SOME THOUGHTS ON CONSERVATION, BIODIVERSITY, MUSEUMS, MOLECULAR CHARACTERS, SYSTEMATICS, AND BASIC RESEARCH

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Conservation biology and molecular genetics are two of the most dynamic and rapidly expanding fields of science. Both fields are complex and interact with many issues that are not only important to mammalogy, but to society in general. Several such issues are addressed including the use of genetic markers in conservation programs, the use of phylogenetic theory and molecular markers in understanding the epidemiology of hantavirus and human immunodeficiency virus, the value of museum collections to society, and techniques that improve the field collection of DNA samples from voucher specimens.

Key words: conservation biology, molecular genetics, biodiversity, systematics

The crush of the human population explosion has resulted in depletion of the ranges and populations of many species (Wilson, 1989), creating a dire need for wise management of the Earth's biodiversity. A resulting science, conservation biology (Soulé, 1986), has developed to provide critical information to address issues related to management of biodiversity. Conservation biology encompasses a wide range of applications including, but not restricted to, breeding strategies for captive populations, models of survival in habitat fragmentation, wildlife forensics, and the maximizing of harvest impact on overpopulated wild populations while minimizing the harvest impact on threatened wild populations. All of these issues are complex, and the discussion of each could occupy entire printed volumes. In this brief paper, I attempt to bring into focus a few issues associated with conservation genetics, molecular biology, and systematics, as well as the implications of these issues to broader problems of society.

GENETIC MARKERS AND THE ROLE OF MOLECULAR BIOLOGY

Evidence of the power of observing changes in genetic character-states was established by Darwin (1859) who used the patterns of stasis and change across taxa, time, and space to develop the theory of evolution. This theory established a framework upon which the current synthesis of biology is founded. In the 100 years following the publication of Darwin's theory, a primary factor limiting the resolution of many biological phenomena has been the dearth of detectable genetic characters. If adequate maternally, paternally, and biparentally inherited genetic markers are available for a particular species, we then should be able to accurately estimate evolutionary history, relative fitness, population subdivision, dispersal, gene flow, effective population size, extended pedigrees, levels of relatedness, natal origin, breeding structure, etc. The availability of large datasets involving many genetic characters would enable scientists to test models of speciation, metapopulational theory, balanced polymorphisms, molecular clock, kin selection, social structuring on rates of evolution, and altruistic behavior, to name just a few. While this grandiose outline of the potential application of genetic markers extends far beyond any single investigator's capabilities, I hope to contribute to the study of
these issues by providing a better understanding of genome organization that will permit the isolation of desired genetic markers and the use of these markers to better understand the biology of natural populations.

The mammalian genome typically consists of ca. 3 billion base pairs, which may be viewed as a formidable haystack or, alternatively, as a gold mine of potential genetic markers. Technological breakthroughs such as the polymerase chain reaction (PCR; Saiki et al., 1988) and improved cloning methods have made finding the desired needles in the genome haystack much more feasible. Development and application of molecular genetic markers is in an awesome expansion phase, and the volume of articles, books (e.g., Avise, 1993; Galbraith, 1994), new journals, and presentations at scientific meetings make it impossible to stay abreast of all of the work in this field.

Numerous techniques have been developed to provide genetic markers including multi-locus techniques such as DNA fingerprinting, which detects variation associated with variable number tandem repeats (VNTRs) of mini- and microsatellite clusters (Burke, 1989; Epplen et al., 1991; Longmire et al., 1991; Jeffreys and Morton, 1987; Jeffreys et al., 1985, 1986, 1990), restriction sites in heterochromatin (Hamilton et al., 1990; Simmons et al., 1992; Van Den Bussche et al., 1993), and retroposons and retrotansposons (Daniels and Deininger, 1983; Daniels et al., 1983; Krane et al., 1991; Lloyd et al., 1987; R. A. Van Den Bussche, in litt.; Vanlerberghe et al., 1993; Wichman et al., 1985). Single-locus methods include restriction endonuclease digestions and Southern blots (Southern, 1975) probed with single-copy DNA (Avise, 1993) fragments to detect restriction fragment length polymorphisms (RFLPs), development of primers for single-copy regions that flank VNTR clusters of micro- and minisatellites using PCR, and development of primers specific for single-copy regions that flank restriction-site variations (Karl and Avise, 1993; Welsh et al., 1991) or variable regions such as introns (Lessa, 1992; Manhart and Palmer, 1990).

