important contribution is that researchers who lack the aptitude and technical ability to design their own primers, but have time, money, and lab space, can do publishable work with very little additional time and money. The system can be used to compare genetic variability among populations, and follow the genetic effects of translocations. These markers can also be used to trace the reproductive success of the Texas male bald eagle which was the first non-Arizona hatched bird believed to have bred in Arizona in modern times. Additional funding will allow several more variable regions to be sequenced and protocols developed for amplifying DNA from both bald eagles and related Accipiters.
Are bald eagles evolving as a species, or are subspecific groups on separate evolutionary paths? Current scientific debate on the evolutionary unit of species partially inspired my inquiry (Erwin 1991; Mallet 1995; Wayne 1992; Zink and McKittrick 1995). I was especially interested in applying the evolutionary unit, also called the Evolutionarily Significant Unit, to the Endangered Species Act (ESA) (Dizon et al. 1992; Moritz 1994; Nielsen 1995; Rojas 1992; Vogler and Desalle 1994; Waples 1991). The fusion of evolutionary biology and wildland management existed prior to the conception of the Evolutionary Unit.

Evolutionary scientists have addressed issues relevant to conservation (Darwin 1858; Stebbins 1977), and some conservation scientists have recognized the value of applying evolutionary principals.

"I propose, only half in jest, that a license to practice conservation biology be required of all agency personnel involved with decision making in regards to biodiversity and endangered species issues. To be granted this license, some level of understanding would be required about Darwinian evolution by natural selection and all it implies regarding coevolution, coadaptation, and adaptive strategies... (Behnke 1995)."
Chapter 3 explains the ESA and its relationship to the evolutionary unit. The ESA recognizes 3 wildlife taxa: species, subspecies, and distinct population segments. A 1996 Congressional amendment defines the latter as an evolutionary unit of a species. The evolutionary unit is a Darwinian concept acknowledging the sub-specific process of evolution. Any species characterized by nonrandom mating and unequal selective pressure will evolve on a sub-specific, not a species level. The terms, “population” and “deme” describing genetically linked panmictic mating groups, are well understood by biologists (see Chapter 2 in Wilson 1975), but adherence to the traditional definition has been lax. Managers find species easier to understand. Species, unlike populations, are described in reference books. ‘Population’ is confounded by widespread colloquial use.

Congress approved the distinct population clause with hesitance and cautioned discretion in its application. Are distinct populations gaining acceptance as a listing taxon? Are distinct populations being defined according to scientific protocols for evolutionary units? I used the definition of evolutionary unit developed by the National Marine Fisheries Service: "Evolutionarily significant units are substantially reproductively isolated, and represent an important component in the evolutionary legacy of the species (Waples 1991)."

I chose to use the bald eagle to examine the concept of the evolutionary unit. All species are evolving. Why study the evolution of the bald eagle? Few endangered or threatened taxa are evolving on a subspecific level. Many are too
reduced to consist of more than one effective population (Shilling 1997; Wilcove et al. 1993; Erwin 1991). Some species have so few remaining individuals that random genetic drift has removed alleles at many loci. In contrast, bald eagles, although a threatened species, may be expanding their range southward.

Applying the evolutionary unit framework requires a delineation of the populations within species. This study started out to determine those deme boundaries. After beginning my study, the U. S. Fish and Wildlife Service (FWS) announced by way of a de-listing procedure, that the demes of bald eagles were known and described in the Congressional Record (USFWS 1995). The opinion of the FWS, that the entire species of bald eagles in the lower 48 comprise a single mating unit, has never been contested.

Why continue to pursue a question that has already been answered? Primarily I was concerned with the implications of the bald eagle listing decision. The designation of a single mating unit justified the commencement of artificial augmentation of wild populations, at a cost of over 1 million dollars per year. In addition, in Chapter 3 I show that most listing decisions involving taxa below the species level involve errors in judgement.

In Chapter 3 I analyze data available at the time of the bald eagle downlisting, and document that the decision-making process was faulty.

In Chapter 2 I develop my hypothesis of bald eagle demes. The hypothesis is based on behavior, ecology, and demographics following principles developed for other species (Barlow 1995; Lande 1988).

Also in the second chapter, I discuss the pursuit of microsatellite DNA as
a means of establishing patterns of reproductive isolation within the bald eagle species. As this project began, it was known that the isozyme techniques developed in the 1970's and 1980's rarely revealed sufficient variation for avian population studies (Ball and Avise 1992; Barrowclough 1992). Clearly, new molecular markers were needed, and microsatellites seemed the most reasonable new marker to develop. Microsatellites are di- and trinucleotide repeats that are hypervariable in the human genome (Stallings et al. 1991). Microsatellites have become popular for revealing more genetic variation than other molecular techniques (Amos and Pemberton 1992; Buchanan et al. 1993).

Almost nothing was known about microsatellites in any avian species at the beginning of the project, and very few people in conservation biology had actually cloned microsatellites for any wildlife taxa (Brooker et al. 1994). Microsatellites cloned by the chicken genome project were not expected to provide primers for the bald eagle genome (Burt et al. 1995).

References cited


Barlow, G. W., 1995 The relevance of behavior and natural history to evolutionarily significant units, pp. 169-175 in J. L. Nielsen, editor. Evolution and the aquatic ecosystem: defining unique units in population
conservation, American Fisheries Society Symposium 17, Bethesda, MD.


Nielsen, J. L., editor. 1995 Evolution and the Aquatic Ecosystem: Defining


USFWS, 1995 Final rule to reclassify the bald eagle from endangered to threatened in all of the lower 48 states. Federal Register 60: 36000 - 36010.


CHAPTER 2

EVOLUTION IN THE BALD EAGLE

Introduction

Bald Eagles: Background

Bald eagles (*Haliaeetus leucocephalus*) are one of 8 species in the Holarctic sea eagle genus. Among sea eagles, bald eagles are most closely related to temperate species, and further removed from the tropical species (Seibold and Helbig 1996). On occasion the temperate species are sympatric, though no hybridization events have been recorded (Bent 1938). Bald eagles are the only sea eagles native to the New World, where they have historically been restricted to North America. If central Mexico harbors resident eagles (USFWS 1995), this is a recent change in distribution (Bent 1938). Bald eagles range north to the Arctic Circle in Canada and Alaska, south to the Gulf of Mexico and Baja California, east to the Atlantic Coast, west to the Pacific Coast, and throughout riparian zones in continental North America. Because they are dependent on water, their natural distribution is patchy. Nesting areas are
concentrated along the Great Lakes, Chesapeake Bay, the Florida peninsula, coastal South Carolina and Louisiana. Sea eagles are considered resident species, although many bald eagles must migrate to remain near open water year round.

Breeding begins between the ages of 5 and 8, and continues until death at 30-35 years. The northernmost populations are the longest lived. Bald eagles are considered monogamous. Mate replacement occurs upon death of a partner. Up to 4 eggs are produced each year in a single clutch. The chicks fledge within 3 months, and permanently leave the nest soon after.

In 1782, when the bald eagle was adopted as our national symbol, between 25,000 and 75,000 bald eagles lived in the conterminous States (University of Nevada-Reno 1998; USFWS 1998). Alaska must have supported a larger pupation since bounties were paid on 50,000 - 70,000 bald eagles between 1917 and 1936 (Bent 1937). The population in the lower 48 dropped to 450 nesting pairs by the 1960's. The current census is 4,500 nesting pairs. The upswing is due to a reduction in the use of DDT and widespread reintroduction coupled with captive breeding programs.

**Evolution: Background**

Evolution may be defined as changes in a population's allele frequencies which result in increased adaptation to the environment (Hartl 1988, Chapter 2). Evolution is also defined as “changes in the (heritable) properties of populations that transcend the lifetime of a single individual” (Futuyma 1986, Chapter 1).
The changes may or may not increase adaptation. Because many environmental conditions are stable, some lineages will become better adapted with time. Alternatively, conditions may change more rapidly than hereditary adaptation permits, and some lineages will disappear. Both processes are difficult to observe. Evolution is cryptic because many conditions must exist before allele frequencies show an adaptive response. Environmental variables must remain stable long enough for genotype frequencies to respond. The boundaries of these environmental variables must overlap, with a great deal of accuracy, the boundaries of breeding groups.

Species are groups of organisms that interbreed. Therefore, breeding groups, broadly speaking, are species. The boundaries of many species are so vast that it is not likely that all members are equally likely to breed with each other. Alternatively, the boundaries may be separated by physical barriers that prevent interbreeding among all members. So species are often arranged into smaller active breeding groups, called demes. Whether called demes, populations, or stocks, these are the units within which evolution occurs. Colloquial usage has driven the terms “population” and “deme” far from their original meanings. “Stocks” is rarely used outside the fisheries community. The term “evolutionary unit” accurately describes the breeding group within which evolution occurs.

Demes can evolve only in the presence of genetic variation. Genetic variation may result from mutation or migration of individuals with different genotypes (Dobzhansky 1937). Gene flow at low levels may increase variation
and enhance evolutionary potential. High levels of inter-deme gene flow may disrupt natural selection because the link between inheritance and ecology is broken.

A Model of Evolution in the Bald Eagle

Bald eagles may not be evolving as a species. Eagles in the southwestern United States appear likely candidates for a population on a separate evolutionary path as they differ in habitat occupied and life history traits. Centered in Arizona along the Salt and Verde Rivers, this population numbers about 60 breeding pairs. Migration into the population is probably less than one effective migrant per generation (Chapter 3), and therefore adaptive traits may develop within this population.

Size differences are most likely a mating barrier for the southwestern bald eagles because they are significantly smaller than eagles to the north (Hunt et al. 1992a). Females normally mate with smaller males, so southwest females are unlikely to mate with males from any other population. In fact no male from outside the southwest has ever been observed mating in this region (Hunt et al. 1992a). The final requirement for evolution, genetic variation, is present in this population even among slowly evolving genes (Chapter 3).

The most significant life history distinction is the early breeding age, a full year earlier than the rest of the species. This suggests limited gene flow into the population. If mature birds from outside were moving in, subadults would not have the opportunity to breed. Secondly, this hints at neotenic development that
may have occurred on 2 other occasions among sea eagles (Seibold and Helbig 1996). Finally, the shorter relative generation time, means that gene flow is less effective at homogenizing this population and more likely that allelic frequencies reflect natural selection (Wright 1931).

One way to validate the model of subspecific evolution within the bald eagle is by examining genetic relatedness using measures of genetic distances (Nei 1972). Isozymes evolve slowly in the bald eagle, and in birds overall (Morizot et al. 1985; Barrowclough and Corbin 1978; Selander 1971). Only about 10% of isozyme loci examined in avian species have been polymorphic (Barrowclough and Corbin 1978). Microsatellites evolve more rapidly, but may evolve so rapidly that even a gene flow of 1 effective migrant per generation will not homogenize the species (Slatkin 1987 and 1995).

In this paper I ask whether microsatellites may provide a means to investigate the possibility of population structure in the bald eagle. I hoped to use microsatellites to test the null hypothesis that there is no genetic difference between southwest bald eagles and other eagles. Studies of microsatellites show a large discrepancy in levels of variability between taxa (Brooker et al. 1994). Few surveys of avian microsatellite variability exist (Brooker et al. 1994) and no microsatellite primer sequences have been published for any Falconiformes.

Other researchers have explored the possibility that the bald eagle is a polytypic species. Hansen suggests differences between "pristine and man-altered environments " may exist (Hansen and Hodges 1985). DNA
minisatellites, tandem repeats of 15 - 30 basepairs, have been unable to shed light on evolutionary patterns in bald eagles (Hunt et al. 1992b). Morizot et al. (1985) suggested a north-south cline of allele frequencies based on his exploratory study with 3 polymorphic isozyme loci. Only 5 of the individuals in the Morizot study were from Arizona. This same study also suggested the possibility that natural selection is responsible for the allele frequencies in the Arizona population. No prior study has shown the existence of separate population units within the species.

