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CHARACTERIZATION AND PHYLOGENETIC SIGNIFICANCE OF A REPETITIVE DNA SEQUENCE FROM WHOOPING CRANES (*GRUS AMERICANA*)

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ABSTRACT.—We surveyed a Whooping Crane (*Grus americana*) genomic library enriched for repetitive clones, and isolated a clone whose insert hybridized stringently to a repeated-DNA family in the genomes of Whooping Cranes, but not Sandhill Cranes (*G. canadensis*). This tandem sequence, repeated approximately 500 times in the Whooping Crane genome, displays taxon-specific properties suggesting that the Common Crane (*G. grus*) is the Whooping Crane's nearest living relative. Low-stringency hybridizations with this repeat produced conserved patterns in all cranes except crowned-cranes (*Balearica*), which indicates an early divergence of the crowned-cranes and the remaining cranes. Sequence and DNA-hybridization analyses imply that this repeat is a satellite sequence of similar complexity and organization to the primate alphoid DNA-sequence family, which also has chromosome and species specificity. *Received 13 December 1990, accepted 30 April 1991.*

A SUBSTANTIAL portion of the eukaryotic genome consists of sequences repeated thousands, to hundreds of thousands, of times. Because most repetitive sequences do not code for genes, they often escape selection and may accumulate mutations rapidly. Consequently, repeats are good candidates for the study of closely related species. Repeats may be interspersed throughout the genome or arranged in long, tandem arrays. Arthur and Straus (1977) found that repeated sequences were not extensively interspersed in the chicken (*Gallus gallus*) genome, and established that avian-genome sequence organization is most similar to that of *Drosophila*. The C_0t curves they produced from DNA samples sheared to different lengths, indicating that both the fold-back component and the moderately repeated component of chicken genomes are primarily tandem repeats.

Tandem repeats are divided into several types (families) based upon length and complexity of the repeating units. The variable-number tandem repeats that yield "DNA fingerprints" in Southern blot analysis are familiar (for examples of their use, see Wetton et al. 1987, Burke and Bruford 1987). These simple repeats contain short sequences, tandemly repeated a moderate number of times and mostly interspersed throughout the genome. The large, complex tandem repeats, which are less studied in birds, are the subject of this paper. We isolated from a Whooping Crane (*Grus americana*) an autosomal, complex tandem repeat with taxon specificity and suggest that it may contribute to the isolating mechanism in cranes.

MATERIALS AND METHODS

Construction of Whooping Crane library.—We extracted high-molecular-weight DNA from female Whooping Crane red blood cells by standard methods (Maniatis et al. 1982). It was sheared by sonication to greater than 1-kilobase (kb) average fragment length, and purified by NACS column chromatography (Bethesda Research Laboratories, Gaithersburg, Maryland). Fragments were end-repaired using the Klenow fragment of DNA polymerase I, and blunt-ligated into the *Sma*I site of pUC118 following the procedures of King and Blakesley (1986). Competent XL-1 Blues (a strain of *Escherichia coli* from Stratagene, La Jolla, California) were transformed with aliquots of the ligation mix, and recombinants were selected by growth on ampicillin plates. The library of 20,000 independent clones contained an average insert size of 0.5 kb, representing a total of 10,000 kb, or approximately 1%, of the crane's genome, assuming a genome the same size as the chicken's (1×10^6 kb; Shields 1983).

Isolation of the whooper repeat.—The specific clone for the Whooping Crane (whooper repeat) was isolated fortuitously during an unsuccessful survey for sex-specific repetitive sequences (data not shown). Clones were tested individually for their ability to

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hybridize to dot blots of male and female crane DNA of both Whooping Cranes and Sandhill Cranes (*G. canadensis*) under high-stringency conditions. We investigated a clone that produced a strong signal with Whooping Crane DNA from both sexes, but not with Sandhill Crane DNA of either sex. This was the whooper repeat or clone.

Preparation of the whooper-repeat fragment.—The whooper clone was double digested with *Eco*RI and *Bam*HI to release the recombinant insert. The digested DNA was electrophoresed through a 2% agarose gel and the 0.2 kb clone fragment collected. This fragment was used to make quantified standards for dot-blot experiments and as probe in all hybridization experiments.

