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DNA EXTRACTION, AMPLIFICATION, AND DIRECT SEQUENCING FROM HORNBILL FEATHERS

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ABSTRACT

Two methods for extracting DNA from feathers for non-invasive multilocus genotyping of birds are described. DNA was amplified by the polymerase chain reaction and the double-stranded products were sequenced directly without the need for cloning. One method uses a single extraction step with Chelex as the extraction medium, thus reducing opportunities for contamination, and eliminating the need to use organic solvents and proteinases. It constitutes a simple, fast and relatively inexpensive method of DNA-level genotyping. Partial mitochondrial cytochrome b *sequences of 7 species of hornbill (Aves: Bucerotidae) and a warbler are reported to demonstrate the method's potential for phylogenetic and genealogical analyses involving free-ranging birds and museum specimens.*

INTRODUCTION

Molecular genetic methods have revolutionized studies of avian sociobiology, ecology, evolution, and systematics.[1,2] There are several methods for determining relationships between individuals, populations and species but until recently all relied on traditional methods of collecting whole birds, tissues or blood samples, storing them in liquid nitrogen or dry ice for transport from the field, and the large scale extraction of high molecular weight DNA. This applied to studies involving restriction fragment length polymorphism (RFLP) analysis of mitochondrial DNA (mtDNA),[3-6] DNA-DNA hybridization[7] and DNA minisatellite fingerprinting.[8-11] Although some recent studies[12-14] have made use of the polymerase chain reaction (PCR) to amplify DNA from smaller amounts of fresh blood or tissue, they still require the direct collection of such tissues, and many lengthy steps of DNA extraction, amplification, cloning, and sequencing multiple copies (which can take weeks) before reliable results are available. In this paper we describe an alternative non-invasive method of genotyping that avoids many of these problems.

At the Biodiversity Seminar held during the annual meeting of the Biology Section of the Science Society of Thailand in 1989 we demonstrated our ability to extract, amplify and directly sequence DNA from shed and plucked feathers.[15] Others have since reported similar success.[16-18] We here report our methods, which provide high quality DNA sequences in a couple of days (rather than weeks), from plucked or naturally shed feathers. This non-invasive feather-based procedure will greatly facilitate genetic studies of free-ranging birds, especially in countries where freezing samples in the field is logistically difficult. Stressful

bleeding and tissue biopsy are no longer necessary; in some cases it will be possible to genotype known individual birds without even capturing and handling them. For the purpose of this demonstration we describe the DNA sequence of a short region of the mitochondrial cytochrome *b* gene which contains numerous variable and phylogenetically informative sites.[19,20]

MATERIALS AND METHODS

Tissue samples

Feathers (primaries and secondaries) of four species of hornbills were collected from the forest floor beneath their nest sites in Khao Yai National Park, Thailand, during the course of ecological studies[21] and stored at ambient conditions. These species are great hornbill, *Buceros bicornis* (2 individual birds), wreathed hornbill, *Aceros [Rhyticeros] undulatus,* Oriental pied hornbill, *Anthracoceros albirostris,* and brown hornbill, *Anorrhinus [Ptiloaemus] tickelli austeni.* Tissues of three additional species were available from captive birds in the collection of the Zoological Society of San Diego: wrinkled hornbill, *Aceros [Rhyticeros] corrugatus* (from Indonesia), rhinoceros hornbill, *Buceros rhinoceros silvestris* (from Indonesia), and Abyssinian ground hornbill, *Bucorvus abyssinicus* (wild caught, Africa). For the wrinkled and rhinoceros hornbills, frozen muscle tissue obtained at necropsy, and for the ground hornbill, feathers, were available. For comparative purposes we also examined DNA from a 2 cm long primary feather of a yellow-browed leaf warbler, *Phylloscopus inornatus,* collected in Kashmir in 1988.

DNA extraction

DNA was successfully extracted from feathers between 1 and 3 years after they were collected using two different protocols. Our original method was to wash a feather tip with 70% ethanol and sterile water, finely chop 5-10 mm of the proximal end of the quill with a sterile razor blade, and digest the material with proteinase-K in a 500 μl volume (60 μg/ml proteinase-K, 50 mM KCl, 10 mM Tris pH 8.3, 2.5 mM $MgCl_2$, 0.1 mg/ml gelatin, 0.45% Nonidet P40, 0.45% Tween 20, 39 mM Dithiothreitol) for 4-12 hours at 56°C. The digest was centrifuged to pellet undigested material, and the supernatant transferred to a 1.5 ml microcentrifuge tube. Standard DNA purification procedures[22] of phenol/chloroform/ butanol extraction were performed followed by microfiltration with Millipore MC-30000 or Amicon Centricon 30 cartridges to desalt and concentrate the DNA to a final volume of about 30-50 μl.