**Materials**

For the DNA library, I removed tissue from the frozen carcasses of banded bald eagles from the Northern Rockies. The birds were in temporary storage at the Montana Department of Fish Wildlife and Parks. DNA was extracted from blood samples from birds hatched in Canada, Florida and Arizona. Samples were between 10 and 15 years old, and stored in unknown solutions. Various organic extraction methods were used. The isolated DNAs were quantified and qualified. Spectroscopy confirmed the removal of protein and RNA, and the concentration of DNA. Size fractionation on 1% agarose confirmed high molecular weight DNA.
Methods

Cloning the Bald Eagle Library

Bald eagle DNA libraries were created following published protocols, with modifications, for blunt (Rassmann et al. 1991) and adhesive ends (Ostrander et al. 1992). The bald eagle and population vector DNAs were prepared, ligated, and electroporated into *E. coli*.

The SURE strain of *E. Coli* was chosen to carry the library because it maintained repetitive sequences longer than other cell lines (Raven, unpublished observations). The SURE phenotype was confirmed before each competence treatment by streaking on a tetracycline plate. Plates were LB/agar with 13 ug/ml tetracycline. A single colony was selected from the tet+ plates and grown in 2 ml. LB for 7 hours at 37°C at 300 rpm. This culture was diluted 1:100 into LB and incubated another 5 hours until the optical density at 600 nm was 0.4. Cells were washed according to standard protocols (Speyer 1990). The final pellet was diluted 1:1 with GYT and transformed within 1 hour.

The competency of the cells was tested with supercoiled plasmid (pUC) to produce 10⁵ or 10⁷ colonies per microgram of plasmid DNA. No transformations were attempted with competencies below 10⁷. Reagents used to prepare the stock concentration of the supercoiled plasmid were not necessarily equivalent to reagents used in preparation of the insert and vector DNAs. Undoubtedly, these reagents, especially alcohol and agarose, contain contaminants which decreased the efficiency of the electroporation. Charged particles affect the
electric current flowing through the cells, and proteins inhibit, or kill, the competent cells.

Both blunt and adhesive end (sticky) libraries were created. For sticky libraries DNA was digested with Sau3AI, and the vector with BamHI. Blunt end inserts were triple digested with Rsa, Alu, and HaeIII, and blunt end vector with Smal. DNA was fractioned on 1.5% FMC NUSIEVE agarose in TAE for the Sau3AI fractions, and 3% for the blunt fractions. Fragments between 300 and 600 base pairs were purified from the agarose. Molecular weight standards were cut from lambda DNA with a variety of enzymes. Size selected DNA was frozen on parafilm and squeeze extracted. The recovered DNA was spun down and washed.

The vector, pUC 18, was cleaved and dephosphorylated. The calf intestinal phosphatase was removed from the vector prior to ligation. The vector and the eagle DNA to be inserted were quantified on 2% agarose prior to the ligation. 200 ng of BamHI digested vector was ligated to 200 - 400 ng of Sau3AI digested insert at 12°C for 24 hours with 200 NEB units of ligase. The ligase was extracted with phenol and chloroform prior to ligation. A sample of the ligation reaction was fractionated prior to electroporation. The pUC/eagle recombinant plasmids were digested with BamHI prior to electroporation to linearize non-recombinants. This is a cost-saving measure which prevents self-ligated pUC from transforming the bacteria cells and obviates the use of X-gal/IPTG on the screening plates.
Cells were electroporated in 50 ul volume with 25 - 100 ng of plasmid DNA using a homemade electroporator (Speyer 1990). The electroporated cells were placed in warm SOC and recovered at 37°C for 1.5 hours. Following recovery, the cells were incubated at 30°C on SOB or 2X LB plates with 100 ug/ml ampicillin.

The colonies were transferred to secondary patch plates, 300 or 400 colonies per plate, along with positive and negative control colonies prior to lifting onto positively charged nylon membranes. Membranes were autoclaved after being cut to fit 8 cm plates. Colonies lifted onto the membranes were denatured with NaOH, neutralized, and wet in 6X SSC. DNA was baked onto the nylon and prehybridized according to standard protocol (Church and Gilbert 1984).

Planning Strategy for the Oligonucleotide Probe

Efficient probes require both a strong signal and a high signal to noise ratio. A strong signal decreases the time required to develop the autoradiograph. Development time can range from 24 hours to 10 days. The quicker the film can be developed, the faster the positive colonies can be cultured. Because the SURE transformants are fragile, long development times may cause colonies to die, mutate, or overgrow. A strong target signal allows the background signal to be strong, too. A visible background is necessary to correctly orient the autoradiograph to the petri dish so that positive colonies can be selected. A high signal:noise ratio with a low signal will have no background, so choosing positively hybridizing colonies is problematic.
The high signal:noise ratio is necessary to detect positives without picking false positives, the sequencing of which is time-consuming and expensive. The signal is increased by increasing the concentration of oligonucleotides in the hybridization solution. This is accomplished by decreasing the volume of the solution, increasing the number of oligos, or adding a filler such as PEG. When the concentration of oligos is too high, a large number of artifacts appear in the autoradiograph. I found the volume could be kept the lowest, without introducing artifacts, by hybridizing in an 8 cm covered glass petri dish.

The signal:noise ratio is increased by decreasing the number of probe:probe hybrid molecules while increasing the number of probe:target DNA hybrid molecules. Hybridization kinetics are difficult to control, because single stranded DNA probes anneal to each other more rapidly than they anneal to target DNA. This effect is magnified with simple repeat probes, as compared with complex sequence probes. To counteract this effect, I synthesized long, variable length probes. When the single stranded probes hybridized to each other, long single stranded “tails” remained free to hybridize to the target DNA.

To create an (AC)\textsubscript{n} probe, I incubated 22 ul of (AC)\textsubscript{25} (240 ng per ul), with 1 ul of T4 kinase for 2.5 hours at 37°C. The kinase was heat killed at 65°C for 20 minutes. I spun out the rATP from the kinase buffer to prevent competition with the [\textsuperscript{32}P] dATP in the labeling reaction. The kinase treated oligo was brought up to 50 ul and 3.5 ul was labeled by incubating with DTT, 10 mM dCTP,
3.3 mM [\textsuperscript{32}P] dATP, and Sequenase (© USB) and incubating at 20°C for 10 minutes. The probe was spun down and brought up to 10 ul in deionized water. To this volume I added ligase and incubated for another 5 hours at 16°C. The probe was then boiled for 3 minutes to separate the strands and added immediately to hybridization solution. I used 10 - 20 ng of labeled probe for each milliliter of hybridization solution. Based on before and after size fractionation with molecular weight standards, I estimated a 25% increase in oligonucleotide would be realized following the extension reaction, such that an initial oligo of 20 ng would be 25 ng when the elongation was completed. When hybridizing in glass petri dishes, 125 ul of hybridization solution was used for each square centimeter of nylon membrane.

Screening the Library

The probe was hybridized to the DNA on the membranes in Church's hybridization solution (Church and Gilbert 1984), and washed in 2XSSC/0.1% SDS at temperatures ranging from 45°C to 56°C, depending on the sequence of the oligonucleotide probe. The molecular weight of sodium phosphate has been recalculated so I used a 0.5 M solution in lieu of Church's 1M solution.

Following hybridization the membranes were exposed on x-ray film for 1-5 days and developed with Kodak GBX developer and fixer. Colonies with a strong radioactive signal were streaked on plates for a second screening. The second screening followed hybridization and probe protocols identical with the
first. Colonies with strong signals relative to the negative controls, were selected for sequencing. The negative controls were a colony with a large poly-dATP region but no microsatellite, and untransformed SURE cells.

Colonies containing plasmids with high signal inserts were incubated until a sufficient quantity of plasmid was extracted. Plasmids were purified on Qiagen Tip 20 columns, and then quantified and qualified prior to sequencing. Plasmids were digested and fractionated to ensure an absence of RNA, chromosomal DNA, and to ascertain the size of the insert.

For cycle sequencing I used unlabeled universal forward and reverse sequencing primers and Perkin Elmer Prism fluorescent dNTPs and a Perkin Elmer 9600 or 480 thermocycler. After sequencing, the reactions were purified on Centri-Sep columns, dried under vacuum and stored at -20°C. Several inserts were read by the Human Genome facility, Los Alamos, New Mexico the remainder by the University of Montana.

I designed primers by hand, e.g. without the use of primer design software. Primers had the following characteristics: no closer than 30 bases to the repeat sequence, no internal homology to prevent the primers from forming loops or hairpins, 16 to 21 base pairs, a nearly equal distribution of G/C and A/T bases, a strong (high G/C content) anchoring end.

**Visualizing the Microsatellites**

DNA was primed in 3 different sets of reactions. First it was run on agarose without a label to confirm the size of the insert. Next on non-denaturing
polyacrylamide stained with SYBR green or with ethidium bromide. A final set was run with one primer each on a 30 cm denaturing polyacrylamide gel using primers labeled with an incorporated radioactive dNTP. Enough primers were purchased for about 50 reactions. M13 was sequenced and run simultaneously to confirm the size of the alleles.

**Results**

From the 2 bald eagle libraries I screened 4 million bases of the bald eagle genome. Using the estimated 360 million bases in the genome, this indicates that about 1% of the bald eagle genome was screened. A total of 22 clones from both libraries were isolated, and 11 of these were sequenced. The majority were CT repeats (Table 1). There was very little variation at the 3 loci examined. All colonies were screened with CA and CT, and half were screened with CTT. The only locus that was polymorphic had a series of fifteen repeat units. Microsatellite regions from the human genome have not been consistently polymorphic below thirteen tandem repeat units. But all microsatellite loci containing more than thirteen tandem repeats have been polymorphic (Weber 1990). Smaller units were sequenced for the bald eagle because there are no data concerning information content for avian microsatellites, and because it's impossible to know which allele a clone contains. The clone from this project contained the smallest allele at locus 6.
Table 1. Repeat regions cloned from bald eagle genomic DNA

<table>
<thead>
<tr>
<th>Repeat pattern</th>
<th>Name</th>
<th>Primer sequence</th>
<th>Alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td>(AC)$<em>7$(TC)$</em>{18}$</td>
<td>3</td>
<td>F: CAGGTTGATGATG</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GGCACTAGATCA</td>
<td></td>
</tr>
<tr>
<td>(CT)$_{10}$</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(CA)$_{10}$</td>
<td>2</td>
<td>F: CAGGTTGATGATG</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GGCACTAGATCA</td>
<td></td>
</tr>
<tr>
<td>(CA)$<em>8$ N$</em>{10}$(CA)$_{16}$</td>
<td>6</td>
<td>F: CTTGATGTATCCT</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: AGAGGCTATGGAA</td>
<td></td>
</tr>
<tr>
<td>(CA)$_{17}$</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(CG)$_5$(CA)$_9$</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(TG)$_8$</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(CA)$_6$</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(CA)$_{12}$-interspersed</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(CA)$_{10}$-interspersed</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(G$<em>{3}$A$</em>{3}$(AG)$_{10}$</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(CTT)$_8$</td>
<td>4</td>
<td></td>
<td>1</td>
</tr>
</tbody>
</table>

Several microsatellite regions could not be primed because the clone did not contain enough sequence outside of the microsatellite region (Figure 1). In order to design a primer, there should be at least 30 base pairs of sequence on each side of the microsatellite, at least half of which should be random sequence. While the shorter clones were problematic for the above reasons, clones with loci 4, 2, and 6, each had to have additional primer sets designed in
order to read through to the microsatellite region. Locus 2 required a third primer set to replace one that was possible forming an internal loop. Although I checked all sequences as I designed the primers to avoid internal complementarity, the high percentages of single base run-ons left few options for optimal primer design.

Frequency of Microsatellites

I found that the bald eagle genome contains about 1 (CA)n every 96,000 base pairs. Interspersed (CA)n runs were considered, but only if at least one run included 6 uninterrupted repeats, for example, plasmids K2 and K3 (Table 1). The calculation was based on the number of positive colonies screened, assuming that each clone contained about 400 base pairs of bald eagle DNA. The average size DNA insert used for the library was 400 base pairs. The eagle DNA for the library was size selected by fractionation with molecular weight standards designed to indicate 300 and 600 base pair lengths. DNA between 300 and 600 base pairs was cut from the agarose and purified. This DNA was size fractionated a second time on agarose and the purified DNA ligated to the vector for transformation.