Dot-blot analysis.—DNA from two male Whooping Cranes, two male Sandhill Cranes, a male hybrid of the two species, and a female of each member of the genus *Grus* (as well as a female chicken) were tested. We sonicated 2.5 μg of DNA in 10× SSC (1.5 M sodium chloride, 0.15 M sodium citrate) to approximately 0.5 kb, boiled for 5 min, and quick-chilled. For quantitation, 2.5 μg aliquots of Hela DNA were spiked with different amounts of the whooper fragment and treated in the same manner. The solution was vacuum dot blotted onto nitrocellulose membrane, then vacuum-baked at 80°C for 2 h.

The whooper-repeat fragment was radiolabeled with α^{32}P dATP by nick-translation (Maniatis et al. 1982). The probe was separated from unincorporated materials by Sephadex G-50 chromatography.

After prehybridization (in 25mM KPO_4, 5× SSC, 5× Denhardt's solution, 50 μg/ml sonicated salmon milt DNA, 50% formamide), the dot blot was hybridized at 42°C overnight in 10 ml of the same solution containing 6×10^6 cpm (0.25 μg) of the boiled probe. The blot was washed twice in 2× SSC, 0.1% SDS at 37°C followed by a wash in 0.1× SSC at 68°C, and autoradiographed.

Southern blot analysis.—Five micrograms of *Hae*III-digested DNA from a female member of each species of Gruidae, a chicken, a parrot *Amazona ochrocephela*, and a hawk *Parabuteo unicinctus* (as well as male Whooping Cranes, Sandhill Cranes, and a hybrid of the two species) were electrophoresed through a 1% agarose gel and transferred to nitrocellulose membrane (Maniatis et al. 1982).

After prehybridization, the blot was hybridized overnight at 42°C in 10 ml of hybridization solution (as before) containing 14×10^6 cpm (0.25 μg) of probe. The blot was washed twice in 2× SSC, 0.1% SDS at 37°C followed by a wash in 0.1× SSC at 60°C. This low-stringency blot was autoradiographed for 24 h, after which it was rewashed in 1 L of 0.1× SSC at 68°C. This high-stringency blot was autoradiographed for a similar length of time.

Sequencing.—The DNA of the whooper repeat was sequenced in both directions by the method of Kraft et al. (1988). Sequenase Version 2.0 (U.S. Biochemical Corp.) was used. Products of the four forward and four reverse reactions were electrophoresed through an 8% polyacrylamide, 7 M urea gel, after which the gel was dried under vacuum and autoradiographed.

RESULTS

Dot blots.—A one-day autoradiograph demonstrated hybridization of the DNAs of the Whooping Crane and the Common Crane (*G. grus*) with intensities equivalent to the control Hela sample (2.5 μg) that was spiked with 250 pg (picograms) of whooper fragment (Fig. 1). This suggests approximately 10^{-4} (250 pg/2.5 μg) of the genomes of these two species contain this repeat. The DNA of the Whooping Crane × Sandhill Crane hybrid displayed an intensity approximately half that of the Whooping Crane DNA. No other crane DNA produced a hybridization signal after a one-day exposure. In follow-up experiments, DNA from all eight Whooping Cranes tested were positive, and all seven Sandhill Cranes tested were negative (data not shown).

A six-day exposure of the same blot revealed weak hybridization with some other species of crane, which indicates the presence of similar sequence(s). Note that this experiment cannot differentiate between copy numbers and degree of sequence similarity. The low signals from the longer exposed dot blot may be due to small numbers of sequences identical to the whooper sequences, or due to a large number of sequences that are similar enough to the whooper sequence to cause a fraction of them to hybridize with the probe. DNA from the Black-necked Crane (*G. nigricollis*) and the Hooded Crane (*G. monachus*) produced signals equivalent to approximately 15 pg of the sequence in the 2.5 μg dots of DNA, and that from the Japanese Crane (*G. japonensis*) contained slightly less. The remaining members of the Gruidae did not hybridize with the whooper probe (at high stringency), nor did the chicken or Hela DNAs, even after six days of exposure.