Alternatively, we have developed a new method for DNA extraction from feathers which is much simpler, cheaper, and involves far fewer opportunities for DNA contamination. This method, modified from Singer-Sam et al.,[23] uses 5% Chelex (BioRad Laboratories) as the extraction medium, and allows rapid extraction without use of proteinase or organic solvents. The feather tip was washed as above and 5-10 mm of the proximal end removed from the rest of the feather. This portion of the feather was ground in a sterile mortar with about 10 ml of liquid nitrogen until powdered. The powder was transferred to a 0.6 ml silanized microcentrifuge tube containing 250 μl sterile 5% Chelex 100 (prepared with

sterile double-deionized water and then UV sterilized for 15 minutes each at 254 nm and 366 nm).[24] Samples were vortexed, incubated at 56°C for 7 hours, vortexed again, incubated at 100°C for 15 minutes, and centrifuged for 2 minutes at 15000 g in a microcentrifuge.

DNA amplification

A 340 base pair (bp) segment of the mitochondrial cytochrome *b* gene was amplified using Kocher's[19] conserved primers L14841 and H15149 without the added restriction sites. Either 1 μl of the DNA extracted by the first method, or 10 μl of the Chelex extract supernatant, was used for amplification, along with several negative controls using water or 5% Chelex instead of feather extract. The reaction conditions for a final volume of 25 μl were: 10 mM Tris, pH 8.3; 50 mM KCl; 0.01% gelatin; 1.5 mM $MgCl_2$; 0.1μg/μl BSA; 0.2 mM of each dNTP; 0.4 μM primer (L14841) (=10 pmol); 0.4 μM primer (H15149) (=10 pmol); and 1 unit AmpliTaq Taq polymerase (Perkin-Elmer Cetus Corp.). Forty cycles of 1 min at 92°C, 1 min at 55°C, and 30 sec at 74°C were performed in a Hybaid Thermal Reactor, after which amplified products were gel purified in 2% low-melt agarose. Each purified PCR product in agarose was melted into 250 μl sterile T.E., pH 8.0, and a 5 μl aliquot was subjected to 20 additional cycles of amplification in a volume of 50 μl. The final PCR product was purified using the GeneClean kit (BIO 101, Inc.), and re-suspended in approximately 25 μl T.E., pH 8.0.

DNA sequencing

Direct sequencing was carried out using a slight modification of the Sequenase (U.S. Biochemical) protocol. In a 0.6 ml microcentrifuge tube, 7.5 μl of double-stranded, purified PCR product was annealed to 100 pg of the H15149 sequencing primer by boiling for 5 min, followed immediately by quick freezing in an ethanol/dry ice bath. Primer/template tubes were centrifuged briefly, then placed on wet ice until the labeling mix with a-$dATP^{32}$ (3000 Ci/mMol) was added. The remainder of the sequencing protocol is as described in the Sequenase kit.

Sequence electrophoresis was carried out on 8% polyacrylamide/50% urea or 6% Long-Ranger (A.T. Biochem) polyacrylamide/50% urea 30 x 60 cm sequencing gels, at approximately 20-30 mA for 3-8 hours. Dried gels were exposed to autoradiography film for 16-72 hours.

Analysis

Gels were scored by eye and the sequences were entered into a Macintosh microcomputer for analysis. The sequences were aligned with respect to the published cyt *b* sequence of the chicken[25] and studied using PHYLIP, PAUP and MacClade software packages.[26,27] Parsimony analysis was performed using the heuristic search algorithm.

RESULTS

Large quantities of high quality mtDNA suitable for direct sequencing were produced after 40 cycles of amplification, or after 20 cycles when preceded by gel purification of the primary product. Sufficient DNA was extracted from each feather by either method to do at least 20 separate PCR reactions. Typical sequence autoradiographs are shown in Fig. 1. The Chelex method is preferred as it has a lower risk of amplifying non-sample DNA (e.g. cyt *b* of humans associated with the sample), the most common source of sequence contamination.

For the hornbills, the actual sequences in a 189 bp segment of cyt *b* for which data were available for all 8 individuals are shown in Table 1. A few sites could not be scored and are regarded as missing data (N). 37 (29.6%) of the 189 sites were variable in these hornbills and transitions out-numbered transversions 5:1. Although 86% of the observed substitutions occurred at generally silent third codon positions, it should be noted that there are 6 variable amino acid sites among the Asian species.