Following electroporation, the recovered colonies were picked and placed onto screening plates with grids drawn on the bottom. Each 8 cm plate was patched with 300 colonies. I was then able to screen about 120,000 bases on each 8 cm nylon. Positively hybridizing colonies were picked and placed onto new plates for a second screening. These secondary plates had both positive
and negative controls. The positive control became available after my first colonies were sequenced. SURE cells transformed with plasmids A1 or B1 were positive controls for (CA). After sequencing plasmids containing long poly A regions, but no microsatellites, I added a poly A as a negative control. Poly A regions provide a strong signal because the probe is labeled with $[^{32}\text{P}]$ dATP. I tried spinning out the unincorporated radioactive nucleotides, but this makes it very difficult to isolate colonies because the background is too low. By leaving the unincorporated nucleotides in the hybridization mixture, I was able to get enough of a background that all of the colonies could be seen on the autoradiograph, and the negative control served as the baseline above which true positives could be determined. A second negative control was untransformed SURE cells.

**Table 2. Distribution of alleles for 22 individuals at locus 6**

<table>
<thead>
<tr>
<th>Allele</th>
<th>Number</th>
<th>Frequency</th>
<th>Size**</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18</td>
<td>0.419</td>
<td>110 base pairs</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>0.140</td>
<td>112</td>
</tr>
<tr>
<td>3</td>
<td>11</td>
<td>0.256</td>
<td>114</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>0.023</td>
<td>118</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>0.023</td>
<td>120</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>0.140</td>
<td>122</td>
</tr>
</tbody>
</table>

TOTAL 43*

* The clone is haploid
** The size is relative to the 110 bp clone repeat region
Table 3. Distribution of alleles at locus 6 by birthplace of individual

<table>
<thead>
<tr>
<th>Location (number)</th>
<th>Allele</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Northern Rockies (1)</td>
<td>1.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Canada (1)</td>
<td>1.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Florida (4)</td>
<td>0.5</td>
<td>0.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Southwest (13)</td>
<td>0.385</td>
<td>0.154</td>
<td>0.385</td>
<td>0.077</td>
<td></td>
</tr>
<tr>
<td>Unknown (3)</td>
<td>0.5</td>
<td>0.17</td>
<td>0.17</td>
<td>0.17</td>
<td></td>
</tr>
</tbody>
</table>

False positives can be the result of heterochromatin or minisatellites. The avian W chromosome has long stretches of non-random “junk” DNA. DNA for my library was isolated from tissue of a male bald eagle to avoid this problem.

The occurrence of 1 (CA)n repeat every 96,000 bases is not unusual among eukaryotes, however, the repeats are more common in all other species reported to date. Similar repeats occur every 65,000 base pairs in the ovine genome (Buchanan et al. 1993), every 18,000 bases in the mouse, every 21,000 bases in the rat (Stallings et al. 1991), and every 7,000 base pairs in the Atlantic cod, *Gadus morhua* (Brooker et al. 1994).
Figure 1: Sequenced microsatellite regions that were not primed. The vector sequence is in italics.

Plasmid H1

TATAAACATGATTACGCAAAGCTTGATGCCTGCGAGGTGACTC
TAGAGGATCT TTGCA
CACTCACAACACACACACACACATCACTGACAGAAAA
TTCAGTTCTTACTACTCTGTCTACAACCTTGCCCCTGGCTCATCCCA
GAGAGTCAGAGGA

Plasmid M4

AGTATGACATGATTACGCAAAGCTTGATGCCTGCGAGGTGACT
CTAGAGGATC TTTTTTCAATCCTTTACTTTACCTCTCTTTTCT
TCTACCTTTTGGGTGTGTGTGTGTGTGTGCTTAATCTCA
TCCTGGCATTTTCATGAAATGCAACATAGCCTTTTCTTTGCTGTCTC
CTCCATCCACTGCATTTTCATGGTACAGTCCCAAAACAAAATTTTT
CA

Regions Associated with Microsatellites

Certain patterns of DNA are believed to be associated with coding and/or non-coding regions in certain species (Tautz 1989). Little is known about the DNA environment around avian microsatellite sequences and nothing about the environment around Accipiter microsatellite regions. Microsatellite regions of avian DNA may be embedded within long stretches of nonrandom or “junk” DNA (Jon Longmire, personal communication). In the avian genome (CG) repeat islands are believed associated with coding regions (McQueen et al. 1996).

An ovine survey found microsatellites positively correlated with the presence of SINES (short interspersed elements) and (CA)n repeats (Buchanan et al. 1993). Microsatellites associate with the AluI repeat in the human genome (Zuliani and Hobbs 1993).
Microsatellites cloned from the bald eagle are highly correlated with 2 patterns of DNA: poly (dA), and interspersed (CTT) runs (Table 4). I considered bases within 75 base pairs of the microsatellite to be “associated” with the microsatellite. Interspersed (CTT) are stretches with at least 90% made of only 2 bases, and are at least 15 bases long. An example is 21 continuous dC or dT bases with 2 other bases interspersed. The correlations with the poly (A) regions may support the hypothesis that microsatellites are sometimes caused by the poly A tails of retroposons that slip during replication (Kaukinen and Varvio 1992).

I did not detect any CG islands, however, this is due in part to the use of a CGCG base cutter to construct one of the blunt end libraries. The same base cutter, Hae III cleaves a bald eagle minisatellite region into fragments of less than 300 base pairs, so I did not clone any of this minisatellite (C. Raven, unpublished data). Hence, I can not comment on an association between SINES and microsatellite regions.

Use of Microsatellites with Related Raptors

Microsatellites are useful for phylogeny reconstruction (Ellegren 1992; Bruford and Wayne 1994; Bowcock et al. 1996). No microsatellites have been cloned from any Accipiter, and yet this group remains one of the most phylogenetically confused among all avian taxa (Feduccia 1996). None of the 9 Passerine flanking sequences could prime DNA from the Accipiter Buteo galapagoensis (Primmer et al. 1996). Most microsatellites primers amplify all
congeneric species, while more distantly related groups are either less variable or not amplifiable (Buchanan et al. 1994; Moore et al. 1991; Pepin et al. 1995). Phylogeny reconstruction is possible if the bald eagle primers amplify microsatellites from related species.

Table 4. Non-random sequences associated with bald eagle microsatellites

<table>
<thead>
<tr>
<th>CLONE</th>
<th>sequence</th>
<th>(CTT) interspersed</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>(A) 7</td>
<td>21 bp run</td>
</tr>
<tr>
<td>6</td>
<td>(A)11 (A)6, 2 sets</td>
<td>18 , 12, 16 bp runs</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>24 bp run</td>
</tr>
<tr>
<td>12</td>
<td></td>
<td>21 bp run</td>
</tr>
<tr>
<td>M4</td>
<td>(A) 6</td>
<td>30 bp run</td>
</tr>
<tr>
<td>5</td>
<td>(A)10, (A)7, (A)5</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>(A) 7</td>
<td>44 base run</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>k2(10)</td>
<td>(A)11</td>
<td>38 base run</td>
</tr>
</tbody>
</table>

I tested my primers with both griffon vulture (Gyps fulvus) and golden eagle (Aquila chrysaetos) DNA. Both species are members of the Accipitridae family. Griffon are Old World vultures whose allied species have been managed through conservation reintroduction in Europe for the past decade (Negro and Torres 1999). The phylogeny of the Falconiformes is contested, but there is general belief that the Old World vultures are the closest relatives of the sea eagle clade,
and the golden eagles are the furthest removed within the family (Olsen 1995; Amadon 1983). I primed, then fractionated on non-denaturing agarose, pooled bald eagle, griffon, and golden eagle DNAs. The golden eagle genome does not have microsatellite sequences at the following bald eagle loci: 6, 4, and 2. The griffon sample can be amplified with all primers. At locus 6, it can not be distinguished from bald eagle.

The implications of the cross-species hybridization are that bald eagle microsatellites should be revealed using primers developed for closely related groups: Old World vultures, and soaring kites (Milvus spp.). If bald eagle microsatellites could be synthesized using primers from any other Accipiters, there is a reduced likelihood that the loci would be informative.

Locus 4 appeared to be polymorphic and was difficult to interpret. The problem occurred because the samples donated by one individual consisted of both bald and golden eagles. Unreliable and poorly documented sampling has been a problem recognized by other investigators who have attempted genetics work with bald eagles (Hunt et al. 1992b; Morizot et al. 1985). The following tests were made to ensure that the purported bald eagle samples were actually golden eagles. First, golden eagle DNA was isolated from samples donated by Dr. Grainger Hunt. An individual golden eagle sample was run with the locus 4 primer set, along with the bald eagles, on a denaturing polyacrylamide gel. A sequenced M13 was run alongside as a size standard. The golden eagle was homozygous at the same size allele as the 2 "bald eagles." Next, DNA from the 2 suspect bald eagles was primed at locus 6, along with 3 golden eagles, and
known bald eagle DNA samples. The suspect bald eagles and the 3 golden eagles samples were all monomorphic at the same size allele. The allele is only 2 base pairs removed from the true bald eagle allele, thus compounding the original confusion. All samples donated by the individual who misidentified the bald eagles have been removed from the analysis because the location of birth, age of individual, and all other information must be considered unreliable.

Discussion

A funding shortage for molecular projects in non-commercial species makes microsatellite assessment of population structure impractical, if not impossible. Locus 6 was cloned in the spring of 1995 (Raven and Vyse 1995b). A funding shortage prevented sequencing this clone and developing primers until the fall of 1995 (Raven and Vyse 1995a).

The inaccessibility of samples renders the microsatellite system moot even in the event that organized samples were available. Microsatellite primers can not assess population structure in the absence of samples. The inability to obtain a permit and samples delayed testing the primer until the spring of 1997, 4 years following the creation of primers which are in fact, able to detect population structure in the species.

In this paper I propose that the southwest group of eagles is relatively reproductively isolated. Alternatively, the species could be panmictic, or, finally, isolated by distance. Different sets of samples are needed to disprove each
alternative model. Samples from Arizona could be compared with samples from the Great Lakes. A resulting genetic distance that is relatively large could disprove panmixia, but will not address the possibility of a cline. The presence of a north-south cline, will contradict the theory of a separately evolving southwestern population. To test the clinal hypothesis, a sample of birds intermediate to the far north and south populations will need to be examined. The only practical possibility for a north-south cline is along the Atlantic Coast and then westward towards Arizona. The Southern Pacific Coastal population has been too depleted to provide emigrants for most of this century. The birth date of individuals in the samples is critical. Birds hatched after hacking commenced will not necessarily represent the evolutionary unit from the area of their birth, because they could be descendants of introduced birds.

The highest fractions of the cost for developing primers are screening with radioactive probes, and sequencing. Sequencing is the most problematic and the most expensive. When the project first began, the University of Montana facility could read 400 base pairs of each sequence per run. I routinely sequenced 700 base pairs; so to get my entire sequence read took at least 2 runs and required the design and purchase of additional primers. Due to normal aging of equipment, the facility dropped to 300 bases per reading. This required 3 reads per sequence just to confirm the presence, location, and size of the tandem repeat. A read which is clear enough to allow unambiguous design of primers required additional readings. I used small sized DNA to allow the cheapest sequencing (Rassmann, Schlotterer and Amos 1991), but the inability
to find good primer sequences in these short stretches makes short clones impractical.

The probe cost is related to the expense of buying the oligonucleotide to screen, and the label. The label expense is compounded by a short half life, and the kinetics of the hybridization reaction itself. Hybridization is most efficient with a high density of probe in the mixture. But the oligonucleotide probe is limiting. The cost of the probe was decreased by increasing the signal:noise ratio in the hybridization mix.

Costs of visualizing the alleles is related to end-labeling the primers. Incorporation of a labeled oligonucleotide is cheaper, but makes interpretation very difficult unless the primers are by chance so specific that there are no other products amplified. Cloning microsatellites and synthesizing primers, and optimizing conditions under which amplified products can be visualized are not a reasonable endeavor for a non-commercial species. The time and effort and cost are greater than an isozyme system. The effort is best spent on organisms for which samples can be made available.

The absence of funding to develop the necessary level of resolution to examine the bald eagle genome does not preclude the understanding of the evolutionary unit. In many cases, behavioral, ecological, and demographic trends are equally important indicators of the evolutionary unit (Avise 1989). Lande (1988) has suggested that genetics is less important than other characteristics for conservation planning (Lande 1988). Because the bald eagle is so mobile, reproductively isolated populations are difficult to find without the
benefit of molecular genetics. However, direct evidence of migration and immigration, coupled with morphological distinctiveness, and unique behavior, exposes the southwest group of bald eagles as an evolutionary unit within the species.

