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ESTABLISHING PATERNITY IN WHOOPING CRANES (*GRUS AMERICANA*) BY DNA ANALYSIS

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ABSTRACT.—DNA fingerprinting was used to study paternity and genetic variability within a captive flock of Whooping Cranes (*Grus americana*). Fingerprint patterns for 42 individuals were obtained by digesting genomic crane DNAs with *Hae*III followed by electrophoresis, blotting, and hybridization to the M13 minisatellite probe. Despite finding reduced levels of genetic variation in the Whooping Crane due to a population "bottleneck," these polymorphisms were successfully used to determine paternity in six of seven cases of captive propagation where the maternal-offspring relationship was known, but where the sire was unknown. These determinations of paternity are required for effective genetic management of the crane flock. These results also revealed a number of heterozygous minisatellite loci that will be valuable in future assessments of genetic variability in this endangered species.
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THE WHOOPING CRANE (*Grus americana*) is a highly endangered species. Today's 221 surviving individuals are all descendants of a single natural population that nests in Wood Buffalo National Park, Canada, and over-winters at Aransas National Wildlife Refuge near Austwell, Texas. The wild Wood Buffalo population reached a low of 15 birds in 1941 and then slowly increased to 48 in 1967, and to 142 in 1990 (Erickson 1968, Ellis et al. 1991). Besides the 142 cranes making up the Wood Buffalo-Aransas population, in 1990 there were 66 individuals in captivity and 13 in an experimental transplant-release program in Idaho. Due to its endangered status, the Whooping Crane has been the subject of intense efforts aimed at preservation and recovery of the species. These efforts have included ecological study, habitat management, and reintroduction (U.S. Fish and Wildlife Service 1986, Canadian Wildlife Service 1988).

A captive propagation program was initiated at the Patuxent Wildlife Research Center in Laurel, Maryland, in 1965. Relying principally on artificial insemination, this project has produced 50 captive-reared cranes since 1975, and has supplied 73 fertile eggs for transplant release. Whooping Cranes represent a substantial captive investment due to slow sexual maturity and low reproductive capacity (only 60% of eight-year-old captive females have produced eggs, and only five or six eggs are produced per female per year). In order to maximize the production of fertile eggs, adult females often were inseminated with semen from several males. In these cases, the female parent was known, but the sire was not known. The unknown paternities pose a significant impediment to future breedings. In order to avoid the possibility of inbreeding, female offspring could not be mated with any of their possible sires. In addition, it was not possible to distinguish sibling and half-sibling relationships among these offspring. Determining paternal relationships, thus, would benefit those involved in the genetic management of the captive Whooping Crane flock.

Although knowledge of paternity has been needed for some time, conventional techniques (such as allozyme analysis) have failed to resolve paternity in most birds, including these cranes. However, the recent development of DNA fingerprinting (or DNA profiling) has made it feasible to perform meaningful paternity testing in avian species. This technique makes use of "minisatellites," which are short tandemly repeated DNA sequences. An interesting feature of minisatellites is that the nu-