One of the most commonly employed mechanisms to develop populational markers involves the use of primers that flank microsatellite VNTR clusters. An important question is how many microsatellite VNTR clusters are available in a typical mammalian genome as potential variable genetic markers? To address this question, we estimated the number of these regions that are available for study in the genome of the white-footed mouse, Peromyscus leucopus (Janecek et al., 1993). These results suggest that within the haploid genome of P. leucopus there are ca. 75,000 (GT)$_n$ clusters, 50,000 (CT)$_n$ clusters, 13,800 (GATA)$_n$ clusters, 6,800 (TCC)$_n$ clusters, and 40 (GC)$_n$ and (AT)$_n$ clusters. This indicates that there are ca. 145,000 of these six microsatellite clusters that are available for the development of variable genetic markers. While other microsatellite clusters occur in the mammalian genome, these six provide an idea of the relative abundance of such microsatellites in the mammalian genome. Of course, all microsatellite clusters may not be sufficiently variable to provide resolution to address evolutionary questions. Nonetheless, it is obvious from the abundance of microsatellite clusters, as well as the potential of techniques noted previously, that adequate genetic variability is detectable with current methods to resolve many biological phenomena in far greater detail than previously was possible. While this is an exciting time from a technical standpoint, caution must be used in applying these data to specific conservation needs.

Molecular Genetics, Distinctness of Taxa, and Hybrid Origins

It is realistic to expect that science can provide a suite of DNA markers that identify the limits of monophyletic groups (taxon-specific markers) and the geographic or-
igin of populations within a species. In a best-of-all-worlds scenario, a single piece of tissue contains more than enough information to identify the taxonomic position and geographic origin of the donor organism. While time, money, and personnel make it impossible to do this for all species in the near future, it is realistic and necessary to build such supporting data for carefully chosen species to be used as model systems.

One of the controversial frontiers to which molecular genetic markers will contribute significantly will be in the estimation of distinctness of populations and whether specific status should be accorded taxa. Such decisions undoubtedly will remain controversial and subjective, nonetheless, empirical data should help remove some subjectivity. Initially, the issue will be difficult to quantify because these markers have the potential to identify every individual in every population. Ultimately, with an adequate understanding of variation of these markers within and among taxa, statistical procedures should be developed that at least provide a defendable working position. Because in nature the limits of species boundaries are so variable that a single concise definition is impossible and because the species concept is poorly formulated in the minds of most biologists, an appropriate amount of controversy and confusion undoubtedly will remain. Some pertinent papers that address these issues include Dowling et al. (1992a), Frost and Hillis (1990), Honeycutt and Yates (in press), Jones et al. (in press), O’Brien and Mayr (1991), and Wayne (1992).

Also linked to the issue of distinctness of populations is another controversial issue that concerns the implications of hybridization to the endangered species act and to the international code of nomenclature (International Commission on Zoological Nomenclature, 1985). Hybrid individuals are not to be protected by the endangered species act and are not to be recognized as formal taxa (Frost and Hillis, 1990; Jones et al., in press; O’Brien and Mayr, 1991). The conservation of the red wolf (Canis rufus) and the Florida panther (Felis concolor) certainly are entangled in this ‘‘hybrid’’ issue. As demonstrated by Dowling et al. (1992b), Jones et al. (in press), Lehman et al. (1991), Nowak (1992), O’Brien and Mayr (1991), Phillips and Henry (1992), and Wayne et al. (1992), molecular genetic markers provide critical resolution concerning the hybrid origin of individuals and toward this end will play a critical role in resolving these issues.