References cited


Buchanan, F. C., L. J. Adams, R. P. Littlejohn, J. F. Maddox and A. M.


Rassmann, K., C. Schlotterer and B. Amos, 1991 Isolation of simple-sequence loci for use in polymerase chain reaction-based DNA


USFWS, 1995  Final rule to reclassify the bald eagle from endangered to threatened in all of the lower 48 states. Federal Register 60: 36000 - 36010.


Wright, S., 1931  Evolution in Mendelian populations. Genetics 16: 97-159.

CHAPTER 3

INTEGRATING SCIENCE AND MANAGEMENT:
EVOLUTION AND THE ENDANGERED SPECIES ACT

Introduction

Sometimes species evolve as a unit, and sometimes populations within species set out on separate evolutionary paths. Sub-specific groups on distinct evolutionary trajectories are "evolutionary units." The species and evolutionary unit are not necessarily equivalent (Selander 1971). In fact, 'population' may be equivalent to the evolutionary unit except that the former term is confounded by extensive colloquial usage. Conservation biologists use taxonomic units to inventory biodiversity, but inventory is just a small part of conservation. Because the environment changes constantly, maintaining biodiversity for the long run requires preserving the evolutionary process by which populations adapt.

One of the most important tools for conservation management is the Endangered Species Act (ESA) of 1973. In 1978 Congress amended the ESA (16 U.S.C. §§ 1532) to define "species" to include "any subspecies of fish or
wildlife or plants and any distinct population segment of any species of vertebrate fish or wildlife (emphasis added)." The amendment did not include an explicit definition of “distinct population segment,” (DPS), nor did it greatly change the reliance on the species taxon. For the period 1985 through 1991, Federal managers considered 9% of vertebrates listed or proposed for listing as distinct population segments (Wilcove et al. 1993). The emphasis on species decreased after 1991 when Pacific salmonids required ESA protection. From 1978 through 1991, only 5% of threatened or endangered fishes were listed as populations. During this period only one salmonid required protection. After 1991, sixteen salmonids, all listed as populations, came under ESA protection.

The shift away from species as salmonid listings increased was not a coincidence. The idea that conservation management must involve the evolutionary process was not new (Allendorf 1983), but the salmon listings exemplified the shortcomings of ignoring that process. Biologists from the National Marine Fisheries Service (NMFS) found species an inadequate unit for a group whose genetic biodiversity is distributed among stocks. Sub-specific units provided a more logical measure of biodiversity, especially for recently diverged forms (Utter 1981; Behnke 1992). NMFS continued to refine the application of the population taxon (Dizon et al. 1992; Johnson et al. 1994; Waples 1991).

In 1991 the National Marine Fisheries Service (NMFS) proposed to resolve controversial listing decisions under the Endangered Species Act (ESA) by deferring to evolutionary principles (Waples 1991). Wildlife biologists received a
similar mandate from the National Academy of Sciences (National Research Council 1995). Congress subsequently amended the ESA to reflect NAS and NMFS policies, (Malakof 1998). Rohlf (1994) suggested that it was not in the best interest of conservation to use only science as a criterion for an evolutionary unit. He suggests that nonscientific criteria may provide the best long-term conservation for salmonids. No comments such as this were included in the Congressional debate on DPS (USFWS 1996).

The amended ESA explicitly defined "distinct population segment" as an evolutionarily significant unit of a species, which in turn was defined according to NMFS policy (USFWS, 1996). A segment of a species must be substantially reproductively isolated from other conspecific population units, and evolutionarily significant to be classified as an EU (Waples 1995). Distinct Population Segment and Evolutionarily Significant Unit are identical legal definitions, meant to capture the biological significance of evolutionary unit. The National Research Council (1995) suggests that the modifier 'significant' is unnecessary because the evolutionary unit as the portion of a species on a unique evolutionary path, is inherently significant.

What is "substantial reproductive isolation?" Species are organized as collections of demes. Demes are groups within which individuals are more likely to mate with each other than with conspecifics outside the deme (Hartl 1987). Within demes, mating is panmictic. Each deme potentially inherits some adaptive traits that are not shared by the rest of the species. Each adaptive trait represents a significant piece of the evolutionary legacy of the species.
Differential adaptation will not occur, despite selective pressure, within a panmictic species. Among evolutionary units of a species some migration occurs but reproductive isolation is sufficient to permit separate lines of adaptation. Immigration that is consistent with differential adaptation has been the subject of many articles (Allendorf and Phelps 1981; Bossart and Prowell 1999; Mills and Allendorf 1996).

Reproductive isolation may be inferred from topography. For confined animals, such as fish in discrete bodies of water, this may be trivial. For extremely mobile animals such as birds reproductive isolation is difficult to document. In these cases geographic isolation is inferred from a combination of topography, distance to nearest conspecific population and dispersal potential. Reproductive isolation is most obtuse among sympatric populations, but it may be deduced from biochemical genetics or behavior.

Genetic markers may reveal a history of reproductive isolation. However, the lack of diagnostic markers cannot rule out reproductive isolation. When a population is suspected of being isolated and diagnostic markers are not observed at one level of the genome, a finer level of resolution can be examined. For example, microsatellite DNA generally provides a finer level of resolution than minisatellite DNA. The development of genetic markers is expensive and should be pursued only where isolation is thought to exist (Ryder 1986). The evolutionary unit, while based on genetic principles, “is not dependent upon biochemical genetics” (Waples 1995).

An important alternative to biochemical genetics is mating behavior. Mating
behavior offers clues to reproductive patterns. Sometimes mate preference
depends upon slight morphological differences that divide sympatric individuals
(Seehausen et al. 1997). Mating behavior can be seasonally variable within
species causing temporal separation between sympatric populations (Barlow
1995). Common among fish, sympatric yet distinct populations have also been
noted among birds. Migratory and resident animals that share a single seasonal
territory are classic examples. Bald eagles in the eastern U.S. winter in Florida.
Some wintering birds are residents, mating in the summer, while others are
migrants from the north and return to spring habitats before mating.

Neither introduced nor reintroduced groups are a priori reproductively
discrete. In both situations gene pools may be similar to native wildlife. Even if
the extent of prior gene flow is unknown, gene flow may commence between
populations after an introduction. The importance of this issue is illustrated by
conservation management of the Florida panther (Puma concolor). When
Central American panthers were introduced into Florida, they bred with native
panthers, but, the introduced animals originated from a separate subspecies
(Hedrick 1995).

Reproductive isolation is the most important prerequisite for incipient
speciation, and thus the most critical element in identifying groups for
conservation. One characteristic that is not a reliable means of detecting
isolation is the presence of differential demographics. The presence of distinct
demography within a species does not necessarily show distinct populations.
Yet, this has been a primary characteristic promoting the use of the DPS taxon. For example, one discrete geographic area may support most individuals while another supports a very low number. Distinct densities may not correlate with distinct demes. Differential demographic trends may result from at least 3 other factors: sources and sinks, differential harvest rates, and differential mortality due to toxins. However, in these cases, panmixia within the species cannot be ruled out.

The second requirement of a EU is significance. Significance implies a unique evolutionary lineage (National Research Council 1995) or incipient speciation (Utter 1981). Unique lineages contribute substantially to the ecological or genetic diversity of the biological species (Myers et al. 1998).

Evolutionary significance does not follow from reproductive isolation; it must be determined separately (USFWS, 1996). A species structured into distinct or discrete mating units may not contain any EUs. Some workers have argued that reproductively isolated groups are inherently significant. The inability of reproductive isolation alone to confer evolutionary significance on a population has been tested with fish (Malakof 1998). Fish stocks, by definition, are reproductively isolated. However, not all fish stocks are considered EUs of their species (Waples 1995). In 1998 Federal biologists showed genetic distinctness among several populations of Atlantic salmon. They further argued that this distinctness conferred EU status to each individual population. Biologists from Maine argued successfully that the populations were not evolutionarily significant, because they were partially composed of hatchery or nonnative
A significant evolutionary legacy implies that the evolutionary process is ongoing. Evolution is driven by genetic variation and natural selection. EUs contain genetic variation that substantially augments the variation within the species. The variations may have resulted from unique selective pressure. This variation can be perceived at the molecular or phenotypic level. Any unique behavior, genes, or traits may suggest important components of the species' diversity. Finally, characters that are heritable, adaptive, and unique identify an evolutionarily important population.

Both small and artificially bred, populations are becoming increasingly common and create unique circumstances by which to judge evolutionary significance. Unique traits that are anthropogenic are not evolutionarily significant because they may reflect artificial not natural selection (Waples 1991). Hatchery-bred salmon are not EUs because they are invariably the product of artificial selection (Allendorf and Waples 1996).

Species consisting of few individuals occupying a very small range are generally panmictic and experiencing homogenous selective pressure. Although uncommon in nature, such species are evolving as a unit. They may themselves be remnants that are no longer adapting to the environment. Even when they are adapting, small populations may experience random genetic changes often indistinguishable from adaptive changes. Extremely small populations may not be worth saving because their long-run prognosis for survival is poor (Erwin 1991). In these cases, the units may not be considered an important
evolutionary legacy. Waples (1995) expressed concern that historically small populations may not be well suited to their environment. The rule of thumb is that populations too small to persist in isolation for a long time are unlikely candidates for EU status. However, historically large isolates, recently diminished, may by EUs despite their current size (Waples 1995).

In this paper I ask whether the use of DPS has increased since the 1995 amendment. Congress expressed concern that the DPS clause would be abused. I investigate these concerns by asking whether the FWS defined distinct population segments as evolutionary units, or whether they relied on a more casual definition of population (Waples 1991). I hope to shed light on the role of evolutionary theory in the conservation and management of threatened fauna.

Other authors asked whether the proportion of species, compared with all other taxon listed, changed between 1985 and 1991 (Wilcove, McMillan and Winston 1993). I am looking at both the quantity and quality of the population taxon. This is an important question because “population” is widely accepted as a colloquial term for virtually any group, and because of the great potential, as noted by Congress, for abuse of this term.

**Methods**

attempting to standardize the use of EUs. Three years later a major conference included workers throughout the conservation profession. The executive summary from this conference became public in 1995. The NAS was actively working on the issue while this was released. Congress amended the ESA in 1996, but sufficient guidance was clearly in place at this time, since the amendment followed the professional consensus.

Actions included fauna listed, delisted, redefined, and proposed for the above. I ignored “similarity of appearance” listings. All taxa and not just those restricted to the U.S. were included. I have 3 categories: 1) species whose evolutionary units were defined 2) Species whose DPS were defined, 3) species listed according to a taxonomic category, either subspecies or species, with no consideration of evolutionary groups. Animals reintroduced or introduced for conservation purposes, which are isolated from conspecifics, are by definition, populations. These experimental populations are not required to meet the criteria of an evolutionary unit. I did not include these when determining the proportion of populations in the time under consideration.

Data was collected from federal register listing notices and, in wildlife for which the NMFS has jurisdiction, data came from Technical Memoranda from the National Oceanic and Atmospheric Administration. When considering whether genetic data supported the FWS or NMFS model of the species distribution, I considered only whether the agency reported such support, not whether the support existed. Since the agency neither prepares nor commissions their own genetic work, they can’t be expected to have the most
current information.

I decided a taxon was an EU if it met 3 criteria: reproductive isolation, genetic variation, and natural selection, the latter two as a means to interpret "important evolutionary legacy of the species."

Results

Eleven actions involved taxonomic units above the level of a population, of which 5 were species and 6 subspecies. None of these were divided into evolutionary categories. One action was a range expansion for the previously listed jaguar. The other 4 species had so few remaining individuals that they necessarily are a single EU. These are perhaps best described as the remnants of a former EU. Recent fragmentation of an anthropogenic origin describes a single, fragmented, EU that may need to be restored. The willow flycatcher, a subspecies, does not have enough individuals, nor a range exceeding its dispersal, and therefore is unlikely to consist of more than a single EU. Four of the subspecies meet the criteria of an EU of a species: kangaroo rat, meadow jumping mouse, and Sonoran tiger salamander. However, none were described as such. The red-legged frog subspecies was divided into native and nonnative groups, all of which may be discrete populations, though they were not labeled as such. Introduced conspecifics were excluded from protection to the effect that the listing split the subspecies into at least 2 distinct populations: native and nonnative.
With purposeful introductions separate population status is granted to each experimental population. Concomitantly, each conspecific native group receives population status. Experimental populations made up 17% of all listings during this period.