Southern blot analysis.—We utilized Southern blot analysis to determine the pattern of restriction fragments containing this repeated sequence (Fig. 2). At low stringency, we observed doublet patterns of fragments in digests of DNAs of most gruids, including the Wattled Crane (*Bugeranus carunculatus*). High-molecular-weight bands were missing or were very light in some species, such as the Japanese Crane. The pattern

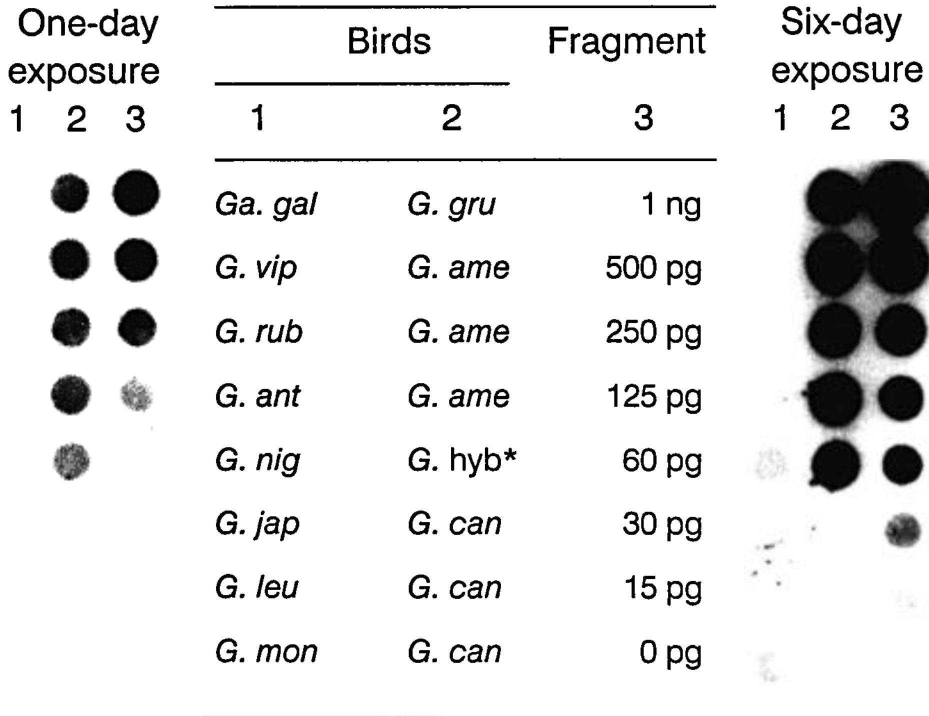

Fig. 1. Dot blots of crane (and chicken) DNAs hybridized with whooper probe. We dotted 2.5 μg of genomic DNA and positive controls of Hela DNA spiked with quantity of Whooping Crane fragment listed onto nitrocellulose paper. Hybridization with radiolabeled whooper fragment (probe) followed by high-stringency wash and autoradiography. Abbreviations: *Ga. gal* = *Gallus gallus* (chicken); *G. vip* = *G. vipio* (White-naped Crane); *G. rub* = *G. rubicunda* (Brolga); *G. ant* = *G. antigone* (Sarus Crane); *G. nig* = *G. nigricollis* (Black-necked Crane); *G. jap* = *G. japonensis* (Japanese Crane); *G. mon* = *G. monacha* (Hooded Crane); *G. gru* = *G. grus* (Common Crane); *G. ame* = *G. americicana* (Whooping Crane); *G.* hyb = hybrid *G. americana* × *G. canadensis*; *G. can* = *G. canadensis* (Sandhill Crane).

for the Sandhill Crane consisted of a few, very small bands (<300 bp) in this low-stringency experiment. Both species of the genus *Anthropoides* (Demoiselle Crane, *A. virgo*; Stanley Crane, *A. paradisea*) produced small, very light bands. DNAs of both species of *Balearica* (Black Crowned-Crane, *B. pavonina*; Gray Crowned-Crane, *B. regulorum*) were negative. Birds from three other avian orders also failed to hybridize with the whooper-repeat probe.

