Isolation of a Sex-Linked DNA Sequence in Cranes

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A female-specific DNA fragment (CSL-W; crane sex-linked DNA on W chromosome) was cloned from female whooping cranes (*Grus americana*). From the nucleotide sequence of CSL-W, a set of polymerase chain reaction (PCR) primers was identified which amplify a 227–230 bp female-specific fragment from all existing crane species and some other noncrane species. A duplicated versions of the DNA segment, which is found to have a larger size (231–235 bp) than CSL-W in both sexes, was also identified, and was designated CSL-NW (crane sex-linked DNA on non-W chromosome). The nucleotide similarity between the sequences of CSL-W and CSL-NW from whooping cranes was 86.3%. The CSL primers do not amplify any sequence from mammalian DNA, limiting the potential for contamination from human sources. Using the CSL primers in combination with a quick DNA extraction method allows the noninvasive identification of crane gender in less than 10 h. A test of the methodology was carried out on fully developed body feathers from 18 captive cranes and resulted in 100% successful identification.

Cranes are the tallest flying birds in the world. Of the 15 extant species of cranes, 7 are considered to be endangered species (IUCN 1990). The current population sizes of several species or subspecies of cranes is very small. For example, the total number of wild whooping cranes (*Grus americana*) was estimated to be about 181 individuals in 1998 (North American Crane Working Group, *The Unison Call* 10[2]:5). Cranes form behaviorally monogamous mating pairs. The sex of a crane cannot be distinguished visually by researchers because there is no difference in morphology between male and female cranes.

The sex determination system of birds is different from that characterized in mammals. In the XY sex determining system of mammals, loci within a small part of the Y chromosome, designated SRY (sex-determining region Y) play a major role in determining sex (Sinclair et al. 1990). Avian sex determination contrasts with mammals in the chromosomal constitution of the sexes. Male birds are homogametic, with sex chromosome constitution designated ZZ, while females are heterogametic, with sex chromosomes ZW (Bloom 1974). Thus far no specific sequence on the W chromosome has been identified which plays a role similar to SRY in mammals. However, because only female birds carry the female-specific W chromosome, sequences on the W chromosome can be used as genetic markers to identify sex in avian species. Identification of the sex of individuals or determination of sex ratio in an avian population is often difficult. Many bird species are morphologically monomorphic for external characters that would differentiate the sexes; sex identification in populations is often problematic. Among the many considerations in the conservation management of endangered species, the balance of the sex ratio in a small population is important. This is especially true when potential reintroduction programs are being considered.

In the field, researchers are able to sex adult cranes during the breeding season according to a unison call (Archibald 1976). Karyotyping individuals was the most widely used traditional method for sexing cranes in the laboratory. Recently restriction fragment length polymorphism (RFLP) analysis of Southern blots using a sex-specific DNA probe has been used to identify sex in birds (Ogawa et al. 1997). There are no reports on the use of RFLP analysis to determine the sex of cranes. Both karyotyping and RFLP analysis usually require fresh tissue or blood, since viable cells or high-quality genomic DNA is needed. Alternative sources of material, such as feathers, are not normally appro-

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priate sources of tissues for these methods, since the number of viable cells and DNA is comparatively low.

