A rapid, non-invasive, PCR-based method for identification of sex of the endangered Old World vultures (white-backed and long-billed vultures) – Implications for captive breeding programmes

Anuradha Reddy[1], Vibhu Prakash[2] and S. Shivaji[1,*]

[1]Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad 500 007, India
[2]Bombay Natural History Society, Hornbill House, S.B. Singh Road, Mumbai 400 023, India

The rapid and inexplicable decline of the Old World vulture populations across the Indian subcontinent is a cause of growing concern among ecologists and conservationists. Survival of these threatened birds may depend on breeding programmes where sex identification plays an important role. Here, a PCR-based method that amplifies a region of the chromo-helicase-DNA-binding gene was modified to identify the sex of individuals of two *Gyps* species. The results were further reconfirmed by amplification refractory mutation system. This study allowed for the rapid and safe assessment of sex and will facilitate all future efforts in the captive breeding of these birds.

Keywords: Captive breeding, old world vultures, sex determination.

OUT of the four species of griffon vultures in India, the Indian white-backed vultures (*Gyps bengalensis*) and the slender-billed vultures (*Gyps indicus*) were commonly sighted during the 1970s and early to mid-1980s, and the densities ranged from 3 nests/sq. km in the city of Delhi[1] to 12 nests/sq. km in Keoladeo National Park, Rajasthan, India (V. Prakash, unpublished). In fact, in 1985 *G. bengalensis* was regarded as possibly the most abundant large bird of prey in the world[2], and flocks of several thousands of birds were present at carcass dumps. During the 90s, the populations of these two species declined suddenly[3] by more than 90%. There are several possible explanations for the high mortality and reduced breeding success of the Indian vultures including food shortage, persecution, contaminants like diclofenac[4], and infectious diseases[5,6]. This catastrophic decline, which has pushed these birds to the brink of extinction, has necessitated immediate conservation efforts either *in situ* (habitat protection) or *ex situ* (captive breeding programmes for eventual reintroduction/assisted reproduction in zoos).

One of the basic problems faced in the conservation of these birds is identifying their sex. Like birds of many other groups, vultures do not exhibit any external sexual dimorphism. The sex of vultures can be determined laproscopically, but this technique involves some amount of risk to the bird. Karyotyping can be done to identify the sex chromosomes (ZZ in males and ZW in females)[7], but this would require blood samples. Other methods like faecal steroid analysis and flow cytometry[8] are also possible, but are complicated and time-consuming. Therefore, there is need for a rapid and more flexible approach. Molecular sexing[9] based on polymerase chain reaction (PCR) is an attractive option, since PCR is simple to perform, rapid and requires only a minute quantity of DNA, which could be obtained from a single feather or a drop of blood. The DNA thus obtained could be used to identify a sex-specific marker. The female bird is heterogametic (ZW), and obviously the W chromosome has to be the source of the sex-linked marker. The first W chromosome gene discovered was the chromobox-helicase-DNA-binding gene (*CHD-W*)[10]. This gene is highly conserved and it has been shown that a single pair of PCR primers (P2 and P8) can be used to sex most of the avian species[11,12]. These primers anneal to conserved exonic regions and amplify across an intron that varies in size between *CHD-W* and *CHD-Z*[12–15]. Gel electrophoresis reveals one band in males and two bands in females in most of the bird species. Unfortunately, in vultures, as in other members of the *Accipitridae* family, the two CHD products (*CHD-W* and *CHD-Z*) are of the same size[16,17], and therefore based on PCR alone it would not be possible to differentiate the two sexes. One way to overcome this problem would be to use a restriction enzyme that would selectively cut *CHD-Z* but not *CHD-W* before gel electrophoresis[10,11,16]. Using this strategy the sexes could be identified, since females would generate three bands and males two bands. In the present study we used *CHD* PCR followed by *Hae*III digestion to sex *G. bengalensis* and *G. indicus* and also compared our results with ARMS (amplification refractory mutation system)[17], another PCR-based technique used for sexing Falconiformes. ARMS is based on the differences in sequences between *CHD-Z* and *CHD-W*, and can amplify specific alleles using a 3′-terminal mismatch primer, that pair with only these specific alleles[18]. There are some nucleotide differences between *CHD-W* and *CHD-Z* fragments. A new primer (MP) was designed in such a way that its 3′-end was on a point mutation and would amplify only part of the *CHD-W* fragment. In this method three primers (P2, NP and MP) are used. P2 and NP are similar to P2 and P8 described by Griffiths *et al.*[12], and amplify both *CHD-W* and *CHD-Z* fragments, while NP and MP only amplify part of the *CHD-W* fragment. Thus gel electrophoresis after all three primers are used together in a single PCR, reveals one band in males and three bands in females.

