STUDIES IN VULTURE CONSERVATION: DEVELOPMENT OF CHROMO-HELICASE-DNA-BINDING GENE BASED MOLECULAR METHOD FOR UNAMBIGUOUS SEX DETERMINATION IN *GYPS INDICUS* AND *GYPS BENGALENSIS*

THESIS

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of

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in

ANIMAL BIOCHEMISTRY

By

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To

DEEMED UNIVERSITY

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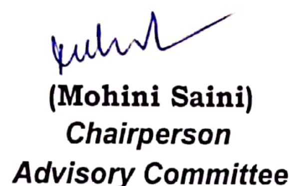

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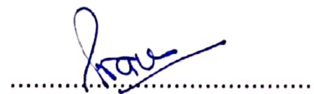
Dr. Praveen K. Gupta, Senior Scientist Division of Animal Biotechnology	
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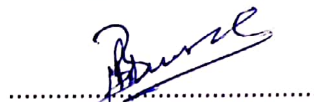
Dr. Sanjeev Bhure, Senior Scientist, Division of Animal Biochemistry	

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Abbreviations

A	-	Absorbance
aa	-	Amino acid
Amp	-	Ampicillin
bp	-	base pair
CHD W	-	CHD gene on W chromosome
CHD Z	-	CHD gene on Z chromosome
DNA	-	Deoxy ribonucleic acid
dNTP	-	Deoxy nucleotide triphosphate
DW	-	Distilled Water
EDTA	-	Ethylenediamine tetra acetic acid
Etbr	-	Ethidium bromide
FAM	-	Carboxyfluorecein
h	-	Hour(s)
Kb	-	Kilo base
L.B	-	Luria Bertini
M	-	Molar
$MgCl_2$	-	Magnesium Chloride
Min	-	Minute(s)
Mol.Wt.	-	Molecular weight
OD	-	Optical Density
PCR	-	Polymerase Chain Reaction
PM	-	Post-mortem
pmol	-	Picomole
RE	-	Restriction Enzyme
rpm	-	Revolutions per minute
RT	-	Room Temperature
RT-PCR	-	Reverse Transcription Polymerase Reaction.
sec	-	Second(s)
TAE	-	Tris Acetate EDTA buffer
TAMRA	-	Tetramethyl-6-carboxyrhodamine
Taq Pol.	-	*Thermus aquaticus* Polymerase
UV	-	Ultra violet
µl	-	Microliter
µM	-	Micro Molar

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I. Introduction

India has 2.4% of world total area with 328.7 million hectare of land mass. Of total land area 23.57% i.e. 77.47million hectare area is under forest cover. There are 606 protected areas comprising 96 national parks and 510 wildlife sanctuaries with 28 tiger reserves and 25 elephant reserves covering 15.59 million hectare and making up about 4.58% of total geographic area and 22.12% of total forest cover of country. There are 150 recognized zoological parks with about 40,000 wild animals in captivity in country. India has vast biodiversity with 350 species of mammals, 1224 of birds, 408 of reptiles, 197 of amphibians, 2546 of fishes, 57548 of insects and 46286 species of plants found in India form 8% of total biodiversity of life forms in world (CZA, 2009). Aves are one of the important class in maintaining ecosystem balance. Avian populations and dependent ecosystem services are declining faster than species extinctions would indicate (Sekercioglu *et al.,* 2004) as evidenced from extinction of only 1.3% of bird species as compared to estimated 20–25% reduction of global number of individual birds since 1500.

Population sex ratio in birds often diverges from parity and is mostly male biased (Trivers, 1971; Bellrose, 1980; Dobson, 1987; Breitwisch, 1989; Sargeant and Raveling, 1992). Monogamy is a form of sexual bonding involving a permanent pair bond between male and female. Falconiformes are mostly monogamous in nature. Population sex ratios in monogamous birds are often male biased. One factor that can affect population sex ratios is sex-biased predation (Nebel *et al.,* 2004).

At the top of ecological food webs are Vultures, eagles, hawks, harriers and buzzard belonging to the nine subfamilies of the family of Accipitridae, order Falconiformes that are very sensitive to environmental changes such as chemical contaminants, food shortages, sex ratio distortion, and habitat destruction. One such evidence is the catastrophic decline in Gyps vulture population in Indian subcontinent by indiscriminate use of diclofenac (an anti-inflammatory drug) in livestock. As recently as the early 1980's, there were approximately 40 million vultures in India and now there are only 60,000 of these important scavengers left. This decline has adversely affected the scavenging on animal carcasses of animals and the disposal of dead bodies as per the religious practices of the Parsi community, thus highlighting the ecological, social and cultural significance of these species (Swan *et al.,* 2006).

1.1 VULTURES IN INDIA

India has nine species of vultures in the wild (Cunningham *et al.,* 2003). These are the Oriental White-backed Vulture (*Gyps bengalensis*), Slender billed Vulture (*Gyps tenuirostris*), Long-billed Vulture (*Gyps indicus*), Egyptian Vulture (*Neophron percnopterus*), Red Headed Vulture (*Sarcogyps calvus*), Indian Griffon Vulture (*Gyps fulvus*), Himalayan Griffon (*Gyps himalayensis*), Bearded Vulture or

Gyps bengalensis

Gyps indicus

Gyps himalayensis

Aegypius monachus

Vulture at VCBC, Pinjore

Lammergeier (*Gypaetus barbatus*) and Cinereous Vulture (*Aegypius monachus*). The population of three species i.e. White-backed Vulture, Slender billed Vulture and Long billed Vulture in the wild has declined drastically over the past decade. The decline of *Gyps* genus in India has been put at 97% by 2005. The latest survey in 2007 indicates that numbers of Oriental white-backed vultures have declined by a staggering 99.9% over the preceding 15 years. Long-billed and slender-billed vultures have decreased by 97% over the same period (Prakash *et al.,* 2007; Pain *et al*., 2008; Cunningham *et al*., 2003). Because of the evidence of widespread and rapid population decline, all three vulture species were listed by IUCN, the World Conservation Union, in 2000 as ‘Critically Endangered’, which is the highest category of endangerment. In 2002, all the three species were categorized under Schedule I of Indian Wildlife Protection Act, 1972.

1.1.1 Status of population of *Gyps* vultures in the Indian subcontinent

Gyps bengalensis occurs in Pakistan, India, Bangladesh, Nepal, Bhutan, Myanmar, Thailand, Laos, Cambodia and southern Vietnam, and may be extinct in southern China and Malaysia. It has been recorded from south-east Afganistan and Iran where its status is currently unknown (IUCN, 2009). *Gyps indicus* breeds in South-east Pakistan (where it is rare although a 200-250 pair colony was discovered in 2003 in Sindh Province, Pakistan) and peninsular India South of the Gangetic plain, north to Delhi, east through Madhya Pradesh, south to the Nilgiris, and occasionally further south (IUCN, 2009). *Gyps tenuirostris* is found in India north of, and including, the Gangetic plain, west to at least Himachal Pradesh and Haryana, south to southern West Bengal (and possibly northern Orissa), East through the plains of Assam, and through southern Nepal, and North and central Bangladesh. It formerly occurred more widely in South-east Asia, but it is now thought to be extinct in Thailand and Malaysia,

and the only recent records are from Cambodia, southern Laos and Myanmar (IUCN, 2009).

Decline of vulture populations was first recorded in India at the Keoladeo Ghana National Park, Rajasthan during mid 1980's to mid 1990's, followed by Northern India road counts. Nepal has also experienced similar reductions. A dramatic decline of two species, *G. bengalensis* and *G. tenuirostris* was noticed in Nepal since the mid-1990s, when an estimated >150,000 pairs of White-rumped Vulture were known to breed (Ministry of Environment and Forests Government of India 2006). There are now less than 1000 pairs of the Slender-billed Vultures in Nepal. The rate of annual decline in Nepal is estimated to be 40% and the rate of decline within a decade is estimated at 90 to 95%. In Bangladesh, the *Gyps bengalensis* is threatened and *Gyps indicus* and *Gyps fulvus* are now rare. Populations of *Gyps bengalensis* and *Gyps tenuirostris* in South-East Asia are low, but declines are thought to have been historical and slower, rather than recent and rapid. The primary reason behind decline in the region is thought to be the demise of large ungulate populations and improvements in animal husbandry resulting in a lack of available carcasses for vultures. Extensive research has identified the non-steroidal anti-inflammatory drug (NSAID), diclofenac, to be the cause behind the rapid population collapse (Shultz *et al.,* 2004; Oaks *et al.,* 2004). This drug, used to treat domestic livestock, is ingested by vultures feeding on their carcasses causing visceral gout leading to renal failure. It is unknown if diclofenac is affecting populations of other vulture species and scavenging birds in the region, but numbers of Red-headed Vulture *Sarcogyps calvus* and Egyptian Vulture *Neophron percnopterus* have recently undergone rapid declines in India and studies have pointed out that not only diclofenac, but also some other NSAIDs are harmful to vultures and other scavenging birds

(Cuthbert *et al.,* 2009). The study by Acharya *et al.*, (2009) demonstrated that the number of Himalayan Griffons has substantially decreased in the Mustang region of Nepal. Populations along transects declined by up to 70% from 2002 to 2005, at an average decline rate of 30% per year a rate of decline comparable with the annual declines of 22% and 48% recorded for the now 'Critically Endangered' Long-billed and Oriental White-backed vultures in India (Green *et al.,* 2004, Green *et al.,* 2006; Green *et al.,* 2007).

The white-backed vultures global population is now estimated to fall within the band 2,500-9,999 individuals, Long-billed vultures recorded 337 individuals along >18,000 km road transects (IUCN 2009).

1.2 CONSERVATION OF VULTURES IN INDIA

In Nepal a project named "Vulture Restaurant" is underway in an effort to conserve the dwindling number of vultures. Government of India has also taken steps in order to preserve wildlife including vultures. India moved a IUCN motion in 2004 for vulture conservation, which was accepted in the form of the IUCN resolution which called upon *Gyps* vulture Range countries to begin action to prevent all uses of diclofenac in veterinary applications that allow diclofenac to be present in carcasses of domestic livestock available as food for vultures; establishment of IUCN south Asian task force under the auspices of the IUCN; Range countries to develop and implement national vulture recovery plans, including conservation breeding and release (Ministry of Environment and Forests, Government of India, 2006)

The last hope of saving these birds from extinction is the captive breeding and reintroduction of the birds after removal of the cause of mortality from the environment. The National Wildlife Plan (2002-2016) of Govt. of India, emphasis the role of Zoos for *ex-situ* breeding

of endangered species of wild fauna and then their rehabilitation in wild as per IUCN guidelines for reintroduction. Vulture Breeding and Conservation Centre had already been established at Pinjore, Haryana in 2001 and at Buxa, West Bengal in 2005 by Bombay Natural History Society (BNHS) in partnership with UK based organizations RSPB, Darwin Initiative for the survival of species, ZSL and National Birds of Prey Trust. The species housed at the centre are white-backed vultures, Long-billed vultures, Slender-billed vultures and the Himalayan griffon (Jakati, 2006). The Central Zoo Authority of India has also established centres in the zoos at Junagadh, Bhopal, Hyderabad, Bhubhaneshwar, Rajabhatkhawa, and Guwahati in 2006-07. These centres also serve as rescue and analysis centres for sick vultures or carcasses sent for treatment and investigations.

1.3 SIGNIFICANCE OF BIRD SEXING

Survival of most endangered birds may depend on breeding programs where sex identification plays an important role for the establishment of breeding pairs or breeding flocks different for either gender (Cheney, 1990; McGill and Perkins, 1993; Stevenson, 1993). The wild population of the oriental white stork (*Ciconia boyciana*) in Japan became extinct in 1971, but captive breeding efforts have been successful (Murata *et al.,* 1998). Knowledge of the sex of individuals is also essential for investigations on evolution and ecology, including sex ratio evolution, sexual selection, parental care strategies, demography and conservation.

In birds that are sexually dimorphic, such as the house sparrow (*Passer domesticus* Linneus), mallard (*Anas platyrhynchos Linneus*) and collared flycatcher (*Ficedula albicollis Temminck*), it is very easy to distinguish between males and females based on the secondary sexual characteristics such as body size, plumage color etc. However,

morphological identification of sex in birds can often be difficult in monomorphic species and sexually immature individuals, and thus molecular sexing techniques are employed (Ellegren and Sheldon 1997; Griffiths *et al.,* 1998; Kahn *et al.,* 1998; Fridolfsson and Ellegren, 1999). Chromosome-helicase-DNA binding protein 1 (or CHD1), the sex-linked gene locus used for these molecular sexing techniques, has contributed to a greater understanding of sex chromosome evolution (Ellegren, 2000). CHD based molecular methods have extensively been exploited for gender identification in number of bird species including raptors and scavengers (Fridolfsson and Ellegren, 1999; De volo, 2005; Reddy *et al.,* 2007).

Molecular sexing has enabled researchers to determine the sex of rare monomorphic birds such as the Po'ouli (*Melamprosops placosoma*) and the Spix macaw (*Cyanopsitta spixii*), a species that is now extinct in the wild, thus aiding conservation efforts in these species (Griffiths and Tiwari, 1995; Groombridge *et al.,* 2004). Likewise, DNA sex identification has played a pivotal role in captive breeding of genetically healthy stocks of penguins in Edinburgh Zoo in UK, thus assisting their care and conservation in the wild (Costantini *et al.,* 2008).

The difficulty of gender identification may hinder both evolutionary studies and human-assisted breeding of vultures. In the colony of birds breeding as monogamous pairs in captive breeding centers, it is highly desirable to know the sex of the individuals of this monomorphic bird to keep a relatively stable 1:1 sex ratio (Griffiths, 2000). Therefore, developing a rapid, economical, and reliable method for vulture gender identification is important, not only for bird management but also to assess environmental impacts. Considering the above facts, the present study has been proposed with the following

OBJECTIVES:

- To clone and characterize the choromo helicase I (CHD I) gene of Gyps indicus and Gyps bengalensis
- To develop CHD-based molecular method for sex identification of Gyps indicus and Gyps bengalensis

2. Review of Literature

The global avifauna is declining at rapid rate overall, 21% of bird species are currently extinction-prone and 6.5% are functionally extinct, contributing negligibly to ecosystem processes. A quarter or more of frugivorous and omnivorous species and one-third or more of herbivorous, piscivorous, and scavenger species are extinction prone. Projections indicate that by 2100, 6–14% of all bird species will be extinct, and 7–25% (28–56% on oceanic islands) will be functionally extinct. Important ecosystem processes, particularly decomposition, pollination, and seed dispersal, will likely decline as a result (Sekercioglu *et al.,* 2004).

There are various orders under class aves which help classify birds. Table 2.1 (Sekercioglu *et al.,* 2004) mentions functional groups of birds accordingly they are classified in different classes.

Vultures are scavenging birds which are mainly of two types, Old age vulture (*Acipitridae*) and New age vulture (*Cathartidae*) (Hertel, 1994). Vulture are biological environment cleaning machines, they

eat dead animal flesh, remove putrefying disease spreading dead carcass off the environment. They very rarely known to attack live animals, mostly in this rare case prey is diseased, weak, moribund animals. They eat dead animal carcass but if carcass skin is tough to pierce by beak then they wait till other predator animals attacks and make carcass open it. Vultures are social creatures they usually found in conspecies. Vultures breed in colonies and mostly can be seen near human habitation. Local names for species of vulture include Gidhad, Gidh. Vultures have various behavioral patterns regarding reproduction like they probably can decide whether to produce male or female. In case of scarcity of food or harsh environmental conditions female of Lesser Black-backed Gull produces more females than males because females are more adaptable for survival in scarcity in them (Trivers and Willard, 1973; Oddie *et al.,* 1998; Wiebe and Bortolotti, 1992).

New World vultures have a good sense of smell, but Old World vultures find carcasses exclusively by sight. A particular characteristic of many vultures is a bald head, devoid of feathers. Old age vultures are classified under Kingdom –Animalia, Phylum – Chordata, Class-Aves, Order- Accipitriformes, Family- Accipitridae, and Subfamily - Aegypiinae (Linnaeus and Linnaeus 1735). Species of vultures are listed in Table 2.2. The vultures in family *Acipitridae* were most abundant prey birds till 1985, but according to IUCN Red List Category (2009), they are now endangered. There are several possible explanations for the high mortality and reduced breeding success of the Indian vultures including food shortage, persecution, contaminants like diclofenac, and infectious diseases (Oaks *et al.,* 2004; Cardoso, 2005). Captive breeding programmes of BNHS, Mumbai are running in India with the hope that the birds will be introduced to the wild after ensuring a diclofenac-free environment for them.

Table 2.1 Ecological and economical contributions of avian functional groups

Functional group	Ecological process	Ecosystem service and economical bene?ts	Negative consequences of loss of functional group
Frugivores	Seed dispersal	Removal of seeds from parent tree; escape from seed predators; improved germination; increased economical yield; increased gene ?ow; recolonization and restoration of disturbed ecosystems	Disruption of dispersal mutualisms; reduced seed removal; clumping of seeds under parent tree; increased seed predation; reduced recruitment; reduced gene ?ow and germination; reduction or extinction of dependent species
Nectari-vores	Pollination	Outbreeding of dependent and or economically important species	Pollinator limitation; inbreeding and evolutionary consequences; extinction
Scavengers	Consumption of carrion	Removal of carcasses; leading other scavengers to carcasses; nutrient recycling; sanitation	Slower decomposition; increases in carcasses; increases in undesirable species; disease outbreaks; changes in cultural practices
Insecti-vores	Predation on invertebrates	Control of insect populations; reduced plant damage; alternative to pesticides	Loss of natural pest control; pest outbreaks; crop losses; trophic cascades
Piscivores	Predation on ?shes and invertebrates and production of guano	Controlling unwanted species; nutrient deposition around rookeries; soil formation in polar environments; indicators of ?sh stocks; environmental monitors	Loss of guano and associated nutrients; impoverishment of associated communities; loss of socioeconomic resources and environmental monitors; trophic cascades
Raptors	Predation on vertebrates	Regulation of rodent populations; secondary dispersal	Rodent pest outbreaks; trophic cascades; indirect effects
All species	Miscellaneous	Environmental monitoring; indirect effects; bird watching tourism; reduction of agricultural residue; cultural and economic uses	Losses of socioeconomic resources and environmental monitors; unpredictable consequences

In 2006, India, Pakistan, and Nepal banned the manufacture of veterinary formulations of the nonsteroidal anti-inflammatory drug (NSAID) diclofenac. This action was taken to halt the unprecedented decline of three Gyps vulture species that were being poisoned by diclofenac residues commonly present in carcasses of domestic livestock upon which they scavenged. To assess the effect of this ban and evaluate residue prevelances of other NSAIDs by liquid chromatography-mass spectrometry method to detect diclofenac and eight more NSAIDs and apply this to 1488 liver samples from carcasses of livestock taken across seven Indian states. Diclofenac was present in 11.1% of samples taken between April and December 2006, and meloxicam (4%), ibuprofen (0.6%), and ketoprofen (0.5%) were also detected. Although meloxicam is safe for a range of avian scavengers, including Gyps vultures, data regarding the safety of other NSAIDs is currently limited. If wild Gyps on the Indian subcontinent are to survive, diclofenac bans must be completely effective, and NSAIDs that replace it within the veterinary drug market must be of low toxicity toward Gyps and other scavenging birds (Taggart *et al.,* 2009).

In Southern Africa, the species of greatest conservation concern is the Cape Griffon Vulture (*Gyps coprotheres*), as only 2900 breeding pairs remain in the wild. Study was taken to test if this species is toxicologically sensitive to diclofenac. In a single dose-toxicity study, two adult Cape Griffon Vultures with severe injuries, that were considered to have a very poor prognostic outcome, were dosed intravenously with diclofenac at 0.8 mg/kg. The changes in the clinical pathology were compared to the normal reference range established for 24 healthy Cape Griffon Vultures. Both birds died within 48 h of dosing. The clinical signs, clinical pathology, gross pathology and histopathological finding were typical for diclofenac toxicity. It would appear that the sensitivity of the Cape Griffon is

similar to that of their Asian counterparts and the African White-backed Vulture (*Gyps africanus*). Diclofenac is almost certainly toxic to all Gyps vulture species and strong efforts must be taken to ensure that veterinary diclofenac products are not licensed or introduced to the African continent (Naidoo *et al.,* 2009a).

Safety testing was undertaken using captive non-releasable Cape griffon vultures (*Gyps coprotheres*) and wild-caught African white-backed vultures (*G. africanus*), both previously identified as susceptible to diclofenac and suitable surrogates. Ketoprofen doses ranged from 0.5 to 5 mg/kg vulture body weight, based upon recommended veterinary guidelines and maximum levels of exposure for wild vultures (estimated as 1.54 mg/kg).Doses were administered by oral gavage or through feeding tissues from cattle dosed with ketoprofen at 6 mg/kg cattle Chapter VIII bodyweight, before slaughter.Mortalities occurred at dose levels of 1.5 and 5mg/kg vulture body weight (within the range recommended for clinical treatment) with the same clinical signs as observed for diclofenac. Surveys of livestock carcasses in India indicate that toxic levels of residual ketoprofen are already present in vulture food supplies.

Consequently, we strongly recommend that ketoprofen is not used for veterinary treatment of livestock in Asia and in other regions of the world where vultures access livestock carcasses. The only alternative to diclofenac that should be promoted as safe for vultures is the NSAID meloxicam (Naidoo *et al.*, 2009b).

The population collapse of resident Gyps vulture species in South Asia, caused by the use of a veterinary drug diclofenac, has highlighted an urgent need to monitor numbers of other vulture species in the region. Population trends of Himalayan Griffon Gyps himalayensis in the mountainous region of Upper Mustang,

Nepal, which is an important breeding area for the species was assessed. Vultures were surveyed in 2002, 2004 and 2005 by recording the number of birds sighted along 188 km of transects, and observing numbers of birds at breeding colonies. The number of birds recorded per day and per kilometre of transect declined by 67% and 70% respectively over the period of study. The number of active nests has been declined by 84% from 2002 to 2005. The veterinary drug diclofenac was available in pharmacies in the Mustang region. Young Himalayan Griffons, which migrate to the lowland areas of Nepal and, in increasing numbers to India, are highly likely to be subject to diclofenac poisoning. If this rate of population decline occurs throughout the Himalayan region, the conservation status of the species will need to be urgently reassessed (Acharya *et al.,* 2009).