Because of these drawbacks, alternative convenient and potentially noninvasive methods are needed to identify a bird's sex, especially using tissue sources such as feather samples. One approach, random amplified polymorphic DNA (RAPD) has been shown to be capable of identifying the sex in some species of birds (Griffiths and Tiwari 1993; Griffiths et al. 1992). The RAPD-PCR method, followed by isolation of a sex-specific DNA fragment, was used to identify the first avian sex-linked gene, the chromodomain-helicase-DNA binding protein (CHD) gene (Ellegren 1996; Griffiths et al. 1996). However, two versions of the CHD gene exist in most birds species, one located on the W chromosome (CHD-W), appearing only in females, and a second copy presents in both sexes and located on the Z chromosome (CHD-Z) (Ellegren and Fridolfsson 1997; Griffiths and Korn 1997). In many bird species these paralogous DNA fragments, from the W chromosome and the Z chromosome, have the same length (Griffiths et al. 1996). Thus DNA sequencing, restriction enzyme analysis, or other alternative methods must be used to analyze the PCR product to determine a bird's sex. Alternative methods for analysis of the CHD region, using primers flanking an intron in the gene have also been developed (Fridolfsson and Ellegren 1999; Griffiths et al. 1998; Kahn et al. 1998). However, if the CHD intron sizes are not substantially different in the male and female, they cannot be separated clearly by agarose-gel electrophoresis without further analysis. One further complication exists, however, since the CHD gene exists in organisms as diverse as yeast and mammals (Woodage et al. 1997), with high sequence conservation and conservation of length between mammals and birds. Contamination can produce PCR products that will appear similar to male avian products when several combinations of PCR primers are used (Ellegren and Sheldon 1997). Potential contamination needs be considered when fully developed feathers are used as a source of genomic DNA. The amount of DNA in fully developed plumage feather samples is much lower than in blood samples, increasing the chance of contamination when conserved primers are used. Because of these limitations, we attempted to identify additional avian-specific sex-linked DNA sequences to use for sex identification using DNA extracted from adult feathers. Here we report the identification of such a region in cranes.

Methods

Sample Collection

Genomic DNA was extracted from fully developed body feathers. The samples include all species from the crane family. The species studied included the black-crowned crane (*Balearica pavonina*), gray-crowned crane (*Balearica regulorum*), Demoiselle crane (*Anthropoides virgo*), blue crane (*Anthropoides paradisea*), wattled crane (*Bugeranus carunculatus*), sandhill crane (*Grus canadensis*), red-crowned crane (*Grus japonensis*), whooping crane (*Grus americana*), common crane (*Grus grus*), hooded crane (*Grus manachus*), black-necked crane (*Grus nigricollis*), sarus crane (*Grus antigone*), Australian crane (*Grus rubicundus*), white-naped crane (*Grus vipio*), and Siberian crane (*Grus leucogeranus*). Feathers were provided from two centers involved in breeding cranes. Plucked small body feathers were obtained from the Patuxent Environmental Science Center, Laurel, Maryland. Samples from the International Crane Foundation, Baraboo, Wisconsin, were usually plucked, fully developed small body feathers, but also included some molt feathers. In addition plucked body feather samples for some specimens were obtained from the Columbus Zoo. Samples from noncrane avian samples were obtained from several sources and supplied as extracted DNA or as whole blood. The species studied included black vultures (*Coragyps atratus*), marabou stork (*Leptoptilos crumeniferus*), albatross (*Diomedea irrorata*), pigeon (*Columba livia*), American kestrels (*Falco sparverius*), turkey (*Meleagris gallopavo*), chicken (*Gallus gallus*), house sparrow (*Passer domesticus*), and takahe (*Notornis mantelli*).

DNA Extraction

Feathers. Crane genomic DNA was extracted from adult feather quills using a sodium hydroxide boiling method (Zhang and Tiersch 1994). Feather quills were cut into small pieces and then boiled in 0.1 N NaOH solution (1 mg feather quill was added to 100 μl 0.1 N NaOH solution) for 40 min, then centrifuged at 10,000 rpm for 10 min. Liquid was transferred into a new tube, and 10× TE (Tris-EDTA) buffer was added to the extraction to a final concentration of 1× TE buffer.

Blood samples. DNA was extracted from blood by a standard phenol/chloroform extraction method. Blood was added to lysis buffer and proteins degraded using a proteinase enzyme (proteinase K) at 55°C for 24 h. Phenol/chloroform extraction was performed, followed by 95% ethanol precipitation of the DNA. Excess ethanol was removed by a 70% ethanol wash, following which the sample was dried and resuspended in 1× TE (Tris-EDTA) buffer overnight at 65°C.