Two to three breast feather tips (calami) were collected from 18 white-backed vultures (*G. bengalensis*) and 18 slender-billed vultures (*G. indicus*), housed at the captive breeding facility at Pinjore, Haryana, India. Total genomic DNA was

*For correspondence. (e-mail: shivas@ccmb.res.in)

isolated by the phenol–chloroform method and used for PCR. The PCR reaction (30 µl) mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 2 mM of each dNTP, 0.01 mg bovine serum albumin (BSA), 50 ng of each primer (P2 and P8)[12], 0.05 units of *Taq* polymerase (Ampli Taq Gold™, Applied Biosystems) and 100 ng of genomic DNA. An initial denaturing step of 94°C for 10 min was followed by 37 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 30 s. A final run of 52°C for 1 min and 72°C for 5 min completed the programme. The PCR products were then digested with 1 unit of *Hae*III (New England Biosystems) under the following reaction conditions: 50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (pH 7.9) and incubated for 2 h at 37°C. The digested products were separated in a 12% non-denaturing acrylamide gel and scored for sex on the basis of number of correct size bands.

PCR was also performed with the primer set P2 and NP[17] essentially as described above, except that the initial denaturing step of 94°C for 10 min was followed by 35 cycles of 94°C for 30 s, 52°C for 45 s and 72°C for 45 s. A final run of 50°C for 1 min and 72°C for 5 min completed the programme. The PCR products were separated in a 12% non-denaturing acrylamide gel.

ARMS was performed using three primers (P2, NP and MP)[17]. PCR amplification was carried out in 30 µl volume as described above using 200 ng of genomic DNA, 50 ng of P2, and 25 ng each of NP and MP primers. The products were separated in a 12% non-denaturing acrylamide gel.

In preliminary experiments, to check the accuracy of the PCR-based method used in the present study, male white-backed vultures were selected based on their ability to ejaculate following manual massage[19], as part of the assisted reproduction programme at the Nehru Zoological Park, Hyderabad, India. Feathers from the selected males were used for the standardizing the PCR-based technique. Later, a modified version of the sexing technique[12] was successfully applied to all the individuals housed in captivity at Pinjore. Each individual was assigned to a particular sex based on the number of DNA bands generated following PCR and restriction digestion. DNA from females generated three DNA bands of 386, 343 and 43 bp respectively, while in males only two bands of 343 and 43 bp respectively, were visualized (Figure 1). The results of all the 36 individuals analysed indicated that in *G. bengalensis* there were 12 males and 6 females, whereas in *G. indicus* there were 4 males and 14 females.

The white-backed vultures (*G. bengalensis*) could also be sexed when P2 and NP primers were used. However, the slender-billed vulture (*G. indicus*) could not be sexed as both the sexes exhibited only a single band (Figure 2). The amplicons were sequenced to investigate the differences between the two species. The sequencing results showed that the size difference between *CHD-Z* and *CHD-W* in *G. bengalensis* is about 9 bp, while that in *G. indicus* is 5–6 bp.

