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Abstract: The U.S. Fish and Wildlife Service (Service) is responsible for recovery of endangered species in the wild and, when necessary, maintenance in captivity. These programs provide an immediate measure of insurance against extinction. A prerequisite inherent in all of these programs is the preservation of enough genetic diversity to maintain a viable population and to maintain the capacity of the population to respond to change. Measures of genetic diversity examine polymorphic genes that are not influenced by selection pressures. Examples of these techniques and those used to determine relatedness are discussed. Studies of genetic diversity, electrophoresis of blood proteins, relatedness, blood typing, and restriction fragment length polymorphisms which are being used by the Patuxent Wildlife Research Center are discussed in detail.

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INTRODUCTION

The Service is responsible for recovery of endangered species in the wild and, when necessary, their maintenance in captivity. The Service, with the help of other conservationists, has established programs that provide insurance against extinction, yield essential management information, preserve extant genetic variation, and provide stock for reestablishing populations. The Service participates in 2 cooperative crane programs, 1 for the Mississippi sandhill (*Grus canadensis pulla*) and 1 for the whooping crane (*Grus americana*). Nineteen of approximately 50 Mississippi sandhill cranes on the Mississippi Sandhill Crane National Wildlife Refuge are captive-reared birds. In addition, 5 of 6 breeding birds were from stock released in 1987 (Mindy Hetrick pers. comm.). Another 50 birds are in captivity at the Patuxent Wildlife Research Center, including 9 breeding pairs. The whooping crane program has developed more slowly. Presently, the species consists of about 200 birds—a captive flock of 42 birds at the Patuxent Wildlife Research Center, a small separate release population of 30 birds in Idaho, and about 135 in the remnant breeding population at Wood Buffalo National Park in Canada.

A prerequisite inherent in all of these programs is the preservation of enough genetic diversity to maintain a vital population and its capacity to respond to change (Mettler & Gregg 1969; Wilcox et al. 1986). Genetic diversity, the presence of alternative forms of genes (alleles) or gene combinations in a population, may not be expressed by obvious signs. However, the lack of diversity often reduces resistance to disease, decreases fertility, increases embryo mortality, and reduces growth rates (Frankel & Soule 1981). In addition, loss of alleles responsible for habitat adaptation during evolution may lead to extinction when the habitat reverts to a previous condition.

In this paper we describe methods used to detect genetic diversity and relatedness in cranes at the Patuxent Wildlife Research Center, i.e., electrophoresis, blood typing, competitive binding immunoassay, and restriction fragment length polymor-

phisms. In addition, we discuss the special use of the histocompatibility complex (blood typing) in the preservation of disease resistance in birds.

METHODS

Electrophoresis

When measuring genetic diversity, one should examine those polymorphic genes that are influenced little by selection pressure (neutral indicators) and examine the gene (DNA) or its direct product. If possible, sampling from the population should be avoided after situations that disrupt gene flow such as (1) nonrandom mating, (2) recent immigration, or (3) conditions that cause genetic drift (Corbin 1983). Most tests estimate, rather than measure, genetic diversity, and vary from those that identify products from a few specific gene loci to those that identify the actual base pair sequence in the entire genome. Most genetic diversity estimates are based on protein electrophoresis of tissue homogenates followed by histochemical localization of specific enzymes. Electrophoresis, a common laboratory procedure, is discussed in detail in Corbin (1983) and many other sources. The banding patterns which result represent the phenotypes at the enzyme locus and yield evidence on the number of alleles segregating at the locus in the population and genotypes of the individuals tested. Unfortunately, only a small part of the genome can be examined by electrophoresis (20 to 30 loci in blood and 50 to 60 loci in tissues), and some alleles are undetectable at those loci that can be analyzed.