2. SEX IDENTIFICATION IN BIRDS

Knowing the sex of organism is basic need in captive breeding programme. Some birds are dimorphic; their sex can be determined by visual examination of bird, like feather colors, gonadal examination. But in case of monomorphic birds there lack a distinct visual traits to identify them as the male or female, same problem arises with dimorphic birds in nestling stage, or after breeding season the gonad of birds regress making sex identification process difficult (Prus and Schmutz, 1987).

Various techniques employed for sex determination (Hu *et al.*, 2005) of monomorphic birds are as follows:

2.1. SURGICAL SEX IDENTIFICATION IN BIRDS

The techniques of surgical intervention for identification of sex mainly consist of laparotomy and laparoscopy, which aims to

observe the gonads directly (Risser, 1971). The male has a pair of testes whereas female with single ovary usually. In laparotomy, large opening sufficient enough to allow insertion of metal probe is made, which displace intestinal tract and allow examination of gonads(s). Based of location of gonads insertion is made, it's on left side of body in female because of left position of ovary in the body. The laparoscopy is more feasible technique than laparotomy as involves use of fibre optic cable inserted to allow gonadal inspection with requirement of less abdominal opening (Richner, 1989). But these methods disturb animals with possible future reproductive decreased performance so there has been a need for simple technique in which animal's avoidable pain and side effects can be avoided (Griffiths, 2000). Also examination of gonads in birds is difficult owing to gonadal regression in non-breeding season and small bird size in nestlings as in case of in case of Ostrich (S*truthio camelus*)(Masoud, 2009).

2.2. KARYOTYPIC ANALYSIS AND FLOW CYTOMETRY

In most of bird cases, the female is herterogametic whereas male is homogametic containing ZW and ZZ chromosomes as sex specific chromosome pair (Boer, 1975; Solari, 1994). Sex chromosome evolves from pair of autosomes (Ohno, 1967; Fridolfsson *et al.,* 1998). In case of W chromosome, most of genes are lost in evolution process so it's a shortened form of chromosome known as microchromosome. The Z chromosome is like chromosome 4 and 5 bigger in size called as macrochromosome (Tegelstrom and Ryttman, 1981).

Bird sexing by cytological approaches using karyotyping consists of isolation of feather base pulp cells, growing them in cell culture and stopping mitotic growth at metaphase stage of cell cycle when chromosomes are viable and visible (Delhanty, 1989). The spreading apart of chromosomes is done for observation of chromosome pattern to

know the presence of female specific W chromosome (Hatzofe and Getreide, 1990).

Flow cytomery is one of the arroach for sex determination in birds (Nakamura *et al.*, 1990). DNA content in the homogametic male of Egyptian Vulture (*Neophron percnopterus*) has been compared to that of the heterogametic female. A karyotypic analysis also was performed in order to confirm the Flow cytometry results. Sex identification by both FCM and cytogenetic analyses was concordant in all cases. The average DNA content in males has been was estimated to be 5.6% higher than in females (De *et al.,* 1994).

2.3 DNA BASED METHODS

In birds, the DNA can be isolated from erythrocytes as they are nucleated or base of feather sample (Chang *et al.*, 2002) that contain DNA sufficient for molecular work. Samples need to be stored in Queen lysis buffer to have a stable DNA. The DNA in ethanol is stable for prolonged period of time for years at -20°C to -80°C. The FTA databasing paper can also be used; the FTA paper card is impregnated with the mixture containing chemicals to allow having a stable DNA for prolonged time period (Smith and Burgoyne, 2004). In DNA hybridization, the sex specific sequence in genome of organism is detected in genomic DNA by complementary probe to that sequence and in PCR based method, the sex specific DNA is located by primers and then amplified.

2.3.1 SEX IDENTIFICATION BY DNA HYBRIDIZATION

W chromosome contains repetitive sequence with in microsatellite 150 bp cluster length with repeat unit upto 13bp. In case of minisatellite, 20kb cluster length with repeat unit upto 25bp is present (Brown, 2002). Sex techniques based on hybridization

consist of identification of large repetitive sequences, which are to be marked as W-linked markers. In this DNA is digested with restriction enzyme e.g. (*Hae* III, *Hinf* I, *Pst* I), electrophoresed in agarose gel, transferred to nylon membrane in Southern blotting, labeled with labelling probe (e.g. ^{32}P or biotin) and detected with autoradiogram. This pattern is compared with known female specific pattern to know sex of bird (Jeffreys *et al.,* 1985).

2.3.2 PCR Based methods:

2.3.2.1 RAPD and AFLP and sex identification

In case of Random Amplification of Polymorphic DNA (RAPD), the randomly chosen primer of approximately 10bp is used. The low annealing temperature (35-40°C) in reaction process reduces specificity with genomic DNA to allow amplification of wide range of genomic fragments (Williams *et al.,* 1990; Tingey and Del, 1993). RAPD does not require prior knowledge of the genome. It is suitable for use in birds whose genome has not been well studied (Hadrys, 1992). RAPD and AFLP may be also used to design the primers that are subsequently used in a standard PCR (sequence characterized amplified region, SCAR) (Dubiec and Neubauer, 2006). Random amplified polymorphic DNA (RAPD) marker has been used to sex individuals within the species *S. cheela hoya* and *Accipiter trivigatus formosae* (Hsu *et al.*, 2009).

Amplification fragment length polymorphism (AFLP) combines PCR with restriction digestion of DNA with 4 and 6 cutter restriction enzymes. Here positive control is kept to see if fragment in female means positive and their absence in male as negative. This PCR reaction is usually applied to Ostrich in which the sex chromosomes are undifferentiated making CHD based sex identification impossible (Griffiths and Orr, 1999). AFLP is more repeatable than RAPD and

allows producing greater number of bands per assay and thus provides more chance of detection of sex specific fragments. But this technique being costlier is rarely used for sex identification.

2.3.2.2 Stringent PCR amplification for sex identification

Low stringency PCR to screen randomly selected primers for their ability to amplify sex specific loci has been used to determine sex of some birds. In this approach, 10-mer primer or mixture of 20-30 mer primers of arbitrary sequence were employed. This allowed reproducible amplification of a range of DNA fragments from the genome wherever opposed priming sites occur within 2-3 kb. Southern blot of genomic DNA was done after digesting DNA sample with restriction enzyme *Pvu I* or *Mbo I.* DNA from jackdaws (*Corvus monedula*) was digested with *Pvu II*, great tit (*Parus major*) digested with *Pvu I*, whereas of zebra finches (*Taenopygia guttata*) digested with *Mbo I*. The digestion products were probed with the female-specific PCR products produced for each species. The banding pattern showed hybridization consistent with a W chromosomal location of the target sequence (Griffiths and Tiwari, 1993).

In case of Gallinaceous birds, there is tandem repeat of DNA on most of portion of W chromosome. In Turkey, 0.4-kb *PstI1* element is repeated about 10,000 times in the female diploid genome but is undetectable as such a unit in males. Use of multiplex polymerase chain reaction has been done to identify sex of turkey based on *Pst*1 repeat. First pair was designed to amplify a region of the *Pst*1 repetitive element, resulting in the production of a 177-bp fragment in females. The other pair was designed to amplify a region of the adenosine triphosphate (ATP) synthase gene, present in both males and females. The simultaneous use of all four primers in the same reaction resulted in the co-amplification of a 177-bp and a 250-bp fragment in

females and a 250-bp fragment in males. This technique was used to verify the sex of 45 adults of known sex and to identify the sex of 74 embryos from day 5 to hatch. This procedure is rapid and permits the sexing of many embryos in a short time and therefore can facilitate studies on avian sex determination (D'Costa and Petitte, 1998).

2.3.2.3 Conserved genes sex specific pattern as an approach to sex identification

The conserved genes are those genes that are present almost in all species of vertebrates and non-vertebrates. These genes show some differences in male and female of same species regarding some Single Nucleotide Polymorphism (SNP). The resulting restriction cut site at particular location in the particular sex of species leads to Restriction Fragment Length Polymorphism (RFLP), finding particular mismatch primer in male and female to make difference between them clear by techniques like Amplification Refractory Mutation System (ARMS) (Reddy *et al.*,2007). CHD gene is the most exploited conserved gene used for sex identification.

2.3.2.3.1 Chromodomain-helicase-DNA-binding protein 1

The CHD family of proteins is characterized by the presence of chromo (chromatin organization modifier) domains and SNF2-related helicase/ATPase domains. CHD genes alter gene expression possibly by modification of chromatin structure thus altering access of the transcriptional apparatus to its chromosomal DNA template. The Chromo domain appears as an N-terminal three-stranded anti-parallel γ-sheet which folds against an N-terminal α-helix. A conserved series of hydrophobic residues, which wind across the face of the γ-sheet, is referred to as *a* sash. Interactions with partner proteins are thought to be mediated by the residues within the hydrophobic sash (http://pawsonlab.mshri.on.ca/index.php).

Domain binding and function

The Chromatin Organization Modifier (Chromo) domain is defined as a 30–70 amino acid residue protein module found in a number of proteins involved in the assembly of protein complexes on chromatin. This domain was first described in Drosophila modifiers of variegation proteins that modify the structure of chromatin to the condensed morphology of heterochromatin, a cytologically visible region within which gene expression is repressed. Examples of chromo-domain-containing proteins include the HP1 the Polycomb (Pc) transcriptional repressors and the human retinoblastoma binding protein (RBP-1). Chromo domains function to promote binding to methylated lysines in the tail region of Histone H3. Chromo domains can function individually or in tandem such as CHD1 to recognize specific methylated Histone tails. Examples of Proteins:

CROMO domain protein	Binding partner
HP1	Histone H3 methylated lysine-9
Polycomb Proteins	Histone H3 methylated lysine-27
CHD1	Histone H3 methylated lysine-4

Thus, Chromo Helicase domain must be conserved as it is essential for survival of living beings (http://pawsonlab.mshri.on.ca/index.php).

2.3.2.3.1a Molecular evolution of CHD and sex identification

CHD gene encodes for an enzyme and is found on W chromosome (Griffiths and Tiwari, 1995) and Z chromosome (Griffiths and Korn, 1997) in birds. The presence of a W unique DNA sequence will identify a bird as female. It is found in almost all bird species except ratites as ratites have undifferentiated sex chromosome (Ellegren, 1996; Griffiths *et al.,* 1996). A W linked gene, named CHD1-W evolves far more slowly than nonfunctional DNA. In avian species,

the frequency of nonsynonymous substitutions (*K*a) for *CHD1W* was found to be higher (1.55 per 100 sites) than for *CHD1Z* (0.81), while the opposite was found for synonymous substitutions (*K*s, 13.5 *vs.* 22.7). A male-biased mutation rate is manifested by the faster rate of neutral evolution (synonymous substitutions) on the Z chromosome than on the female-specific W chromosome (Fridolfsson and Ellegren, 2000).

The first method of choice in molecular sexing of birds is CHD based sexing because it's easy, relatively cheaper and fast as sample processing including DNA extraction, PCR and resolution of PCR products on gel take less than 5 hrs.

2.3.2.3.1b Intronic length polymorphism and ARMS

CHD is functional DNA that contains at least two introns. Primer pair is designed to flank fragment of gene with intron. The intron regions of the CHD-W and CHD-Z genes vary between male (ZZ) and female (ZW) individuals (Kahn *et al.*, 1998).

In case of Magellanic penguin (*Spheniscus magellanicus*), less sexual dimorphism is seen, in adult males are larger than female but in case of chicks sex identification is difficult. CHD specific primers 2917F/3088R (Ellegren, 1996), P2/P8 (Griffiths, *et al.,*1998) and 2550F/2718R (Fridolfsson and Ellegren, 1999), designed to detect intronic size differences, were tested initially on four breeding pairs whose members showed large size differences, and whose sex could therefore be confidently determined in Magellanic Penguins (Bertellotti, 2002). In case of Cockatiel (*Nymphicus hollandicus*), sex is determined based on intronic length variation between chromo helicase DNA binding protein (CHD1) or ATP synthesis α-sub unit (ATP5A1W) gene which has similar homologues on both Z and W chromosome(Garcia M.J and Mindell, 2000; Harun and Kozet, 2007).

To identify sex in falconiformes, two alternative methods have been employed- Intronic length variation or the actual sequence differences between CHD-Z and CHD-W. From sequence analysis, a 3' terminal mismatch primer on point mutations (Newton, 1989) conserved among falconiformes was designed and identification of sex with the Amplification Refractory Mutation System (ARMS) was performed. This approach is also known by different names like Allele specific PCR (ASPCR), Mismatch Amplification Mutation Assay (MAMA) and PCR amplification of Specific alleles (PASA). As the 3'-mismatch primer was designed on the point mutations conserved in falconiformes, ARMS with these primers may identify sex in all the falconiformes.

In case of Accipitridae species, use of P2/P8 primer pair yields DNA fragments varying 2-8 bp in size between derived CHD-W and CHD-Z, against 20 bp in falconidae. The sequence information of the PCR products of CHD genes for tested Accipitridae species is presented as GenBank accession numbers of CHD-Z/CHD-W genes, followed by the length difference between P2/P8-amplified PCR products of these two genes: *A. gularis* (3 bp), *A.soloensis* (6 bp), *A. trivirgatus* (2 bp), *A. virgatus* (4 bp), *B. indicus* (2 bp), *P. ptilorhyncus* (19 bp), *S.c. hoya* (13 bp), and *C. gallicus* (9 bp) (Chou *et al.*, 2010).

ARMS with three primers (P2, NP and MP) provided reliable method for CHD based identification of sex as MP primer detects female sequence but not male due to 3' mismatch (Ito *et al.,* 2003). The use of CHD gene which is applicable for sex identification in most birds is not of use for ostrich owing to almost equal intrinsic size between CHD-Z and CHD-W genes.

2.3.2.3.1c PCR-RFLP

As there is very little base pair difference in the CHD-W and CHD-Z amplicons using P2 and P8 primer pair so they produce band

at same position in gel electrophoresis. Therefore, the PCR product from CHD gene is digested with restriction enzyme prior to running the gel. With *Hae* III enzyme specific for CHD- Z only and *Asp 700I specific* for CHD-W, PCR-RFLP has been used successfully for sex identification of Short-toed Eagle (*Circaetus gallicus*), an endangered Accipitridae species living mainly in southern Europe and Asia (Sacchi *et al.*, 2004).

After P2/P8 PCR amplification and 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, 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 CHDZ (e.g. *Hae III*) but not CHD-W before gel electrophoresis. 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 in *Gyps bengalensis* and *Gyps indicus* and analysed by electrophoresis in 12% polyacrylamide gels (Reddy *et al.*, 2007).

2.3.2.3.1d Sex specific PCR

Sex specific PCR relies on primer CHD-ZW-common binding both male and female region of CHD and CHD-W-specific binding female CHD region. Using the same reverse primer P2, the PCR length for primer sets of P2/CHD-ZW-common and P2/CHDW is 148-bp and 258-bp respectively for *S. cheela hoya*. The alignment of Z CHD and W CHD sequences of several other species of raptor birds showed corresponding binding sites for ZW Common and W specific portion of CHD and suggested the application of sex specific PCR for other birds (Chang *et al.*, 2008c).

In Zebra finches to amplify the female-specific CHD W, the combination of W1 and W2 primers were used giving 179 bp fragment. To amplify a fragment of the CHD gene from the Z chromosome of both male and female genomic DNA Z1 and Z2 primers were used giving 242 bp fragment. A 1.2% agarose gel was used (Soderstrom et al., 2007).

2.3.2.3.1e Real time PCR

High-throughput gender identification of Accipitridae eagles *Spilornis cheela hoya* and *Pycnonotus sinensis* by SYBR green (Chang *et al.,* 2008a) and TaqMan probes-based real-time PCR employing CHD-specific primers and probes (Chang *et al.,* 2008b) has been reported.

TaqMan probes-based real-time PCR exploit use of region of common probe binding e.g. probe (HEX) binding both Z CHD and W CHD region and probe (FAM) binding only W CHD region. So female with presence of both Z and W CHD gene region was expected to give fluorescence for both HEX and FAM probe whereas male with only Z CHD genes was expected for HEX probe fluorescence. In real-time PCR and owing to ease of handling many samples can be analyzed by real-time PCR. One of the benefit of this approach is there is no need to run gel of samples directly by auto-calling option of real-time we get all results in one place (Chang *et al.,* 2008b).

Based on hypothesis that proposed CHD-W-specific and CHD-ZW common TaqMan probes for *S. c. hoya* can be applied to determine the gender of Accipitridae species with the same or similar sequences to these probes. The Z CHD and W CHD region of *S. c. hoya* analyzed by BLAST (McGinnis and Madden, 2004) to know other bird's species with similar sequences to Z CHD and W CHD gene region respectively. The species such as *Accipiter virgatus (A. virgatus), Accipiter gularis (A.*

gularis;), Butastur indicus (B. indicus;), Accipiter soloensis(A. soloensis), Pernis ptilorhyncus (P. ptilorhyncus), Accipiter trivirgatus (A. trivirgatus), and *Spilornis cheela hoya (S. c. hoya)* were used for sex determination in them by TaqMan probe as validation of working of TaqMan probe in Accipitridae species. It has been found that the nucleotide mismatches to the regions for CHD-W-specific and CHD-ZW-common S. c. hoya probes are as follows: C. gallicus (0 vs. 0, respectively), *A. gularis, A. soloensis, A. trivirgatus, A. virgatus,* and *B. indicus* (1 vs. 0), and *P. ptilorhyncus* (2 vs. 1). It is possible that the corresponding nucleotides "T" and "G" of CHD-W-specific probe (complementary nucleotides "A" and "C" is base-wobbling to "G" and "T," respectively). As the Accipitridae is a diverse avian family, comprising up to 14 subfamilies, 65 genera, and 231 species, the suitability of *S. c. hoya* CHD W-specific and CHD-ZW-common TaqMan probes cannot be used for evaluation of other non-Accipitrinae subfamilies until their CHD genes have been sequenced and submitted to GenBank (Chou *et al.*, 2010).

2.3.2.4 Use of sex linked genes (DMRT 1, PKCI and HINT) for sex determination

Sex in birds is chromosomally based, as in mammals, but the sex chromosomes are different and to determine mechanism of avian sex determination many efforts has been done (Ellegren, 2000; Mizuno *et al.,* 2002; Smith *et al.,* 2007). Two hypotheses have been proposed for the mechanism of avian sex determination. The W (female) chromosome may carry a dominant-acting ovary determinant (Smith *et al.,* 2007; Arlt *et al.,* 2004). Alternatively, the dosage of a Z-linked gene may mediate sex determination, two doses being required for male development (ZZ) (Smith *et al.,* 2004). A strong candidate avian sex-determinant under the dosage hypothesis is the conserved Z-linked gene, DMRT1 (double-sex and mab-3 related transcription

factor1) (Naidoo *et al.,* 2000; Shan *et al.,* 2000; Smith *et al.,* 1999). Here RNA interference (RNAi) has been used to knock down DMRT1 in early chicken embryos. Reduction of DMRT1 protein expression *in ovo* leads to feminization of the embryonic gonads in genetically male (ZZ) embryos. Affected males showed partial sex reversal, characterized by feminization of the gonads. The feminized left gonad showed female-like histology, disorganized testis cords and a decline in the testicular marker, SOX9. The ovarian marker, aromatase, was ectopically activated. The feminized right gonad showed a more variable loss of DMRT1 and ectopic aromatase activation, suggesting differential sensitivity to DMRT1 between left and right gonads. Germ cells also showed a female pattern of distribution in the feminized male gonads. These results indicated that DMRT1 is required for testis determination in the chicken. This support the Z dosage hypothesis for avian sex determination (Smith *et al.,* 2009).

In Chicken (*Gallus gallus*) Z-linked DMRT 1 gene, is conserved across phyla as a gene involved in sex differentiation. DMRT 1 is involved in development of Wolffian duct, after onset of sexual differentiation, it becomes testis specific and in adult male exclusively expressed in testis. The presence of single copy in female of DMRT 1 in not sufficient to allow male phenotype to develop. Identification of W-linked PKCIW (protein kinase C inhibitor) gene is female sex specific. PKCI-W forms a heterodimer with PKCI-Z and inhibits the biological activities of PKCI-Z in vitro, supporting the predicted role of PKCI-W in sex determination in birds (Moriyama *et al.,* 2006). The PKCIW expression is seen mainly in urogenital tract, genital ridge, mesonephric duct development and ovary development. As DMRT 1 and PKCIW are genes involved in gonad development they can be used for sex determination in birds (Ellegren, 2001).

Histidine triad nucleotide binding protein (HINT) of female can be used as marker for sex identification in females. In case of female, gene for sex identification is HINTW whereas in case of males it's HINTZ (Ceplitis and Ellegren 2004).

In addition, EE0.6 W-located conserved sequence on ratitae and carinatae is used to identify sex (Ogawa, 1997; Ogawa, 1998; Itoh *et al.*, 2001).

Sex typing in owls is used for extension of DNA fragment from W chromosome that for female birds is specific. The use of SS primer that is specific primer for W chromosome and for female sample produces 648 bp fragments. OSFES primer produced fragment with 432 bp length. This fragment is present only in female ostrich (Masoud, 2009).

2.3.3 Oligonucleotide Microarrays

In owls (Family *Strigidae*), sex identification by use of oligonucleotide microarray has been done in which several sex specific probes operated complementary or in concert (Wang *et al.,* 2008).

2.4 UV- Resonance and Raman Spectroscopy

This is minimum invasive technique for sex identification in birds like chicken. This method uses deep UV excitation wavelength to yields selective enhancement of macromolecules such as aromatic amino acids in proteins as well as DNA bases. The scattering intensity increases up to 10^6 by the electronic resonance enhancement, thus improving the signal-to-noise ratio significantly. Raman difference spectrum by subtracting the female from the male UVRR spectrum was calculated identifying the most pronounced differences between

female and male feather pulp UVRR spectra in the wave number region between 600 and 1800 cm-1. The sample preparation is simple, and the sample extracts can directly be analyzed to achieve a Raman fingerprint spectrum within 1 min. Raman spectra of the extract material were compared to those of pure DNA and proteins (Harz *et al.,* 2008).