**PHYLOGENETIC THEORY, GEOGRAPHIC VARIATION AND DNA ZIPCODES**

As much as any scientific discipline, mammalogy has been concerned with patterns of geographic variation within species and subspecies (Hall and Kelson, 1959). The advent of molecular techniques such as those described previously undoubtedly will provide considerable resolution to our understanding of patterns of geographic variation. We use the term ‘‘DNA zipcode’’ to describe DNA-based markers that resolve geographic patterns within species. Although geographic regions where the boundaries of many zipcode markers are concordant would likely be considered as a potential subspecies boundary, as envisioned, zipcode markers should provide far greater resolution of geographic subdivisions within a species than would be appropriate for any type of taxonomic consideration. The development of a hierarchical scheme where zipcode markers are ranked by the size of the geographic range that the marker resolves would be analogous to the way postal codes are established. An example of how restriction-site analysis in satellite DNA was used as a tool to broadly define the geographic origin of individuals within a species is provided by Simmons et al. (1992) for P. leucopus (Fig. 1). More restricted zipcode markers for P. leucopus are described for mitochondrial DNA (Nelson, 1988). It is probable that markers from both the nuclear and mitochondrial ge-
to determine that a man who lived in Arizona and died with the virus actually had been infected with the hantavirus in Colorado.

In the next example discussed, the sequence data fall outside the definition of zipcode markers, unless you consider individual humans as geographic locations and viruses as species. Nonetheless, this specific example is important because biologists who work on basic research for the sake of knowledge, such as patterns of variation in nature, often are asked to justify the value of their work. The biology of systematics not only forms the foundation for classifying forms of life, understanding biodiversity, and a logical framework for understanding the origin and evolution of molecules to life forms, it also is the foundation upon which complex societal issues will be decided. In this specific case, phylogenetic theory is used as a critical data point in understanding the relationships between healthcare workers and their patients. Ultimately, these results impact liability, right to privacy of the patient and of the healthcare worker, and the evaluation of disease risk factors. Using phylogenetic methods to resolve applied problems is an excellent example of how such “esoteric” systematic studies can be valuable to other issues of society. D. M. Hillis and his colleagues (Ou et al., 1992; D. M. Hillis and J. P. Huelsenbeck, in litt.; D. M. Hillis et al., in litt.) have analyzed sequences from the provirus stage of the human immunodeficiency virus (HIV) with bootstrap analysis (Felsenstein, 1985) using PAUP (Swofford, 1991) and their knowledge of basic systematic theory to provide probability statements on the transmission of HIV in a case that involved a healthcare worker (dentist) in Florida. Their work established that five individuals who had no other risk factors for HIV and were patients of this particular dentist had HIV sequences that clustered closely with the sequences of HIV that were isolated from the dentist. However, other patients of this dentist who

Fig. 1.—Geographic distribution of *Peromyscus leucopus* (shaded area). The solid circles represent localities where individuals have a 1.4 kilobase DNA zipcode. Open circles with dots represent localities where individuals do not possess this particular DNA marker. In this study, all individuals were scored for this fragment without reference to geographic locality. Details of the procedure are outlined in Simmons et al. (1992). Reprinted from *Molecular Ecology*.

nomes will complement each other in the use of geographically diagnostic markers.

The hantavirus issue also can provide some insight into the potential of zipcode-type data. The recent events relating to the hantavirus, which infects the deer mouse, *Peromyscus maniculatus*, are having an important impact on many facets of society. The hantaviruses are rodent borne, belonging to the family Bunyaviridae, and their genome is RNA-based rather than DNA-based. Nonetheless, sequence data of the hantavirus genome have been analyzed using phylogenetic analyses developed for studying systematic relationships among species (PAUP; Swofford, 1991). These data revealed subclusters of the virus that were related to geographic populations of *P. maniculatus* (Nichol et al., 1993). Using these data, Nichol et al. (1993) were able
had additional risk factors such as intravenous drug users or male homosexuals had HIV strains whose sequences clustered with sequences from local controls rather than those of the HIV isolated from the Florida dentist. Without a baseline understanding of the biodiversity of HIV and the development of phylogenetic theory, the empirical basis upon which these societal issues will be decided would have been tremendously weakened. This significant contribution to legal, social, and medical issues has been provided as a result of basic research on phylogenetic theory, biodiversity, and computer algorithms to analyze complex sequence data.