Patuxent has conducted 3 electrophoresis studies of diversity in captive endangered birds, 2 in cranes. Dr. Don Morizot at the University of Texas completed a masked bobwhite (*Colinus virginianus ridgwayi*) blood study in 1983, finding that the substantial variation found in masked bobwhite blood isozymes was as great as that found in any other bobwhite (D. Morizot pers. comm.). These results substantiated the effectiveness of our outbreeding program and the use of inbreeding coefficients in mate selection. Genetic diversity in Patuxent's captive quail flock is probably greater than in the native population (Gable & Gee 1987). Dr. Ray Morgan at the University of Maryland examined 10 to 15 crane blood loci in 1975 and found a limited amount of variation, indicating a need for more study, but he appeared to be limited by available techniques and equipment. We started a second study of genetic diversity in cranes with Dr. Herbert Dessauer at the Louisiana State University (LSU) Medical Center in New Orleans in 1985. That study included 4 species of cranes—whooping, sandhill, sarus (*Grus antiqone*), and Siberian (*Grus leucoqeranus*)—and 4 subspecies or races of sandhill cranes—greater (*Grus canadensis tabida*), Florida (*Grus canadensis pratensis*), Georgia (*Grus canadensis pratensis*), and Mississippi (*Grus canadensis pulla*). Preliminary evidence indicates a near normal level of variation in whooping cranes and a reduced level of variation in the greater sandhill from Idaho (Dessauer & Gee 1986). Other species of cranes are being added to the study, and completion is anticipated in 1991.

Breeding programs are the essential management tool used to maintain genetic diversity and depend on an established pedigree in the captive flock to function properly. Diversity estimates can indicate the effectiveness of a breeding program to maintain diversity and can be useful in determining pedigree when they measure differences between individual animals.

Blood Typing

Phenotypic expressions such as eye color and plumage patterns are of limited use in establishing pedigree. There are few of them, and their expression can be difficult to interpret because of interactions with the environment and with other genes. Blood type, various immunoassays, amino acid sequencing, and other tests that isolate specific gene loci can be used to identify family lines. However, most of these tests are of limited use in nondomestic animals because techniques or reagents are not available. Blood typing or immunogenetics of blood, identifies substances on the red cell surface (antigens) and uses molecules of gamma globulin (antibodies) to identify them. Each red cell antigen is controlled by a gene belonging to 1 of several systems. Each system is under the control of an individual gene locus which segregates independently of individual loci controlling other systems. Blood typing is used widely in human medicine and in domestic animal breeding. Even in chickens, where selection pressures have been intense, many blood types segregate independently and can be used to estimate heterozygosity and relatedness. Reagents (antibodies) are prepared by making injections of blood from 1 bird into different birds (preferably of many different blood types). After the birds have produced the antibodies, a large blood sample is taken and the

serum harvested (reagent). Blood cells, with antigens reactive with the reagent, clump together as a result of cross-linkage by antibody molecules (agglutinate). These reagents are most effective in the species from which the blood was taken but can be effective in other species.

Alloantigen research in the domestic chicken offers considerable promise in other nondomestic birds by providing serological reagents useful in determining relatedness and in detecting certain antigenic markers of immune response genes. The prefix *allo-* indicates that there exist alternate antigenic types within the species, each determined by different genes of the same genetic system or locus. The most readily identified alloantigens are those on the surface of red cells, which cause reactive cells to agglutinate or clump together. Thirteen separate genetic loci or systems of erythrocyte alloantigens are currently evaluated each generation in an experimental colony of chickens at Northern Illinois University (Briles 1984; Briles & Briles 1987). The individual chickens are used as recipients and donors in appropriately matched immunizations to produce antisera containing antibodies useful in typing the individual alloantigens.

The detection of alloantigen differences within the whooping crane began in 1985. The red blood cells of 33 whooping cranes were tested with 41 reagents shown to be specific in chickens for individual antigens, each belonging to 1 of 12 systems— *A, B-F, B-G, C, D, E, H, I, J, K, L,* and *P.* Different reactivities of the cells from individual cranes were shown by 17 of the 41 reagents. Six cranes appeared to be much more reactive than the others to alloantigens of the *B, C, D, E, I, K,* and *P* systems. Thus, cells of these 6 cranes appeared to be agglutinated by an antibody in these sera in addition to the system-specific antibodies against the chicken alloantigens. Recently, we found that "normal" chicken serum may agglutinate the red cells of several crane species. These "normal" antibodies can be removed by absorbing reagents with the red cells of 1 or more individual cranes. Certain anti-chicken antibodies may remain in the absorbed reagent and exhibit differential reactivity with other members of the same crane species supplying the cells used in the absorption.