3. *Materials & Methods*

The research work in this thesis encompasses the characterization of CHD gene of Z and W chromosome to aid in sex determination in *Gyps indicus* and *Gyps bengalensis*. The strategy and the methods employed are detailed below:

3.1 MATERIALS

3.1.1 Tissue sample/blood sample

The post-mortem tissue samples (36) were collected from BNHS Vulture Conservation & Breeding Centre, Pinjore, Haryana. The blood samples collected in 2006 from 10 live birds during our previous project on "Meloxicam safety testing in Gyps vultures" were used for genomic DNA isolation. New BP blade and separate pair of gloves was used to process every post-mortem sample to avoid the cross-contamination.

3.1.2 Culture Medium

For bacterial culture, Luria Bertani Agar and LB Broth (Hi Media, India) were used.

3.1.3 Enzymes

Taq polymerase were from Genei, (Bangalore), Restriction Enzymes *Bam* HI, *Rsa* I, and *Dra* I from MBI Fermentas (Lithuania) were used along with their respective buffers.

3.1.4 Bacterial Strains/ Bacterial DNA

The competent *Escherichia coli* strain, DH5α available in Vector Development Laboratory (Animal Biotechnology, Division) was used for cloning.

3.1.5 Oligonucleotide Primers

Following sets of oligonucleotide primers were synthesized from Genscript USA.

3.1.6 Commercial kits

Genomic DNA purification Kit (Qiagen), Gel extraction Kit (Qiagen), plasmid isolation Kit (Promega, USA), PCR product cloning kit including pJET1.2 as a blunt end cloning vector (MBI, Fermentas) were employed during this work.

3.1.7 Chemicals / Reagents

All the chemicals/reagents used for the preparation of buffers/ media were of either of AR/GR grade or molecular biology grade. 100 bp plus DNA ladder, loading dye were from MBI, Fermentas. The chemicals like phenol, tris, EDTA, potassium acetate, sodium acetate, and sodium chloride, ampicillin, chloroform, isopropanol, ethanol, agarose, ethidium bromide, bromophenol blue were obtained from Qiagen (GmbH, Germany), Sigma (USA), SISCO Research Laboratory (India), and BDH Merck (India).

3.1.8 Glassware and Plastic wares

The glassware was from Scott Duran, and Borosil and plasticware was from Labware (USA), Nunc (Denmark), Thermo Fisher Scientific (Denmark) and Axygen Scientific Inc (USA) were employed during the research work. For DNA work, DNase, RNase-free plasticwares and glasswares were used.

3.1.9 Equipments

Laminar flow (Klenzaids, Russia), swinging bucket rotor centrifuge (Remi; India), micro pipettes (Axygen, USA and Thermo labsystems, Denmark , Nichiryo Japan), thermo cycler (Techne, U.K. Model 312), Analytical balance (Model GR202, A&D Co. Ltd, Japan), agarose gel electrophorectic apparatus (Genei, Bangalore), vortex machine (Tarsons) and microcentrifuge (Sigma, USA), water bath (Julabo; Germany), refrigerated microfuge (Hetich; Germany, -80°C ultra low freezer (Nuaire), -20°C deep freezer (Blue Star, Denmark), refrigerator (LG, India), Hot air oven (Yorco instruments, Bombay), ice flaking machine (Model-IM-70, Harrison Scientific Instruments Co Delhi), microwave oven (LG-electronics India), alphaimager gel documentation system (Alpha InnoTech Corporation (USA), stratagene Mx3000P Realtime PCR machine (with MxPro™ QPCR software) were used during research.

3.1.10 Computer Aided Molecular Biology Software

The alignment of vulture sequences and prediction of restriction sites was done using Laser gene (DNAstar Inc., USA). Phylogenetic tree based on evolutionary distances was constructed from nucleotide sequences using Mega 4.1 software.

3.2 METHODS

3.2.1 Genomic DNA isolation

Genomic DNA from the 36 tissues was isolated using DNeasy tissue kit and from 10 blood (erythrocyte) samples using QIAamp

DNA Blood Mini Kit as per manufacturer's (Qiagen, Valencia, CA, USA) instructions.

3.2.2 PCR amplification of partial CHD gene using P2/P8 primer pair

Based on the reported sequence and P2, P8 primers (Griffiths *et al.*, 1998), partial CHD Z and CHD W gene portion as a conserved gene fragment important in sex identification in birds, was amplified. The PCR reaction mixture of final volume 25 µl was made which consisted of P2 (forward primer) and P8 (reverse primers) (50pmol) 1.0 µl each; Template genomic DNA 1.0 µl; dNTP mix (10 mM) 0.5 µl; 10 X Taq Buffer (without $MgCl_2$) 2.5 µl, 25 mM $MgCl_2$ 1.5 µl and Taq DNA Polymerase (1U/µl) 1.0 µl. The final volume of 25 µl made with nuclease free PCR grade water.

The PCR conditions employed initial denaturation at 94°C for 4 min, followed by 5 repeated cycles of 94°C for 30 sec, 49°C for 30 sec, 72°C for 30 sec. Then 49 repeated cycles of 94°C for 30 sec, 48°C for 20 sec, 72°C for 20 sec. Final extention at 72°C for 5 min was followed by final hold at 10°C. The amplicon (approximately 400 bp) along with the 100 bp DNA ladder were resolved by 3% agarose gel electrophoresis and the ethidium bromide stained gel was visualized in Gel documentation system.

3.3 CLONING AND CHARACTERIZATION OF CHROMO-HELICASE-DNA BINDING PROTEIN (CHD) GENE (PARTIAL)

3.3.1. CHD amplicons of different vulture species

Genomic DNA of *Gyps indicus* (P10), *Gyps bengalensis* (P17), *Gyps hymaleyensis* (P30) and *Aegypius monachus* (P49) was used for amplification of P2-P8 PCR products of the respective species as described above. PCR products were purified by Gel extraction kit ((Qiagen, USA). The concentration and purity of the eluted PCR amplicon was checked by running in 1% agarose gel.

3.3.2 Cloning of amplicon in pJET1.2/blunt cloning Vector

PCR product cloning kit (MBI, Fermentas) was used as per manufacturer's instructions. The ligation reaction mixture of total volume 20 µl was made in an ice-cold 0.5 ml microfuge tube with the following components: 2X Ligation buffer (10 µl), Insert (Purified PCR product, 1-2 µl depending upon the concentration; (insert: vector molar ratio 3:1 was found to give optimum ligation efficiency), pJET1.2/blunt cloning Vector (1.0 µl), T4 DNA Ligase [3U/µl] (1.0 µl). The volume was adjusted to 20 µl with Nuclease-free water. The ligation reaction was carried out at 22°C for 30 min.

3.3.3 Preparation of Competent cells

The competent cells were prepared by the method of Chung *et al.* (1989). A single colony of *E. coli* DH5α was picked from a LB agar plate and grown for overnight in 5 ml of SOB without any antibiotic at 37°C with vigorous shaking at 180-200 rpm in an orbital shaker. This overnight grown culture was diluted 1:100 in freshly prepared LB broth without antibiotics and incubated at 37°C with vigorous shaking at 180-200 rpm till the OD_{600} of the culture reached 0.25-0.35. The bacteria were pelleted by centrifugation at 2,500 rpm for 10 min. The supernatant was discarded by complete decantation so that pellet should not have trace amount of the media and the pellet was kept on ice. Chilled TSS was added to the pellet approximately 1/10th volume of the original culture. The bacterial pellet was carefully resuspended by slow pipetting 3 to 4 times. The tubes were incubated in ice for 1 h, at the end of incubation the cells were gently mixed and 200 µl of the competent cell suspension was dispensed using a cut tip in pre-chilled, sterile labeled 1.5 ml microfuge tubes.

Amplification strategy for cloning insert in vector

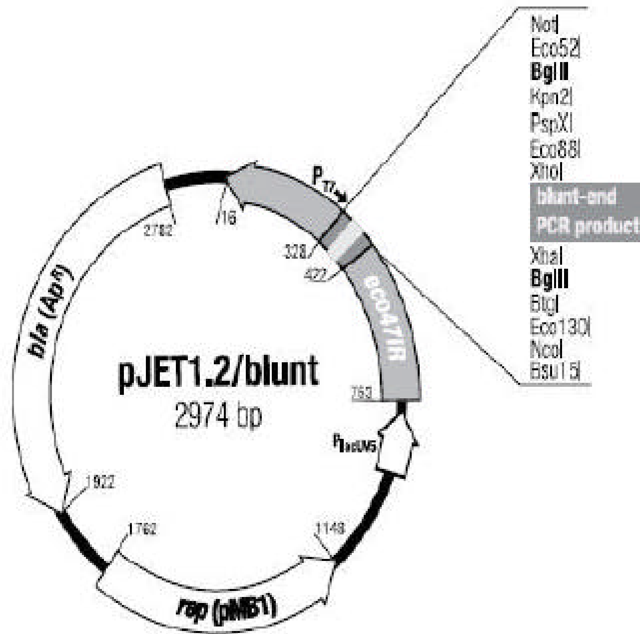


Fig. 1. pJET1.2/blunt Vector Map.

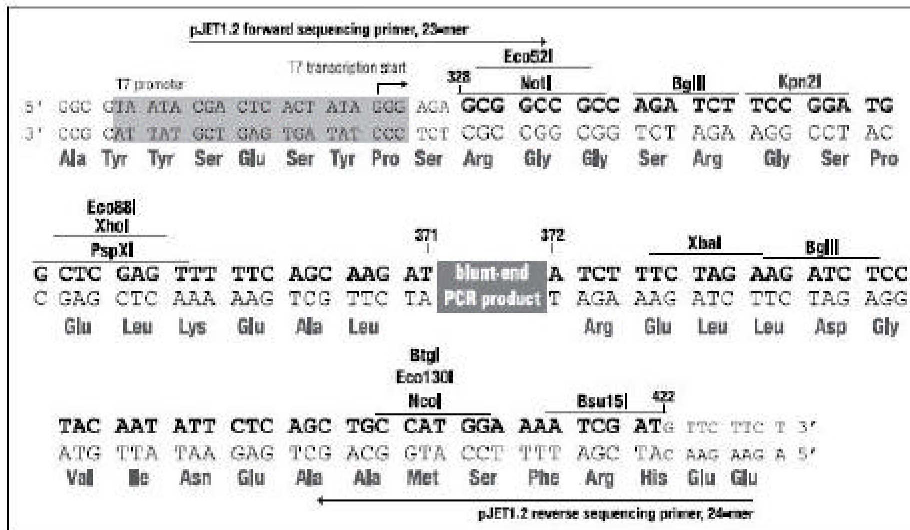


Fig. 2. DNA Sequence of MCS region.

3.3.4 Transformation

The transformation was carried out in DH5α competent cells prepared as above. Ligation product was directly added to 200 µl competent cells and mixed without touching the pipette at the bottom and was kept on ice for 45 min. Heat shock was given at 42°C for 45 sec in a water bath and the cells were immediately transferred on chilled ice for 5 min. 800 µl of SOC (ice cold) was added to the tubes. The cells were then incubated at 37°C with slow shaking for 1 h to allow the expression of ampicillin resistance genes and recover from stress caused by the heat shock. The bacterial culture was pelleted by centrifugation at 4,000 rpm for 5 min at room temperature. Approximately 800 µl of supernatant was discarded and the pellet was resuspended in rest of the supernatant by slow pipetting and plated on LB agar plate containing ampicillin (100 mg/ml) added to agar plate. The plates were incubated at 37°C in an inverted position for 12 -18 h until the colonies of the transformed bacteria appears on the surface of the LB agar plate.

The pJET1.2/blunt end cloning vector is designed such that after overnight incubation of plate, only the recombinant clones grow thus omitting the need for blue white screening. Colonies were picked up in LB medium and grown overnight in shaking water bath at 37°C.

3.3.5 Plasmid isolation

For initial screening of recombinants, plasmid isolation from overnight grown culture was done by alkaline lysis method (Birnboim and Dolly, 1979) with little modification as described by Sambrook and Russell (2001). The overnight grown culture (1.5 ml) was transferred to microfuge tube and centrifuged at 13,000 rpm for 1min. The supernatant broth was discarded meticulously by leaving the bacterial pellet as dry as possible. Then 300 µl of ice-cold P1 solution

was added and bacterial pellet was resuspended by vigorous vortexing assuring all the bacterial pellets get suspended. To this, freshly prepared 300 µl of P2 solution (room temperature) was added and mixed by inverting only. Finally, 300 µl of ice-cold P3 solution was added, mixed by inverting and incubated in ice for 10 min. Centrifugation was carried out at 13,000 rpm for 15 min at 4°C to pellet the precipitated chromosomal DNA, protein and RNA. The supernatant containing plasmid DNA, without any traces of white precipitate was transferred to an autoclaved fresh microfuge tube. Equal volume of phenol:chloroform (1:1 v/v) was added to the contents and briefly vortexed. The tube was centrifuged at 13,000 rpm for 5 min at 4°C resulting in the separation of yellowish organic phage in the bottom and clear upper aqueous phase. The aqueous phase was transferred to a fresh tube. Nearly 0.7 volumes of ice cold isopropanol was added and kept in ice for half an hour. Then plasmid DNA was precipitated by centrifuging the tube at 13,000 rpm for 15 min at 4°C. The supernatant was decanted and the pellet was washed twice with 70% ethanol and the air-dried plasmid DNA was resuspended in nuclease-free water or TE buffer. The recombinant plasmids were analyzed for the presence of CHDZ and CHD W gene insert respectively.

3.3.6 Characterization of recombinant plasmids

The recombinant plasmids were characterized by ARMS PCR (Section 3.2.3) and CHD-W PCR (Section 3.3.4) for the presence of CHD-Z or CHD-W insert using plasmid as template.

Amplification with T7 promoter based primer pair was performed to confirm the presence of insert as well as its orientation in the recombinant vector. The T7 primer matching vector sequence with either of primer P2 or P8 assisted to detect insert orientation.

Two separate reaction of T7 promoter primer with either P2 or P8 primer determined to know sequence orientation of insert in vector. The PCR reaction mixture of final volume 25 µl was made which consisted of P2 and T7 promoter primer (50pmol) 1.0 µl each; Template genomic DNA 1.0 µl; dNTP mix (10 mM) 0.5 µl; 10 X Taq Buffer (without $MgCl_2$) 2.5 µl, 25 mM $MgCl_2$ 1.5 µl and Taq DNA Polymerase (1U/µl) 1.0 µl. The final volume of 25 µl made with nuclease free PCR grade water.

The PCR reaction mixture of final volume 25 µl was made which consisted of P8 and T7 promoter primer (50pmol) 1.0 µl each; Template genomic DNA 1.0 µl; dNTP mix (10 mM) 0.5 µl; 10 X Taq Buffer (without $MgCl_2$) 2.5 µl, 25 mM $MgCl_2$ 1.5 µl and Taq DNA Polymerase (1U/µl) 1.0 µl. The final volume of 25 µl made with nuclease free PCR grade water.

The PCR conditions employed: initial denaturation at 95°C for 2 min, followed by 5 repeated cycles of 95°C for 30 sec, 49°C for 30sec, 72°C for 30 sec. Then 49 repeated cycles of 95°C for 30 sec, 48°C for 20 sec, 72°C for 20 sec. Final extention at 72°C for 3 min was followed by final hold at 10°C. The amplicon along with the 100 bp DNA ladder were resolved by 3% agarose gel electrophoresis and the ethidium bromide stained gel was visualized in Gel documentation system.

3.3.7 Sequencing of CHD gene (Partial)

After confirmation of the inserts, the recombinant plasmids encoding the Z CHD gene and W CHD gene fragments of all the species were sequenced using T7 promoter primer in Automatic Sequencer by Chromous Biotech Pvt. Ltd, Bangalore.

3.3.8 Sequence Analysis

After obtaining the sequence of CHD Z and CHD W of *Gyps bengalensis, Gyps indicus*, *Gyps himalayensis* and *Aegypius monachus*,

BLAST with each sequence was done to get related sequences. The sequences of other raptor birds that showed the sequence homology more than 92% were aligned by the Lasergene software (DNAstar Inc, USA) with respect to CHD Z and CHD W sequence obtained in this study. Phylogenetic tree based on evolutionary distances, nucleotide and identity with respect to species showing greater than 92% sequence homology was constructed from nucleotide sequences of these species using Mega 4.1 software.

3.4 STANDARDIZATION OF MOLECULAR METHODS FOR SEX IDENTIFICATION IN VULTURES

The CHD Z and CHD W sequences obtained above from different vulture species were aligned independently. The primers and probe binding sites from the previous studies (Ito *et al.* 2003 , Chang *et al.,* 2008a and Chang *et al.,* 2008b) were found conserved on these sequences. Additionally, to use PCR-RFLP for sex discrimination, restriction enzymes sites were predicted for CHD Z and CHD W specific sequences. The results of these tests expected as bands of different size to be visualized in agarose gel were predicted. Genomic DNA isolated from known male and female birds of *Gyps indicus* (P 33, P10) and *Gyps bengalensis* (P35, P17) and only available sample of *Gyps himaleyensis* (P30) and *Aegypius monachus* (P49) were employed for standardization of the test.

3.4.1 P2/P8 amplicon-based tests for sex identification

P2/P8 based PCR (Griffiths *et al.*, 1998) was applied and the products were run in 3% agarose gel elctrophoresis as described earlier (Section 3.2.2).

3.4.1.1 Restriction Analysis of the PCR Product

PCR RFLP was performed for sex specific differentiation of the P2 P8 PCR products. The sequences of *G. indicus* and *G. bengalensis* available in Genbank were initially analyzed for CHD Z and CHD W

specific restriction patterns in PCR RFLP. Restriction enzymes *Dra I*, *Rsa I* and *Bam HI* were picked up for the purpose.

The restriction digestion was performed at 37°C in a reaction mix of 20µl which contained 10 X Restriction enzyme specific buffer 2 µl, Restriction enzyme 1 µl, PCR product 5 µl. The final volume of 20 µl made with nuclease free PCR grade water.

3.4.2 Tests based on Allelic discrimination

3.4.2.1 Amplification Refractory Mutation System (ARMS)

Amplification Refractory Mutation System (ARMS) based on 3'-terminal mismatch primer (MP primer) on point mutation conserved among Falconiformes due to differences between CHD W and CHD Z sequences (Ito *et al.* 2003) was used to identify sex in vultures with some modifications. In this PCR, NP was used as reverse primer, P2 and MP as forward primers. MP, a mismatch primer for CHD Z, amplified only female specific fragment derived from W chromosome.

The PCR reaction mixture of final volume 25 µl was made which consisted of P2 (forward primer), MP (forward primer) and NP (reverse primers) (50pmol) 1.0 µl each; Template genomic DNA 1.0 µl; dNTP mix (10 mM) 0.5 µl; 10 X Taq Buffer (without $MgCl_2$) 2.5 µl, 25 mM $MgCl_2$ 1.5 µl and Taq DNA Polymerase (1U/µl) 1.0 µl. The final volume of 25 µl made with nuclease free PCR grade water.

The PCR conditions employed initial denaturation at 94°C for 1 min 30 sec, followed by 35 repeated cycles of 94°C for 30 sec, 50°C for 45 sec, 72°C for 30 sec. Final extention at 72°C for 5 min was followed by final hold at 10°C. The PCR products along with the 100 bp DNA ladder were resolved by 3% agarose gel electrophoresis and the ethidium bromide stained gel was visualized in Gel documentation system.

3.4.2.2 W specific and ZW common PCR

Female specific identification method as described by *Chang et al.* (2008a) was used. In this method, W specific primer was used as reverse primer in combination with P2 forward primer to detect females and in separate reaction the ZW common primer used as reverse primer with P2 forward primer to detect both the sexes.

The ZW common.P2 primer PCR reaction mixture of final volume 25 µl was made which consisted of P2 (forward primer) and ZW common (reverse primers) (50pmol) 1.0 µl each; Template genomic DNA 1.0 µl; dNTP mix (10 mM) 0.5 µl; 10 X Taq Buffer (without $MgCl_2$) 2.5 µl, 25 mM $MgCl_2$ 1.5 µl, DMSO 1.5 µl and Taq DNA Polymerase (1U/µl) 1.0 µl. The final volume of 25 µl made with nuclease free PCR grade water.

The W specific.P2 primer PCR reaction mixture of final volume 25 µl was made which consisted of P2 (forward primer) and W specific (reverse primers) (50pmol) 1.0 µl each; Template genomic DNA 1.0 µl; dNTP mix (10 mM) 0.5 µl; 10 X Taq Buffer (without $MgCl_2$) 2.5 µl, 25 mM $MgCl_2$ 1.5 µl, DMSO 1.5 µl and Taq DNA Polymerase (1U/µl) 1.0 µl. The final volume of 25 µl made with nuclease free PCR grade water.

The PCR conditions employed initial denaturation at 94°C for 3 min, followed by 45 repeated cycles of 94°C for 30 sec, 56°C for 30 sec; 72°C for 20 sec. Final extention at 72°C for 5 min was followed by final hold at 10°C. The amplicon was resolved in 3% agarose gel electrophoresis along with the 100 bp DNA ladder and the ethidium bromide stained gel was visualized in Gel documentation system.

3.4.2.2 Realtime PCR with TaqMan Probes

The method of Chang *et al.* (2008b) using TaqMan chemistry based realtime PCR for sex identification in *S. cheela hoya* was employed. In case of vultures, the P2/P8 primer amplified product gives 389bp fragment corresponding to CHD W and 383bp to CHD Z

so this length difference of 6bp is not useful to identify sex in vultures. But considerable difference in sequence composition of CHD W and CHD Z was observed when aligned using Megalign (DNA Star). Based on this differences, a CHD W specific probe (5'- FAM-TGTGCCATGTGTGAAAACCACCCA-TAMRA) which binds only CHD W region at position 122-145bp whereas one ZW common probe (5'-HEX-CCCTTCACTTCCATTAAAGCTGATCTGG-TAMRA) which binds both Z and W CHD chromosome regions at 234-261bp in CHD W and 228-255 bp in CHD Z was found useful.