RAPD

Polymorphic DNA was amplified according to a modification of the original RAPD technique (Williams et al. 1990). Amplification reactions were performed in 25 μl reaction volumes containing 2.5 μl 10× RAPD buffer (1 ml 10× buffer consisted of 670 μl 1 M Tris pH 8.8, 67 μl 1 M $MgCl_2$, 83 μl 2 M ammonium sulfate, 7 μl 14 M beta-mercapto-ethanol, 150 μl glycerol, and 23 μl H_2O), final concentrations of 500 μM of dNTPs, 15 pmol of a chosen RAPD primer (Operon Technologies), 1 unit of *Taq* DNA polymerase (BRL Inc.) and 50 ng of genomic DNA. A negative control reaction was essential to avoid misinterpretation of the RAPD patterns due to contamination or other artificial factors. The control reactions contained all the components except template DNA. The RAPD amplification was carried out in a Perkin-Elmer Cetus DNA TC1 thermal cycler. RAPD conditions were as follows: initial denaturation for 3 min at 94°C, followed by 45 cycles of 30 s at 94°C, 1 min at 35°C, 2 min at 72°C, and a final 10 min extension at 72°C.

The RAPD products were separated on a 2% agarose gel. Gels were run for 1.5 h at 6 V/cm. To compare the size of the products, a 123 bp DNA marker (BRL Inc.) was used as a molecular size marker in each gel. Gels were stained with ethidium bromide (EB) and photographed under ultraviolet illumination. The reproducibility of banding patterns was determined by repeating RAPD amplification under the same conditions.

Cloning and Sequencing

The putative female-specific RAPD products from three different female whooping cranes were isolated from an agarose gel. The bands were then cloned into a vector by the TA cloning method (Mead et al. 1991) using a TA cloning kit (Invitrogen Corp.), following the manufacturer's instructions. The size of the insert was checked by using the restriction enzyme *Eco*RI to cut the vector at both ends of the insertion. The inserted fragments were the

same size as the initial female-specific RAPD fragment. The sequences of the inserted fragments were obtained using primers M13 forward and M13 reverse, which were complementary to vector sequences. Sequencing was performed using a dsDNA cycle sequencing system (Gibco BRL, Inc.). Sequencing primers were labeled with ^{32}P-ATP with 1 unit of T4 polynucleotide kinase in a 5 μL reaction. Sequencing reactions were performed following the manufacturer's instructions. Sequencing products were separated on an 8% polyacrylamide gel. The gel was exposed to X-ray film and the DNA sequence was read manually.

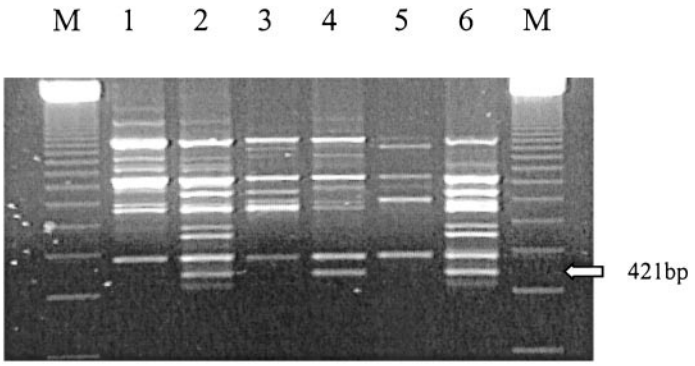

Figure 1. Results of RAPD amplification with primer OF-11 from genomic DNA extracted from feathers of three crane species: whooping crane (*G. americana*; lanes 1 and 2), hooded crane (*G. monachus*; lanes 3 and 4), and sandhill crane (*G. canadensis*; lanes 5 and 6). The arrow indicates the band corresponding to the crane sex-linked DNA fragment, which has a size of 421 bp in whooping crane and hooded crane and 424 bp in sandhill crane. M is a marker lane (123 bp ladder). Even number samples are females, odd number samples are males.