In a recent collaborative investigation with Claire Mirande and Scott Swengel of the International Crane Foundation, Sue Jarvi and Elwood Briles differentially absorbed *B-F* and *B-G* chicken anti-chicken reagents with the blood of individual Siberian cranes and used these reagents to detect at least 2 major histocompatibility (*B*) haplotypes among 6 birds tested (unpublished). Thus, with appropriate absorption of chicken anti-chicken reagents with the blood of individual members of another bird species, it is now feasible and practical to prepare reagents capable of detecting individual haplotypes of the major histocompatibility complex (MHC) in a species of special interest.

In 1989, 6 different haplotypes were isolated using intrafamilial blood injections (Briles 1984), in Florida sandhill cranes (Gee et al. unpublished). The first use of these reagents effectively separated paternity in young Siberian cranes (*Grus leucogeramus*). An expansion of the typing study based on the 1989 results should isolate other haplotypes.

The *MHC*, or *B* system as it is genetically designated in avian species, plays an essential function in the immune response at the population level (Klein 1986). Therefore, maintenance of haplotype diversity in a population preserves a higher level of immune response, resulting in a greater capacity to resist pathological organisms. The *MHC*, as well as other alloantigen systems found to be segregating in a crane species, may also serve to identify familial relationships.

Competitive Binding Immunoassay

Many other immunoassays use blood serum or tissue reactions to identify individuals or groups of animals. Most of these use an antibody or antigen specially labeled with a dye, radioisotope, or fluorescent chemical to detect the immune reaction. One of the more complex of these, competitive binding immunoassay (e.g., Lowenstein 1985; Sarich 1976), is being used to estimate diversity and relatedness in fish by Dr. Simon at the National Fisheries Research Center in Leetown, West Virginia. Attempts to use this system to determine relatedness in cranes have been unsuccessful thus far.

The competitive binding immunoassay is not as well known as other procedures described here. In the first phase of the competitive binding immunoassay (reagent production), an individual component in the blood serum is chemically and physically separated, and used as antigen in the assay. Albumin is injected into a rabbit or some other suitable animal to produce reagent. Even 1 component will have several identifying antigens, similar to alloantigens of blood group systems. We chose albumin in whooping crane blood serum as our antigen source because it was easily isolated and possessed a limited number of antigenic poly-

morphisms.

In the first step of the testing phase of the immunoassay, an exactly measured amount of reagent (anti-albumin) is added to an unknown antigen. In the second step of the testing phase, wells in plates are coated with homologous albumin (albumin used in the injections used to make the reagent in the first phase). The incubated reagent-antigen mix prepared in the first step is added to the plate and the excess washed from the plate after an appropriate incubation time (24 hours for cranes). A color reaction is produced, stained antibody against the anti-albumin antibody, to determine the amount of antibody present. Plates without stain result when homologous albumins are used.

The results are simple to interpret. Plates from closely-related animals stain lightly, distantly related individuals stain strongly, and the others are intermediate. Families of animals tend to cluster together, and these clusters can be tested again with a new reagent to separate the clusters.

Restriction Fraqment Lenqth Polymorphisms

A better understanding of maternity, paternity, and relatedness is especially important for the management of the Patuxent whooping crane flock. Although most founder animals used in the captive flock are taken from nests of known parents, the relatedness of neighboring pairs and the continuity of the birds at the nest site are unknown. Also, some birds were taken after fledging and cannot be identified by nest territory, and several birds produced in captivity are of unknown paternity. Semen from more than 1 male has been used for insemination when semen from the regular donor male was unavailable. More intensive identification of birds on the breeding grounds in Canada will eliminate much of that confusion in birds taken from the wild. But, if propagation programs hope to obtain 90+% fertility of eggs laid, the occasional use of semen from a male other than the designated donor will be necessary.