The PCR reaction mixture of final volume 20 µl was made which consisted of P2 (forward primer) and P8 (reverse primers) (50 pmol) 1.0 µl each; Template genomic DNA 1.0 µl; dNTP mix (10 mM) 0.4 µl; 10 X Taq Buffer (with 15mM $MgCl_2$) 2 µl, and Taq DNA Polymerase (1U/µl) 1.0 µl, W specific probe (200nM) 2 µl, ZW common probe (200nM) 2 µl. The final volume of 20 µl was made with nuclease free PCR grade water. The Allelle-specific PCR was performed using appropriate controls. DNA template was excluded from NTC (no template control) whereas probe was excluded from NPC (no probe control). In addition, known male and female DNA sample was used in every run as positive control for the test. Two step PCR conditions employed initial denaturation at 94°C for 4 min, followed by 50 repeated cycles of 92°C for 15 sec, 60°C for 1 min. The results were recorded as amplification plot, text report and gender discrimination.

3.5 APPLICATION OF THE TESTS ON POST-MORTEM AND LIVE BIRDS SAMPLES

All the standardized tests were applied for sex identification in 36 post-mortem and 10 live bird samples obtained from VCBC, Pinjore. The sex identified was reported to the centre and the results were validated for the known sex birds.

4. Results

This chapter has documented the outcome of present study, which covers the cloning and characterization of CHD Z and CHD W gene (partial) in Indian vulture species and development of molecular tests for sex identification in *Gyps bengalensis, Gyps indicus*, and *Gyps himalayensis* species to support ex-*situ* vulture conservation. The sequence difference in CHD Z and CHD W alleles has been exploited in developing ARMS Nested PCR, W-specific PCR or Real time for male and female bird identification.

4.1 GENOMIC DNA ISOLATION

Genomic DNA was isolated from various tissues like pectoral muscle, testes, ovary, crop, gizzard collected from post-mortem samples of 36 birds. The genomic DNA isolated from the erythrocytes of 10 vultures at time of meloxicam safety trial of IVRI at VCBC, Pinjore in 2006 was used as source of DNA sample for live bird sex identification. The purity of DNA and yield was obtained in range of 0.2-11.9 µg/ml for majority of samples. As evident from agarose gel

electrophoresis results provided in Fig. 1, 10 post-mortem samples either did not yield genomic DNA or the quality was very poor. These unsuitable samples included P6, P18, P19, P25, P26, P32, P42, P44, P46 and P47 and accordingly did not yield results in various test.

4.2 PCR AMPLIFICATION OF PARTIAL CHD GENE

P2/P8 primer pair amplified nearly 390 bp region in CHD gene from all the good quality post-mortem and live bird samples. As a result, in agarose gel electrophoresis, only one clear band of the corresponding amplicon was observed for all the samples, whether they belonged to male or female bird. The results are documented in the Fig 2. No amplification in 'No template control' ensures the absence of contamination in each run.

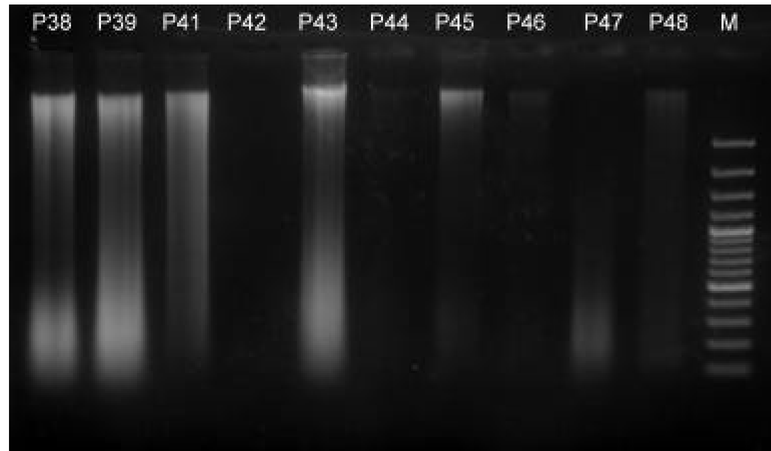
P2/P8 PCR was carried out on all the samples, out of these sample numbered P6, P18, P19, P25, P26, P32, P42, P44, P46 and P47 having poor genomic DNA samples did not yield a product and simply served as additional negative controls.

4.3 CLONING OF PARTIAL CHD GENE AND CHARACTERIZATION OF CHD Z AND CHD W SEQUENCES

Among the post-mortem samples, the sex of some of the birds was known from the tissues obtained i.e. male from testes and female from ovary. For cloning and characterization of CHD gene, genomic DNA obtained from female sample P10 of *Gyps bengalensis* and P17 of *Gyps indicus* was employed. In addition, DNA corresponding to P30 of *Gyps himaleyensis* and P49 of *Aegypius monachus* were also taken, although the sex of these two birds was unknown initially.

4.3.1 PCR amplification of CHD gene using P2/P8 primers and cloning

For obtaining PCR product to be used in cloning, proofreading DNA Polymerase was used for amplifying CHD gene from genomic



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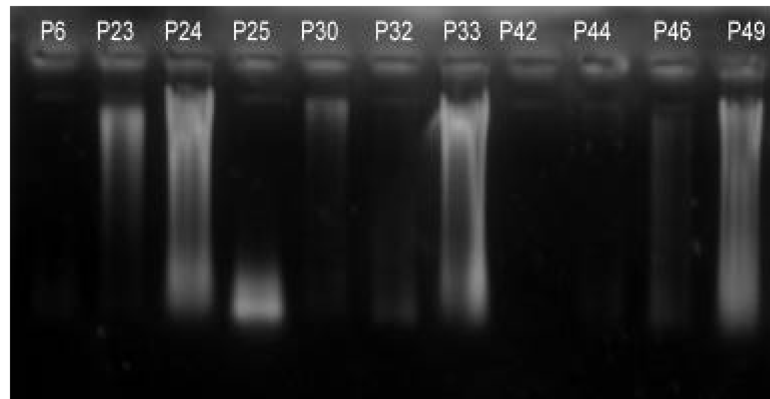

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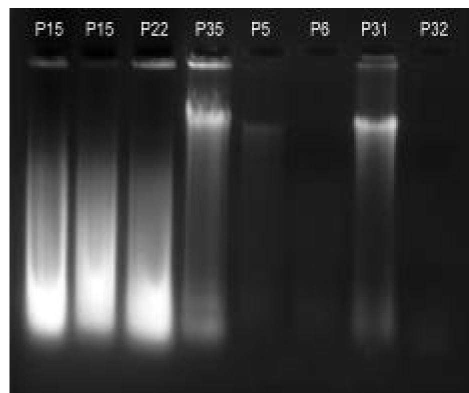
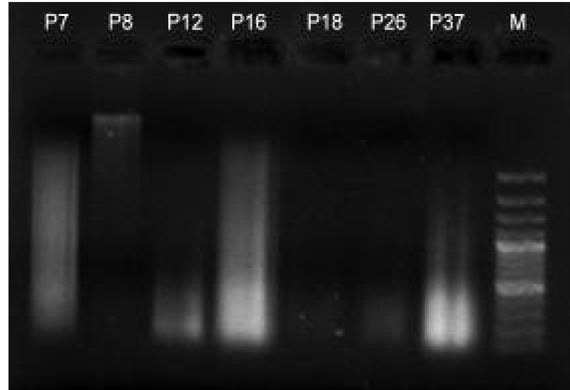


C

FIG.1(A) : VULTURE POST-MORTEM SAMPLE GENOMIC DNA ELECTROPHORESED ON 0.8% AGAROSE GEL

LANE P: **POST-MORTEM SAMPLE**

LANE M : **100BP PLUS DNA LADDER**



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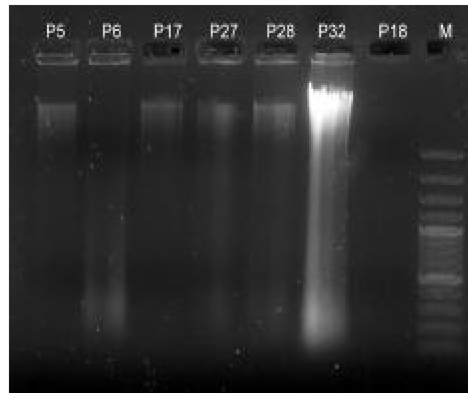

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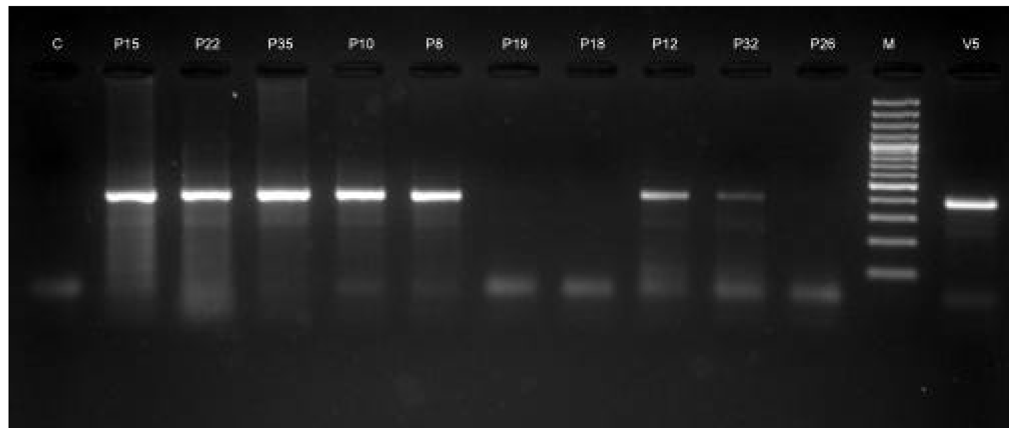
FIG.1(B) : VULTURE POST-MORTEM SAMPLE GENOMIC DNA ELECTROPHORESED ON 0.8% AGAROSE GEL

LANE P : **POST-MORTEM SAMPLE**

LANE M : **100BP PLUS DNA LADDER**



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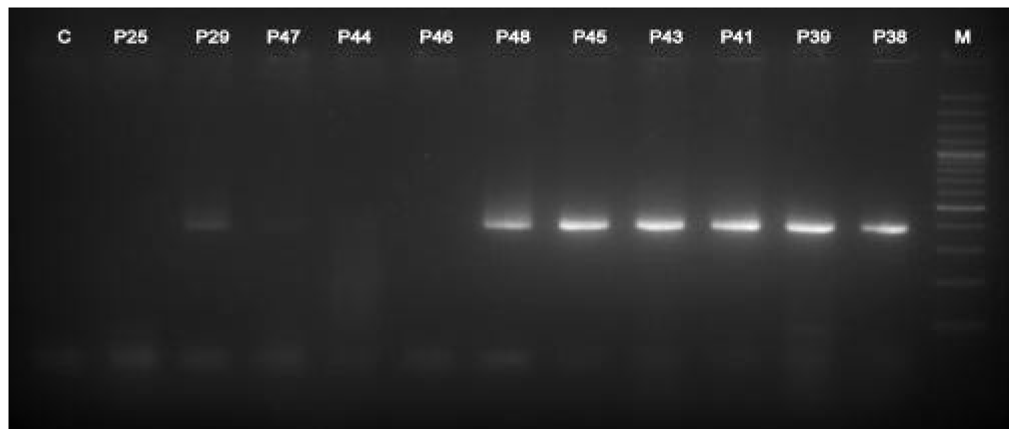

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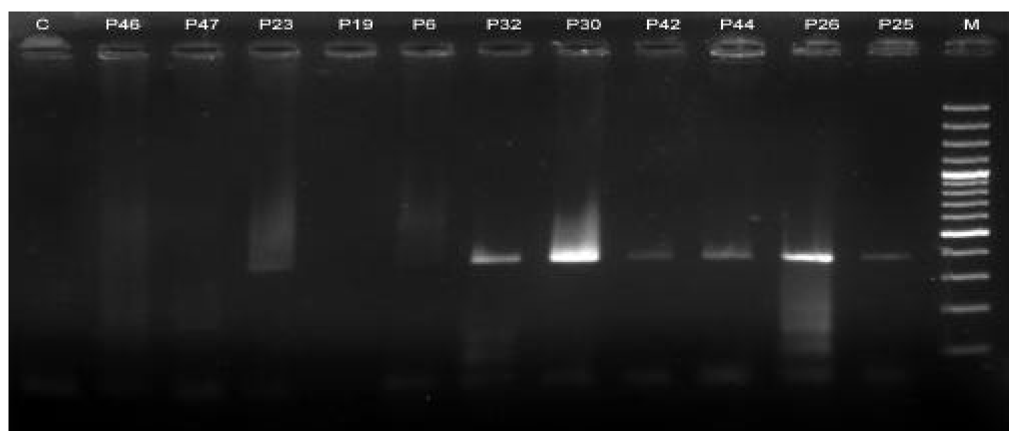

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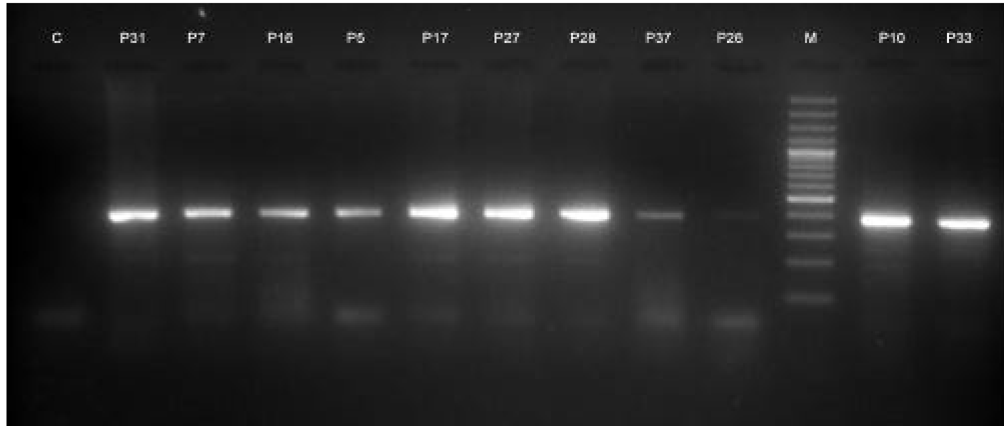
Fig.2(a) : **PCR amplification of CHD genes using P2/P8 primers and electrophoresed in 3% agarose gel**

Lane C : No template control
Lane P : Post-mortem sample
Lane V : Live bird sample
Lane M : 100bp plus DNA ladder



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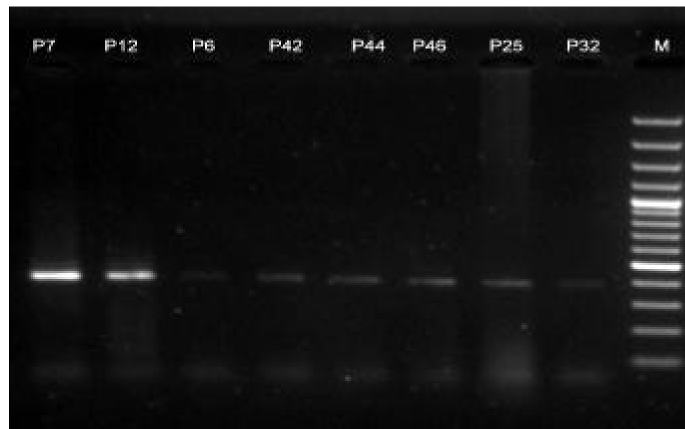

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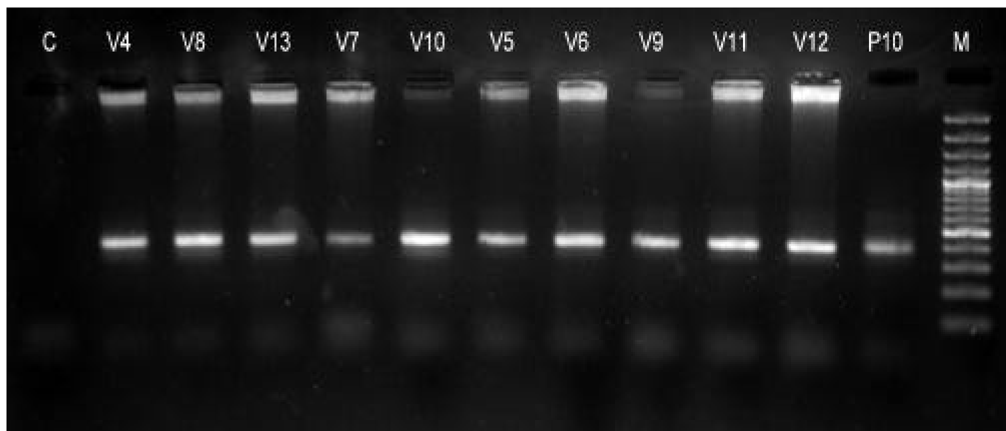

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Fig.2(b) : PCR amplification of CHD genes using P2/P8 primers and electrophoresed in 3% agarose gel

Lane C : No template control
Lane P : Post-mortem sample
Lane V : Live bird sample
Lane M : 100bp plus DNA ladder

DNA samples. DNA of female birds P17 and P10 endowed with both CHD Z and CHD W alleles was suitable template for *Gyps indicus* and *Gyps bengalensis*. However, in case of other two species (P49 and P30), there was no choice for selecting the sample. The resulting amplified product of respective species was gel purified and used for cloning into pJET 1.2/blunt end cloning vector. The ligated product transformed in *E.coli* DH5α competent cells yielded corresponding recombinant colonies, which in turn were employed for plasmid isolation and characterization.

4.3.2 Characterization of recombinant plasmids encoding CHD Z and CHD W

Several plasmids isolated from recombinant colonies for each species were screened for the presence of CHD Z or CHD W insert by sequence differences indicated in *Gyps bengalensis* and *Gyps indicus* and related allelic discrimination PCR approaches reported earlier for various other species by several workers.

The results of the characterization of recombinant plasmid encoding *Gyps bengalensis* CHD Z and CHD W have been provided in Fig. 3. *Bam HI* site was absent in plasmid vector but it's presence at single site in CHD Z insert was used as test to detect whether the recombinant plasmid encoded CHD Z or CHD W. The CHD Z containing recombinant plasmid was cut at insert site and became linear which resolved as single band in agarose gel (Fig. 3a, L2) in comparison to uncut plasmid of CHD W. The CHD W containing plasmid was run as intact plasmid having three bands in it owing to open circular, close circular and supercoiled nature of natural intact circular plasmid (Fig. 3b, L2). ARMS amplification produced single band of near 380 bp for CHD Z insert containing plasmid (Fig. 3a, L3) whereas two bands close to 380bp and 280bp were obtained for CHD

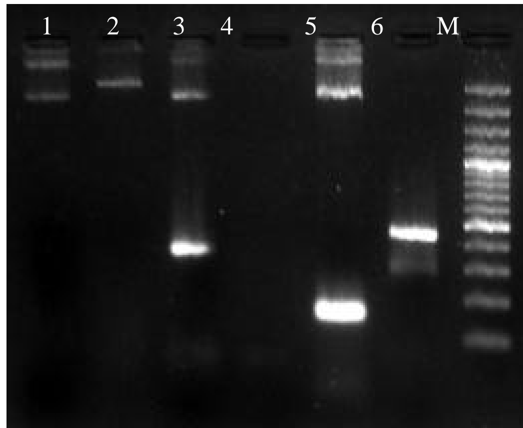


Fig.3(a) : Characterization of recombinant plasmid encoding CHD-Z specific P2/P8 PCR product of *Gyps bengalensis*

Lane 1 : Intact CHD Z plasmid
Lane 2 : *BamH*I digested plasmid (Digested & Run as single band)
Lane 3 : ARMS specific amplification of CHD Z (372bp)
Lane 4 : P2/W Specific amplification of CHD Z (No amplification)
Lane 5 : P2/ZW Common specific amplification of CHD Z (153bp)
Lane 6 : T7 promoter based amplification (450bp)
Lane M : 100bp plus DNA ladder

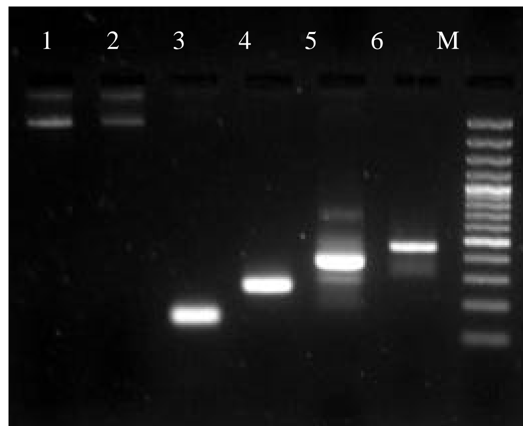


Fig.3(b) : Characterization of recombinant plasmid encoding CHD-W specific P2/P8 PCR product of *Gyps bengalensis*

Lane 1 : Intact CHD W plasmid
Lane 2 : *BamH*I digested plasmid (Remain undigested)
Lane 3 : ZW Common/P2 amplification of CHD W (153bp)
Lane 4 : W Specific/P2 amplification of CHD W (263bp)
Lane 5 : ARMS specific amplification of CHDW (378bp & 293bp)
Lane 6 : T7 promoter based amplification (456bp)
Lane M : 100bp plus DNA ladder

W containing plasmid (Fig. 3b, L5). P2/W specific PCR yielded no amplification in CHD Z containing plasmid (Fig. 3a, L4) whereas band of nearly 250bp was obtained in case of CHD W recombinant plasmid (Fig. 3b, L4). Presence of nearly 150bp band in ZW/P2 amplification yielded in both plasmids encoding CHD Z (Fig. 3a, L5) as well as CHD W (Fig. 3b, L3) inserts. Insert orientation was checked using T7 promoter primer which acted as reverse primer and based on orientation of insert in vector, it was expected to give amplification of nearly 460bp with either P2 or P8 primer. The *Gyps bengalensis* recombinant plasmids encoding both CHD Z (Fig. 3a, L6) and CHD W (Fig. 3b, L6) yielded P2 primer-specific amplification in combination with T7 promoter primer thus, indicating the forward orientation of the insert.

The results of selected recombinant plasmid characterization in case of *Gyps indicus* are depicted in Fig. 4. It is obvious from the gel photographs (Fig 4a for CHD Z and Fig 4b for CHD W) that the patterns of bands were similar to those observed in case of *Gyps bengalensis* CHD Z and CHD W, respectively. However, the product of orientation check with P8 primer was obtained in combination with P8, suggesting reverse orientation of the insert in the recombinant plasmids.