ARMS with three primers (P2, NP and MP) clearly resolved the differences between males and females of both species (Figure 3), since in both species the males showed one band and females showed three bands. Also in the case of *G. indicus*, the amplicons were run for a longer time on acrylamide gel to separate out the bands amplified by P2 and NP primers. All the amplicons were sequenced using an ABM 3700 automated sequencer (GenBank accession nos DQ156153–6). BLAST analysis of the sequences showed a similarity of 97–98% with the partial *CHD* gene sequences of other bird species in Accipitridae like golden eagle, mountain hawk-eagle, sparrow hawk, black kite, goshawk and marsh harrier. Figure 4 shows the alignment and sequence differences between *CHD-W* and *CHD-Z* genes of *G. bengalensis* and *G. indicus*, and also a comparison with the sequence of the same genes of the mountain hawk-eagle.

In order to have a successful breeding programme, it is essential to know the sex ratio of the captive population and the corresponding number of potential breeding pairs. This becomes even more important in small populations of endangered or threatened species in which sex ratio is

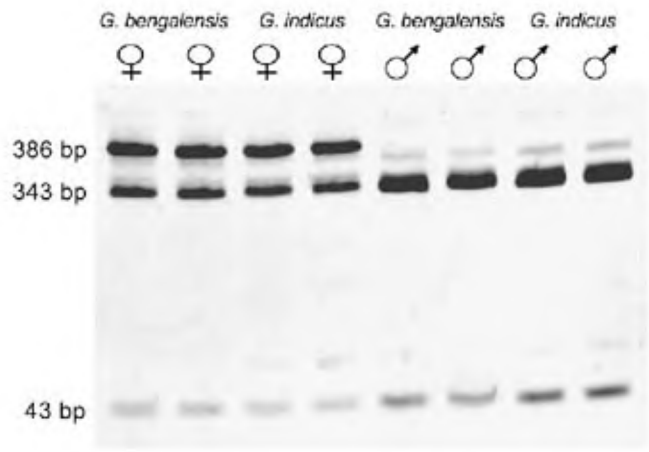

Figure 1. Identification of sex of *Gyps bengalensis* and *Gyps indicus* by *Hae*III restriction digestion of chromo-helicase-DNA-binding gene. CHD markers were amplified by PCR using P2/P8 primer pair, digested with *Hae*III and analysed by electrophoresis in 12% polyacrylamide gels.

Figure 2. Identification of sex *G. bengalensis* and *G. indicus* by PCR amplification of the chromo-helicase-DNA-binding gene using P2/NP primer pair. PCR amplification yielded two bands in females of *G. bengalensis* (380 and 371 bp) and one band in the males (371 bp). In *G. indicus*, both sexes yielded a single band (375 bp).