Recent developments in DNA analysis make it possible to determine paternity and maternity and estimate relatedness between individuals. The new method of DNA analysis allows the researcher to detect variations in DNA structure. Enzymes, known as restriction endonucleases, cut chains of DNA at specific sites and the restriction fragments of DNA obtained are identified with radiolabeled complementary probes. In man, Jeffreys et al. (1985) described regions of tandemly repeated sequences in these restriction fragments that were highly polymorphic (restriction fragment length polymorphisms or RFLP). A radiolabled complementary RFLP probe was developed to identify these fragments. The level of polymorphism detected by the probe was sufficient to provide a set of genetic "fingerprints" for all individuals tested. Although originally described in humans, hypervariable regions (highly polymorphic RFLP) have been found in a number of vertebrate species.

In addition to the species-specific probe, a probe from a tandemly repeated sequence in DNA from the M13 bacteriophage can be used to identify the hypervariable regions in several animals (Vassart et al. 1987). J. Longmire at the Los Alamos National Laboratory has found that this same M13 sequence detects hypervariable RFLP in a number of avian species, including the peregrine falcon (*Falco peregrinus*), the Mauritius kestrel (*Falco punctatus*), and the whooping crane (Longmire, in press). Longmire also found that the M13 probe can be used to identify sex in the peregrine falcon and the Mauritius kestrel. Also, O. Ryder at the San Diego Zoo has used the M13 probe to identify hypervariable RFLP's in the California condor (Gymnogyps californianus) (Ryder pers. comm.). In addition, J. Longmire at Los Alamos has isolated tandemly repeated sequences from large blocks of heterochromatin localized around the centromeres of merlin (*Falco columbarius*) chromosomes. Longmire and co-workers have used this probe to measure relatedness between individuals and populations of peregrine falcons (Longmire in press; Longmire et al. 1988).

In summary, at least 2 distinct classes of tandemly repeated sequences can be used to prepare these probes: (1) dispersed, such as the M13 phage and (2) heterochromatic, such as the merlin probe. Patuxent is cooperating in 3 crane RFLP studies, 2 to determine relatedness and diversity and 1 to determine sex. The Los Alamos National Laboratory is conducting a relatedness and diversity study of captive whooping cranes using a species-specific probe developed from the whooping crane red blood cell DNA. This analysis is similar to the one accomplished with the peregrine falcon (Longmire in press). Another crane relatedness study is being conducted at the LSU Medical Center using a probe developed from the M13 phage similar to techniques developed by others (Vassart et al. 1987). Also at the LSU Medical Center, RFLP's are being screened to find ones that are unique to the w-chromosome to develop a probe for sexing cranes. Los Alamos has found that the M13 probe

allows sex determination in the peregrine falcon and the Mauritius kestrel.

A special RFLP probe for the whooping crane has been isolated at the LSU Medical Center in New Orleans that separates whooping cranes from all other cranes and cranes from all other species. Using this probe to estimate systematic distance between cranes, the crown cranes (*Balearica regulorum* and *B. pavonina*) appear to be the most distantly related to the whooping crane and all Grus species to be closely related except the sandhill crane (Love 1990).

CONCLUSIONS

A prerequisite inherent in endangered species programs is the preservation of genetic diversity. Estimates of diversity and relatedness among nondomestic birds in captivity and intensely managed wild populations may be accomplished by a variety of techniques. Several such techniques have been employed, successfully and unsuccessfully, with the captive crane population at Patuxent.

Most diversity estimates are based on protein electrophoresis of serum protein or tissue homogenates. Although limited to 20 to 30 genetic loci in blood and so to 60 loci in tissues, electrophoresis is a relatively inexpensive and rapid diversity estimate. Serum protein electrophoresis provides an effective estimate of genetic diversity in cranes.