The screening of *Gyps himalayensis* recombinant plasmids for CHD Z and CHD W insert indicated both types, representing the sex of P30 bird to be female. The subsequent characterization of the selected plasmids encoding CHD Z (Fig 5a) and CHD W (Fig 5b) resulted in band pattern similar to those obtained for above mentioned two species. The screening of *Aegypius monachus* recombinant plasmids revealed the presence of only one type of insert encoding CHD Z suggesting the sex of the bird to be male. The selected plasmid

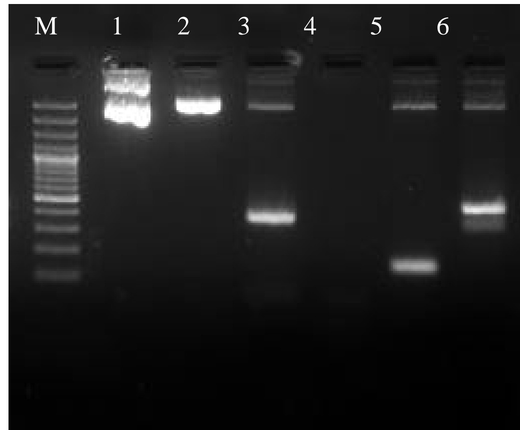


Fig.4(a) : Characterization of recombinant plasmid encoding CHD-Z specific P2/P8 PCR product of *Gyps indicus*

Lane M : 100bp plus DNA ladder
Lane 1 : Intact CHD Z plasmid
Lane 2 : *BamHI* digested plasmid (Digested & Run as single band)
Lane 3 : ARMS specific amplification of CHD Z (375bp)
Lane 4 : P2/W Specific amplification of CHD Z (No amplification)
Lane 5 : P2/ZW Common specific amplification of CHD Z (153bp)
Lane 6 : T7 promoter based amplification (453bp)

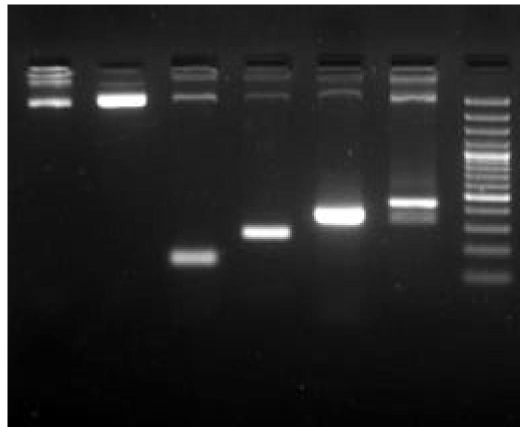


Fig.4(b) : Characterization of recombinant plasmid encoding CHD-W specific P2/ P8 PCR product of *Gyps indicus*

Lane 1 : Intact CHD W plasmid
Lane 2 : *BamHI* digested plasmid (Remain undigested)
Lane 3 : ZW Common/P2 amplification of CHD W (153bp)
Lane 4 : W Specific/P2 amplification of CHD W (263bp)
Lane 5 : ARMS specific amplification of CHDW (378bp & 293bp)
Lane 6 : T7 promoter based amplification (456bp)
Lane M : 100bp plus DNA ladder

was characterized fully as demonstrated in Fig 6. The inserts in the recombinant plasmids of both these species were found in reverse orientation, as in case of *Gyps indicus*.

4.3.3 Sequencing of CHD Z and CHD W gene

Final characterization of all the seven recombinant plasmids was completed by their sequencing using T7 promotor primers. The sequences so obtained were aligned with P2 and P8 primers to define CHD gene-specific sequence and rest of the vector sequences were removed. Thus, partial sequence of CHD Z and CHD W gene of *Gyps bengalensis*, *Gyps indicus* and *Gyps himalayensis* was obtained whereas only CHD Z sequence could be obtained for *Aegypius monachus*.

4.4 DEVELOPMENT OF MOLECULAR METHODS FOR SEX IDENTIFICATION

Various approaches have been tried for sex identification in vultures in present study to avoid any ambiguity in results and to cross check the result of one test with another test. The methods included, PCR-RFLP by *BamHI* and *RsaI*, TaqMan probe based Real time PCR, Amplification Refractory Mutation System (ARMS) based PCR amplification and Sex specific PCR approach.

4.4.1 Prediction of primers, probes and restriction enzyme recognition sites

The sequences for CHD Z and CHD W of the four species were aligned independently. The alignment report for CHD Z and CHD W has been provided in Fig.7a and Fig 7b, respectively. Primer recognition sites for P2, P8, NP, MP, ZW Common, W specific primers and restriction site for *BamHI* as well as *RsaI* were mapped on the alignment report. The recognition site for MP and W specific primers were found in CHD W but not in CHD Z sequences. *BamHI* restriction site was found specific for CHD Z but not CHD W.

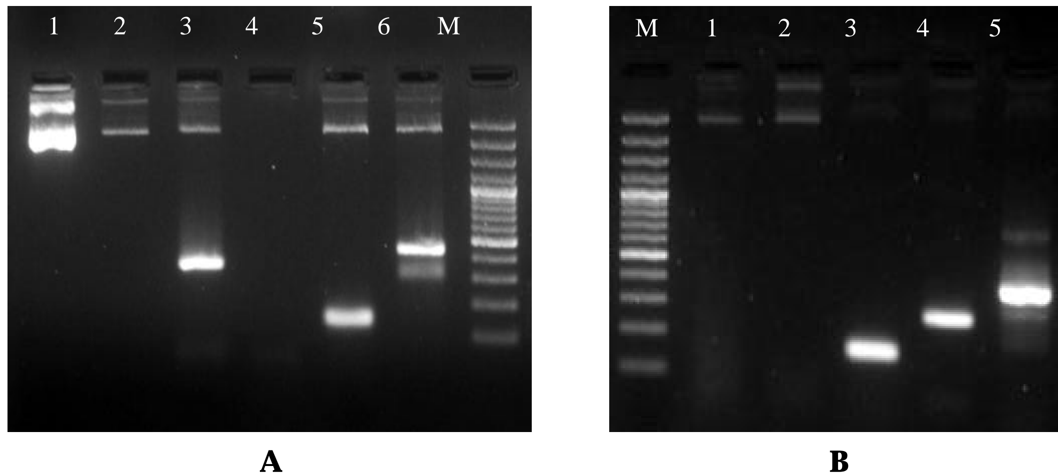


Fig.5(A) : Characterization of recombinant plasmid encoding CHD-Z specific P2/P8 PCR product of *Gyps himalayensis*

Lane M : 100bp plus DNA ladder
Lane 1 : Intact CHD Z plasmid
Lane 2 : *BamHI* digested plasmid (Digested & Run as single band)
Lane 3 : ARMS specific amplification of CHD Z (375bp)
Lane 4 : P2/W Specific amplification of CHD Z (No amplification)
Lane 5 : P2/ZW Common specific amplification of CHD Z (153bp)
Lane 6 : T7 promoter based amplification (453bp)

Fig.5(B) : Characterization of recombinant plasmid encoding CHD-W specific P2/ P8 PCR product of *Gyps himalayensis*

Lane M : 100bp plus DNA ladder
Lane 1 : Intact CHD W plasmid
Lane 2 : *BamHI* digested plasmid (Remain undigested)
Lane 3 : ZW Common/P2 amplification of CHD W (153bp)
Lane 4 : W Specific/P2 amplification of CHD W (263bp)
Lane 5 : ARMS specific amplification of CHDW (378bp & 293bp)

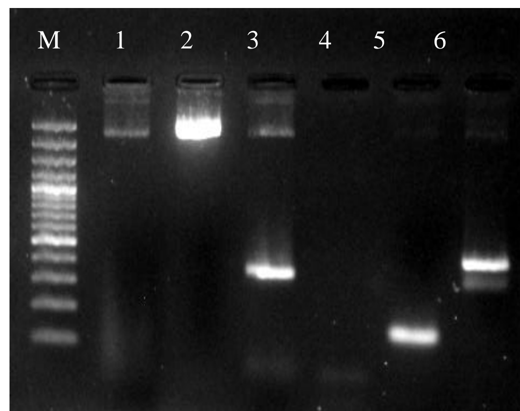


Fig.6 : Characterization of recombinant plasmid encoding CHD-Z specific P2/P8 PCR product of *Aegypius monachus*

Lane M : 100bp plus DNA ladder
Lane 1 : Intact CHD Z plasmid
Lane 2 : *BamHI* digested plasmid (Digested & Run as single band)
Lane 3 : ARMS specific amplification of CHD Z (369bp)
Lane 4 : P2/W Specific amplification of CHD Z (No amplification)
Lane 5 : P2/ZW Common specific amplification of CHD Z (153bp)
Lane 6 : T7 promoter based amplification (449bp)

4.4.1.1 CHD Z sequence analysis

Based on CHD Z sequence analysis (In DNAstar software), exact product length expected in corresponding PCR reaction was predicted. Accordingly, P2/P8 amplicon size of 383bp, 386bp, 386bp, 380bp and, P2/NP amplicon size of 372bp, 375bp, 375bp and 369bp was expected in CHD Z of *Gyps bengalensis*, *Gyps indicus*, *Gyps himaleyensis* and *Aegypius monachus,* respectively. P2/ZW Common amplicon of 153bp was expected in all four species; however, P2/W specific and MP/NP specific products were not expected in the case of male bird.

Restriction enzyme *BamHI* was found to have one site in CHD Z cutting P2/P8 amplicon into two fragments, 283bp+100bp in case of *Gyps bengalensis,* 286bp+100 bp in *Gyps indicus* as well as *Gyps himaleyensis*, and 280bp+100bp in *Aegypius monachus*. Restriction enzyme *RsaI* was found to have one site in CHD Z giving two fragments, 327bp+56bp in case of *Gyps bengalensis*, 330bp+56 bp in *Gyps indicus* as well as *Gyps himaleyensis*, 324bp+56bp in *Aegypius monachus*. The expected fragments obtained from all these tests to be visualized in 3% agarose gel electrophoresis are recorded in Table 1.

Binding site for HEX-labeled ZW Common probe could be located on CHD Z, however binding site for FAM labeled W specific probe was found absent in all the species (Fig.7a).

4.4.1.2 Sequence analysis for CHD W

As per sequence analysis in DNAStar softaware (Fig. 7b), CHD W product length of P2/P8 amplicon of 389bp, P2/NP amplicon length of 378bp and P2/ZW common amplicon length of 153bp, NP/MP amplicon length of 293bp and P2/W specific amplicon length of 263bp was expected in *Gyps bengalensis*, *Gyps indicus*, and *Gyps himaleyensis.* As evident from the alignment report (Fig 7b), on CHD

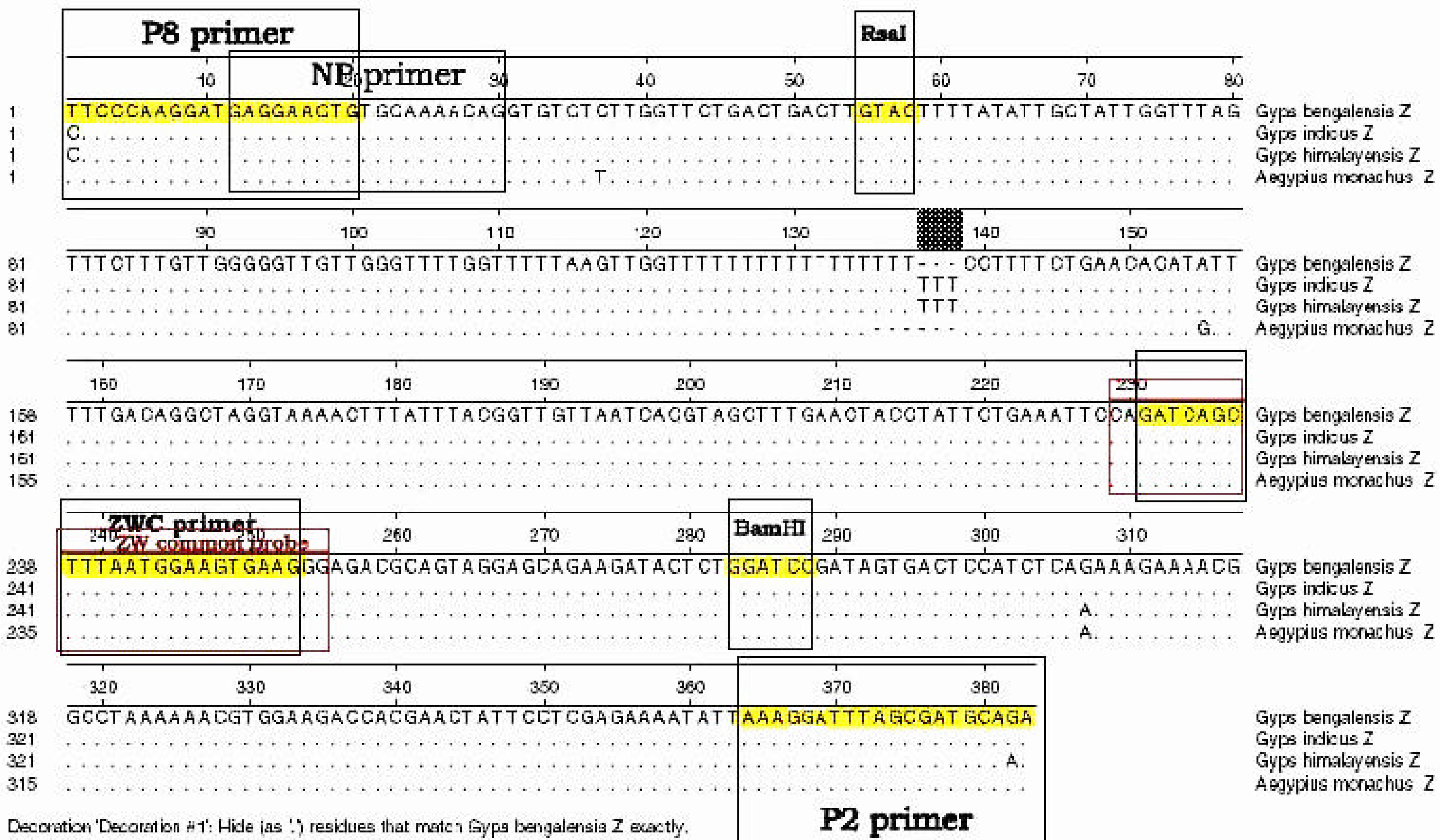


Fig. 7a: Alignment Report of CHD Z Nucleic acid sequence by ClusterW (MegAlign DNAStar)

Fig. 7 b: Alignment Report of CHD W Nucleic acid sequence by ClusterW (MegAlign DNAStar)

W sequence, *BamHI* restriction enzyme site was absent whereas one site for *RsaI* was expected to cut P2/P8 amplicon of the above three species into two fragments of size 278bp and 111bp. All the expected fragments to be visualized in 3% agarose gel electrophoresis are listed in Table 1.

In addition, binding site for FAM labeled W specific probe could also be located on the CHD W sequence of all the three species, suggesting the suitability of this probe for sex identification of the vulture samples (Fig. 7b).

4.4.2 Standardization of PCR based methods for sex identification

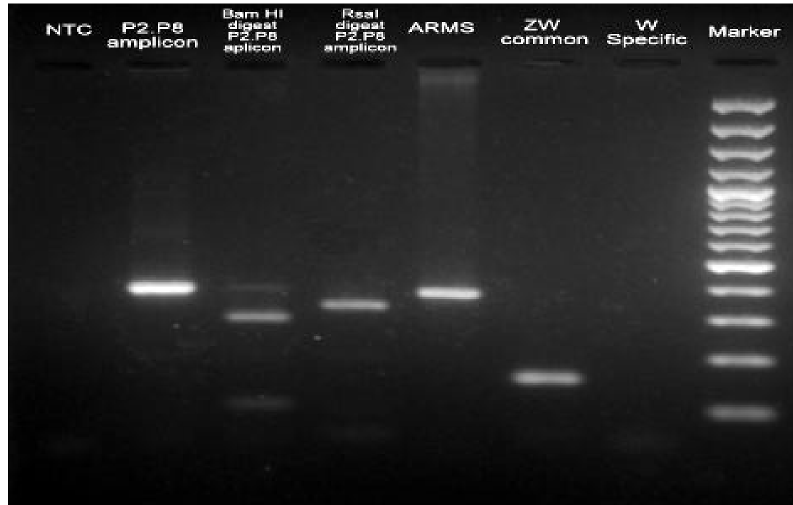
For standardization of the tests, known female (P10) and male (P33) samples of *Gyps bengalensis*, likewise female (P17) and male (P35) samples of *Gyps indicus* were used. P49 and P30 served as the only sample for *Gyps himalayensis* and *Aegypius monachus*, respectively. As it can be noticed from sequence analysis (Fig. 7) and predicted banding patterns (Table 1), any test employed in sex identification in *Gyps bengalensis*, *Gyps indic*us, or *Gyps himalayensis* is expected to produce similar pattern on agarose gel. Therefore, to avoid duplication of the figures, the results of application of the above methods have been exhibited only for *Gyps bengalensis* male (P33) and female (P10) (Fig. 8a and 8b).

4.4.2.1 P2/P8 PCR

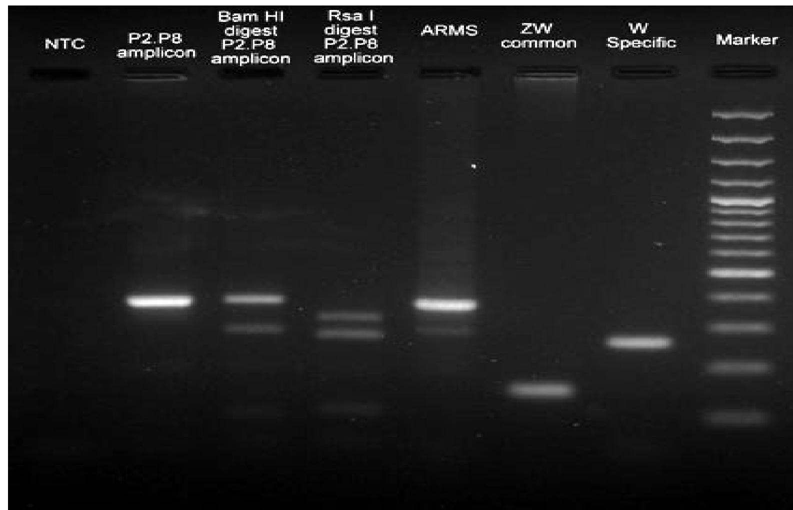
As expected for *Gyps bengalensis*, amplified product obtained by P2/P8 primer pair was 383bp in case of CHD Z (Fig. 8a, L2) and 389bp in case of CHD W (Fig. 8b, L2).

4.4.2.2 PCR-RFLP of P2/P8 amplicon of CHD gene

Digestion of the P2/P8 amplicon of G*yps bengalensis* female CHD gene with *Bam HI* produced three bands of size 389bp+283bp+100bp (Fig. 8b, L3) whereas in case of male bird of the same species, restriction



A



B

Fig.8 : PCR based methods for sex identification of Gyps bengalensis , PCR product run in 3% agarose gel
Lane NTC : No template control
Lane M : 100bp plus DNA ladder

	Fig.8 (A): Male(P33) amplicon (size in bp)	Fig.8 (B): Female(P10) amplicon size in bp
P2/P8 amplification	383	(389bp + 383bp)
BamHI digest of P2/P8 amplicon	283 & 100	389bp, 283bp & 100bp
RsaI digest of P2/P8 amplicon	327 & 56	327bp, 278bp, 111bp & 56bp
ARMS amplicon	372	378bp + 372bp and 293bp
ZW Common/P2 amplicon	153	153
W specific/P2 amplicon	No amplification	263

digestion yielded two bands of size 283bp+100bp. Faint band of 383bp could result from incomplete digestion of the PCR product. (Fig. 8a, L3). Upon restriction digestion of G*yps bengalensis* female P2/P8 amplicon by *RsaI*, four bands of size 327bp+278bp+111bp+56bp (Fig. 8b, L4) were visualized whereas in case of males of two bands of size 327bp+56bp (Fig. 8a, L4) were observed.

4.4.2.3 Amplification Refractory Mutation System (ARMS) PCR

ARMS PCR from male bird (P33) yielded single band of 372bp (Fig. 8a, L5) and from female bird (P10) produced three products which appear as two band (378bp+372bp)+293bp in 3% agarose gel (Fig. 8b, L5).

4.4.2.4 Sex specific primers PCR

The ZW common primer anneals to both Z and W specific sequence and in combination with P2 as forward primer generated 153bp product (Fig. 8a, L6 and Fig. 8b, L6) but W specific primer anneals to only female specific CHD W not to CHD Z so single band of 263bp product was obtained only in female (Fig, 8a, L7 and Fig. 8b, L7).

4.4.3 Standardization of TaqMan probe based Real time PCR for sex identification

Using genomic DNA of known female (P10) and known male (P33) birds of *Gyps bengalensis*, TaqMan Probe based Real-time PCR was standardized using two step thermal set up (Fig. 9 a). Both HEX- and FAM-specific fluorescence was detected in case of female bird whereas only HEX-specific fluorescence was observed in case of male (Fig.9 b). Real-time PCR is based on allele discrimination option i.e. CHD Z and CHD W alleles are detected specifically by HEX- and FAM-labeled probes. The dual color plot provided male-female distinction in graphical format (Fig.9 c) whereas text report directly auto-calling based male-female discrimination (Fig. 9 f).