Actions involved 9 taxa listed as DPS: pygmy-owl (*Glaucidium brasilianum cactorum*), 3 populations, nineteen individuals in Arizona, Texas, South of the U.S./Mexico border; peninsular bighorn sheep (*Ovis canadensis*), 2 populations, California, Mexico; Canada lynx (*Lynx canadensis*), 2 populations, Canada, U.S. lower forty-eight states; bog turtle (*Clemmys muhlenbergii*), 2 populations; northern and southern; bald eagle (*Haliaeetus leucocephalus*), 2 populations; U.S. lower 48 and Canada plus Alaska; Steller’s eider (*Polysticta stelleri*), 2 populations Alaska and Russia; copperbelly water snake (*Nerodia erythrogaster neglecta*), 2 populations, northern and southern; Arkansas River shiner (*Notropis girardi*), 2 populations, introduced and native; and Bull trout (*Salvelinus confluentus*), 2 populations, Klamath and Columbia Rivers.

For the 9 species listed as population segments, (Table 5), I first ask if the substructure were genetically determined. This is not asking whether any genetic studies have been completed, but whether cited genetic studies support the proposed model. Listing notices cite genetic studies for the following: bald eagle, bog turtle, bighorn sheep and bull trout. Only the bull trout had prior genetic work that supported the distinction among the 2 listed populations (Leary *et al.* 1993). Using protein electrophoresis Leary established genetic
characteristics of bull trout in the Klamath River and Columbia River drainages. Based on this data, the 2 populations of bull trout are reproductively isolated and evolutionarily distinct. The bald eagle is divided into 2 populations: the coterminous 48 states, and Alaska plus Canada. The genetic studies cited in support of this distribution did not ask whether these 2 populations are isolated from each other, nor whether either population is panmictic (Hunt et al. 1992). The bog turtle genetic studies cited disproved the proposed species structure. The bighorn studies cited could not show that the U.S. segment of the species is isolated from the Mexican segment (Boyce et al. 1997).

Instead of genetic data, the FWS cited political boundaries, geographic isolation, natural history, or demographics to divide species into populations. Natural history was cited due to anthropogenic introductions in the case of the Arkansas River shiner.

Demographic discreteness was cited when differences in abundance could be prescribed to specific geographic areas, regardless of whether there was gene flow between these areas. This describes the bog turtle, lynx, bald eagle, and eider. Six species were divided into populations based partially upon international borders (Table 6). Geographic isolation was cited as a mechanism of reproductive isolation for the bog turtle. With the copperbelly water snake, modern agricultural practices have divided the species, so geographic isolation is a recent phenomenon, commencing with the U.S. population explosion in the 1960's (USFWS 1997). The owl is not completely fragmented. If one considers the species south of the U.S. border, the east and west population are
contiguous. A population recently isolated as the result of human activity probably does not play an integral role in maintaining ecological or genetic diversity of the species. The same might be true of some natural isolates, particularly those of recent origin (Waples 1991).

Table 5. Listing taxa in ESA decisions from 1995 through 1999.

<table>
<thead>
<tr>
<th>Common name</th>
<th>Scientific name</th>
<th>Taxon</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Topeka shiner</td>
<td>Notropis topeka</td>
<td>species</td>
</tr>
<tr>
<td>2 Jaguar</td>
<td>Panthera onca</td>
<td>species</td>
</tr>
<tr>
<td>3 Alameda whipsnake</td>
<td>Masticophis lateralis euryxanthus</td>
<td>species</td>
</tr>
<tr>
<td>4 Guajon (frog)</td>
<td>Eleutherodactylus cooki</td>
<td>species</td>
</tr>
<tr>
<td>5 Barton springs salamander</td>
<td>Eurycea sosorum</td>
<td>species</td>
</tr>
<tr>
<td>6 SW willow flycatcher</td>
<td>Empidonax traillii extimus</td>
<td>subspp</td>
</tr>
<tr>
<td>7 Merriam's kangaroo rat</td>
<td>Dipodomys merriami parvus</td>
<td>subspp</td>
</tr>
<tr>
<td>8 Red-legged frog</td>
<td>Rana aurora draytonii</td>
<td>subspp</td>
</tr>
<tr>
<td>9 St. Andrew's beach mouse</td>
<td>Peromyscus polionotus peninsularis</td>
<td>subspp</td>
</tr>
<tr>
<td>10 Meadow jumping mouse</td>
<td>Zapus hudsonius preblei</td>
<td>subspp</td>
</tr>
<tr>
<td>11 Sonoran tiger salamander</td>
<td>Ambystoma tigrinum stebbinsi</td>
<td>subspp</td>
</tr>
<tr>
<td>12 Timber wolf (Southwest)</td>
<td>Canis lupus</td>
<td>X pop</td>
</tr>
<tr>
<td>13 Blackfooted ferret</td>
<td>Mustela nigripes</td>
<td>X pop</td>
</tr>
<tr>
<td>14 Whooping crane</td>
<td>Grus americana</td>
<td>X pop</td>
</tr>
<tr>
<td>15 Red wolf</td>
<td>Canis rufus</td>
<td>X pop</td>
</tr>
<tr>
<td>16 Condor (Arizona)</td>
<td>Gymnogyps californianus</td>
<td>X pop</td>
</tr>
<tr>
<td>17 Bog turtle</td>
<td>Clemmys muhlenbergii</td>
<td>DPS</td>
</tr>
<tr>
<td>18 Canada lynx</td>
<td>Lynx canadensis</td>
<td>DPS</td>
</tr>
<tr>
<td>19 Bighorn sheep</td>
<td>Ovis canadensis</td>
<td>DPS</td>
</tr>
<tr>
<td>20 Bald eagle</td>
<td>Haliaeetus leucocephalus</td>
<td>DPS</td>
</tr>
<tr>
<td>21 Steller's elder</td>
<td>Polysticta stelleri</td>
<td>DPS</td>
</tr>
<tr>
<td>22 Copperbelly water snake</td>
<td>Nerodia erythrogaster neglecta</td>
<td>DPS</td>
</tr>
<tr>
<td>23 Arkansas River shiner</td>
<td>Notropis girardi</td>
<td>DPS</td>
</tr>
<tr>
<td>24 Pygmy-owl</td>
<td>Glaucidiolm brasiliiana cactorum</td>
<td>DPS</td>
</tr>
<tr>
<td>25 Bull trout</td>
<td>Salvelinus confluentus</td>
<td>DPS</td>
</tr>
<tr>
<td>26 Steelhead</td>
<td>Oncorhynchus mykiss</td>
<td>EU</td>
</tr>
<tr>
<td>27 Coho salmon</td>
<td>Oncorhynchus kisutch</td>
<td>EU</td>
</tr>
<tr>
<td>28 Umpqua River Cutthroat</td>
<td>Salmo clarki</td>
<td>EU</td>
</tr>
<tr>
<td>29 Steller sea-lion</td>
<td>Eumetopias jubatus</td>
<td>EU</td>
</tr>
</tbody>
</table>

Xpop = experimental population
Evidence that distinct populations are historically isolated, and self-sustaining, was presented for the bull trout (Leary, Allendorf and Forbes 1993), and the bog turtle. The other 7 DPSs are not isolated either, or, if isolated, not self-sustaining. The lynx population, as listed, in the Northern boreal forest contains “one to several” individuals. In most of the states where the lynx is proposed for lists there are not believed to be any lynx. Reintroduced lynx, if they become a viable population, are considered “experimental,” and not distinct populations. To the extent that any lynx survive in the eastern coterminous U.S., the animals must be coming down from Canada. Yet the listing record claims that any lynx outside of the coterminous U.S. are a discrete population.

The Bighorn sheep on the Baja peninsula are panmixtic across the U.S./Mexican border, and therefore the U.S. group is not a distinct population (Boyce et al. 1997). The Steller’s eider may alternate nesting within a generation between Alaska and Russia, so it is unlikely that the Alaska and Russian groups

---

**Table 6: Reproductive isolation within distinct population segments**

<table>
<thead>
<tr>
<th>Taxon</th>
<th>genetic data</th>
<th>data in lieu of genetics</th>
<th>isolated</th>
<th>panmictic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bog turtle</td>
<td>NO</td>
<td>topographic separation</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>Canada lynx</td>
<td>NO</td>
<td>inti boundary</td>
<td>NO</td>
<td>NO</td>
</tr>
<tr>
<td>Bighorn sheep</td>
<td>NO</td>
<td>inti boundary</td>
<td>NO</td>
<td>YES</td>
</tr>
<tr>
<td>Bald eagle</td>
<td>NO</td>
<td>inti boundary, demographics</td>
<td>NO</td>
<td>NO</td>
</tr>
<tr>
<td>Steller’s eider</td>
<td>NO</td>
<td>inti boundary</td>
<td>NO</td>
<td>NO</td>
</tr>
<tr>
<td>Copperbelly</td>
<td>NO</td>
<td>topographic separation</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>Shiner</td>
<td>NO</td>
<td>temporal</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>Pygmy-owl</td>
<td>NO</td>
<td>inti boundary, topography</td>
<td>NO</td>
<td>NO</td>
</tr>
<tr>
<td>Bull trout</td>
<td>NO</td>
<td>inti boundary</td>
<td>NO</td>
<td>NO</td>
</tr>
</tbody>
</table>
represent separate demes (USFWS 1997c). The copperbelly water snake has been fragmented in the last thirty years into several isolated groups, all of which may be destined to extinction if they remain isolated. However, the northern and southern populations described in the listing report, are neither historically, nor naturally isolated (USFWS 199a). The Arkansas shiner was divided into 2 populations because 1 reproductively isolated patch is a 20 year old illegal or accidental transplant population (USFWS 1998). The 20 year divergence does not seem to clearly indicate a reproductively isolated group; the gene frequencies are expected to be identical. The pygmy-owl was divided into 3 populations. North of the Mexican border, the east and west groups are physically separated. However, in Mexico the west and east populations converge, forming a ‘V’ shaped range opening to the north. The north western most tip of the range, near Tucson, Arizona, consist of only twenty or so individuals. These individuals, the only population of pygmy-owl which has been declared endangered, are clearly not self-sustaining, and not historically isolated (USFWS 1997b).

Among the population segments only the bull trout meet the criteria of evolutionary significance. Each river population contains unique genetic characteristics. None of the DPS for any of the nine species possess diagnostic behavioral, or morphological characteristics.
Table 7: Reproductive isolation within DPS listings

<table>
<thead>
<tr>
<th>Description and number of DPS</th>
<th>Time DPS became isolated</th>
<th>Isolating mechanism</th>
<th>Distance/ dispersal</th>
<th>uniqueness/ significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bog turtle (2)</td>
<td>Pleistocene</td>
<td>Climate change</td>
<td>4</td>
<td>percent of total species range</td>
</tr>
<tr>
<td>north/south</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Canada lynx (2)</td>
<td>Not applicable</td>
<td>political</td>
<td>≤ 1</td>
<td>none</td>
</tr>
<tr>
<td>U.S./Canada</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bighorn sheep (2)</td>
<td>Not applicable</td>
<td>political</td>
<td>≤ 1</td>
<td>none</td>
</tr>
<tr>
<td>U.S./Mexico</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bald eagle (2)</td>
<td>Not applicable</td>
<td>political</td>
<td>≤ 1</td>
<td>none</td>
</tr>
<tr>
<td>lower 48/ Ca+ Ak</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Steller’s eider (2)</td>
<td>Not applicable</td>
<td>political</td>
<td>≤ 1</td>
<td>political</td>
</tr>
<tr>
<td>Alaska/Russia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Copperbelly (2)</td>
<td>beginning in 1960’s</td>
<td>Anthropogenic: agriculture</td>
<td>2.5</td>
<td>Percent of total species range</td>
</tr>
<tr>
<td>No/So</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shiner (2)</td>
<td>twenty years</td>
<td>Anthropogenic: introduction</td>
<td>N/A</td>
<td>ecological</td>
</tr>
<tr>
<td>ARS/New Mexico</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pygmy-owl (3)</td>
<td>possibly contiguous or throughout evol.</td>
<td>Uncontiguous plateau, un- genetic</td>
<td>2</td>
<td>ecological</td>
</tr>
<tr>
<td>Az/ Tx/ Mexico</td>
<td>time</td>
<td>suitable habitat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bull trout (2)</td>
<td></td>
<td>Anthropogenic genetic</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>Klamath/Columbia</td>
<td>Pleistocene</td>
<td>genetic</td>
<td>N/A</td>
<td>genetic</td>
</tr>
</tbody>
</table>

N/A = not applicable. Distance/dispersal is a ratio of the closest distance between 2 DPS divided by the distance over which the organism may disperse.