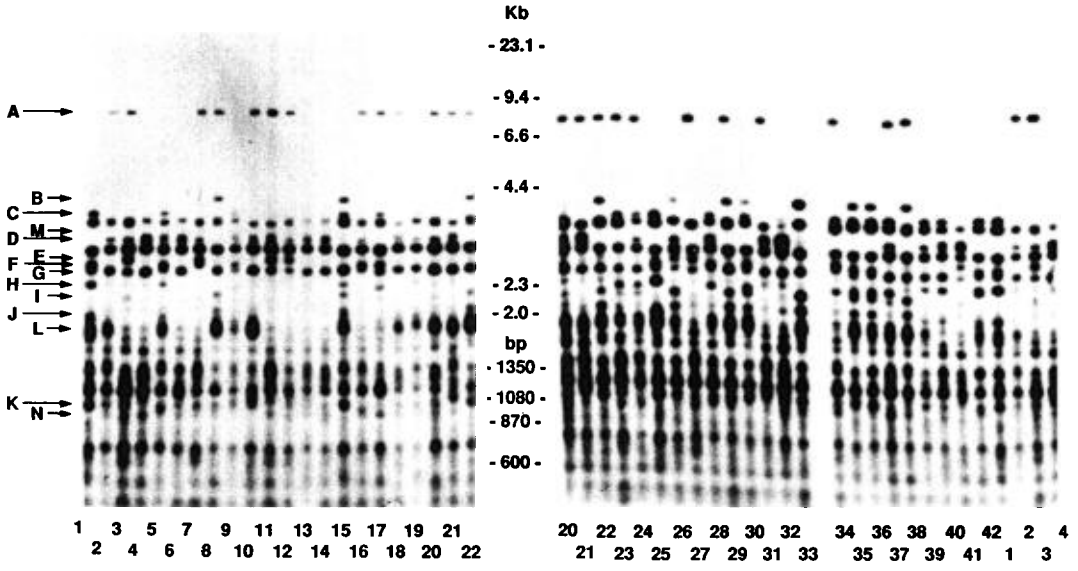

Fig. 1. Hybridization of M13 repeat to *Hae*III digested Whooping Crane DNAs. Of each DNA, 5 μg was digested with excess *Hae*III and electrophoresed within 0.8% agarose gels. Resulting blots were hybridized to M13 minisatellite probe. Arrows indicate designations and positions of fragments scored. Size standards, as indicated in kilobase (kb) pairs, derived from *Hin*dIII digested bacteriophage lambda DNA, and from *Hae*III digested OX174 DNA.

cleotide sequences of the core repeats are conserved and occur in many diverse species. Because of this, minisatellites cloned from the DNA of one species can be used as a probe in other species. The first sequence to be used in this way was a 33-bp repeat isolated from an intron of the human myoglobin gene (Jeffreys et al. 1985a). The myoglobin repeat has been used in studies of humans (Jeffreys et al. 1985b), mice (Jeffreys et al. 1987), sparrows (Wetton et al. 1987, Burke and Bruford 1987), wrens (Rabenold et al. 1990), and various domestic animals (Georges et al. 1988). Other minisatellite probes have since been described (Jarman et al. 1986, Longmire et al. 1990), including one from the bacteriophage M13 (Vassart et al. 1987).

When used as probes, minisatellites have revealed high levels of genetic variation among conspecific vertebrates. This variation, which results from differences in the number of core repeats within allelic clusters, is sufficient to generate individually diagnostic DNA fingerprints (Jeffreys et al. 1985a, b). In this study, we used the M13 probe to generate DNA fingerprints for 42 of the captive Whooping Cranes. The DNA profiles revealed a significant degree of genetic individualization, and has enabled paternity assignments to be made in several cases.

Methods

Blood collection and DNA isolation.—Between 0.25 and 1.0 cc of peripheral blood (drawn from the jugular vein) was added to a sterile 15-ml polypropylene tube containing 5 ml of lysis buffer (0.1 M Tris-HCl pH 8.0, 0.1 M EDTA, 0.01 M NaCl, and 0.5% w/v SDS). Removing this volume of blood in this manner was not deleterious to the birds, and the amount of DNA isolated from 0.5 cc of bird blood is sufficient for numerous independent analyses. Samples were shipped to the laboratory at ambient temperature. For the purification of DNA, proteinase K was added at 0.5 mg per ml, and the samples were incubated overnight at 37°C on a tube rotator. The samples were extracted one time with an equal volume of phenol that had been saturated with TE buffer (0.01 M Tris-HCl pH 8.0, 0.001 M EDTA). This extraction step was carried out for 60 min on a tube rotator at 37°C. Following centrifugation for 10 min at 800 × *g*, the aqueous phases were dialyzed at 4°C for approximately 24 h against three changes of TE (or until the odor of phenol could no longer be detected). Final purified DNAs were quantitated by UV spectroscopy.

DNA restrictions, Southern blotting, and hybridization.—Five micrograms of each DNA were digested with a 10-fold unit excess of *Hae*III using buffer conditions recommended by the supplier (New England Bio Labs). Restricted DNAs were electrophoresed in 20 × 25 cm 0.8% agarose gels at 35 volts for approximately 30 h and, subsequently, transferred onto Zetabind nylon membranes. The loading order of sam-

TABLE 1. Representation of scored fragments in 42 Whooping Cranes.