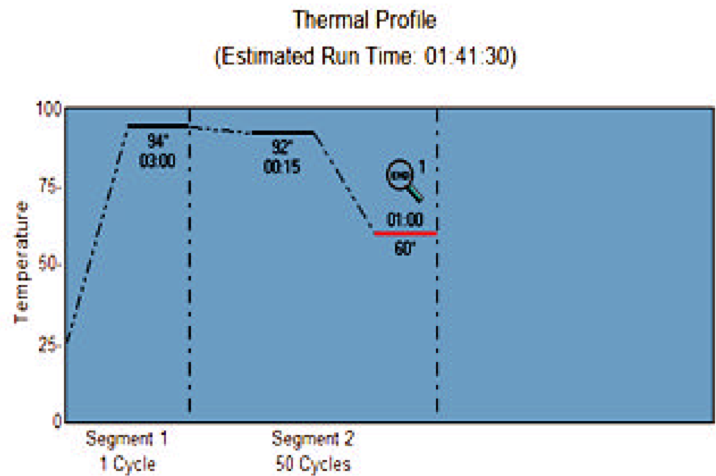


Fig.9 a : Two Step PCR thermal profile set up for Probe based Real-time PCR

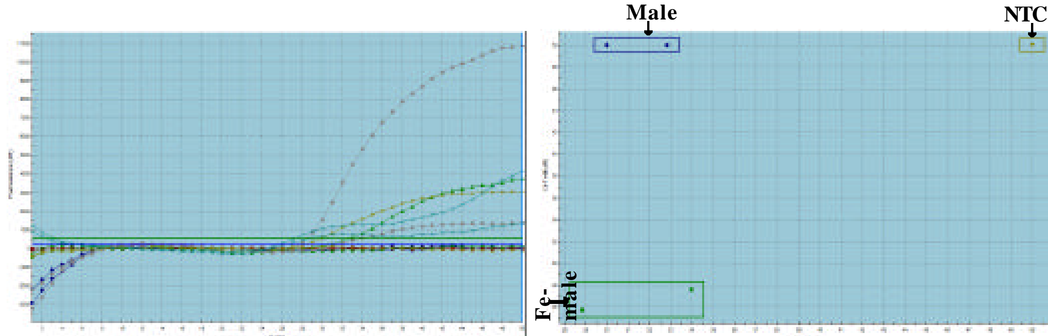


Fig.9 b : Amplification plot CHD at time of Real-time PCR standardization (Allele Discrimination)

Fig.9 c : Dual Color Scatter Plot for CHD at time of Real-time PCR standardization (Allele Discrimination)

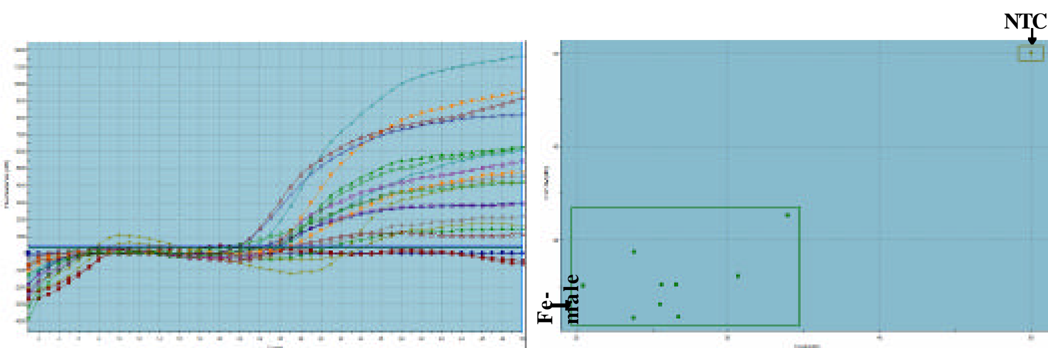


Fig.9 d : Amplification plot CHD of Real-time PCR sample testing (Allele Discrimination)

Fig.9 e : Dual Color Scatter Plot for CHD at time of Real-time PCR sample testing (Allele Discrimination)

Fig.9 : Sex identification by TaqMan probe based Real time PCR

Replicate	Dye	Well Type	Threshold (dR)	Ct (dR)	Final Call (dR)	Genotype (dR)
1	HEX	NTC	553.665	No Ct	-	None
1	FAM	NTC	209.436	No Ct	-	None
2	HEX	NPC	553.665	No Ct	-	None
2	FAM	NPC	209.436	No Ct	-	None
3	HEX	Unknown	553.665	32.81	+	Allele A
3	FAM	Unknown	209.436	No Ct	-	Allele A
4	HEX	Unknown	553.665	33.98	+	Both
4	FAM	Unknown	209.436	27.61	+	Both
5	HEX	Unknown	553.665	29.99	+	Allele A
5	FAM	Unknown	209.436	No Ct	-	Allele A
6	HEX	Unknown	553.665	28.82	+	Both
6	FAM	Unknown	209.436	25.70	+	Both

Fig.9f. Text Report in Real-time PCR by auto-calling option at time of Real-time PCR standardization (Allele Discrimination)

Allele A: HEX; CHD-ZWcommon probe

Both: For combined HEX; CHD-ZWcommon probe and FAM; CHD-W-specific probe

NTC: No Template Control

NPC: No Probe Control

Fig. 9D Text report

Replicate	Dye	Well Type	Threshold (dR)	Ct (dR)	Final Call (dR)	Genotype (dR)
1	HEX	NPC	307.738	No Ct	-	None
1	FAM	NPC	380.052	No Ct	-	None
2	HEX	NTC	307.738	No Ct	-	None
2	FAM	NTC	380.052	No Ct	-	None
5	HEX	Unknown	307.738	30.66	+	Both
5	FAM	Unknown	380.052	26.19	+	Both
7	HEX	Unknown	307.738	23.80	+	Both
7	FAM	Unknown	380.052	28.74	+	Both
12	HEX	Unknown	307.738	33.95	+	Both
12	FAM	Unknown	380.052	32.70	+	Both
15	HEX	Unknown	307.738	25.52	+	Both
15	FAM	Unknown	380.052	23.13	+	Both
17	HEX	Unknown	307.738	25.62	+	Both
17	FAM	Unknown	380.052	25.26	+	Both
22	HEX	Unknown	307.738	26.55	+	Both
22	FAM	Unknown	380.052	25.26	+	Both
31	HEX	Unknown	307.738	23.74	+	Both
31	FAM	Unknown	380.052	21.65	+	Both
41	HEX	Unknown	307.738	26.73	+	Both
41	FAM	Unknown	380.052	21.79	+	Both
48	HEX	Unknown	307.738	20.43	+	Both
48	FAM	Unknown	380.052	25.12	+	Both

Fig.9g: Text Report by Auto-calling in Real-time PCR (Allele Discrimination) at time of sample testing

Allele A: HEX; CHD-ZWcommon probe

Both: For combined HEX; CHD-ZWcommon probe and FAM; CHD-W-specific probe

NTC: No Template Control

NPC: No Probe Control

4.5 APPLICATION OF THE MOLECULAR METHODS FOR SEX IDENTIFICATION

We had received 36 post-mortem and 10 live bird samples for sex identification. Twenty six post-mortem and ten live bird samples were tested by the all the molecular methods described above. Poor genomic DNA samples did not yield clear results, although some signal of their sex was obtained on sex-specific PCR.

4.5.1 Sex identification by P2/P8 PCR-based methods

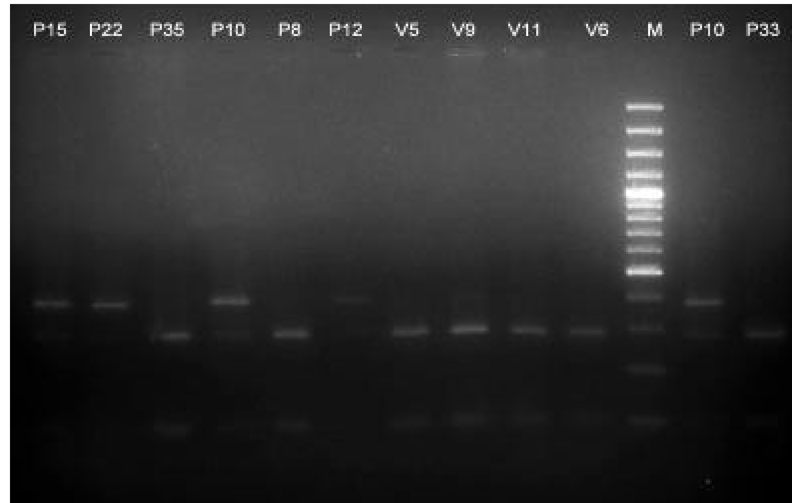
As depicted in Fig 2, one single band of P2/P8 specific PCR Product was obtained in all those samples which contained good quality genomic DNA. Due to only 306 nucleotide intronic length variation, females and males could not be discriminated.

However, the intronic variation in CHD Z and CHD W sequence was utilized in PCR-RFLP. The results of *BamHI* and *Rsa I* digestion of the P2/P8 amplicon of all the samples tested in this study were in accordance with Table 1 and the gel photographs are provided in Fig.10 and Fig.11, respectively.

4.5.2 Sex identification by allellic discrimination

As demonstrated in (Fig.12), Multiplex PCR approach in ARMS was found successful in discriminating the sex of the birds as female (two bands) or male (one band) in all the samples analyzed by this method. Accordingly, female samples showed two bands of approx. 378bp and 293 bp whereas males yielded single band of 372bp in *Gyps bengalensis*, 375bp in *Gyps indicus* as well as *Gyps himalayensis*, and 369bp in *Aegypius monachus* as predicted in Table 1.

Likewise, sex specific PCR approach employing ZW Common/W specific primer amplification in independent reactions proved useful in identifying female birds. Amplification of the ZW common product authenticated the CHD specific product obtained genomic DNA (Fig.13a)



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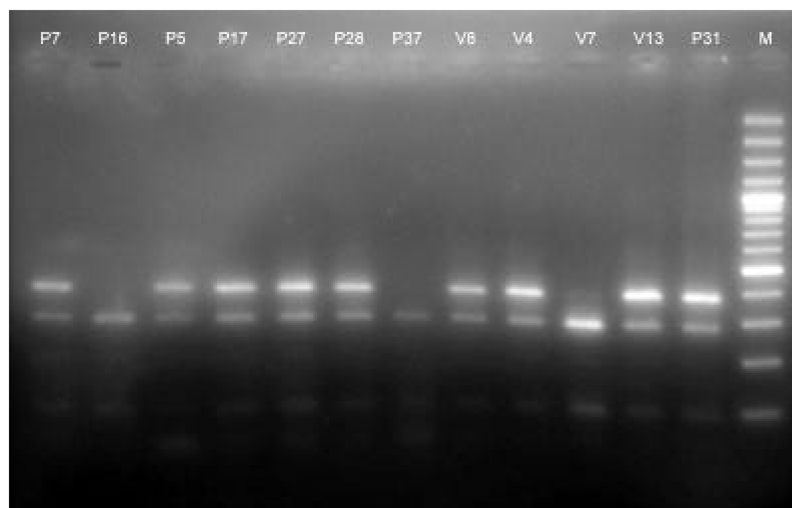
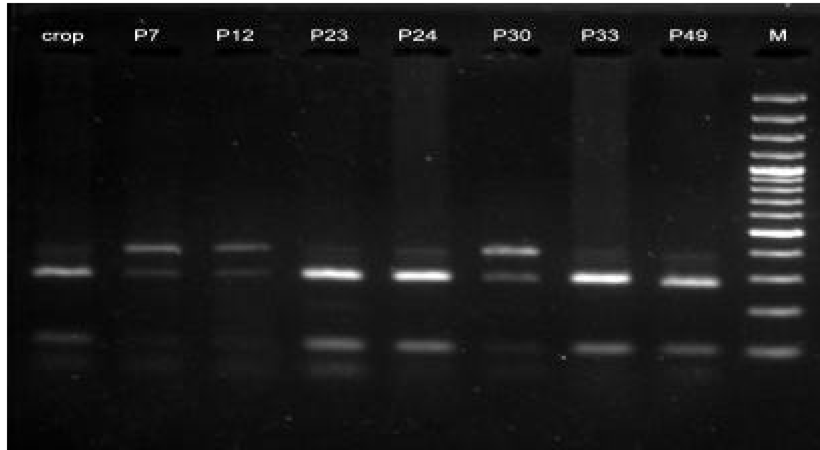


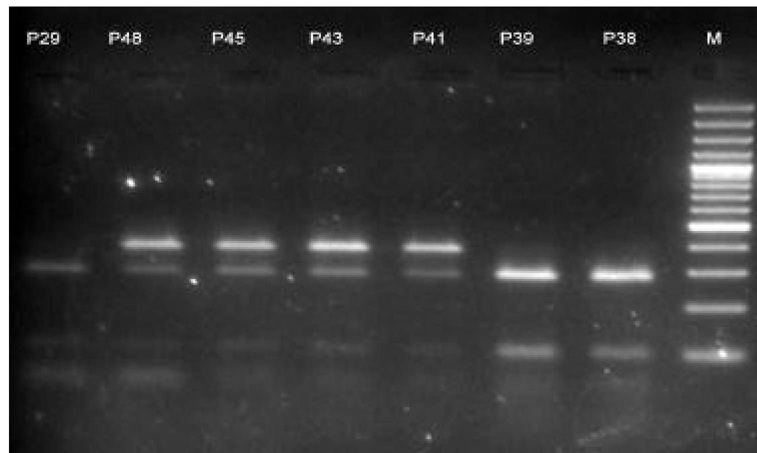
B

Fig.10(A,B) : PCR-RFLP of P2/P8 amplicon of CHD gene using *Bam*HI digestion product run in 3% agarose gel reveal three band for females and two bands for male

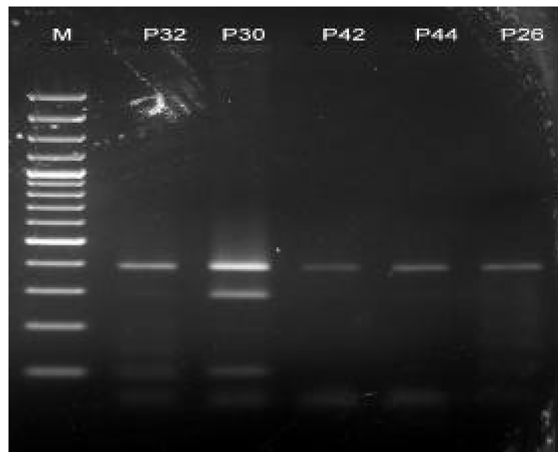
Lane M : 100bp plus DNA ladder
Lane P : Post-mortem sample
Lane V : Live bird sample



C



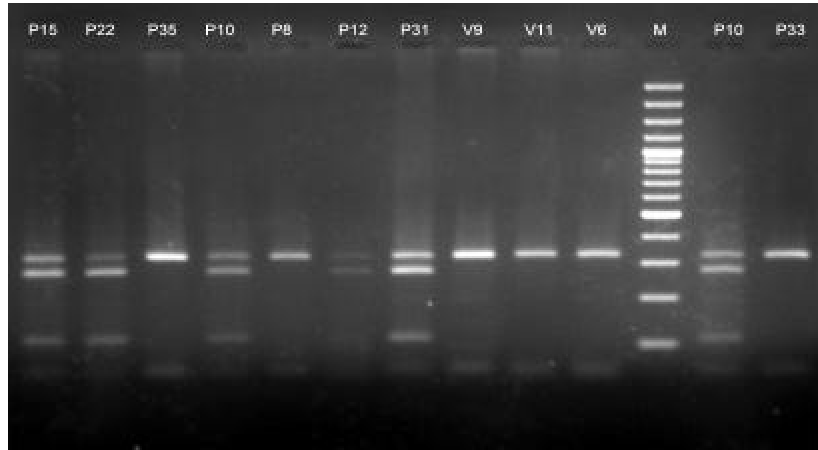
D



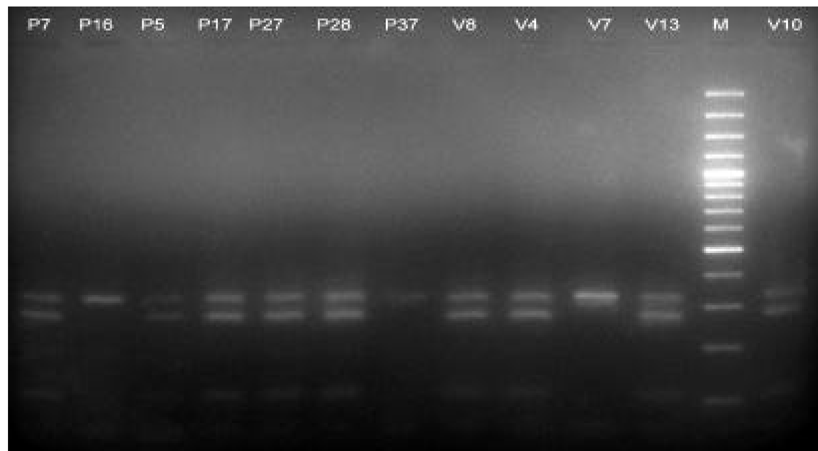
E

Fig.10(C-E) : PCR-RFLP of P2/P8 amplicon of CHD gene using *Bam*HI digestion product run in 3% agarose gel reveal three band for females and two bands for male

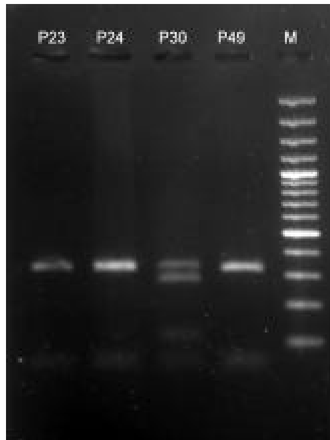
Lane M : 100bp plus DNA ladder
Lane P : Post-mortem sample
Lane V : Live bird sample



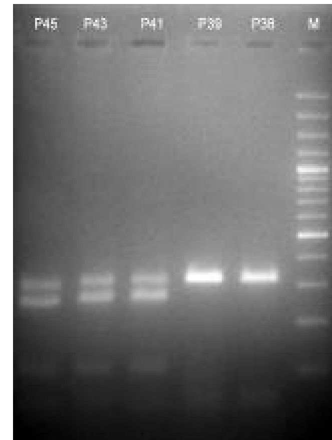
A



B



C



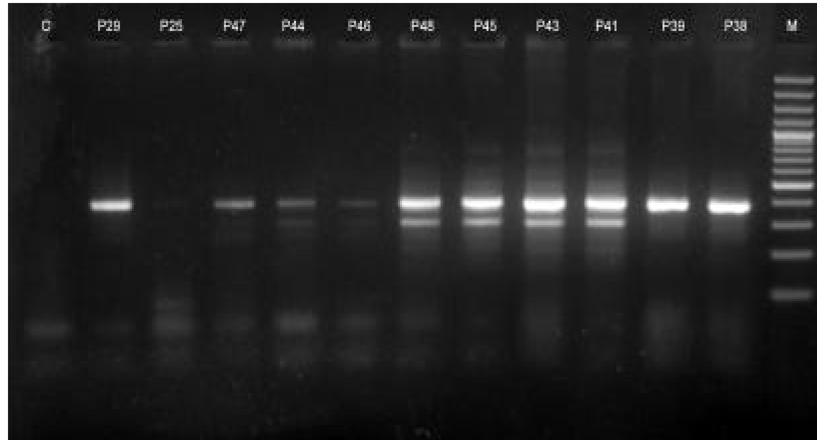
D

Fig.11(A-D) : PCR-RFLP of P2/P8 amplicon of CHD gene using *Rsa*I digestion product run in 3% agarose gel reveal four bands for females and two bands for male

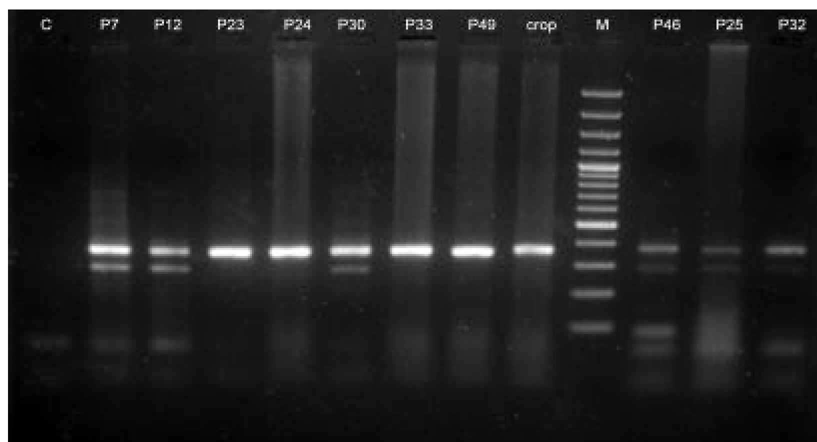
Lane M : 100bp plus DNA ladder

Lane P : Post-mortem sample

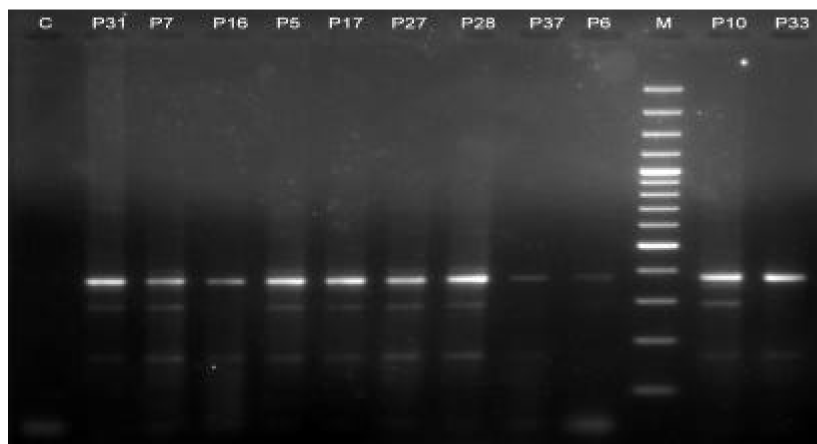
Lane V : Live bird sample



A



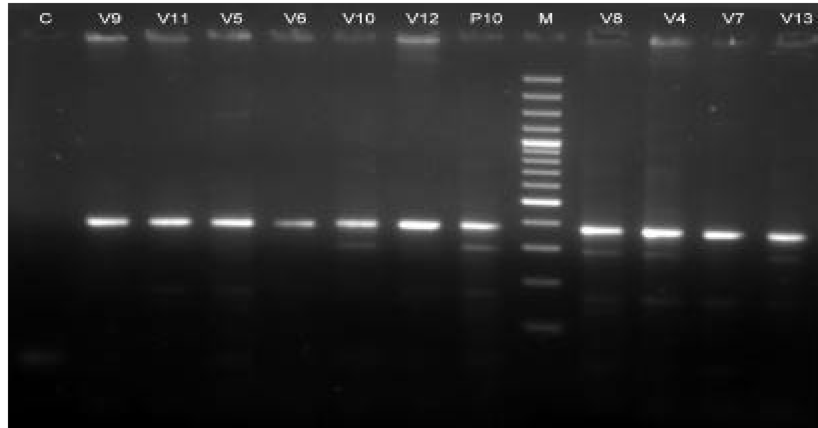
B



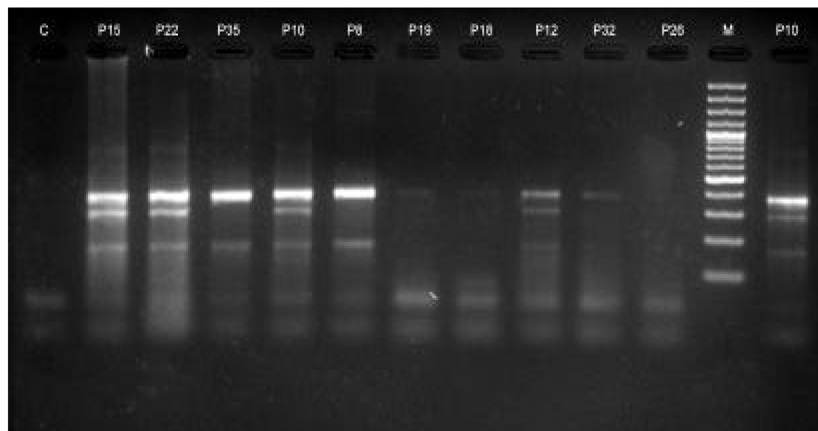
C

Fig.12(A-C) : PCR amplification of CHD gene using P2/MP/NP primers as visualized in 3% agarose gel, two bands in female & male reveal on band