Blood typing, although of limited use in this study, can be an effective way to determine relatedness in many animals. A recent technique modification, absorption of chicken reagents with blood of individual members of another bird species, makes it practical to prepare reagents capable of detecting haplotypes of the major histocompatibility complex in cranes. Maintaining histocompatibility diversity, a part of the individual's immune mechanism, results in a greater capacity to resist disease.

Competitive binding immunoassay can detect differences suitable to determine relatedness in some species, but was found to be an ineffective tool in cranes. Relatedness estimates are being generated for the whooping crane using restriction fragment length polymorphisms (RFLP). RFLP's provide a genetic "fingerprint" of the individuals tested. The "fingerprint," first reported in 1985 in man, was recently reported in birds and other animals. Probes developed and being used in the whooping crane reveal the same polymorphic patterns obtained from the other animals tested. In some species, the probes have been useful in sex determination. Although more expensive than most other procedures, RFLP's would provide the most powerful tool to estimate relatedness and diversity in cranes.

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LITERATURE CITED

Briles, W. E . 1984 . Early chicken blood group investigations . Immunogenetics 20: 217-226.

Briles, W.E. & R.W. Briles. 1987. Genetics and classification of major histocompatibility complex antigens of chickens. Poultry Sci . 66:776-781.

Corbin, K.W. 1983. Genetic structure and avian systematics. Pp. 211-244 *in* R.F. Johnson (ed.), Current Ornith., Plenum Press, N.Y. & Lond., 425p.

Dessauer, H.C. & G.F. Gee. 1986. Estrase-D and 6 phosphogluconate dehydrogenase polymorphisms in whooping cranes. Isozyme Bull. 19:41.

Frankel, O.H. & M.E. Soule (eds.). 1981. Conservation and evolution. Cambridge Univ. Press, N.Y.,327p.

Gable, R.R. & G. F. Gee. 1987 . Genetic management of endangered species at the Patuxent Wildlife Research Center. Pp. 1-8 *in* Proc. 2nd Jean Delacour/IFCB Symp. Breeding Birds in Captivity, 571p.

Jeffreys, A.J., V. Wilson & S.L. Thein. 1985. Hypervariable "minisatellite" regions in human DNA. Nature 314: 67-73 .

Klein, J. 1986. Natural History of the Major Histocompatibility Complex. John Wiley & Sons, N.Y., 776p.

Longmire, J.L. 1988. Identification and development of population-specific DNA polymorphisms within the genome of Falco peregrinus. Pp. 779-788 *in* T. Cade (ed.) Peregrine Falcon Populations: Their Manage. & Recovery, Proc. Int. Symp. Peregrine Falcon, 1985.

Longmire, J.L., A.K. Lewis, N.C. Brown, J.M. Buckingham, L.M. Clark, M.D. Jones, L.J. Meincke, J. Meyne, R.L. Ratliff, F.A. Ray, R.P. Wagner & R.D. Moyzis. 1988. Isolation and molecular characterization of a highly polymorphic centromeric tandem repeat in the family Falconidae. Genomics 2:14.

Love, J. 1990. Avian Repetitive DNA. Ph.D. Thesis, La. State Univ. Med. School, 133p.

Lowenstein, J.M. 1985. Molecular approaches to the identification of species. Am. Scientist (Nov.-Dec.):541-547.

Mettler, L.E. & T.G. Gregg. 1969. Population Genetics and Evolution. Prentice-Hall, Englewood Cliffs, N.J., 212p.

Sarich, V.M. 1976. Rates, sample sizes, and the neutrality hypothesis for electrophoresis in evolutionary studies. Nature 265(5589):24-28.

Wilcox, B.A., P.F. Brussard & B.G. Marcot. 1986. The management of viable populations. Cen. Conserv. Biol., Stanford Univ., Cal., 188p.

Vassart, G., M. Oeorges, Monsieur H. Brocas, A.S. Lequarre & D. Christophe. 1987. A sequence in M13 phage detects hypervariable minisatellites in human and animal DNA. Sci. 235:683-684.