The high-stringency wash removed the signal from DNAs of all but the Common Crane and the Whooping Crane. The smallest doublets, approximately 0.38 and 0.44 kb, were clearly visible on autoradiographs. The DNA of the hybrid crane produced a pattern identical to that of the Whooping Crane, but of half the intensity. The pattern for the Common Crane had a slight smear as well as the doublet pattern (the smear may represent an experimental artifact). An 11-day exposure of the high-stringency blot revealed small, very light bands for some of the other cranes, congruent with findings from the dot-blot experiment.

Sequence analysis.—The Southern analysis indicated that the sequence of the 135 base-pair whooper-repeat clone (Fig. 3) represents only a portion of the overall repeat sequence (see Discussion). The whooper-repeat sequence consists of 51% G+C and its structure contains no apparent structures of simple internal repeats. We compared the sequence, in both directions, to sequences stored in the European Molecular Biology Organization's (EMBL) databanks (updated August 1989) using the fast-N-scan program with a K-tuple value of 6 and 3 (Intelligenetics). No significant similarities were found to any other sequence. The sequence was sub-

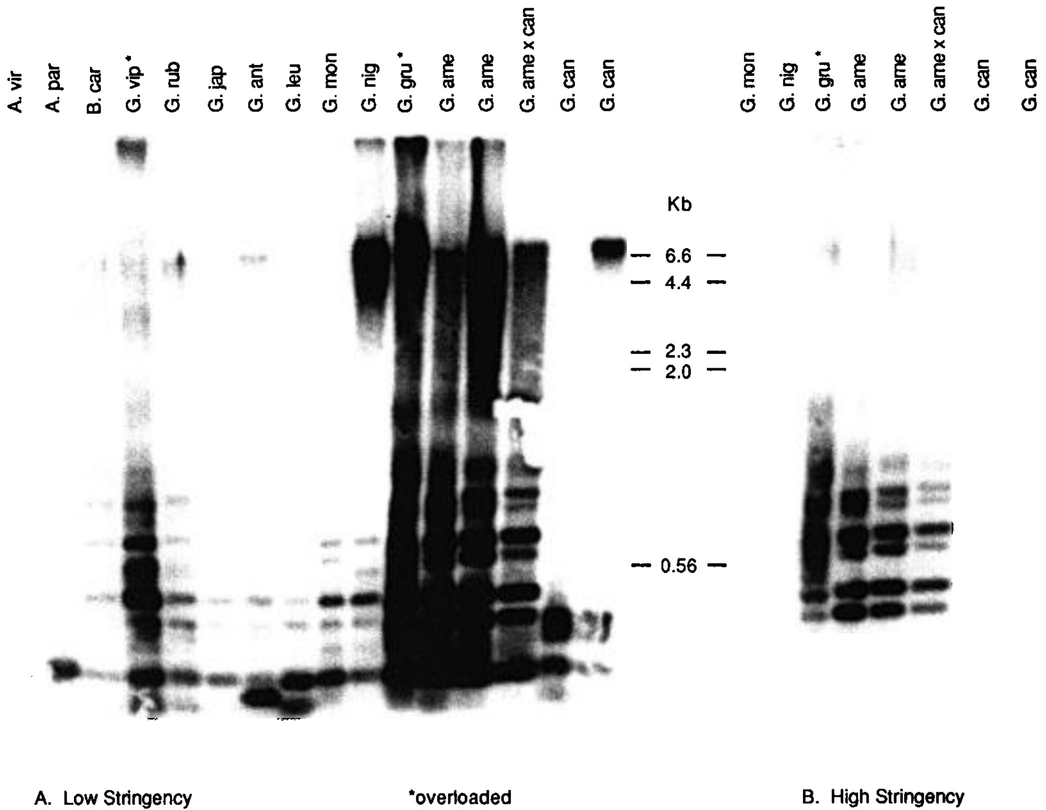

Fig. 2. Autoradiographs of crane DNA hybridized with Whooping Crane fragment. Genomic DNA from various species digested with *Hae*III and analyzed by Southern analysis using whooper fragment as probe. (A) Low-stringency washes and (B) high-stringency washes. Abbreviations: A. vir = *Anthropoides virgo* (Demoiselle Crane); A. par = *A. paradisea* (Stanley Crane); B. car = *Bugeranus carunculatus* (Wattled Crane); G. vip = *Grus vipio* (White-naped Crane); G. rub = *G. rubicunda* (Brolga); G. jap = *G. japonensis* (Japanese Crane); G. ant = *G. antigone* (Sarus Crane); G. leu = *G. leucogeranus* (Siberian Crane); G. mon = *G. monacha* (Hooded Crane); G. nig = *G. nigricollis* (Black-necked Crane); G. gru = *G. grus* (Common Crane); G. ame = *G. americana* (Whooping Crane); G. ame × can = *G. americanus* × *G. canadensis* hybrid; G. can = *G. canadensis* (Sandhill Crane).

mitted to EMBL and is assigned the accession number X54174.