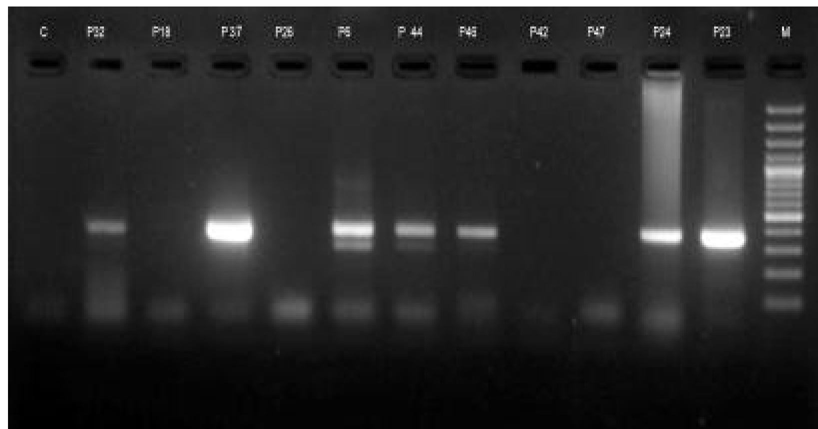
Lane C **:** No template control
Lane M **:** 100bp plus DNA ladder
Lane P **:** Post-mortem sample
Lane V **:** Live bird sample



D



E



F

Fig.12(D-F) : PCR amplification of CHD gene using P2/MP/NP primers as visualized in 3% agarose gel, two bands in female & male reveal on band

Lane C : No template control
Lane M : 100bp plus DNA ladder
Lane P : Post-mortem sample
Lane V : Live bird sample

Table 1; Agarose gel banding pattern for various sex determination approaches in (size in bp)

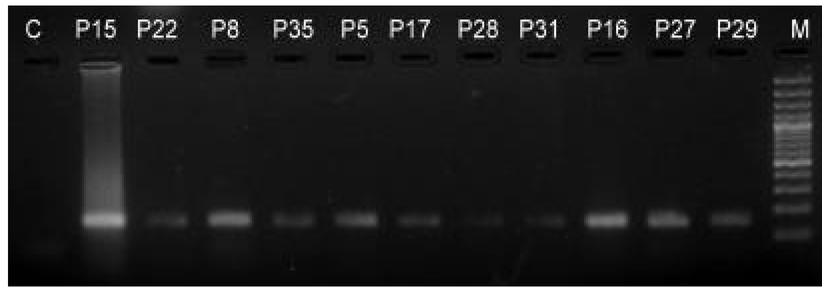
P2/P8		*BamHI* digest of P2/P8 amplicon		*RsaI* digest of P2/P8 amplicon		P2/NP/MP		P2/ZW common		P2/W specific	
Gyps bengalensis											
Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male
389(W) 383(Z)	Nil 383(Z)	389 283 100	283 100	327 278 111 56	327 56	378(P2/NP-W) 372(P2/NP-Z) 293(MP/NP-W)	372(P2/NP-Z) Nil	153(W) 153(Z)	153(Z)	263(W)	Nil
Gyps indicus											
389(W) 386(Z)	Nil 386(Z)	389 286 100	286 100	330 278 111 56	330 56	378(P2/NP-W) 375(P2/NP-Z) 293(MP/NP-W)	375(P2/NP-Z) Nil	153(W) 153(Z)	153(Z)	263(W)	Nil
Gyps himalayensis											
389(W) 386(Z)	Nil 386(Z)	389 286 100	286 100	330 278 111 56	330 56	378(P2/NP-W) 375(P2/NP-Z) 293(MP/NP-W)	375(P2/NP-Z) Nil	153(W) 153(Z)	153(Z)	263(W)	Nil
Aegypious monachus											
-	380(Z)	-	280 100	-	324 56	-	369(P2/NP-Z)	-	153(Z)	-	Nil

Table 2: Validation of the CHD based molecular tests in sex identification of vultures

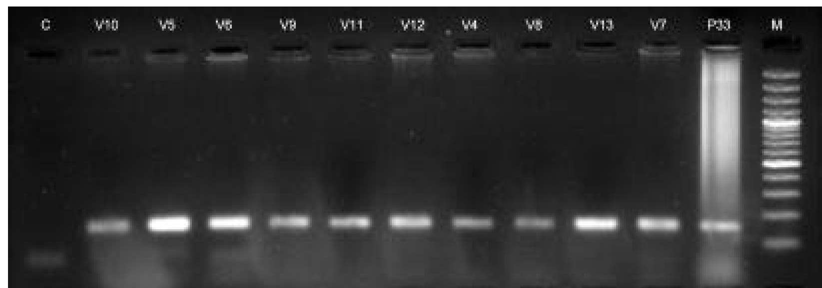
Sample Code	Sex identified in this study							Sex informed by VCBC Pinjore
	P2-P8 PCR based methods				ARMS PCR	CHDZW /CHD W-specific PCR	Con-sensus	
	Presence of P2-P8 amlicon	PCR-RFLP		Real time PCR				
		Bam HI	*Rsa* I					
Gyps bengalensis								
PM Samples								
P10	Yes	Female	Female	Female	Female	Female	Female	Female
P12	Yes	Female	Female	Female	Female	Female	Female	**Male**
P15	Yes	Female	Female	Female	Female	Female	Female	Female
P22	Yes	Female	Female	Female	Female	Female	Female	Female
P29	Yes	Male	-	-	Male	Male	Male	Male
P33	Yes	Male	Male	Male	Male	Male	Male	Male
P35 (A06)	Yes	Male	Male	-	Male	Male	Male	Male
P39	Yes	Male	Male	-	Male	Male	Male	Male
P41	Yes	Female	Female	Female	Female	Female	Female	Female
P43	Yes	Female	Female	Female	Female	Female	Female	**Male**
P45	Yes	Female	Female	-	Female	Female	Female	Female
P48	Yes	Female	-	Female	Female	-	Female	**Male**
Live Birds								
A04 (V6)	Yes	Male	Male	Male	Male	Male	Male	Male
A06(V12)	Yes	-	-	-	Male	Male	Male	Male
A07(V10)	Yes	-	Female	-	Female	Female	Female	Female
A12(V11)	Yes	Male	Male	-	Male	Male	Male	Male
Gyps indicus								
PM Samples								
P5	Yes	Female	Female	Female	Female	Female	Female	Female
P7	Yes	Female	Female	Female	Female	Female	Female	**Male**
P16	Yes	Male	Male	Male	Male	Male	Male	Male
P17	Yes	Female	Female	Female	Female	Female	Female	Female
Live Birds								
B09 (V7)	Yes	Male	Male	-	Male	Male	Male	Male
Aegypius monachus								
PM sample P49	-	Male	Male	-	Male	Male	Male	Male

Table 3: Application of the CHD based molecular tests in sex identification of vultures

Sample Code	Sex identified in this study						Consensus
	P2-P8 PCR based methods				ARMS PCR	CHD Common /CHD W-specific PCR	
	Presence of two bands	PCR-RFLP		Real time PCR			
		Bam HI	*Rsa* I				
Gyps bengalensis							
PM Samples							
P8	Yes	Male	Male	-	Male	Male	Male
P23	Yes	Male	Male	-	Male	-	Male
P24	Yes	Male	Male	-	Male	-	Male
P33	Yes	Male	Male	-	Male	Male	Male
P35	Yes	-	Male	-	Male	Male	Male
P38	Yes	Male	Male	-	Male	Male	Male
Live Birds							
V5	Yes	Male	-	-	Male	Male	Male
V9	Yes	Male	-	-	Male	Male	Male
Gyps indicus							
PM Samples							
P27	Yes	Female	Female	-	Female	Female	Female
P28	Yes	Female	Female	-	Female	Female	Female
P31	Yes	Female	Female	Female	Female	Female	Female
Live Birds							
B03 V13)	Yes	Female	Female	-	Female	Female	Female
B04 (V8)	Yes	Female	Female	-	Female	Female	Female
B06 (V4)	Yes	Female	Female	-	Female	Female	Female
G. himalayensis							
PM sample 309	Yes	Female	Female	-	Female	Female	Female



A



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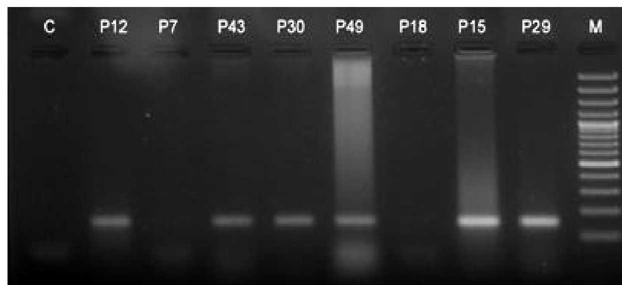
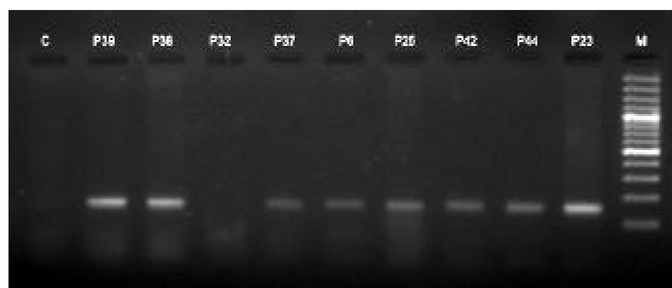


C

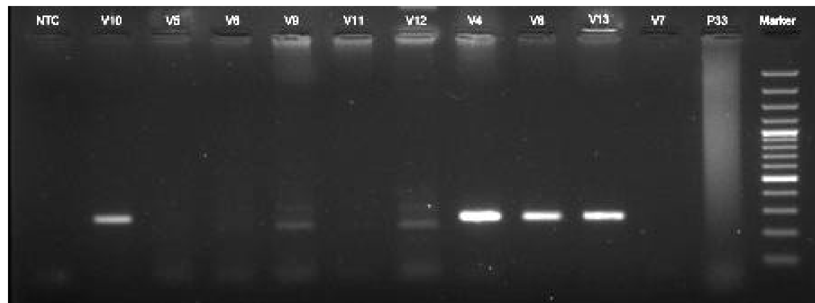


D

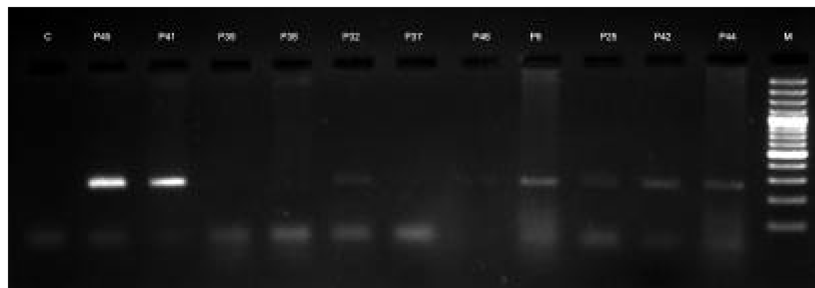
Fig.13(A-D) : Amplifying CHD-Z and CHD-W by P2/ ZW Common primers as visualized in 3% agarose gel

Lane C : No template control
Lane M : 100bp plus DNA ladder
Lane P : Post-mortem sample
Lane V : Live bird sample

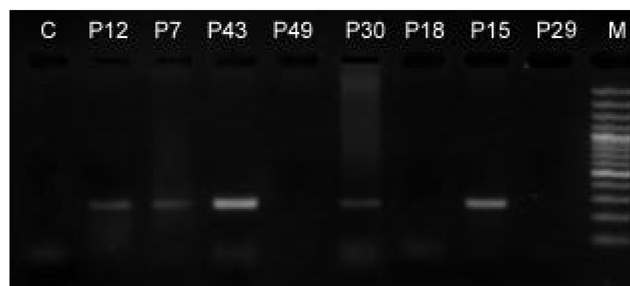
E



F



G



H

Fig.13(E-H) : Identification of sex in vultures using P/CHD W specific amplification of 263bp product in females and no product in males

Lane C : No template control

Lane M : 100bp plus DNA ladder

Lane P : Post-mortem sample

Lane V : Live bird sample

to discriminate no amplification in "NTC" from no CHD-W specific amplification in males. One band of 263 bp belonging to P2/W-specific product was visualized in all the female samples (Fig.13b). Also, this method was found useful in analyzing samples even with degraded DNA.

In another approach of allelic discrimination (Section 4.4.3), TaqMan probe based Real time PCR could successfully identify sex of tested samples for which an amplification curve, dual color plot and text report has been provided in Figs. 9d, 9e and 9g, respectively.

4.5.3 Compilation of results

The results of 26 post-mortem and 10 live bird sex identification interpreted from Fig. 2, Fig.9 and Figs. 10-14 were analyzed. In fact, after reporting the results to VCBC Pinjore, they confirmed the sex of some of these birds. Accordingly, we have arranged our analysis in following sections:

4.5.3.1 Validation of test results on birds of known sex

The summary of sex of 17 post-mortem and five live birds as identified by all the methods and final verification of our results from the breeding centre is provided in Table 2. Sex identified by us for 13 post-mortem samples matched with the known sex, whereas four samples displayed mismatch. P7, P12, P43 and P48 were found females by all the methods whereas the centre reported them to be males. In case of live birds, the sexes of all the five birds (A04, A06, A07, A12, and B09) matched with that reported by the centre.

4.5.3.2 Identification sex of birds with no records

It is evident from the Table 3, that ten post-mortem and five live birds could be identified as males or females based on the results of molecular methods, which were useful in identifying sex of *Gyps indicus, Gyps bengalensis* and *Gyps himaleyensis.*

5. Discussion

Chromohelicase-DNA binding protein gene is a conserved gene present on both Z and W chromosome of the avian species. Due to allelic difference in intronic length in CHD Z and W, this gene has been largely exploited for sex identification in several bird species. CHD gene based sex identification has been carried out in birds such as *Accipiter nisus (A. nisus), Spizaetus nipalensis (S. nipalensis), Aquila chrysaetos (A. chrysaetos), Circus spilonotus (C. spilonotus), and Milvus migrans (M. migrans)* etc. For this purpose, the P2/P8 primers originally designed by Griffiths and Tiwari (1995) have been useful in sexing most of the bird species. However, in certain bird species including vultures, the intronic length variation is found between 2-6 nucleotides, thus, not sufficient enough to be used as a sex identification test in these species by PCR and normal agarose gel elctrophoresis.

In the present study, the emphasis for development of molecular method for sex identification has been laid for four out of nine species of vultures found in India namely, Oriental White-backed Vulture

(*Gyps bengalensis*), Long billed Vulture (*Gyps indicus*), Himalayan Griffon (*Gyps himalayensis*), Cinereous Vulture (*Aegypius monachus*). *Gyps bengalensis* and *Gyps indicus* are 'critically endangered' species in the wild and *ex-situ* conservation through assisted reproduction is in progress at Vulture Conservation and breeding Centre, Pinjore. To assist the captive breeding in maintaining 1 male: 1 female sex ratio of these monogamous birds, we have tested a battery of molecular methods for unambiguous sex identification in these species. To meet the objectives, first of all, the sequence of CHD gene targeted in testing various sex determination approaches was characterized for all these species.

5.1 CHARACTERIZATION OF CHD GENE IN VULTURES

The source for obtaining CHD gene was the genomic DNA isolated from post-mortem sample of *Gyps bengalensis* and *Gyps indicus* vulture of known sex. In this way, one single PCR could yield both CHD Z and CHD W products for cloning. The recombinant plasmids were sequenced to obtain CHD sequences of vulture species. Alignment of all these nucleotide sequences by MegAlign program of DNAstar software revealed that CHD Z sequence of *Gyps bengalensis* shows 99.7%, 99.2%, 99.2% similarity with those of *Gyps indicus, Gyps himalayensis* and *Aegypius monachus,* respectively. *Gyps indicus* CHD Z has 99.5% similarity with *Gyps himalayensis* CHD Z and 98.9% with *Aegypius monachus* CHD Z. *Gyps himalayensis* CHD Z demonstrated 98.9% similarity with *Aegypius monachus* (Fig 5.1). On the other hand, CHD W sequence of *Gyps bengalensis* showed 100% and 99.7% homology with CHD W sequences of *Gyps indicus* and *Gyps himalayensis*, respectively. Likewise, CHD W sequence of *Gyps himalayensis* exhibited 99.7% homology with that of *Gyps indicus* (Fig

5.1). Such high level homology between the sequences of this species justifies that the method developed for one of these species, can be tested on the other species as well.

Using NCBI resources, Blast analysis was performed on CHD W and CHD Z sequences obtained for all the four species. The sequence of those raptor bird species was retrieved which revealed more than (92 %) identity with the corresponding sequences of vultures in our study. Phylogenetic trees based on evolutionary distances at CHD Z (Fig 5.2 a) or CHD W (Fig. 5.2 b) nucleotide sequence level were constructed (Mega 4.1 software) and the bootstrap values are indicated on the roots. All the Indian vulture species were found to be closely related among themselves and to other raptor bird species like Eagle and Falcon for which the primers and probes tested in this study are already employed for sex identification.

5.2 NECESSITY FOR SEQUENCE CHARACTERIZATION

Partial sequences of *Gyps indicus* and *Gyps bengalensis* CHD Z (NCBI Accession DQ156155 and DQ156156) as well as CHD W (NCBI Accession DQ156153 and DQ156154) were characterized earlier by Reddy *et al.* (2007) for sex identification in these species. Initially we used these sequences for standardization of PCR RPLF of using P2/ P8 amplicon for sex identification in these two species. However, the restriction sites of *Dra* I and *Rsa* I predicted from these sequences did not result in expected fragments on the gel. The sequences variation in *Rsa* I and *Dra I* recognition sites necessitated the need for correct sequence determination of CHD Z and CHD W gene pertaining to the allelic discrimination. Therefore, for mapping the primer and probe binding sites and predicting restriction enzymes specific for CHD Z and CHD W amplicon , we have used our own sequences, not the published ones.

5.3 SEQUENCE DIFFERENCE EXPLOITED FOR SEX DISCRIMINATION

5.3.1 P2/P8 based sex determination in birds

5.3.1.1 Intronic length variation

Sex identification based on P2/P8 primer pair is originally developed by Griffith (Griffith and Tiwari, 1993). Intronic length variation in P2/P8 PCR products in male and female allows their differentiation as two band in female (ZW) and one band in male (ZZ). But we found one band in both male and female birds of known sexes in all the vulture species. From sequence analysis it was observed that intronic length difference was 6 nucleotides in *Gyps bengalensis* and only 3 nucleotides in *Gyps indicus* and *Gyps himaleyensis.* The small difference in the expected products does not allow discrimination between males and female vultures as reported by Reddy *et al*. (2007). Alternatively, SSCP analysis of the P2/P8 amplicon has been suggested in case of *Accipiter cooperii* (Ramos *et al*., 2009)

5.3.1.2 PCR-RFLP

Restriction site of *DraI* was predicted in reported sequence of CHD Z only. PCR RFLP of P2/P8 amplicon in case of *Gyps bengalensis* was expected to yield 115bp and 271 bp fragments of CHD Z due to one site and 62, 52, 272bp fragments were expected in case of *Gyps indicus* due to two recognition sites. So this enzyme was selected for sex determination as well as *Gyps bengalensis* and *Gyps indicus* species differentiation. But *DraI* enzyme was unable to cut CHD fragment in either species. After sequencing, the absence of DraI site was confirmed on CHD Z and CHD W sequences of both *Gyps bengalensis* and *Gyps indicus*.

Similarly, the *Rsa*I enzyme was expected to have one restriction site in CHD W but no site in CHD Z, suggesting use in sex

determination in through PCR RFLP. But in case of male (ZZ), expected band of 383bp was not observed, but alternate band of size 327 bp was observed in 3% agarose gel. The prediction of this restriction site in CHD Z sequences obtained in our study confirmed the digestion of CHD Z amplicon into 327+56 bp fragments. The shorter fragment being smaller in size may or may not be visible in the gel. Thus, digestion of P2/P8 amplicon by *RsaI* produces four fragments in case of female and two fragments in case of male.

Likewise, the analysis of our sequences revealed *BamHI* restriction site only in CHD Z but not in CHD W and this prediction for PCR RFLP of P2/P8 amplicon justified three fragments in female whereas two in case of male when run on 3% agarose gel. PCR RFLP proved useful discrimination tool between male and female vultures in the present study and the results were validated on the birds of known sexes. This approach has earlier been used successfully in case of Short-toed Eagle (Sacchi *et al.*, 2004).