Gel lane	Crane identification no.	Fragment													
		A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	83001			1			1	1	1		1	1	1		
2	82001	1			1			1				1	1		
3	64001	1			1	1		1		1		1			1
4	82002				1			1							1
5	85007			1	1		1		1		1	1	1		
6	84003				1			1							1
7	87027	1			1	1	1					1			
8	86033	1	1					1		1	1	1	1		
9	85004			1			1	1				1		1	
10	87034	1						1				1	1	1	
11	84002	1			1	1		1				1		1	1
12	85005	1			1	1		1							1
13	83004				1			1							1
14	68001				1			1							
15	68003		1	1				1	1	1	1	1	1		
16	74001	1			1		1	1							
17	87043	1		1			1	1							1
18	13005	1			1			1				1	1		
19	85001			1	1			1				1	1		1
20	77001	1			1			1		1		1	1		
21	69003	1						1					1	1	1
22	82003	1	1					1		1	1	1	1		
23	69002	1		1				1	1	1	1	1	1		1
24	86027	1		1	1			1	1	1			1		1
25	83010			1		1	1	1				1	1		
26	79001		1		1	1		1		1	1	1	1		
27	68002	1				1		1			1	1			1
28	69001			1	1		1		1		1	1	1	1	
29	84004	1	1					1		1	1	1	1		1
30	83008		1	1			1	1	1	1	1	1	1		
31	71001	1			1			1			1			1	
32	87042				1			1			1	1		1	1
33	87041		1					1		1	1	1	1		1
34	84001	1		1		1	1		1		1	1			1
35	83006		1	1			1	1	1	1	1	1	1		
36	85006		1	1			1	1	1		1	1	1		
37	82004	1		1	1			1	1	1	1		1		1
38	87033	1	1					1		1	1		1		1
39	83005			1	1			1	1		1	1	1		
40	85002			1	1			1	1		1	1		1	
41	83003				1	1		1		1				1	
42	85003			1			1	1	1		1	1	1		

ples on the gels was random in that no attempt was made to group adult females, offspring, and potential sires. However, certain samples were run in duplicate to provide a measure of standardization between gels. Resulting blots were prehybridized for 4 h at 42°C in 6X SSC, 40% formamide, 0.005 M EDTA (pH 8.0), and 0.25% w/v powdered milk (Vassart et al. 1987). Hybridization was in the same solution containing 1×10^6 cpm per ml probe. The 282-bp *Hae*III-*Cla*I double-digest fragment of M13mp8 was gel purified and labeled with [^{32P}]dCTP to specific activities greater than 1×10^8 cpm per μg using the primer extension method described by Feinberg and Vogelstein (1983). Posthybridization washes were as follows: two times 15 min at 22°C in 2X SSC, 0.1% SDS; and twice for 15 min at 50°C in 1X SSC, 0.1% SDS. Washed blots were autoradiographed at −70°C in cassettes containing intensifying screens.

Data scoring and analyses.—Original autoradiographs and large contact-sized prints were scored. This was found to be much more accurate than attempting to score the data after it had been reduced in size for publication (as in Fig. 1). The migration of each scored fragment was measured relative to other fragments in the same gel lane and in other gel lanes.

TABLE 2. Mendelian inheritance of minisatellite fragments within a defined family of Whooping Cranes. Fragments identified as being heterozygous are designated "1h."

Fragment	Dam 71001	Progeny 85005 (M)	Progeny 83003 (M)	Progeny 82002 (F)	Progeny 84003 (M)	Sire 64001
A	1h	1				1h
B						
C						
D	1	1	1	1	1	1
E		1h	1h			1h
F						
G	1	1	1	1	1	1
H						
I			1h			1h
J	1h					
K						1h
L						
M	1h		1h			
N		1h		1h	1h	1h

RESULTS

Figure 1 shows the fingerprinting patterns. The primary data illustrated were converted to binary form to represent the scored fragments in the patterns of the 42 cranes (Table 1). The patterns were quite variable, and the level of polymorphism seemed sufficient for paternity testing.

TABLE 3. Paternity determination: males 64001, 68001, and 68002 excluded by their lack of fragment F; 69001 identified as real sire. Informative fragments underlined in progeny and potential sires. Fragments identified as heterozygous are designated "1h."