Discussion

Population has become a more common listing taxa since the 1996 Congressional Amendment provided a definition for DPS. The definition stated a DPS was an evolutionary unit of a species. The evolutionary unit is a biological
idea. Under the ESA, the EU has both a biological and a political definition. In spite of increased use of DPS, most population listings use political, and not biological criteria. The political criteria allow population borders to coincide with international borders. Among taxa involved in actions since 1995, only one under the jurisdiction of the FWS had demes identified before the petitioning process.

A consequence of ignoring the evolutionary unit is the loss of genetic integrity whenever artificial augmentation is employed as a conservation measure. The true population approach is preferable to the species approach because the former preserves genetic integrity within the species, while the latter does not (Eisner et al. 1995). A breeding cluster has genetic integrity if it maintains all the genes that made up its historical lineage, and no additional genes. Overall, conservation biologists seek to maintain genetic integrity because over time genes expressing adaptive traits increase, while those that are maladaptive decrease. Genetic integrity may be destroyed in the presence of one-way gene flow. One-way gene flow occurs when animals from one population introgress another population. Diminished genetic integrity may have the following effects: 1) adaptive genes will be lost, and 2) adaptive gene complexes will be broken up (Lynch 1998).

The conservation unit influences listing, delisting, and recovery efforts. One approach to improving the status of threatened and endangered species has been artificial augmentation of a wild threatened population with animals from another growing population. This process is also known as "stocking," or
“hacking.” Transferring chicks or eggs from one wild population to another depleted wild population has been common in bald eagle management since the late 1970's. Because EUs are not recognized, managers can augment the endangered populations with any conspecific birds.

If the entire species is considered a single population, then bald eagles from any part of the country can be moved to any other part in a restoration effort. This destroys genetic integrity of the population receiving the transplants. A reduction in fitness is often associated with this type of outbreeding. The outbreeding depression is expected to be greatest when the population sizes are smallest, (a population of 1000 individuals may be immune to outbreeding depression) (Hartl 1987; Lynch 1998). One of the purposes of the ESA is maintaining genetic diversity. Because species' demes are not recognized, the recovery mandated by the listing can decrease a species' genetic biodiversity in order to increase absolute numbers of individuals.

Most of the political boundaries are international (Table 6). This is surprising since the intent of the Endangered Species Conservation Act of 1969, the predecessor to the ESA, was the prevention of worldwide extinctions. This act led to the CITES convention and the beginning of international collaboration on species conservation. The ESA of 1973, following immediately behind CITES, allowed all Threatened and Endangered species to be combined on a single list. Listings from the past 4 years are fragmenting natural populations to coincide with political jurisdiction.
Table 8: Species recorded with their Evolutionary Units

<table>
<thead>
<tr>
<th>Species and Number and description of EUs</th>
<th>genetic support</th>
<th>geographic isolation</th>
<th>significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coho salmon (6)</td>
<td>YES</td>
<td>YES</td>
<td>ecological and environmental</td>
</tr>
<tr>
<td>Central Ca./No. Ca/Or./SW Wa/Olympic/Puget sound</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Steelhead (7)</td>
<td>YES</td>
<td>YES</td>
<td>genetic</td>
</tr>
<tr>
<td>Ca coast/Ca/So Ca/Snake/Upper &amp; lower Columbia/SoCentral Ca coast</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Umpqua river cutthroat (1)</td>
<td>YES</td>
<td>NO</td>
<td>genetic</td>
</tr>
<tr>
<td>naturally spawning in Umpqua River</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Steller sea-lion (2)</td>
<td>YES</td>
<td>NO</td>
<td>genetic</td>
</tr>
<tr>
<td>western/eastern</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Recommendations**

The ESA has increased public awareness of species' fragility. The ESA can increase public acceptance of evolution. One way to do this is to increase the emphasis on the biological criteria of the evolutionary unit. The taxon of choice for conservation listings under the ESA should be the evolutionary unit. Taxon expressed as evolutionary units should receive higher priority for final decisions to encourage the progression of evolutionary thought. Having this level of information in advance would speed up the listing process.

Monitoring of candidate and recovered species is required by the ESA.
Monitoring could include a determination of the demes as a prelude to modeling the EUs. Monitoring should also include follow-ups on genetic introgression (or swamping) whenever the FWS has used stocking to augment populations.

References cited


USFWS, 1996 *Policy regarding the recognition of distinct vertebrate population segments under the Endangered Species Act.* The Federal Register 61: 4721 -4725

USFWS, 1997a *Final Rule (copperbelly water snake).* The Federal Register 62: 4183-4192


Waples, R. S., 1995 *Evolutionarily significant units and the conservation of biological diversity under the Endangered Species Act,*
CHAPTER 4

EVOLUTIONARY UNITS AND CONSERVATION BIOLOGY:
ANALYZING THE BALD EAGLE PARADIGM

Introduction

Evolution and Ecology of Sea Eagles

Bald eagles (*Haliaeetus leucocephalus*) belong to the sea eagle genus and the Accipitridae family. All Accipitrids hunt and scavenge. The oldest Accipitrids, Old World Vultures, are primarily scavengers, while the most recently evolved, the true eagles and hawks, are primarily hunters. Sea eagles, as both predators and scavengers, occupy an intermediate, generalist, niche. Specialized for survival in an aquatic ecosystem, sea eagles belong to the rare guild of aquatic raptors. Among the 65 genera of Accipitridae only 3 are aquatic: *Haliaeetus*, *Ichthyophaga*, and *Busarellus* (Brown and Amadon 1968). Osprey, (Pandionidae family) and sea eagles are the only obligate aquatic raptors in North America. *Busarellus nigricollis* inhabit South America and are the only other aquatic raptors in the New World. *Ichthyophaga* are African fishing eagles.
The genus *Haliaeetus* occurs on all continents except Antarctica and South America. The bald eagle is the only member of the genus endemic to the New World. Eight distinct color morphs within the genus are essentially allopatric. Each of these eight plumage patterns has species status (Table 9). Several authors suspect that some of these distinct morphs should be combined into a single species (Olsen 1995, Seibold and Helbig 1996).

### Table 9. Characteristics of *Haliaeetus* species

<table>
<thead>
<tr>
<th>SPECIES</th>
<th>COMMON NAME</th>
<th>ADULT PLUMAGE</th>
<th>DISTRIBUTION, STATUS</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. leucocephalus</em></td>
<td>American bald eagle</td>
<td>brown with white head and tail</td>
<td>Inland and coastal North America US ESA Threatened</td>
</tr>
<tr>
<td><em>H. pelagicus</em></td>
<td>Stellar’s sea eagle</td>
<td>brown with white legs and tail</td>
<td>Coast of Siberia IUCN Red list</td>
</tr>
<tr>
<td><em>H. albicilla</em></td>
<td>White-tailed eagle</td>
<td>brown with white head and creamy tail</td>
<td>Greenland, Northern Europe Aleutian Islands IUCN Red list</td>
</tr>
<tr>
<td><em>H. leucoryphus</em></td>
<td>Pallas’ eagle</td>
<td>Brown with a white tail band</td>
<td>Central Asia IUCN Red list</td>
</tr>
<tr>
<td><em>H. vociferoides</em></td>
<td>Madagascar fish eagle</td>
<td>brown with white tail and streaked head</td>
<td>Inland and coastal Madagascar IUCN Red list US ESA Endangered</td>
</tr>
<tr>
<td><em>H. vocifer</em></td>
<td>African fish eagle</td>
<td>chestnut with white head, neck, and tail</td>
<td>Inland and coastal Southern Africa</td>
</tr>
<tr>
<td><em>H. sanfordi</em></td>
<td>Solomon’s sea eagle</td>
<td>reddish brown</td>
<td>Papua New Guinea, Solomon Islands IUCN Red List</td>
</tr>
<tr>
<td><em>H. leucogaster</em></td>
<td>White-bellied sea eagle</td>
<td>white with brown back</td>
<td>Coasts of Australia, India, Ceylon Possibly conspecific w/ <em>H. sanfordi</em></td>
</tr>
</tbody>
</table>
Isozyme frequencies reveal sea eagle species form clusters characterized by the climate zone of their breeding area (Seibold and Helbig 1996). Tropical sea eagles are the oldest lineage, and temperate the most recently diverged. The bald eagle and another temperate sea eagle, *H. albicilla*, show little morphological divergence. Occasionally the 2 are sympatric, though hybridization has not been recorded (Bent 1938).

Bald eagles exploit all types of aquatic habitats. If water is present, eagles are not restricted by climatic or latitudinal extremes and do not distinguish between artificial and natural aquatic habitats. They are as likely to reside near reservoirs as natural lakes. As obligate aquatics, temperate zone bald eagles must migrate when water in their breeding area freezes. In southern temperate, coastal, and tropical zones, mature eagles remain near their nests throughout the year. Even among migratory bald eagles, mature birds rarely nest farther than 100 miles from their natal area (Bent 1938).

Like most large carnivores, eagles are naturally rare throughout their range. Typical of their trophic level, they are becoming increasingly less common as their habitat shrinks. Eager refuse feeders, bald eagles appear to have adapted to man's domination of the natural world.

From the middle of this century through the present, bald eagle ecology is influenced by the biocide DDT (dichlor-diphenyl-trichlor-ethane). Feeding high on the aquatic food chain, raptors are subjected to pesticide bioaccumulation. Poorly understood physiological traits prevent raptors from metabolizing DDT as efficiently as other birds (Redig 1993). DDT, and a breakdown product, DDE,
impair the eagle hen's egg shell production. Thin-shelled eggs are likely to break before chicks are ready to hatch. Bald eagles have an unusually high hen weight to egg weight ratio (Bent 1938), thus increasing the likelihood that a fragile egg will be crushed by its mother.

The drastic population declines of bald eagles in the 1970's elicited a heavy-handed management response. Artificial augmentation commenced. Wild populations were stocked with birds hatched in breeding facilities or transferred from the wild. Available records show that wild chicks or eggs were collected throughout North America, with the majority coming from Alaska (Nye 1988).

**History of Bald Eagle Management Under the Endangered Species Act**

Avian management can have different goals: harvest, damage control, or conservation. Bald eagles have never been managed for harvest. Damage control guided management policy in the 1800's and much of this century, but the current focus is conservation. The overall purpose of bald eagle management is to reverse the downward trend in population size. One type of conservation management is taxonomic conservation by which a morphologic type with species or subspecies status is preserved. Short term conservation may include any means by which the biomass of a desired animal is increased. Long term conservation may require preservation of the evolutionary process, and the genetic traits that enhance adaption.

Currently, bald eagle management policies are not concerned with preserving
an evolutionary process. The United States Fish and Wildlife Service (FWS) claims to be managing the bald eagle according to distinct population policy (USFWS 1995). Their claims have been widely accepted (Pennock and Dimmick 1997). In fact, the bald eagle throughout the lower 48 states is managed by the FWS as a single species.

The FWS and the National Marine Fisheries Service jointly administer activities of the Endangered Species Act of 1973 (ESA). Bald eagles are the responsibility of the FWS. In 1978 the FWS listed all bald eagles in the lower 48 states as either threatened or endangered. Eagles in Minnesota, Michigan, Wisconsin, Washington, and Oregon, were “threatened.” In the remaining States eagles were endangered (Federal Register 43: 6233). When listings split a species into 2 or more ESA categories, as with the bald eagle, animals are only protected when in a protected zone. If a bald eagle from Minnesota summers in Alaska, it loses all protection. Likewise, eagles from Alaska that migrate to Montana, become endangered species, though they are unprotected in Alaska. For vagile species such as the bald eagle, the policy has serious consequences. Rare populations of eagles may not receive protection on their wintering grounds or migration stopovers, when these occur in unprotected lands.