**Molecular Genetics, Captive Breeding Programs, and Maintenance of Genetic Diversity**

An example of the difficulty in using data from molecular genetic markers can be visualized in developing a breeding strategy for a founding captive population. The goals of such a captive breeding program would include 1) increasing the number of individuals, 2) maintaining maximum genetic diversity within the population (Maltbie, 1992), and, of course, 3) using these colonies to re-establish viable natural populations. Strategies to maintain genetic diversity within the population have been developed, which include breeding for maintenance of rare alleles identified by genetic markers, selecting crosses between the most divergent individuals, and breeding for maximum genetic diversity in the histocompatibility genes (Gilpin and Wills, 1991; Haig et al., 1990; Hughes, 1991; Miller and Hedrick, 1991; Vrijenhoek and Leberg, 1991). While all this may seem simple enough, two types of fitness depression (Soulé, 1986) can be associated with captive breeding programs. The first of these is inbreeding depression (Chessser, 1991; Laikre and Ryman, 1991; Noble et al., 1990; O'Brien et al., 1985; Ralls et al., 1986; Ryder, 1993; Wildt et al., 1987), which certainly would be avoided by breeding the most distantly related individuals within the captive population. Outbreeding depression (Templeton, 1986), however, could be a problem when using a strategy that attempts to maximize genetic diversity in the offspring of captive individuals.

Outbreeding depression occurs when local adaptations and isolating mechanisms (premating and postmating) have evolved within subunits of a species. One set of circumstances under which outbreeding depression might occur is when individuals in the captive population come from different geographic regions. Zoo programs have been concerned with these issues for years because the composite zoo populations of various megavertebrate species usually come from a wide variety of geographic localities. In most examples, there probably will not be adequate breeding data from natural populations to determine the extent to which geographically divergent samples are genetically compatible (Greig, 1979). However, karyotypic analysis and other genetic markers may provide some mechanism of identification of trial crosses from which the offspring may be evaluated for fecundity.

A specific example of outbreeding depression is found in the parapatrically distributed taxa of the plains pocket gopher, *Geomys*, which are highly variable across their range. In the latest revision of *Mammal Species of the World* (Wilson and Reeder, 1993), these gophers, which range from Manitoba and Indiana to Louisiana, western Texas and eastern New Mexico, were recognized as a single species, *G. bursarius* (Patton, 1993). Our field studies in western Texas over the past 25 years have indicated significant changes in the geographic range of *Geomys*, and, in many regions, *Geomys* is being replaced by populations of *Cratogeomys*. Taxa at the southern end of the distribution of the species such as *knoxjonesi*, *texensis*, and *llanensis* may be particularly impacted by agricultural and environmental changes (Jones et al.,
in press). It is possible that some populations ultimately will be considered for protection and maybe even for captive breeding programs. In such examples as Geomys, it is critical to know the species concept used by the systematist before any captive breeding program is established. Data from a natural hybrid zone between knoxjonesi and major (Baker et al., 1989; Bradley et al., 1991) indicate that potential F₁ crosses between males of knoxjonesi and females of major do not occur in nature, which would eliminate 50% of the potential crosses. Additional data suggest that crosses between males of major and females of knoxjonesi produce F₁ males that are sterile and females that also have a low fertility level. If the captive population of Geomys included representatives of these two taxa and biologists bred these taxa for maximum genetic diversity, the end result would be disastrous because offspring production would be low and most viable F₁ individuals would be infertile and would have reduced fertility.

**Value of Museum Collections To Society**

Much of the order that the scientific community has relative to biodiversity and systematics has its foundation in the museum concept. With the advent of PCR, which can amplify DNA from small amounts of skin, bones, and tissue, the value of skins and tissues stored in museums has been significantly amplified (Diamond, 1990; Higuchi et al., 1984, 1987; Pääbo, 1989; Pääbo et al., 1988; Thomas et al., 1990; Yates, 1987). This increase in value also has produced a dilemma for the museum community because examination of the material requires that at least some destructive procedures be applied to valuable museum specimens. A number of museums have committed considerable resources to the collecting, cataloging, and maintenance of frozen tissues (Dessauer and Hafner, 1984) such as livers, kidneys, muscles, and hearts, with knowledge that the ultimate purpose is destruction for molecular studies of various sorts. Our museum at Texas Tech University is among those committed to such collections.