DISCUSSION

The whooper-repeat probe could be useful to biologists involved in the Whooping Crane recovery program and law enforcement. For example, cross-fostering is one aspect of the recovery program that could benefit from the availability of a method for identifying Whooping Cranes and Whooping Crane × Sandhill Crane hybrids. Female Sandhill Cranes artificially inseminated with sperm from Whooping Cranes produce hybrid offspring, although the sperm concentration of the hybrid is low (George Gee, pers. comm.). The whooper probe can distinguish between these two North American cranes and their hybrids. Wildlife officers may find the probe valuable as a means of identifying evidence. Recent advances in probe technology may allow its use in identification of dried feather pulp or droppings. Field biologists may wish to use the probe in a similar manner.

Gelter and Tegelstrom (1990) used the patterns of tandem repeats produced after electrophoresis of endonuclease-digested DNA to compare genomes of different species. Due to the relatively low copy number of the whooper

1

GGGCTGTGAATGGGACCATGGTAGAGGTTTCAGGAAAGCAAGAGCATTCG

51

GGGCTGGGATGTTTTCCTTGGGAGCTGGGTCTGGATGTTTGCAGTTTTGA

101

GGCTTGAATCTCGACTATGGCTAGAGAGCTGCTAA

Fig. 3. Sequence of Whooping Crane Repeat from forward primer starting at point of insertion (reading 5′ to 3′).

repeat (500 copies, see below), tandem blocks were not visible under ultraviolet light after staining with ethidium bromide. However, a pair of bands belonging to another tandem block (a different family of repeats) was observed in DNA cleaved with *Hae*III from all cranes, including crowned-cranes, but not the chicken, parrot, or hawk (data not shown).

The whooper sequence is probably part of a tandem repeat. Doublets were produced by *Hae*III, the bands consisting of fragments of approximately 0.86, 0.80, 0.65, 0.59, 0.44, 0.38 and 0.2 kb (Fig. 2); the smallest band disappeared at the higher stringency. The pairs in each doublet were separated by approximately 0.06 kb. Bands in each doublet were separated from their respective band in other doublets by 0.21 kb. A possible explanation for these patterns is that the Whooping Crane DNA contains a 215 base-pair tandem repeat with two *Hae*III sites, 60 base pairs apart. Random point mutations would introduce a ladderlike pattern as restriction sites were lost over time. This tandem repeat should give rise to a 215-bp ladder when cleaved with endonucleases found once in the sequence, and a high molecular-weight band when not cleaved at all. *Msp*I, *Pst*I and *Hin*fI, produce simple ladder patterns, each band in multiples of 0.2 kb. *Eco*RI and *Bam*HI produced a high molecular-weight band (data not shown).

The dot blots revealed the quantity of sequences within each genome that hybridized to the Whooping Crane sequence. The cloned whooper fragment represented 135 bp of the overall 215 bp repeat. If one assumes that the crane genome is the same size as a chicken's (10^9 bp, Shields 1983), one single whooper repeat (of 215 bp) would make up 2.15×10^{-7} of the genome. Because the Whooping Crane (and Common Crane) produced an intensity equivalent to 10^{-4} of the genome, we estimate this sequence to be present in about 500 copies per diploid genome of the Whooping Crane or Common Crane.

The DNA hybridization of cranes with the whooper probe provides evidence of crane relatedness. In concordance with other recent investigations (Ingold et al. 1989, Krajewski 1989), the failure of the DNAs of species of crowned-cranes to hybridize to the whooper repeat following a low-stringency wash supports the distinctiveness of *Balearica*. Overall, the large range of hybridization signals obtained with DNAs of different cranes suggests that crane repeats may offer an effective approach for estimating phylogenetic affinities of crane species.