Besides the legal zones established through Congress under the ESA, the bald eagle is divided into management zones. To supervise bald eagle recovery the FWS divided the threatened and endangered eagles into five recovery zones: Pacific, Northern, Chesapeake Bay, Southeast, Southwest. Most states’
boundaries are respected in the recovery. Texas and Oklahoma are exceptions.
Texas is split along a straight line exactly perpendicular to the Oklahoma
panhandle. Eastern Texas is in the Southeast Recovery Zone and Western
Texas is in the Southwest Recovery Zone. Bald eagles in the recovery zones or
the legal sections are often called populations, but these are not biologically
separate populations. Colloquial use of this term is widely accepted in wildlife
sciences.

Casual use of “population” does not satisfy ESA listing requirements. A
biological population, is the equivalent of a deme. It is the interbreeding unit of
the species within which mating is panmictic (Imhof et al. 1980). The biological
population is the unit within which evolution occurs, so it has been called
“evolutionary unit.” Political, management, and legal categories of the bald
eagles are all useful for some purposes, but the FWS has a mandate, under the
ESA, to categorize species in a biological sense, not political. The ESA requires
that listed taxon be either species, subspecies or distinct population segments of
species. In this sense, population has been defined by Congress as the
evolutionary unit of the species. The establishment of the true evolutionary units
should be a goal, instead of political and economic units (Futuyma 1995).

In 1994, the FWS chose to reclassify most of the endangered bald eagles in
the lower 48 as threatened. The southwest group was to remain endangered
“Based on evidence that it might be reproductively isolated (USFWS 1995).” In
1995, the FWS reversed this decision because they “decided the Southwest
Recovery Region to be part of the same bald eagle population as that of the
remaining lower forty-eight states (USFWS 1995).” The decision to reclassify southwestern bald eagles relied almost exclusively on an Arizona study (Hunt et al. 1992a, Hunt et al. 1992b, Hunt et al. 1992c). In support of the above decision, the FWS cited the following:

- 1) In 1994, a single male, hatched in 1988 in southeast Texas, was nesting in Arizona. “This is the first known bald eagle to breed within Arizona’s boundaries that originated in a different state and a different recovery region (italics added) (USFWS 1995).” No evidence of offspring existed at the time of the downlisting, although 6-year-old males rarely breed successfully. Moreover, eastern Texas was arbitrarily included in the Southeast Recovery Region and Arizona in the Southwest region.

- 2) Unpublished sight records from 1994 suggest that bald eagles fledged in Texas may enter breeding populations throughout the southern United States (USFWS 1995).

- 3) Neither enzyme electrophoresis nor DNA fingerprinting resolved any specific genetic markers from which Arizona eagles could be differentiated from those of other populations (USFWS 1995). The genetics reports referred to never received peer review. They were both parts of a report from a private consulting firm, which was commissioned by the Bureau of Land Management (Hunt et al. 1992c).

- 4) Both of the molecular techniques described above, failed to show that Arizona bald eagles are inbred (USFWS 1995).
In this paper I analyze the data used by the FWS to support designating the bald eagle a single population. If bald eagles in the lower 48 are a panmictic mating unit, then the FWS policies of reclassification and translocation may be justified. However, if bald eagles are not panmictic then the policies may be detrimental to the long-term survival of the bald eagle. I analyze all of the supporting data about hacking and preservation of the southwestern eagles. In analyzing the bald eagle's status, managers asked questions whose answers had no direct bearing on the policies under consideration. I ask, and answer, an appropriate set of questions. The FWS declaration of panmixia within the bald eagle species has not, to my knowledge, been challenged.

Two of these genetics briefs, commissioned by the U.S. Bureau of Reclamation, focused on ecology of bald eagles in Arizona (Hunt et al. 1992c).

**Results**

**Gene Flow Into Arizona**

According to the Final Report on Reclassifying the bald eagle, a 6-year-old male bald eagle, banded after hatching in Texas, occupied a nest in Arizona. This first sighting led the FWS to reverse its prior opinion that the Arizona group was reproductively isolated. The failure of Arizona to appear completely reproductively isolated caused it to lose status as a distinct population segment
of a species. Genetic data from the Reclamation report does not allow the hypothesis that birds from Texas have historically been interbreeding with birds from Arizona to be rejected (Figures 1 and 2).

Will Arizona's status as an evolutionary unit be affected if the Texas adult sires offspring in Arizona? Distinct populations of species are not completely reproductively isolated from other populations. Complete isolation is not required by either the biological or the legal definition of distinct population of a species (Chapter 3).

How isolated must a population be to evolve separately from the rest of its species? Is Arizona sufficiently isolated to evolve as a distinct population if the Texas bird nests there? Whether a species evolves as a unit or as separate populations depends upon genetic drift, migration (gene flow), and selection. Selection and gene flow act to maintain identity of the populations. Gene flow among semi-isolated populations may keep species evolving as a unit.

One immigrant producing offspring in each generation, will offset the effects of genetic drift (Spieth 1974; Wright 1931). The 0.33 migrants per generation into Arizona is probably not enough to counteract the effects of drift and selection (Slatkin 1987). A gene flow below 1 per generation will allow, but certainly not cause, heritable adaptations to local conditions, without being swamped by outbreeding from non-adaptive birds (Allendorf and Phelps 1981; Storfer 1998).
Lack of Genetic Markers

The FWS claimed that the lack of diagnostic genetic markers proves that the lower forty-eight are a panmictic population (USFWS 1995). Only 2 methods have been attempted with bald eagles: allozymes and minisatellites (Hunt et al. 1992c; Morizot et al. 1985). Diagnostic markers in either of these methods would be highly unexpected (Barrowclough 1992; Avise 1989; Barrett and Vyse 1982). Allozymes do not reveal all of the variation at each locus. Allozymes reveal variation only within some structural genes. Not surprisingly, reproductively isolated populations may fail to show significant allozyme differences or private alleles (Utter et al. 1992). Like large mammals, birds are generally monomorphic regarding these allozyme systems, possibly due to the fine-grained variability of their environment and their high mobility (Cameron and Vyse 1978). At least 2 microsatellite markers had already been developed at the time of the reclassification, though lack of funding prevented development of the system (Raven and Vyse 1995). Lack of samples also prevents demonstrating private alleles characteristic of reproductive isolation.

Arizona Birds are Inbred: No Evidence of a Bottleneck Due to High Heterozygosity

The FWS claims that heterozygosity within the population of Arizona birds proves the population is not inbred. Lack of inbreeding was then used to support their claim that Arizona could not be considered a distinct population (USFWS 1995).
Population heterozygosity, the proportion of heterozygous individuals at each locus, is considered positively correlated with higher fitness (Allendorf and Leary 1986). No pattern of heterozygosity has been discovered among endangered, as opposed to common species to allow a general rule about the relationship between heterozygosity and population bottlenecks (Avise and Hamrick 1996, Avise 1994). However, the mean heterozygosity reported in the Reclamation report is an order of magnitude higher than the avian average for non endangered species (0.065) (Evans 1987).

The Reclamation report includes allozyme frequencies for Arizona, Maryland, Florida, Washington, California, Texas, and Michigan at six loci: ESD, esterase-D; GLO, glyoxalase-1; GPI, glucosephosphate isomerase; MPI, mannose-phosphate isomerase; NP, nucleoside phosphorylase; and PEPA, peptidase-A. Among the 3 informative loci, data from Alaska are available for 2: ESD and GLO. Most bald eagles live north of the 40th parallel. Therefore, only loci with data from northern birds should be included when calculating Arizona's relative percent variability. Using the formula $H = 2pq$, the average weighted heterozygosity for the species at the ESD locus is 0.32. The weight takes account of the proportion of the species represented by each population. At the GLO locus the weighted heterozygosity is 0.18. The respective heterozygosity for the Arizona birds is 0.46, and 0.47. The Arizona birds are significantly more variable at both loci ($p < .0005$ and .005). Non-uniform heterozygosity has been linked to population structure with other avian species (Siegel-Causey 1997).

The Reclamation report used isozyme data to answer the question."Is any
population significantly more fixed, or less heterozygous than average?" Their answer, probably correct, was "no." However, they asked the wrong question. The question should be whether the Arizona group contained a higher than expected proportion of heterozygotes. That this would be so is evident from looking at the Alaska frequencies. The Alaska population is the largest component of the species and is nearly fixed at both these loci. The Arizona group is nearly at maximum heterozygosity (p=0.64, and 0.62). Therefore, a bird randomly chosen from Arizona is more likely to be heterozygous than a bird randomly chosen from within the species boundary.

The mean heterozygosity of the species in the lower forty-eight is 0.1436. Excluding the southwest group (Texas and Arizona), the heterozygosity is 0.088. Therefore, 62% of the total heterozygosity in the lower forty-eight is due to the southwest group. Arizona has 0.21 MHL (median heterozygosity at each locus), and the average of the remaining populations is 0.097.

**Population Genetic Structure**

Genetic variation expressed as heterozygosity is the variation within individuals. The total variation in a species is partitioned among intra-individual, and intra-population variation. The partitioning of the variation is the population genetic structure. Population genetic structure was not analyzed in the Reclamation report, nor addressed in the Final Rule to Reclassify the Bald Eagle (USFWS 1995). I use the allozyme data reported in the Reclamation report and in an earlier study to show partitioning of genetic variation within the species
(Morizot et al. 1985).

One way to segregate this variation is with principal components analysis (Allendorf and Leary 1988, Leary 1987). Principal components analysis using all six polymorphic loci showed that all of the variation was contained in only 3 loci. This is clear from the frequency distributions. Although the Reclamation report called six allozyme loci polymorphic, 3 were fixed in all but one or 2 states, and nearly fixed in the polymorphic group. These 3 loci should have been considered monomorphic; they are not appropriate to decide variation. The most common allele in one polymorphic locus was present in 99.989% of individuals. Many geneticists would not consider this locus to be polymorphic (Hartl and Clarke 1997).

I examined variation among the seven states using 3 informative loci: MPI, GLO, and ESD, and 2 data sets. The first set, from 1990, clearly shows at least 2 clusters: the Southwest states, and the virtually identical mid-Atlantic States (Figure 1). Allozyme frequencies from 1985 show more obvious grouping between Texas and Arizona, on one extreme, and the mid-Atlantic states on the other (Figure 2). The Arizona group accounts for most of the variation in the entire species.

**Genetic Distance**

The Reclamation report calculates distance among the states’ eagle populations using Nei’s standard measure. This is an unconventional usage of Nei’s standard distance. Wright’s F statistics should be used as a measure of
intra-species structure. Nei’s distance does not provide a measure of the
distance between populations related by migration (Nei 1972). Nei’s measure is
most appropriate for measuring distance between completely diverged groups
(Nei 1987), whereas Wright’s F statistics measure distance among interbreeding
populations (Wright 1931). I calculated Wright’s $F_{st}$ at one locus using allostyme
data from 1985 and 1990 (Morizot et al., 1985, Hunt, 1992b). The $F_{st}$ in 1990 is
0.167. Using 1985 data, the $F_{st}$ is 0.335. Both numbers are consistent with an
immigration rate near one per generation. Nevertheless, the higher $F_{st}$ reflects
an immigration rate of less than one, which is too low to overcome effects of drift
at neutral alleles.

The authors of the Reclamation report compare the obtained Nei’s pairwise
difference with all other vertebrate species, and declare the distances within the
eagles to be “average.” This is a mistake. As for allozymes, bird taxa are more
likely to be monomorphic and manifest low Nei’s distances than other animal
taxa (Avise 1994). A comparison with other species of Haliaeetus identifies a
Nei’s distance of 0.04 separating bald eagles from H. albicilla. However, a
comparison with populations of other species shows a Nei’s distance of 0.032
between 2 bull trout populations determined to be distinct population segments
(Leary et al. 1993). The Nei’s distances (although the wrong statistic) can only
be interpreted as high divergence between Arizona and other populations. Yet
the authors of the Reclamation report state “The genetic distances between the
populations in this study are well within the range of distances for a diverse
group of animals (Hunt et al. 1992c).” This is an error. The reported Nei’s
Figure 2. Principal Components of 1990 isozyme frequencies
Figure 3: First two principal components of 1985 allozyme frequencies
distances are in fact within the range separating separate avian species (Table 10). Nei’s distance is most useful for phylogenetic inference of groups that have already separated, for example, the bald eagle and allopatric members of its genus (Table 10).