From a biodiversity standpoint, these collections are valuable because DNA from small amounts of tissue can be cloned into libraries or used for PCR amplification without any damage to the voucher specimen. When tissues are available to bona fide researchers as well as exchanges between museums, this reduces the need for field trips and additional collecting. When a specimen of a rare or endangered species becomes available through death due to natural causes or whatever reason, as much tissue as is possible should be saved in these frozen museum collections in order that samples will be available for future studies without any impact on the remaining living individuals. Although it is unrealistic to expect that all mammal collections would have a frozen tissue division, it is time for cooperative efforts to be developed among museums so that when scientific collecting occurs, frozen tissues from these specimens will be deposited in an accredited, systematically arranged, and computerized museum collection. Because of the large number of curatorial problems associated with frozen tissues, there is a need to develop new methods to preserve these molecules that circumvent these unique collection-management problems (see next section).

There are many potential uses for frozen-tissue collections involving systematics, toxicology, forensics, population genetics, and medicine, as well as studies on temporal changes in populations as associated with human disturbances. But as with the HIV example given previously, I would like to choose an extreme example for discussion because the limits of use for such collections are set by our technology and our ability to organize scientific experiments. The example that I have chosen, however, is of extreme importance to mammalogists. The hantavirus outbreak previously mentioned has caused considerable health concerns for humans, and implications to mam-
malogy have been difficult to understand. Specifically, it would be very beneficial to be able to understand how the infection is transferred from deer mice to susceptible individuals, including mammalogists, and this information will ultimately form the foundation for decisions relating to how we practice mammalogy and what risks are associated with various activities of professional mammalogists. According to the Center for Disease Control (CDC) in Atlanta, the primary vector of this virus is *P. maniculatus*, and the mode of transmission is breathing the airborne dust of dried feces. Considering the typical precautions (or the lack thereof) of many mammalogists conducting field studies, undoubtedly many that have worked in the range of *P. maniculatus* have inhaled mega-doses of potential hantavirus-laden particles. An important question is have mammalogists caught the hantavirus? If not, is it because 1) the virus has recently invaded *P. maniculatus* and mammalogists have not previously been exposed, 2) the virus has been common all along but new mutations have produced a more virulent strain, or 3) the virus has been common all along but it is difficult to transmit the virus to humans when they do the kinds of things field mammalogists do? If options 1 or 2 are true, then mammalogists need to implement new protective measures for fieldwork. If option 3 is correct, then mammalogists are relatively safe using previously acceptable practices. There are a variety of ways to address this question, but let me outline some potential experiments involving frozen-tissue museum collections that might shed light on this matter.

I recall a field trip for a mammalogy class in 1989 when we visited a grassland field that contained hundreds of large, round hay bales. We collected rodents by lifting the bales with a tractor and catching by hand the mice that were exposed. Each time a bale was lifted, many rodents were exposed, most of which were *P. maniculatus*. In some cases, >30 individuals might be exposed under a single bale. The hay bales were old, with burrows and nests common throughout. When a bale was lifted, a cloud of dust and falling debris, much of which must have contained old nest material and mice droppings, showered the students as they collected the mice, but did not slow the chase. A few of the several hundred mice that were captured during this trip were returned to Texas Tech University and prepared as museum specimens with frozen tissues and vouchers deposited in the museum. As I recall, none of us became sick with hantavirus-like symptoms. Although none of this was done with forethought about experimental design and hantavirus, tissues were saved; we now have an opportunity to determine if the *P. maniculatus* in these bales were infected with the hantavirus (by examining the frozen tissues), and we also can determine if any of the students on the field trip have antibodies against the virus. If the virus is present in the *P. maniculatus*, then it can be amplified from the tissues, sequenced, and compared to those isolated from people demonstrating the symptoms to determine the extent to which the two vary. In December 1993, CDC announced that it is now possible to isolate the hantavirus. If the mice collected on the 1989 field trip have the virus, it may be possible to isolate the virus from the frozen tissues and to test how virulent this strain is compared to those involved in the current outbreak in the four-corners area (Grady, 1993).

The frozen-tissue collection at Texas Tech University contains >350 specimens of *P. maniculatus* including representatives from 19 states in the United States, five states in Mexico, and two provinces in Canada. These specimens, along with others in museums that have large holdings of *P. maniculatus* (e.g., University of New Mexico, Texas A&M University, University of California at Berkeley, and Louisiana State University), should be able to provide considerable insight into the geographic range of the hantavirus. Additionally, tissues from
other taxa also should be valuable in determining the taxonomic range of hantavirus. Unfortunately, frozen tissues were not extensively collected prior to the late 1970s, but those earlier specimens may provide particularly valuable information. Of course, if it proves possible to amplify, by PCR, the hantavirus from museum skins, then the ability to study this disease both temporally and geographically would be increased tremendously.