The very weak signals produced by DNA of other cranes in the longer exposures of the dot blot may have phylogenetic relevance. We believe the Common Crane is the nearest living relative to the Whooping Crane. A list of the genus *Grus*, according to their relatedness to the Whooping Crane sequence would rank them as: (1) Common Crane and Whooping Crane (very similar, approximately 100%); (2) Black-necked Crane and Hooded Crane (both approximately 6%); (3) Japanese Crane ($<6\%$); and (4) other *Grus* (produced no measurable signal).

Krajewski (1989) placed these five members of the genus *Grus* in a single Species Group Grus. We can resolve Krajewski's Species Group Grus with respect to the Whooping Crane. According to the dot-blot experiments, the Whooping Crane's nearest living relative is most likely the Common Crane. The Hooded Crane and Black-necked Crane are the next closest, and the Japanese Crane is the most distant of the group.

The whooper repeat is a member of a repeat family that is found in all cranes except crowned-cranes (although lower-stringency hybridizations might reveal its repeats). This suggests a divergence or amplification of this sequence occurred after the Balearicinae/Gruinae divergence.

Vogt (1990) presented evidence to support the argument that large, complex, tandemly repeated sequences are involved in chromatin folding prior to chromosome condensation and cell division. The alphoid DNA sequence family of tandem repeats, first identified in African green monkeys (*Cercopithecus aethiops*), are found in man (*Homo sapiens*), many subfamilies of which are chromosome specific. Indeed, most human chromosomes have chromosome-specific sequences composed of members of this alphoid family. Vogt (1990) suggested each tandem block folds into a specific structure recognized by specific DNA-binding proteins that stabilize the structure and (perhaps) identify it during meiosis. These attributes cause Vogt (1990) to postulate that tandem repeats may act as "species barriers" in sympatric populations.

Sex-chromosome specific sequences are more easily identified than autosomal ones, and a *W*-chromosome-specific sequence has been found in chicken (Tone et al. 1982). Subsequent workers (Tone et al. 1984, Kodama et al. 1987) have reported the sequence to be present in approximately 20,000 copies (46% of the *W*-chromosome) and to display unusual electrophoretic mobility due to its ability to form DNA curvatures (tertiary structures). The chicken *W*-repeat hybridizes exclusively to female *Gallus* at high-stringency conditions, and other females of the order Galliformes using low-stringency conditions. Harata et al. (1988) isolated a DNA-binding protein from chicken livers, showing high affinity for the *W*-chromosome-specific repeat of chickens. Griffiths and Holland (1990) recently isolated a *W*-chromosome-specific repeat from the Lesser Black-backed Gull (*Larus fuscus*).

The hypothesis that species-specific tandem repeats and the proteins that bind them coevolve to regulate chromosome recognition and meiosis is consistent with the fact that the Whooping Crane × Sandhill Crane hybrid (produced by artificial insemination at Patuxent National Wildlife Center) is of low fertility. The hybrid crane contains repeats from both species and a mix of (hypothetical) proteins that bind them. The protein that binds the chicken *W*-repeat is a homo-multimeric structure (Harata et al. 1988). Hetero-multimers of DNA-binding proteins composed of Whooping Crane and Sandhill Crane repeat-binding proteins may not be effective at binding the repeat blocks of either species. During meiosis, these chromosomes might be unable to pair correctly at the metaphase plate hindering gametogenesis. The low sperm count noted for the Whooping Crane × Sandhill Crane hybrid (0.1 of normal numbers; George Gee, pers. comm.) could result from this factor. Conversely, we predict a Whooping Crane × Common Crane hybrid would be of normal fertility, because its repeats are so similar that its proteins would behave similarly. Unfortunately, such a hybrid does not exist.

Future experiments to isolate the Gruinae repeat from each species of Gruinae may allow a reconstruction of the group's phylogeny. Thermal-stability experiments with radiolabeled Gruinae repeats as a probe could be used to quantify its divergence among the cranes. Finally, isolation of proteins that bind to this repeat family may allow comparisons of the coevolution of these two biomolecules and their contribution to species isolation.

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