Table 10. Survey of Nei’s distances. The reference from Barrowclough was offered as a comparisons in the Reclamation report.

<table>
<thead>
<tr>
<th>TAXA</th>
<th>LEVEL</th>
<th>DISTANCE</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>bull trout</td>
<td>DPS</td>
<td>0.032</td>
<td>Leary, et al 1993</td>
</tr>
<tr>
<td>crowned sparrows</td>
<td>sibling species</td>
<td>0.012</td>
<td>Barrowclough and Corbin, 1978</td>
</tr>
<tr>
<td>bald eagle</td>
<td>Az. - Fl.</td>
<td>0.034</td>
<td>Reclamation report</td>
</tr>
<tr>
<td>bald eagle</td>
<td>Az. - Wa.</td>
<td>0.04</td>
<td>Reclamation report</td>
</tr>
<tr>
<td>bald eagle</td>
<td>Az. - Ak.</td>
<td>0.092</td>
<td>Reclamation report</td>
</tr>
<tr>
<td>bald eagle</td>
<td>Az. - Tx.</td>
<td>0.035</td>
<td>Reclamation report</td>
</tr>
<tr>
<td>bald eagle</td>
<td>Az. - Ca.</td>
<td>0.03</td>
<td>Reclamation report</td>
</tr>
<tr>
<td>bald eagle</td>
<td>Az. - Mi.</td>
<td>0.03</td>
<td>Reclamation report</td>
</tr>
<tr>
<td>bald eagle</td>
<td>Az. - Md.</td>
<td>0.03</td>
<td>Reclamation report</td>
</tr>
<tr>
<td>bald eagle and</td>
<td>H. albicilla</td>
<td>0.04</td>
<td>Schreiber and Weitzel, 1994</td>
</tr>
<tr>
<td>H. leucogaster</td>
<td>inter-species</td>
<td>0.173</td>
<td>Schreiber and Weitzel, 1994</td>
</tr>
<tr>
<td>H. Leucoryphus</td>
<td>inter-species</td>
<td>0.171</td>
<td>Schreiber and Weitzel, 1994</td>
</tr>
</tbody>
</table>

Samples

The bald eagle samples in the Reclamation study were not collected to assess species structure. Multiple problems, all recognized by the authors, follow from the lack of experimental design for sample collection. Neither genetics study from the Reclamation report received acceptance in a peer-reviewed journal. The dubious nature of the samples may be at least partially responsible for the inability of the reports to gain acceptance into peer-reviewed
The author of the fingerprint paper offers the following: "We know nothing of the sampling regimes, and have no way of determining the potential for relatedness among samples, except a few samples that may be Arizona nestlings. Natal origin could not be specified for the adult samples since only capture location was known (Hunt et al. 1992c).

The authors of the isozyme study have a similar complaint: “Sampling the birds within a state and calling that sample a population while the birds from the same population that happen to be in an adjacent state are labeled as different population based on convenient political boundaries can produce misleading results. Similarly, sampling from 2 different populations in a large state and lumping all the samples together into what is called a single population can produce equally misleading results (Hunt et al. 1992c).” Ideally, managers would have the following information:

1) Did all the samples represent birds hatched in the area under which they are delineated?

2) Are the samples part of a nuclear family? full-siblings? partial siblings?

3) When were the samples collected? Are all individuals across all states the same age, or are confounding temporal effects present?

Discussion

Allozyme data show that the Arizona population accounts for most of the
genetic variation of the species, and therefore meet the ESA requirements for a
distinct population segment. The mid-Atlantic birds also cluster together, and to
a lesser extent, the Pacific Northwest birds (Figures 1 and 2). The bald eagle
species may be structured into more than a single population unit, or perhaps
birds from further southwest in Texas are part of the Arizona evolutionary group.
Violating the genetic integrity of these units with reintroduction threatens the
long-term health of the species (for a review of issues associated with
translocation see Lynch 1996).

The data presented by the FWS does not necessarily suggest isolation in the
southwest. An equally justifiable conclusion is that the data is inconclusive and
we know almost nothing about population structure within the species. Either
way, prudence suggests erring on the side of conservation (Hunt 1992a). Both
conclusions suggest the following management actions: 1) commence full-scale
protection for possible subpopulations until further evidence becomes known,
and 2) prohibit cross population reintroductions.

The opposite, and I believe, unjustified, management actions are being taken
by the FWS. This is not due to a lack of scientific ideas. Hunt (1992a), and
Morizot (1985) warned about the necessity for more research to examine
species substructure. Morizot recommended not reintroducing any bald eagles
until genetic studies explored the possibility of local, heritable, adaptations
(Morizot et al. 1985).

The choice to downlist the Arizona population and hack birds from unknown
genetic stocks may have resulted from 3 decision making errors: 1) genetic data
available at the time of the decision was not analyzed, and no further genetic studies were commissioned, 2) questions appropriate to the problem at hand were not asked and, 3) politically popular translocations may take precedence to science.

Using translocations to augment endangered populations is a relatively recent technique characterized by a shortage of empirical data. Reintroduction theory cautions against the possibilities of outbreeding depression within groups that may be adapted to local conditions (Templeton 1986; for a current review of the literature see Storfer 1999). Storfer (1998) showed that outbreeding caused by normal gene flow decreases the ability of populations to adapt to local environments.

High heterozygosity in Arizona birds, relative to other bald eagles may be due to the following characteristics: shorter generation time, smaller bodies, habitation of southern latitudes, and resident, as opposed to migratory, lifestyle. All 4 of these characteristics have been correlated with higher relative heterozygosity (Avise 1994, page 24). A correlation between southern climates and increased variability has also been noted in fish (Kellogg 1999). If a warmer climate correlates with higher heterozygosity, then Texas eagles should exhibit similarly high heterozygosity, and they do.

The Reclamation report asked specific questions. The answers, sought with molecular genetic techniques, have no bearing on the issue of translocation nor Arizona's status as a distinct population. Geneticists did not have access to samples that could answer this question. Important questions, which could be
answered with the available data, were left unasked. The Reclamation report asked the following questions:

► 1. Are there any unique genetic markers in the Arizona population?
► 2. Is the Arizona group suffering from a lack of heterozygosity typical of an inbred population?
► 3. Is Arizona genetically distant from all other groups of bald eagles? Why were the authors asked to keep Texas and Arizona separate? Is Texas part of the Southwest group?

Those questions, though answered correctly, had no bearing on whether the Arizona group is a distinct population, nor on whether panmixia exists to an extent compatible with large-scale hacking.

Genetic distance and genetic diversity are different measures and must be addressed individually. Genetic distance, relatively unimportant, was calculated, while genetic variation, was not calculated. Genetic distance measures divergence between 2 groups that have in fact, diverged. The genetic diversity of the species is its potential to evolve, or to adapt instead of becoming extinct with the inevitable variation in the environment. The diversity of a species is structured into that within each individual and that between the populations. Both levels of genetic diversity need to be conserved.

Managers should have delineated possible answers to the questions being asked of researchers, and speculated how those answers would influence the eagle policy. Consequences of alternative actions were never considered. What if Arizona is not a true population but protected as such? What if it is a true
population and is allowed to become extinct because the FWS believes the area will be recolonized by essentially similar bald eagles? What if translocations are held up or only involve adjacent areas and the population is panmictic? What if the population is not panmictic and we alter the gene pools? The answers seem obvious. The conservative approach could have been taken, until genetic studies using a finer level of resolution was reviewed, or until the isozyme data were expanded to include more samples.

The FWS interpreted negative data as evidence of panmixia within the lower 48. All of the authors of the genetics papers recommended prudence because of the inconclusiveness of the data. The FWS, ignored the opinion of the scientific community. The Society of Conservation Biology has suggested increasing the amount of scientific peer review (Meffe and Boersma 1998). Could peer review have reversed the downlisting or prevented artificial augmentation? I believe so. The scientific community has long warned of the potential disasters of poorly planned translocations and the genetic effects of stocking (Allendorf and Waples 1996; Griffith et al. 1989; Leberg 1993; Lynch 1996).

References cited


Conservation Biology 12: 268 - 270.


USFWS, 1995  Final rule to reclassify the bald eagle from endangered to threatened in all of the lower 48 states. Federal Register 60: 36000 - 36010.


Wright, S., 1931  Evolution in Mendelian populations. Genetics 16: 97-159.
As I complete my university degree, the role of the evolutionary unit in conservation biology and wildland management remains controversial (Pennock and Dimmick 1997; Gustafson et al. 1997; Malakof 1998; Peterson and Navarro-Siguenza 1999; Price 1996; Waples 1998). Specifically, the relative importance of genetics to determine conservation priorities is being discussed (Soulé and Mills 1998). I expect that Chapter 2 will receive acceptance as a contribution to the ongoing discussions.

Chapters 2 and 3 contradict 2 widespread beliefs about the evolutionary unit and populations. First, I show that the ESA listings which divide species into individual populations are rarely more than artificial species divided by political or economic boundaries (Pulliam and Babbitt 1997). Secondly, I show that the bald eagle, despite widespread beliefs to the contrary, is not being managed as
populations, but as a single panmictic mating unit. The bald eagle listing has never been officially contested, and Federal managers have become more persistent in their beliefs in the single mating unit within the lower 48 states.

In Chapter 2, I reviewed the Federal Register reports for all 29 vertebrate taxa listed, downlisted, or redefined from January 1, 1995 through December 31, 1998. Subspecies is the listing taxa 21% of the time. Among subspecies, 67% meet the criteria for an evolutionary unit, but are not so identified. The remaining listing taxa are evolutionary units, 14%, and distinct population segments, 31%. None of the population segments satisfy the criteria for evolutionary units. Among distinct populations, 38% are not panmictic and 75% are not reproductively isolated, or have only been isolated in the last half of this century. Failure to recognize the evolutionary unit is largely due to the use of political boundaries as surrogate biological boundaries. Natural boundaries are replaced with international borders 67% of the time, and with economic borders 22% of the time. By failing to recognize incipient speciation as the conservation unit, land managers have traded science for politics.

Evolutionary units are consistently applied to marine fishes and mammals. The application of this unit has been recent and has greatly improved the science of the ESA. For terrestrial taxa, and fishes not under the jurisdiction of the NMFS, evolutionary units are most often invoked for political reasons. They are used instead of, rather than support of, science. This too, has been a recent development.

The bald eagle has become the poster child for success of the Endangered
Species Act. The population structure is based on assumptions of migration and mating patterns that have not been tested. Conservation of the bald eagle is an important and timely issue upon which millions of dollars have been devoted. Yet the information upon which conservation has been based had never been systematically explored until this time.

A lack of funding and the inability to get samples has been a great disappointment. DNA was isolated from more than one hundred samples, with a variety of methods depending on funding and the condition of the sample. Each was quantified and qualified, fractionated, and photographed. In Chapter 4, I present what I believe is the first microsatellite region ever cloned from any Accipiter. During the preparation of the bald eagle library and microsatellite clones, I gathered much new information about a technology that now seems "old" to me. Countless numbers of experiments were completed. Library cloning is a rigorous undertaking for an unfunded project. New protocols were written to keep up with changes mandated by supply shortages, or just to modify published protocols to my supply situation. The technology I learned is still new for most conservation biologists, but many microsatellites have been cloned in the years since the project began.

I hope that investigation into the evolutionary unit of the bald eagle continues. More rigorous data collection is needed to support behavioral differences among the southwest birds. More data about the possible development of neoteny should be gathered. Plumage patterns of breeding birds should be recorded. Because most birds from that population were banded upon hatching, trapping
will allow the breeding adults to be aged. Evidence of direct migration into the southwest population should continue. Combined with population genetics theory, this will provide the single best information about whether the southwest bald eagles are on an evolutionary path distinct from the species overall.

References cited