PCR

PCR primers were designed based on the common sequence of the cloned putative female-specific RAPD bands of three female whooping cranes. The program OLIGO (Rychlik and Rhoads 1989) was used to identify potential primer sequences with similar annealing temperatures. PCR amplification conditions were 95°C for 3 min, followed by 38 cycles of 95°C for 1 min, 55°C for 2 min, and 72°C for 2.5 min, and a final extension step of 72°C for 10 min. The amplification was conducted in a 25 μl reaction containing 400 μM dNTPs, 1× PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl; BRL Inc.), 2 mM $MgCl_2$, 4 pmol of each primer and 25–100 ng of genomic DNA. By using the female-specific primers, only the sex-specific target sequence, which appeared in the original multibanded RAPD gel, should be amplified. A combination of other primers was also identified which was able to produce a different size product, which would appear by using DNA from either males or females, and which could be used as a positive control for PCR.

Based on the complete sequence of CSL-W, several PCR primers were designed to amplify portions of the sequence. Some primer pairs amplify a product from both males and females. For example, the combination of primers CSL06 (5-ACCCCAGTACAGAATATGGAGTT) and CSL290 (5′-TCAAACTCTTGCTTGTGCATCT) yielded amplification products from both males and females. A failure of the PCR reaction for this primer pair should result in a blank sample. To detect any contamination, a negative control including all reagents except the DNA sample was used in each set of amplifications.

Results

The sodium hydroxide boiling method extraction procedure yields large amounts of DNA from either plucked or molted body feathers that can be used for either RAPD-PCR or traditional PCR amplification. To test the initial quality of the DNA produced by this extraction, we amplified and sequenced a 1185 bp DNA fragment of the ribosomal RNA 18S subunit gene and a 312 bp fragment of the mitochondrial cytochrome *b* gene from several crane species (data not show). In both cases, sequences were obtained that were identical to those obtained from blood samples.

In searching for sex-specific fragments, 70 RAPD primers were used to screen individuals of known sex from whooping crane (*G. americana*), sandhill crane (*G. canadensis*), Siberian crane (*G. leucogeranus*), black-necked crane (*G. nigricollis*), sarus crane (*G. antigone*), and red-crowned crane (*G. japonensis*). Three primers (OM-14, OM-7, OF-11) produced patterns in at least one species suggesting that they might amplify sequences from loci located putatively on sex chromosomes. However, only one primer (OF-11, 5′-TTGGTACCCC) produced a female-specific amplification product from several species. The product, finally designated OF11-421, was found to be have a size slightly larger than 400 bp in whooping cranes and several other crane species (Figure 1). Since the female-specific product amplified by OF-11 was found in several species, we pursued further characterization of this fragment.

The female-specific PCR product OF11-421 was band isolated from an agarose gel and cloned separately from three female whooping cranes. All three clones were sequenced and sequences were compared. The inserts each consisted of a 421 bp sequence, which were identical in all three cases. The female-specific clone has been designated the crane sex-linked DNA at W chromosome (CSL-W). The CSL-W sequence from the whooping crane has GenBank accession number AF333793. The homologous non-W sequence from whooping crane has also been deposited in GenBank with accession number AF333794.

Based on the complete sequence of CSL-W, several additional PCR primers were designed to amplify portions of the sequence. One set, consisting of primers CSL65 (5′-TTGCTAAAAGCTCACTTGGTGT) and CSL290 (5′-TCAAACTCTTGCTTGTGCATCT) yielded amplification products of 227–230 bp, which were female specific in all species of cranes. The product was found to be 227 bp in size in whooping crane (*G. americana*), black-necked crane (*G. nigricollis*), hooded crane (*G. monachus*), and common crane (*G. grus*) and 230 bp in the remaining crane species.

Some potential primers, which were identified within the CSL-W sequence, were found to amplify DNA segments in both male and female cranes. This indicates that there is a duplicate locus, which is paralogous to the CSL-W region, and which exists in both male and female cranes. The copy of the sequence which is common to both male and female cranes was designated CSL-NW (the CSL on non-W chromosome). The existence of the CSL-NW product can also be used as a positive control for PCR in any sex determination assay because it must appear in all individuals. The combination of primers CSL06 (5′-ACCCCAGTACAGAATATGGAGTT) and CSL290 was found to amplify a 289–293 bp CSL-NW fragment from both male and female cranes, serving as a positive control.