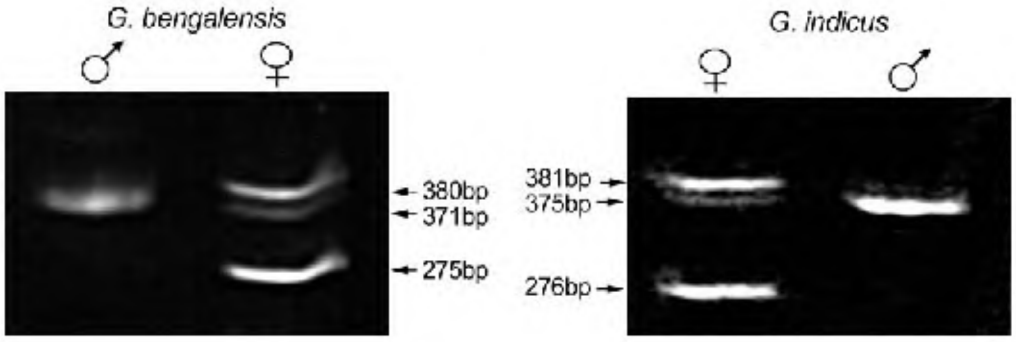

Figure 3. Identification of sex of *G. bengalensis* and *G. indicus* by amplification of the chromo-helicase-DNA-binding gene using ARMS technique. Females of both species yielded three bands (380, 371 and 275 bp; and 381, 375 and 276 bp respectively), while males yielded one band (371 and 375 bp respectively).

```
          1                                                30
Gb-W  CTCCCAAGGATGAGGAAACTGTGCAAAACAGGTATCTTCTGGGTTTTGACCAACTAAATT
Gb-Z  -----C-A-GG-G--------------A---GG------T----C-----G--CTGG--
Gi-W  ------------A-------------------------A------A-------------
Gi-Z  TC---C---GG---------TG----------GG----T-T---------GG---TG---
Mh-W                               ------------------------------C--
Mh-Z                               ------------G------T----C----TG--------

Gb-W  TGATTTTTTTGTTGTTG---TTTGTTTGTTTTTTCATTGCTGTTGTTTTGTCTTGTACTT
Gb-Z  -A-C---AA-A---GCAT---GG---A-------TT---GG-GG-GG-G-GG--TGGG--
Gi-W  ----------A------TGG-----------------A----------------------
Gi-Z  -A-C----AA----CAA----GGT--A-------TT---GG-GG-G--G-GG--T-GG--
Mh-W  ----------T------TTG-----------------------------------------
Mh-Z  ---C--G-AC---TA-A-C-C-AT-GGT--AG--TC--TG--GG-GG----TG-TG-G--

Gb-W  TTGGGTTGGGTGGTTTTCACACATGGCAC-ACCCCCCCATTCCCCCATTTTTGACAGGCT
Gb-Z  --AAAG-----TT----TTTTTT-TTTTT-C-TTTT-TGAA-A-AT--------------
Gi-W  ----------------------------------------------------------
Gi-Z  --TAAA-----TT----TTTTTT-TTTTTTC-TTTT-TGAA-A-AT--------------
Mh-W  ---------------------------------G----------------------
Mh-Z  ----T--TTAA-T-G-GGGGTTT-TTTTTTT-TTTT-T-AA-A-AT--------------

Gb-W  AGATAACATATTAATAAAATGTTTTAGTCACGTAGCTTTGAACTACTTAATCTGAAATTC
Gb-Z  --G---A-CT---T-T-CGGT-G---A-------------------C--T----------
Gi-W  ----------------------------------------------------------
Gi-Z  --G---A-CT---T-T-CGGT-G---A-------------------C--T----------
Mh-W  --------C--------------------------G----------------------
Mh-Z  A-G---AG-T---T-T-TGGT-G---A------------C------C--T----------

          244
Gb-W  CAGATCAGCTTTAATGGAAGTGAAGGGAAACGCAGTAGGAGCAGAAGATATTCTGGATCT
Gb-Z  ----------------------------G----------------------C--------C
Gi-W  ----------------------------------------------------------
Gi-Z  ----------------------------G----------------------C--------C
Mh-W  ----------------------------------------------------------
Mh-Z  ----------------------------G----------------------C---------

Gb-W  GATAGTGACTCCATCTCAGAAAGAAAACGACCAAAAAAACGTGGACGACCACGAA-TACT
Gb-Z  ----------------------------G--T-----------A---------C--T-
Gi-W  ----------------------------------------------------------
Gi-Z  ----------------------------G--T-----------A---------C--G-
Mh-W  ----------------------------------------------------C--T-
Mh-Z  ----------------------------G--T-----------A---------C----

                                                    395
Gb-W  CGTCGAGAAAATATTAAAGGATTTAGCGATGCAGA
Gb-Z  -C--------------------------------
Gi-W  ----------------------------------
Gi-Z  -C--------------------------------
Mh-W  -C-------------
Mh-Z  -C-------------
```

Figure 4. Partial sequences of the *CHD-W* and *CHD-Z* genes in *G. bengalensis* (Gb), accession numbers DQ156153 and DQ156154. *G. indicus* (Gi), accession numbers DQ156155 and DQ156156, and the mountain hawk-eagle (Mh), accession numbers AB096149 and AB096150. The exon (1–30, 244–395) is given in bold. Only those bases that differ from the sequence of the *CHD-W* gene of *G. bengalensis* are indicated.

one of the stochastic events that can drastically affect the size of the next generation. Accurate and non-invasive sex determination of vultures is one of the basic steps towards planning a viable breeding programme or assisted reproduction technique in zoos. In this study genomic DNA isolated from 2–3 feather tips was sufficient for correct sexing. Sexing of adults based on DNA recovered from feather tips minimizes handling compared to what is required in collecting blood. This in turn decreases stress and increases the chances of successful breeding.