Fragment	Dam 68003	Progeny 85007 (F)	Possible sires 64001	68002	69001	68001
A			1	1		
B	1h					
C	1	1			1	
D		1h	1		1	1
E			1	1		
F		1h			1	
G	1h		1	1		1
H	1	1			1	
I	1h		1			
J	1	1		1	1	
K	1	1	1	1	1	
L	1	1			1	
M					1	
N			1	1		

TABLE 4. Paternity determination: males 68001, 68002, and 64001 excluded by their lack of fragment F; 69001 identified as sire. Informative fragments underlined in progeny and sire. Fragments identified as being heterozygous are designated "1h."

Fragment	Dam 68003	Progeny 85006 (F)	Possible sires 68001	68002	69001	64001
A				1		1
B	1	1h				
C	1	1			1	
D			1		1h	1
E				1		1
F		1h			1	
G	1	1h	1	1		1
H	1	1			1	
I	1h					1
J	1	1		1	1	
K	1	1		1	1	1
L	1	1			1	
M					1h	
N				1		1

Genetic polymorphisms must be inherited in a Mendelian fashion to be useful in studies of maternity and paternity. A defined family of cranes with known maternity and paternity was examined to investigate the inheritance characteristics of the fragments that were detected by the M13 probe. As can be seen in Table 2, Mendelian inheritance was observed (i.e. all

TABLE 5. Paternity determination: male 64001 excluded by fragment F; 69001 identified as sire. Informative fragments underlined in progeny and sire. Fragments identified as being heterozygous are designated "1h."

Fragment	Dam 68003	Progeny 85003 (F)	Possible sires 64001	69001
A			1	
B	1h			
C	1	1		1
D			1	1h
E			1	
F		1h		1
G	1	1h	1	
H	1	1		1
I	1h		1	
J	1	1		1
K	1	1	1	1
L	1	1		1
M				1h
N			1	

TABLE 6. Paternity determination: males 68002, 64001 and 68001 excluded by fragment M; 69001 identified as sire. Informative fragments underlined in progeny and possible sires. Fragments identified as being heterozygous are designated "1h."

Fragment	Dam 68003	Progeny 85002 (F)	Possible sires 64001	69001	68001	68002
A			1			1
B	1h					
C	1	1		1		
D		1h	1	1	1	
E			1			1
F				1h		
G	1	1h	1		1	1
H	1	1		1		
I	1h		1			
J	1	1		1		1
K	1	1	1	1		1
L	1h			1h		
M		1h		1		
N			1			1

fragments present in offspring can be traced back to at least one of the two parents). Analysis of this family also indicates heterozygosity at some of the minisatellite loci. In adults, heterozygosity was identified when a particular fragment was not inherited by all of that individual's offspring. In the offspring, heterozygous fragments are those bands that could have been inherited from only one parent. These determinations of heterozygosity are limited. Fragments that are not observed to be heterozygous are not necessarily homozygous. In these very small families, it is possible that all offspring will inherit a fragment even though that fragment is heterozygous in the parent. Likewise, fragments that are displayed by the offspring and both of the parents can be heterozygous in the offspring if one or both parents are heterozygous for that fragment. Thus, these analyses identify only a subset of fragments that are heterozygous, and homozygosity cannot be determined. Complete assessments of homozygosity and heterozygosity would require much larger multigeneration families.

Tables 3 to 9 show paternity assignments that were made from the fingerprint data. Informative fragments present in the offspring (but absent in the female parent) must have been inherited from the true sire. Other paternal candidates were excluded when they were found to be lacking one or more of the informative fragments.

Once paternity was determined, the families were examined for indications of heterozygous loci. Although the true sire was not determined in the family shown in Table 9, it was still possible to identify some fragments as being heterozygous in the female parent and the offspring of that family.

TABLE 7. Paternity determination: male 68002 excluded by fragment D. Males 68001, 69001, and 74001 excluded by fragment N. Male 64001 identified as the sire.[a] Informative fragments underlined in progeny and possible sires. Fragments identified as being heterozygous are designated "1h."

Fragment	Dam 68003	Progeny 85001 (F)	Possible sires 64001	68001	68002	69001	74001
A			1h		1		1
B	1h						
C	1	1h				1	
D		1h	1	1		1	1
E			1h		1		
F						1	1
G	1	1	1	1	1		1
H	1h					1	
I	1h		1h				
J	1h				1	1	
K	1	1	1		1	1	
L	1	1				1	
M						1	
N		1h	1		1		

[a] This paternity assignment must be considered tentative since absence of fragment N in 68001 cannot be absolutely determined due to a smear at that position in lane 14 (Fig. 1).