Because the primer pair CSL65 and CSL290 amplifies only a female-specific product, and the primer pair CSL06 and CSL290 amplifies a fragment from both

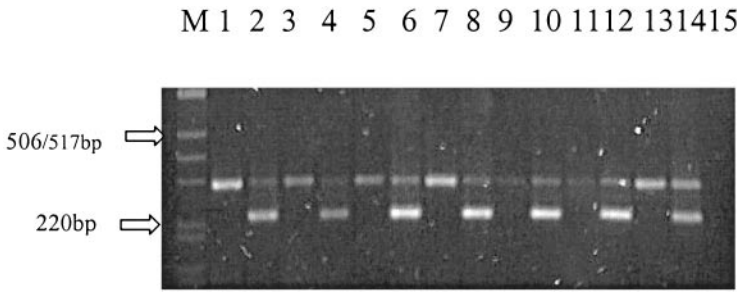

Figure 2. Primers CSL65 and CSL290 were used to amplify a CSL-W fragment (227–230 bp; the bottom band) from seven species, which represent possible products from all crane taxa. A positive control product fragment (the top band; about 290 bp was amplified with primers CSL06 and CSL290) from both male and female cranes indicates the success of PCR. The PCR products were separated in 2% agarose. Even number samples are females, odd number samples are males. Species: gray-crowned crane (*Balearica regulorum*; lanes 1 and 2), blue crane (*Anthropoides paradisea*; lanes 3 and 4), wattled crane (*Bugeranus carunculatus*; lanes 5 and 6), Siberian crane (*G. leucogeranus*; lanes 7 and 8), white-naped crane (*G. vipio*; lanes 9 and 10), whooping crane (*G. americana*; lanes 11 and 12), and sandhill crane (*G. canadensis*; lanes 13 and 14). Lane 0 is a size marker and lane 15 is a negative control.

male and female cranes, the combination of primers CSL06, CSL65, and CSL290 can be used for gender identification for all crane species (Figure 2). The positive control product (the top band in Figure 2), of about 290 bp, was amplified with primers CSL06 and CSL290. This band represents the paralogous non-W sequence present in both males and females. The PCR products in Figure 2 were separated in 2% agarose. These conditions do not provide reliable separation of fragments that differ by less than approximately 10–15 bp, so interspecific differences in the size of the CSL-W and CSL-NW bands are not noticeable. CSL primers do not produce any amplification products when applied to human and bovine samples, indicating that the possibility of false-positive results from human contamination is limited.

The internal portions of the CSL-W region (227–230 bp) and its paralogous locus, the CSL-NW (231–235 bp), have been sequenced from all crane species. Nucleotide similarity between the two copies is 86.3% in whooping cranes (Figure 3). Results from the DNA sequencing indicate that the internal CSL-NW segment is 4 bp longer than the CSL-W in whooping crane, common crane (*G. grus*), hooded crane (*G. monachus*), and black-necked crane (*G. nigricollis*). The CSL-NW is 1 bp longer than CSL-W in all remaining crane species except the crowned crane. Detailed interspecific comparisons of the sequences and a phylogenetic analysis combining these data with those of other sequences will be presented elsewhere.

To examine the accuracy of sex identification using the CSL primers, a blind test was designed by the Patuxent Environmental Science Center on material obtained from 18 cranes of known sex (10 whooping cranes and 8 sandhill cranes). PCR amplification using primers CSL06, CSL65, and CSL290 resulted in correct sex identification of these unknown samples in all 18 tests. By chance, the probability of correctly identifying all 18 individuals, if the sequence were not sex linked, is 4×10^{-6}. This blind test demonstrates that CSL-W DNA is sex linked and is not due to individual polymorphism. This test demonstrates that the method is both reliable and accurate.