5.3.2 Allelic discrimination approaches

The CHD Z and CHD W sequences, although nearly 99% conserved, but minor differences due to point mutations have been exploited for sex determination in several species of birds by designing primer binding to CHD W allele but not CHD Z allele. For W-specific assays viz. ARMS and CHD ZW common/CHD W, primers binding to both CHD W as well as CHD Z allele are also included so as to confirm the integrity of the DNA sample and discriminate male (ZZ) and no template control revealing no amplification. Both reactions can be run independently as in case of **sex specific PCR amplification** (CHD ZW common/CHD W). This method has been reported for successful sex identification in *Spilornis cheela hoya* where CHD ZW Common/P2 primer pair gives amplification of 153 bp from both Z

and W alleles. However, W-specific primer binds to only CHD W allele and in combination with P2 primer gives amplification in case of females not in males (Chang *et al.,* 2008c). The primers reported in this study were mapped on vulture sequences in this study and were found suitable for molecular discrimination of *Gyps indicus*, *Gyps bengalencis* and *Gyps himaleyensis* male and female birds. This test was found most suitable for two reasons, the ease of interpretation of results in agarose gel as presence or absence of CHD W-specific band and the smaller size of the PCR products in ZW common (153 bp) and W-specific PCR (263bp) facilitates the analysis of highly degraded DNA samples. In our study, we could not get results in P2/ P8 primer based tests due to expected larger size product (nearly 390 bp) in samples numbered (P46, P44, P42, P47, P37) with poor genomic DNA. However, this sex specific approach could prove useful in identifying sex of these PM samples.

In second approach, both reaction can be run in multiplex PCR in **ARMS** with forward primer P2 allow to produce CHD W corresponding band in female only whereas CHD Z corresponding band in male and female both. Three primers set NP/MP/P2 are employed in single reaction, where MP is 3'-mismatch primer in CHD Z sequence, but it binds CHD W sequences, so in case of female bird, two bands were expected in agarose gel whereas in case of male bird one single band is expected. This method was used for sex identification in Faconiformes species such as Black kite (*Milvus migrans*), Northern gosahawk (*Accipiter gentiles*), Estern marsh harrier (*Circus spilonotus*), Golden eagle (*Aquila crysaetos*), Eurasian sparrowhawk (*Accipiter nisus*), mountain hawk eagle (*Spizaetus nipalensis*), Peregrin falcon *Falco peregrinus*) and common kestrel (*Falco tinnuculus*) (Ito *et al.,* 2003). The primers sequences used in this study were also found matching on vulture CHD Z and CHD W sequences;

therefore we included them for developing molecular method for vulture sex identification. The amplification and gel running conditions described in our testing were found appropriate for reliable male and female bird identification in post-mortem as well as live bird samples. Although, Reddy *et al*, 2007 indicated the use of this test for the same purpose, but we have strengthened the application of this test to *Gyps indicus* and *Gyps bengalensis* birds through validation of the results on 17 birds with known sex.

TaqMan probe based real-time approach could determine sex of multiple samples at a time and there is no need for running agarose gel too. This approach has been used for sex identification in case of *Spilornis cheela hoaya* (Chang *et al.,* 2008b). Alignment of vulture sequences and mapping the probe binding sites demonstrated that the same probes can also be used for *Gyps indicus, Gyps bengelensis* and *Gyps himaleyensis* species sex identification. The probe binding site has same sequence as ZW Common and W specific primers plus additional nucleotides. Using P2/P8 primes in single reaction containing both the probes, fluorescence for both HEX and FAM probe was detected in female birds and only HEX fluorescence was observed in case of male birds. We have standardized Real-time PCR using these probes and applied it to selected PM and live bird samples. This is a quick and robust method for unambiguous sex determination in birds.

5.4 ASSISTING IN CONSERVATION BREEDING

All the above methods were employed to identify male and female birds among 36 postmortem specimens and 10 live birds being reared at the Vulture Captive Breeding Centre, Pinjore and produced similar results. The sex identified by us for 17 post-mortem samples was found correct for 13 samples by the VCBC, Pinjore. Repeated

testing on 4 mismatch samples produced same results. The mismatch could be attributed to contamination or any discrepancy due to autolysis in recording the sex of bird carcass at the time of post-mortem. Although the primers and probes used in this work span through the entire P2/P8 amplicon, the complete sequencing of these products could confirm our results. To resolve the doubt of contamination at the laboratory level, testing of the fresh tissue obtained from the centre is recommended. The outcome of the study was very encouraging as sexes identified by us for five live birds of *Gyps indicus* and *Gyps bengalensis* were found correct by the centre. The sex for rest of the five birds was not known to the centre and we have provided the results to them. The finding that same test can be applied to *Gyps himaleyensis* sex identification also strengthens the outcome of the present study. Although male bird of *Aegypius monachus* can be identified but the sequence of CHD W in has to be characterized before standardizing these tests for sex discrimination in this species.

6. Summary and Conclusions

Gyps indicus and *Gyps bengalensis* vultures are 'critically endangered' species in India and veterinary drug Diclofenac, was reported as a major cause of their catastrophic decline. This drug has been banned by Government of India for veterinary use to safeguard the environment for vultures in free-range. Captive breeding centres have also been established for *ex-situ* conservation of these species. Like many other birds, vultures are monogamous so 1 male: 1 Female ratio should be maintained in captivity for successful breeding. However, it is difficult to maintain this ratio due to difficulty in identifying sex from external features. Genetically females are heterozygous (ZW) and males are homozygous (ZZ). The present study has attempted to standardize a number of tests employing chromohelicase-DNA binding-protein gene sequence from Z and W alleles. For this purpose, partial CHD Z and CHD W sequences of *Gyps bengalensis*, *Gyps indicus*, *Gyps himaleyensis* and CHD Z sequence of *Aegypius monachus* were obtained after cloning of P2/P8 PCR product of the respective species.

The results obtained are summarized as follows:

6.1 CLONING AND CHARACTERIZATION OF CHD Z AND CHD W GENE (PARTIAL)

- Genomic DNA was isolated from 36 post-mortem and 10 live bird samples of above mentioned vulture species. Ten samples did not yield genomic DNA of quantity/quality suitable for molecular methods. Sex of some of the birds was known from the origin of tissue sample (testes or ovary).
- A segment of CHD gene was amplified using P2/P8 primer pair from genomic DNA of female birds (P10 for *Gyps bengelensis* and P17 for *Gyps indicus*) and the only available sample of *Gyps himaleyensis* (P30) and *Aegypius monachus* (P49).
- The purified amplicon (nearly 390 bp) of respective species was ligated to blunt cloning vector, pJET 1.2 and transformed into *E. coli* DH5á competent cells.
- Colonies were screened by Ampicillin antibiotic selection, the vector was expected to give only positive colonies.
- Several colonies were picked from every plate and grown in LB medium. Plasmids from the cultures grown overnight were isolated by alkaline lysis (mini prep) protocol.
- Recombinant plasmids were screened for the presence of CHD Z or CHD W insert by restriction analysis and nested PCR.
- The selected plasmids encoding CHD W and CHD Z were further characterized by ARMS PCR, W-specific PCR, restriction analysis for CHD Z and CHD W fragment inserts using enzymes *Rsa I* and *Bam HI*.
- The orientation of insert was checked using T7/forward primer and T7/ Reverse primer in combination with P2 forward primer.

- Characterization of CHD Z and CHD W recombinant plasmid was completed by sequencing using T7 promoter primers and analysis of the sequence by DNAstar software.
- The size of P2/P8 specific region of CHD Z was 383bp in *Gyps bengalensis*, 386bp in *Gyps indicus as well as Gyps himalayensis*, and 380bp in *Aegypius monachus.* On the other hand, size of the corresponding region in CHD W was 389bp in *Gyps bengalensis*, *Gyps indicus* as well as *Gyps himalayensis. Aegypius monachus* being male bird (ZZ), CHD W sequence of this species could not be obtained.
- More than 99% similarity was observed between these sequences and phylogenetic tree made from CHD Z or CHD W sequences revealed close relationship among these Indian vulture species and other raptor birds.

6.2 DEVELOPMENT OF TEST FOR SEX IDENTIFICATION

- The sequences of CHD Z and CHD W were aligned independently and the position of primers and probes and the restriction sites for enzymes predicted from related published sequences. Then corresponding PCR products and PCR RFLP fragments expected were predicted.
- A segment of CHD gene was amplified using P2/P8 primer pair from genomic DNA of male (P33) and female (P10) birds for *Gyps bengelensis* and Male (P35) and female (P17) for *Gyps indicus* and the only available sample of *Gyps himaleyensis* (P30) and *Aegypius monachus* (P49). Nucleotide difference of only 3-6 nucleotides was obtained between the PCR products of Gyps species vultures.
- However, PCR RFLP performed on CHD Z and CHD W amplicons using *BamHI* and *RsaI* clearly discriminated male and female birds.
- ARMS PCR (using P2/MP/NP primers) based on 3'- terminal mismatch for MP primer, yielded two detectable bands for

female and single band for male birds in all the *Gyps* species employing the conditions standardized in this study.

- In sex specific PCR run in two reactions independently, P2/W specific primer amplified CHD W specific product of 263bp only in female birds when P2/CHD ZW Common primer amplified 153 bp products both in male and female birds.
- Using female (P10) and male (P33) *Gyps bengalensis* samples, TaqMan probe based Real time PCR was standardized for allelic discrimination between CHD Z and CHD W P2/P8 amplicons. FAM- labeled probe detected W-specific allele whereas Hex-labeled probe detected both the alleles.
- All test gives consistent identification of sex with no variation.

6.3 APPLICATION OF THE MOLECULAR METHODS FOR SEX IDENTIFICATION

- All these standardized tests were applied for sex identification of 26 post-mortem samples and 10 live bird samples obtained from VCBC Pinjore.
- Out of 16 post-mortem samples yielding suitable quality genomic DNA, sex identification of 13 samples matched with sex confirmed by VCBC Pinjore. Nine matching samples belonged to *Gyps bengalensis*, three to *Gyps indicus* and one to *Aegypius monachus*.
- Sex of five live birds identified by molecular methods was found same as confirmed by the breeding centre. Out of these four birds belonged to *Gyps bengelensis* and one to *Gyps indicus* species.
- Additionally, the sex of 10 post-mortem samples from the field (6 *Gyps bengelensis*, 3 *Gyps indicus* and one *Gyps himaleyensis*)

and five live birds (2 *Gyps bengelensis* and 3 *Gyps indicus*) being reared at the Conservation Breeding Centre has been identified.

CONCLUSIONS

Present study has characterized the CHD gene sequences of four vulture species found in India. It is inferred from the molecular methods standardized in this study that any one of the tests including PCR RFLP of P2/P8 amplicon using *BamHI* or *RsaI* and allelic discrimination based methods like ARMS, Sex specific PCR amplification or TaqMan probe based real-time PCR can be employed for reliable sex identification in vultures, although a combination of tests will help in cross validation. The outcome of the present work will facilitate the *ex-situ* vulture conservation efforts in India.

SUGGESTIONS FOR FUTURE WORK

The present study has paved a way for better understanding of different sex identification strategies in case of vultures. CHD W sequence of *Aegypius monachus* needs to be identified for sex discrimination in this species. Tests should be applied to *Gyps indicus* and *Gyps bengalensis* live birds in captivity to assist the ongoing breeding programmes. More number of *Gyps himaleyensis* samples should be tested and validated.

7. Mini Abstract

Chromo-helicase-DNA-binding protein (CHD) conserved gene sequence has been employed for sex identification in birds owing to it's presence on both Z and W chromosomes, with independent evolutionary sequence divergence and considerable sequence difference in Z and W alleles. The present study has standardized a number of tests based on CHD gene specific PCR for sex identification in *G. indicus* and *G. bengalensis*. For this purpose, partial sequence of CHD gene on Z and W chromosome of these two species was characterized. Genomic DNA was isolated from the post-mortem tissue specimens and blood samples from live birds obtained from BNHS VCBC Pinjore, Haryana. A portion of CHD gene was amplified with a proof-reading DNA polymerase and P2 P8 primers from genomic DNA of known female bird of each species, which contains both CHD Z and CHD W alleles. The amplicons of respective species were cloned into pJET1.2 cloning vector. Likewise, partial CHD amplicon of was also cloned from genomic DNA of *Gyps himalyensis* and *Aegypius monachus.* The recombinant plasmids were screened for the presence of CHD Z or CHD W inserts by restriction analysis and PCR. Sequencing of the characterized recombinant plasmids of each species and the subsequent analysis revealed a close phylogenetic relationship among these four Indian vulture species. The size of P2 P8 amplicon was found 389bp (W) and 383 bp (Z) in *G. bengalensis*, 389bp (W) and 386 bp (Z) in *G. indicus* and *Gyps himalyensis. Aegypius monachus* recombinant plasmid yielded only 380 bp (Z) insert as the bird was found to be male. The positions of P2, NP, MP primers used in ARMS PCR, CHD ZW common and CHD W primers in sex-specific PCR and restriction sites of enzymes *Bam*HI and *Rsa* were mapped on CHD-Z and CHD-W aligned sequence of these species and the fragment size to be visualized on 3% agarose gel were predicted to develop a battery of tests for sex identification. Location of CHD common and CHD W specific probes to be used in Real time PCR was also mapped on the obtained sequences. For validation, these tests were employed on 12 post-mortem and 4 live bird samples of *G. bengalensis* and 4 postmortem and one live bird sample of *G. indicus* of known sexes. All the five tests standardized in this study produced similar results although the identification matched with it 13 out of 17 post-mortem samples (including *Aegypius monachus*) and all five live birds of known sex. Additionally, the tests were also employed to 10 post-mortem samples and 5 live birds of unknown sex. The sex mismatch on four samples has to be confirmed by sequencing of the corresponding PCR products. We found the tests suitable for sex identification in *G. himalensis* but CHD W sequence of *Aegypius monachus* needs to be characterized. Further validation is suggested on more samples from the vultures of all these species. It is inferred from that characterized CHD sequences of four species of Indian vultures and sequence related PCR-based methods standardized in this study can be employed for reliable sex determination and thus assist in captive breeding of this critically endangered species.

८. लघु सारांश

क्रोमोहेलिकेस डीएनए बन्धन प्रोटीन (सीएचडी) के संरक्षित जीन क्रम का उपयोग पक्षियों में लिंग पहचान के लिये किया जाता है क्योंकि यह 'जेड' व 'डब्ल्यू' दोनों गुणसूत्र पर पाया जाता है, दोनों का स्वतन्त्र विकासक्रम पथन्तरण होता है तथा 'जेड' व 'डब्ल्यू' विकल्पों में पर्याप्त अन्तर पाया जाता है। प्रस्तुत शोध में सीएचडी जीन विशेष श्रृंखला क्रिया द्वारा जिप्स इंडीकस व जिप्स बेंगालेन्सिस प्रजाति के गिद्धो में लिंग पहचान के लिये कई जांच विधियों का मानकीकरण किया गया। गिद्ध प्रजनन केन्द्र, पिंजौर से प्राप्त मरणोत्तर ऊतक नमूनों व सजीव पक्षियों के रक्त से डीएनए को पृथक किया गया। पी2/पी8 प्राईमर–युग्म व प्रूफरीडिंग डीएनए पोलिमरेस द्वारा मादा पक्षियों के डीएनए से सीएचडी जीन के एक खण्ड का श्रृंखला क्रिया द्वारा सवंर्धन किया गया, क्योंकि मादा पक्षी के गुणसूत्र में 'जेड' व 'डब्ल्यू' दोनों विकल्प होते हैं। संवर्धित उत्पादों को पी जेट–12 क्लोनिंग वेक्टर में एक पूंजक किया गया। नमूनों में उपलब्ध जिप्स हिमालयोन्सिस व ऐजिपियस मोनाकस प्रजाति के पक्षियों से प्राप्त डीएनए से संवर्धित उत्पादों को भी एक पुंजक किया गया। हर रिकाम्बिनेंट प्लाज्मिड़ में 'सीएचडी जेड' या 'सीएसडी डब्ल्यू' खण्ड की उपस्थिति के लिये पोलिमरेस श्रृंखला क्रिया द्वारा जांच की गई। जांच के उपरान्त चुने हुये सात प्लाजिमड़ का क्रमण किया गया, क्रमों के आंकलन में इन सभी गिद्ध प्रजातियों में समीपवर्ती विकास क्रम पाया गया। पी2/पी8 संवर्धित उत्पाद का आकार जिप्स वेन्गेलेन्सिस गिद्ध में 389 बेसयुग्म (डब्ल्यू) व 383 बेसयुग्म (जेड) था जबकि जिप्स इंडीकस व जिप्स हिमालयेन्सिस गिद्धों में यह 389 बेसयुग्म (डब्ल्यू) व 383 बेसयुग्म (जेड़) पाया गया। ऐजिपियस मोनाकस के रिकॉबिनेन्ट प्लाज्मिड में 380 बेसयुग्म का 'सीएचडी जेड' खण्ड प्राप्त हुआ। इन सभी क्रमों को क्रमबद्ध करके सीमित ऐन्जाईम विश्लेषण में प्रयुक्त 'बैमएच1 व 'आरएसए–1' इन्जाईम का तथा 'एआरएमएस' पीसीआर में प्रयुक्त 'पी2/एमपी/एनपी' प्राईमरस का, लिंग विशेष पीसीआर में प्रयुक्त 'सीएचडी–जेड डब्ल्यू कॉमन' व 'सीएचडी डब्ल्यू' प्राईमर युग्म का तथा 'रियल टाईम पीसीआर' में प्रयुक्त 'प्रोब' का पहचान स्थल आंकलन किया गया। तत्पश्चात् इन सब जांच विधियों का प्रयोग करने पर एगारोज जेल ईलेक्ट्रोफोरेसिस में दिखाई देने वाली पट्टियों के आकार का पूर्वआंकलन किया गया। इन जांच विधियों का प्रयोग मरणोपरांत उत्तक नमूनों व सजीव पक्षियों के रक्त के नमूनों से लिंग पहचान के लिये किया गया। सभी जांच विधियों में लगभग एक जैसे परिणाम पाये गये। विधिमान्यकरण के लिये उन नमूनों को मान्यता दी गई, जिनका लिंग गिद्ध प्रजनन केन्द्र द्वारा ज्ञात हुआ। जिप्स बेगेलेन्सिस प्रजाति के 16 में से 12 तथा जिप्स इंडीकस प्रजाति के 5 गिद्ध एजिपियस मोनाकस प्रजाति के एक पक्षी का लिंग हमारे परिणाम से मेल खाया। विश्लेषण जिन पाँच नमूनों के परिणाम मे विषमता पाई गई, उनकी पुष्टि सी0एच0डी जीन खण्ड के क्रम द्वारा की जाये प्रस्तुत शोध में दर्शाये गये सीएचडी जीन क्रमों तथा उसमें जुड़ी जाँच विधियों का प्रयोग सभी चार प्रजातियों की लिंग पहचान के लिए किया जा सकता है। ऐसा पाया गया कि सभी जाँच विधियाँ जिप्स हिमालयेन्सिस प्रजाति की लिंग पहचान के लिए भी उपयुक्त हैं परन्तु ऐजिपियस मोनाकस प्रजाति के सीएचडी डब्ल्यू क्रम ज्ञात करने की आवश्यकता है। इनके अधिकाधिक नमूनों पर विधिमान्यकरण किया जाना चाहिए ताकि भरोसेमन्द लिंग पहचान के द्वारा इन विलुप्त होनी प्रजातियों के प्रजनन में सहायता की जा सके।

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Appendix

50X TAE BUFFER

Tris base	242 g
Glacial Acetic Acid	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml

Distilled water upto 1000 ml

TRIS EDTA (TE) BUFFER 1M (PH 8.0)

Tris-HCl (pH 8.0)	10 mM
EDTA (pH 8.0)	1mM

GEL LOADING DYE (6X)

Tris-HCl (pH 7.6)	10 mM
Bromophenol blue	0.03%
Xylene cyanol	0.03%
Glycerol	60%

EDTA60 mM

ETHIDIUM BROMIDE (10 MG/ML)

Ethidium bromide	10 mg
Distilled Water	1 ml

Mix well, store at 4°C in dark. Handle with gloves and avoid inhalation.

REAGENTS FOR PLASMID ISOLATION

i) Resuspension buffer (P1)

Tris-HCl (pH. 8.0)	25 mM
Glucose	50 mM

EDTA10 mM

Add RNase 100 μg/ml. P1 can be prepared in batches of approximately. 100 ml autoclaved for 15 minutes at 15 lb/sq in liquid cycle and stored at 4°C

ii) Lysis buffer (P2)

NaOH ((Freshly diluted from 10 N stock)	0.2N

SDS 1%

iii) Neutralization buffer (P3)

5 M Potassium acetate	60 ml

Glacial acetic acid	11.5 ml
Distilled water	28.5 ml

The resulted solution is 3 M with respect to Potassium and 5 M with respect to acetate. Adjust pH to 5.5 by adding glacial acetic acid.

Luria Bertani (LB) Broth

Bact. Tryptone	1g
NaCl 1g	
Yeast Extract	0.5 gm

Distilled Water to make up to 100 ml. Adjust pH to 7.5 by NaOH. Sterilize by autoclaving

Luria Bertani (LB) Agar

LB Agar was prepared by adding 1.5% agarose to LB medium

SOB Medium (per 100 ml)

Bacto-Tryptone	2.0g
Yeast Extract	0.5 g
NaCl 100 mM	
KCl 2.5 mM	
$MgCl_2$	10 mM
$MgSO_4$	10 mM

SOB medium was prepared without Mg^{++} and autoclaved. After autoclaving 20mM Mg^{++} (By mixing equal amount of 1 M $MgCl_2$. $6H_2O$ and 1 M $MgSO_4$. $7H_2O$, 2 M Mg^{++} stock was prepared and sterilized by filtration) was added.

SOC Medium

SOB medium	980 ml
20 mM Glucose	20 ml

TSS (For 75ml)

30% PEG	25 ml
DMSO	3.75 ml
1M $MgCl_2$	4.5 ml
2x LB medium	37.5 ml
Distilled water	4.25 ml

Sterilized by filtration through 0.22 μ millipore filter and stored

at 4°C.

Ampicillin (100 mg /ml)

Ampicillin powder	100 mg
Sterile distilled water	1 ml

Sterilize by filtration and store at 4°C.

EDTA (Ethylene diamine tetra acetate) (0.5 M, pH 8.0)

EDTA18.61 gm

Distilled water	80 ml

Stir vigorously on magnetic stirrer to mix. Adjust pH to 8.0 and make the volume up to 100 ml. Autoclave and store at room temperature.

☑☑☑

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