The sexing technique used here involves a simple PCR protocol to amplify a conserved pair of *CHD* genes[11]. The modified version of this protocol presented here, followed by restriction digestion with *Hae*III, could be a valuable tool for various *ex situ* conservation efforts in vultures. Although some groups have argued that PCR–RFLP method based on *Hae*III may suffer from incomplete digestion[16], we have ruled out all possibilities of ambiguous results, as the amount of enzyme used was more than sufficient to digest the amplified products in the given incubation period. Further, the results were also cross-checked and confirmed using the ARMS technique[17]. Although the P2/NP primer pair can detect stronger signal band compared to P2/P8, the results can still be erroneous if the separation of the bands is not done properly, due to small size differences between the *CHD-Z* and *CHD-W* amplicons. Further, the ARMS technique requires a high yield of pure genomic DNA[17], which is normally obtained from blood or muscle. In the present study the ARMS technique was only successful when DNA from blood was used (Figure 3). DNA from feathers was not suitable for the ARMS technique (data not shown). In contrast, in the present PCR-based method genomic DNA from feathers yielded PCR products of similar quality to those derived from blood/muscle samples.

PCR-based sexing was successfully applied to the captive populations of *Gyps* vultures at Pinjore with minimum stress to the birds, and will facilitate all future efforts in breeding these birds in captivity. This technique would also be useful in establishing the sex ratio of these birds in the wild.

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High frequency plant regeneration via somatic embryogenesis in *Podophyllum peltatum* L., an important source of anticancer drug

Y. S. Kim[1,3], S. Lim[1], Y. E. Choi[2] and V. Ramesh Anbazhagan[2,*]

[1]Laboratory for Plant Biotechnology, Pharmax Co, Ansung 456-756, Korea
[2]Forest Resources Development, Division of Forest Resources, College of Forest Sciences, Kangwon National University, Chunchon 200 701, Kangwon-do, Korea
[3]Present address: Research and Education Center for Genetic Information, Nara Institute of Science and Technology, Nara 630-0192, Japan

***Podophyllum peltatum* (L.) is an important medicinal plant that produces podophyllotoxin with anti-cancer properties. In the present study, we report the establishment of plantlet regeneration of *P. peltatum* via somatic embryogenesis. Somatic embryos differentiated directly from cotyledon explants of zygotic embryos on Murashige and Skoog (MS) basal medium supplemented with 21.6 µM α-naphthaleneacetic acid after 8 weeks of culture. An embryogenic callus developed from cotyledon explants of somatic embryos on MS medium + 6.78 µM 2,4-dichlorophenoxy acetic acid under continuous darkness after 6 weeks of culture. When the embryogenic callus was first grown on MS basal medium + abscisic acid (11.35 µM) for three weeks, followed by sub-culturing onto plain MS basal medium, it led to the development of high frequency somatic embryogenesis. Germination of cotyledonary somatic embryos was noticed on MS basal medium with the addition of 2.89 µM gibberellic acid after two weeks. The germinated embryos grow into plantlets with well-developed roots. Rooted plantlets were acclimatized in soil. The present protocol can be widely used for micropropagation and metabolic engineering.**

Keywords: Anticancer drug, mayapple, medicinal plant, podophyllotoxin, plant regeneration, somatic embryogenesis.

PODOPHYLLUM peltatum (L.) commonly called mayapple, belongs to family Berberidaceae and is native to southern Canada and northeastern USA. It is a perennial and grows in patches with a branched rhizome system. The rhizome contains podophyllotoxin with anticancer properties. The semi-synthetic derivatives of this compound like etoposide, etophos, and teniposide, are used clinically as chemotherapeutic agents for a variety of tumours, including small cell lung carcinoma, testicular cancer and malignant lymphoma[1]. Both *P. peltatum* and *P. emodi* Wall. ex Royle (syn.

*For correspondence. (e-mail: vrameshanbazhagan@yahoo.com)