Additional analysis suggests that the CSL sequence is also conserved across some other non-Gruiformes taxa. Because of the limitation of sample collection, we were able to test the primers on only a limited number of noncrane species. Several primers derived from the CSL-W sequence amplify female-specific fragments from black vultures (*C. atratus*), marabou stork (*L. crumeniferus*), albatross (*D. irrorata*), pigeon (*C. livia*), and American kestrel (*F. sparverius*). However, the CSL primers reported in this study did not produce products useful for sex identification in turkeys (*M. gallopavo*), chicken (*G. gallus*), house sparrows (*P. domesticus*), or takahe (*N. mantelli*). Without additional analysis of flanking sequences, and identification of the full coding sequence of this locus, it is not clear whether the CSL sequence is universally present in avian species.

The functional significance of the sequence is not known. Further analyses of the genetic region around CSL-W need to be conducted in hopes of determining whether the sequences represent coding regions of the crane genome.

The sequence of CSL-W and CSL-NW does not show significant similarity to any sequences that currently have been identified and deposited in the reference DNA sequence databases (as assessed by conducting a BLAST search of the GenBank database species).

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        CSL65

Female  TTGCTAAAAGCTCACTTGGTGTCCTCTAAAATTTTATAGGTAGGTAATGCAAATACCTTG   60

Male    ----------GAA-A---TCA--G-------------------C-----------TT---   60


Female  CACTATCAAAAGAACACATTTTATGAAATTA***TGTTTCAAAGCAATTGCTATATATGA  117

Male    ------A-------TT------G-A------CTG------------------------C-  120


Female  TATTACCTTTCCAGGAGTCATCTGATTTTATTGCTGTAATTTTATAACTATTTTGAATGA  177

Male    -----A----AA---CA-------------C-----C-G-G---C-----G-------A-  180


Female  AATGGCTTTGAT*AAAAATGCATCGTTTTAGATGCACAAGCAAGAGTTTGA           227

Male    ------------A------A---A-------------------------            231

                                                        CSL290
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Figure 3. Comparison of the nucleotide sequence of the crane sex-linked DNA (CSL) between male and female whooping cranes (*G. americana*). Dashes denote identical sequence. Asterisks denote deletions. Primers CSL65 and CSL290 (underlined), which were designed based on the female sequence, are shown on the diagram.

Discussion

For the management and conservation of avian species, for the study of animal ecology, behavior, population structure, and life history, and for captive management, sex identification is necessary. A search for sex-specific PCR products in cranes

generated by RAPD-PCR produced a conserved avian W chromosome-linked DNA sequence unlike any previously reported. Whether this sequence contributes in any way to avian sex determination is not known. Molecular techniques have been used to identify sex in various studies of bird species (Bradbury and Griffiths 1997; Ellegren et al. 1996; Griffiths and Tiwari 1995; Itoh et al. 1997; Komdeur 1996; Lessells and Mateman 1998; Millar et al. 1996; Sheldon and Ellegren 1996). The techniques that have been used include searching for the female-specific variable number of tandem repeats (Rabenold et al. 1991), identifying female-specific products using RAPD (Griffiths and Tiwari 1993), and using DNA probes that were selected from an avian clone library enriched for the W chromosome and hybridizing these clones to genomic DNA belonging to other avian species or groups. The most attractive molecular approaches for gender identification rely on simple PCR techniques (Ellegren and Sheldon 1997), since PCR requires only minute samples for genetic analysis.