The critical point is that a few years ago, there were little or no empirical data to justify collecting these specimens and tissues with the intent of addressing a current health problem that could potentially affect the hundreds of millions of people that live within the geographic range of *P. maniculatus*. Nonetheless, systematic collections of specimens preserved in museums serve society in this and many other valuable ways.

*Note added in galley:* Information provided by Zaki et al. (in press) document that activities typical of field mammalogists do make individuals at risk for hantavirus. Zaki et al. (in press) report that a 23-year-old graduate student in Kansas, who routinely handled wild rodents, died in 1983 with a viral pulmonary edema and subsequently tested positive for hantavirus. Zaki et al. (in press) report that the individual retained Sherman live-traps with rodent excrement in his living quarters. The significance of this report is that at least some activities place mammalogists at risk for hantaviral infections; therefore where appropriate care and precautions such as those outlined by the Centers for Disease Control and Prevention should be followed.

**Collection of Tissues for DNA Samples**

I would like to use this opportunity to call attention to a different method of collecting tissues for DNA samples that does not require refrigeration. The procedure that we commonly use (J. L. Longmire and R. J. Baker, in litt.) involves macerating the liver, kidney, brain, or other appropriate tissue into a paté and mixing these macerated tissues in a lysis buffer. With the exception of scissors and forceps, all material involved is disposable, and, with reasonable caution, there is little possibility of cross contamination of samples that might give false amplifications when using PCR. After the tissue sample is removed from the specimen it takes <5 min to get the cells mixed in lysis buffer. Once the tissue is mixed in the lysis buffer, little care is needed, and samples have been kept for >1 year before finishing the process of DNA isolation without any detectable degradation of DNA quality. The laboratory procedures associated with this technique involve digestion with Proteinase K, a single phenol extraction, and dialysis.

Another positive aspect of this procedure is that it yields the highest molecular weight and the greatest volume of DNA of any method that we have attempted in our laboratory. The method yields both mitochondrial and nuclear DNA. We have further adapted this method to the shipping and receiving of samples from the frozen-tissue collection that are to be used for DNA analysis. In these cases, the tissue sample is thawed, macerated, placed in the lysis buffer, and shipped by standard mail.

We consider this procedure a great advantage for opportunistic sample collections because a relatively small field kit is all that is required rather than the equipment needed for either liquid nitrogen or dry ice. There are some negative aspects associated with this procedure. Tissues collected by this method do not work for protein electrophoresis, and it is doubtful if these samples would have value in detecting antibodies, pesticides, and possibly other complex molecules. It is yet to be determined to what extent RNA that is isolated by these procedures is valuable for other procedures, such as cDNA libraries. Nonetheless, this field method should prove valuable for some situations involving collection of museum specimens, especially at localities where dry ice or liquid nitrogen is
difficult to obtain or transport. It also is valuable for reducing costs to frozen-tissue collections in cases where frozen tissues will be used for studies such as those previously listed. I would be pleased to supply people with the detailed protocol and list of equipment for this procedure.

A CALL FOR COOPERATION

The crisis that we face in conservation of biodiversity is exceedingly complex, and it is highly probable that many of the complexities are yet to be minimally visualized. Conservation biology is expensive, labor-intensive, often tedious, and requires considerable expertise. Most strategies are developed with a low confidence level of success, in many cases because of the lack of knowledge. If we are to address these issues, it will require cooperation among diverse scientists including conservation biologists, systematists, molecular biologists, statisticians, natural historians, reproductive biologists, and ecologists, to name a few. A lot of time and energy will be spent developing strategies for preservation and recovery of biodiversity, and I hope that these strategy sessions will involve our best scientists from the appropriate diverse fields. Also, because many procedures are so expensive, financial constraints will make it impossible for excessive duplication of effort. This means that cooperation among scientists and laboratories will be needed to most expeditiously address these issues. We should all carefully consider how important biodiversity is to society and cooperate in an appropriate manner.

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