TABLE 8. Paternity determination: male 67001 identified as sire (even though no DNA was available from this deceased individual), since 68001 was excluded by fragments E and F, 69001 was excluded by fragment E, and 64001 was excluded by fragment F. Informative fragments underlined in progeny and possible sires. Fragments identified as being heterozygous are designated "1h."

Fragment	Dam 68003	Progeny 83010 (F)	Possible sires: 64001	68001	69001	67001 (no DNA)
A			1			
B	1h					
C	1	1			1	
D			1	1	1	
E		1h	1			
F		1h			1	
G	1	1	1	1		
H	1h				1	
I	1h		1			
J	1h				1	
K	1	1	1		1	
L	1	1			1	
M					1	
N			1			

TABLE 9. Paternity determination: The actual sire could not be identified in this family since none of the three potential sires could be excluded (254 is deceased, and both 64001 and 69001 are positive for the informative fragment D). Informative fragments underlined in progeny and possible sires. Fragments identified as being heterozygous are designated "1h."

Fragment	Dam 68003	Progeny 85003 (M)	Possible sires: 64001	69001	254 (no DNA)
A			1		
B	1h				
C	1	1		1	
D		1h	1	1	
E			1		
F				1	
G	1	1	1		
H	1	1		1	
I	1h		1		
J	1	1		1	
K	1	1	1	1	
L	1	1		1	
M				1	
N			1		

DISCUSSION

The ability to determine paternity and maternity in avian species has been limited due to the fact that birds are unusually monomorphic at the allozyme level (Barrowclough et al. 1985). DNA techniques using probes that detect restriction-fragment-length polymorphisms (RFLPs) arising from base substitutions have proven useful. Quinn et al. (1987) used low-copy-number sequence probes isolated from a genomic library made from Lesser Snow Goose (*Anser caerulescens*) DNA to detect multiple RFLPs in that species. Analysis of those polymorphisms enabled the identification of "illegitimate" offspring in nests of Snow Geese. A heterochromatic tandem-repeat sequence was cloned from Merlin (*Falco columbarius*) DNA, and this probe has been applied to family and population studies in the Peregrine Falcon (*F. peregrinus*; Longmire et al. 1988). Although RFLP approaches are informative, they are not presently in widespread use because molecular cloning is often required to obtain suitable probes for each species of interest. In addition, the generally low level of genetic variation detected especially by single-copy probes is more appropriate for studies at the population level.

Maternity and paternity analyses have become more feasible in nonhuman species due to the recent development of DNA fingerprinting. In this study we used the M13 minisatellite probe to investigate paternal relationships in a captive flock of Whooping Cranes. Our results show a somewhat reduced level of genetic variation in comparison to that of other avian species. The mean frequency of the polymorphic minisatellite fragments among these Whooping Cranes was 0.42, whereas in the sparrow study conducted by Burke and Bruford (1987) it was 0.28. In a study of Peregrine Falcons, the frequency of minisatellite fragments was 0.12 (Longmire et al. 1991). An allozyme study of this same Whooping Crane population also indicates reduced variation in comparison to other large and contiguous crane populations (H. Dessaure and G. Gee, unpubl. data). The relatively low level of genetic variation observed in these cranes most likely is attributable to the severe population "bottleneck" that this species has gone through. Nevertheless, the crane DNA fingerprint patterns were still sufficiently vari-

able to determine paternity in six of the seven cases that were studied. These results provide further evidence of the usefulness of DNA fingerprinting in clarifying very close genetic relationships, even in species with reduced genetic variability. The ability to determine paternity provides information that is much needed for the effective genetic management of the captive Whooping Crane flock.

Our analysis identified several restriction fragments originating from heterozygous minisatellite loci within the crane genome. This set of heterozygous loci, together with measured allele frequencies, can be used in quantitative comparisons of the level of genetic diversity remaining in captive and wild Whooping Cranes, and to make comparisons between the Whooping Crane and other endangered and nonendangered crane species.

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