The CHD gene was the first gene positioned on the avian W chromosome (Griffiths et al. 1996). The highly conserved nature of the paralogous CHD-W and CHD-NW loci (92% nucleotide sequence homology in chicken; Ellegren and Sheldon 1997), however, causes some complications for sex identification using the CHD gene. To identify the sex of a bird, the DNA must be either digested with a restriction endonuclease (Griffiths et al. 1996) or the DNA sequence must be obtained. Alternatively, if a product obtained from CHD-W has a similar size to the paralogous CHD-Z fragment, analysis to detect single-strand conformation polymorphisms (SSCPs) can be applied to analyze the CHD PCR products (Ellegren 1996). More recently a method using primers flanking a CHD gene intron were developed which yield CHD-W and CHD-Z products of different lengths that can be used for sex identification (Fridolfsson and Ellegren 1999; Griffiths et al. 1998; Kahn et al. 1998). If the CHD intron size difference between male and female birds is not significant in a particular species, however, the CHD-Z intron and CHD-W intron cannot be separated clearly by agarose-gel electrophoresis. For example, some terns, pukeko, most owls, and hawks show very similar sizes of the W-linked and Z-linked introns (Griffiths et al. 1998; Kahn et al. 1998).

A further problem in the identification of sex using DNA obtained from feathers analyzed by PCR is the potential for erroneous results due to contamination. There is a major risk of contamination using feathers to sex birds if a set of PCR primers is universal to birds and mammals. Griffiths and Tiwari (1995) demonstrated that feathers from a Soix's macaw (*Cyanopsitta spixii*) could be used for sex identification using a nested PCR technique combined with restriction enzyme cleavage methods. One potential disadvantage for using CHD primers to sex birds is that human contamination will mimic a positive male avian sample (Ellegren and Sheldon 1997). Human contamination has also been reported to produce a false pattern when avian sex was identified by differences in the CHD intron. We recommend great caution in the use of human and avian universal primers to type a bird with DNA extracted from feathers.

In addition to the studies on CHD, a pair of PCR primers (USP1 and USP3) has been used to amplify a female-specific DNA fragment from a range of Carinatae birds (Ogawa et al. 1997). However, the USP primers fail to identify sex in a range of non-Galliforme birds, for example, the white stork (*Ciconia boyciana*) (Ogawa et al. 1997), old world vultures (*Aegypius monachus, Gups fulvus,* and *Torgos tracheliotus*) (Wink et al. 1998), all cranes, and American kestrel (*F. sparverius*) (unpublished results from our laboratory).

Primers CSL65 and CSL290 were designed to amplify female-specific DNA in cranes, but these primers can also amplify other non-Gruiformes species, such as female black vultures and female marabou storks. It is expected that the primer pair CSL65-CSL290 can be used for sex identification of some other Ciconiiformes species. CSL primers do not produce any amplification products when applied to human and bovine samples, so a human contamination is limited.

This study clearly highlights the advantages of using PCR to analyze DNA obtained from feathers. In addition to examination of sequences that can be used for sexing of avian species, feathers are a good source for both nuclear and mitochondrial DNA. DNA extracted from feathers can be used for PCR of unique gene sequences, RAPD, and microsatellite studies. Ellegren reported (1991) that a swallow feather specimen collected in 1860 was useful for microsatellite analysis. Feathers are easy to collect, and easily stored and transported. However, many feathers contain only minute amounts of DNA, especially shed feathers. In order to obtain enough PCR product for analysis, more than 30 PCR cycles or the use of nested PCR is necessary. The current study illustrates that the use of a simple NaOH boiling extraction method allows DNA to be isolated from a feather in less than 1 h. PCR amplification can be done in 5 h. Obtaining information on the sex of a bird in less than 10 h using feathers is clearly practicable.

Blood samples from a crane or other avian species can obviously also be used for sex identification with the CSL DNA primers. Because blood contains a higher quality and quantity of DNA than feathers, and the blood cell membrane is much easier to lyse, conditions should be altered. For instance, the boiling time for DNA extraction, PCR annealing and extension time, and PCR cycles should be reduced.

The sequences reported here can be used to evaluate the sex of individuals in many avian species, especially in cranes, for which obvious external differences between the sexes is not apparent. The molecular methods that we used are easily performed, are less invasive than blood typing, and can be done at a very early age. The low cost, speed, and ease of collection, storage, and transport of feather samples are the major advantages. Like other sex identification methods, this technique also provides the ability to monitor sex ratios in crane populations, and could be very useful to captive management, population biology, and conservation biology studies.

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