To illustrate how such data may be used in phylogenetic analyses we summarize the genetic differences between each pair of birds (Table 2) and present a rooted most parsimonious gene tree corrected for missing data (Fig. 2). The two great hornbills are nearly indistinguishable, differing by a single silent transition. Each of the two pairs of congeners cluster together, as expected, differing by 7-11 transitions and no transversions. The intergeneric distances are not clearly resolved with these very short sequences but the overall relationships suggested by this preliminary analysis are in close agreement with cladograms based on non-genetic data.[28] As expected, the African hornbill (Family Bucorvidae) sorts as an outgroup to the six Asian hornbills (Family Bucerotidae).

For the small *P. inornatus* feather, 200 bp (read from a single gel) were found to perfectly match a consensus sequence derived from multiple clones of the same region amplified from DNA extracted from muscle.[14]

DISCUSSION

Previous studies have shown that bird and mammal mitochondrial cytochrome *b* sequences are useful for phylogenetic studies.[12,14,17,19,20] For the purposes of this demonstration, we amplified a 340 bp region involving 3 transmembrane, one outer surface, and one inner surface domain between the Q_i and Q_0 redox centers.[29] These transmembrane regions contain numerous variable sites, in contrast to the more highly conserved redox sites.[20] Our determinations of these sequences in 7 hornbill species show that variation in this very short (1% of the mitochondrial genome) maternally transmitted fragment may be useful in elucidating phylogenetic relationships among Asian hornbill species. Studies of infraspecific variation will, however, have to be based on more variable sequences like the mtDNA control region (D-loop).[30,31]

The sequences presented were confirmed by duplicate determinations but must be regarded as preliminary until more individuals are studied. As 200-300 nucleotides were interpretable on a single gel, longer sequences should not be difficult to produce. Although such full sequences are far more informative, it is also possible to analyse the amplified

Table 1. Sequence of a 189-bp segment of the cytochrome *b* gene for 8 individual hornbills aligned with that of a chicken. Species names are given in the Methods. Abbreviations: ab g: Abyssinian ground hornbill; N: missing data.

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chicken    ACT TGC CGG AAC GTA CAA TAC GGC TGA CTC ATC CGG AAT CTC CAC GCA AAC GGC GCC

ab_g       ACA TGT CGC GAC GTA CAA TAC GGC TGA CTA ATC CGC AAC CTC CAT GCT AAC GGA GCC
great      ... ..C ... A.. ... ... ... ... ... ... ... ..T ... ... ... ..C ... ... ...
great      ... ..C ... A.. ... ... ... ... ... ... ... ..T ... ... ... ..C ... ... ...
wreathed   ... ..C ... A.. ... ..G ... ... ... T.. ... ..T ... ... ... ..C ... ... ...
pied       ... ... ... A.. ... ..G ... ... ... ... ..T ... ... ... ..C ..C ... ... ...
brown      ... ... ... A.. ... ... ... ... ... ... ..T ..T ... ... ..C ..C ... ... ...
wrink      ... ..C ... A.. ... ..G ... ... ... T.. ..T ... ... ... ... ..C ... ... ...
rhino      ... ..C ... A.. ... ... ... ... ... T.. ... ... ... ... ..C ..C ... ... ...


           TCA TTC TTC TTC ATC TGT ATC TTC CTT CAC ATC GGA CGA GGC CTA TAC TAC GGC TCC

           TCA TTA TTC TTT ATC TGC ATC TAC CTT CAC ATC GGA CGA GGA CTC TAC TAC GGA TCT
           ... ..C ... ..C ... ..T ... ..T ..G ... ..T ... ... ... ..T ... ..T ..G ..C
           ... ..C ... ..C ... ... ... ..T ..G ... ..T ... ... ... ..T ... ..T ..G ..C
           ... ..C ... ..C ... ... ... ... ..G ... ... ... ... ... ... ... ... ... ..C
           ... ..C ... ..C ... ... ... ... T.A ... ... ... ... ... ... ... ... ... ..C
           ... ..C ... ..C ..N ..N ... ... ..C ... ..T ..G ... ... ..T ... ... ... ..A
           ..G C.C ... ..C ... ... ... ... ..A ... ... ..G ..G ... ..T ... ... ..G ..C
           ... ..C ... ..C ... ..T ... ..T ..A ... ..T ... ... ... ..T ... ... ... ..C


           TAC CTC TAC AAG GAA ACC TGA AAC ACA GGA GTA ATC CTC CTC CTC ACA CTC ATA GCC

           TAC CTC TAN AAA GAA ACC TGA AAC ACA GGC ATC ATC CTT CTN NTC ACA CTC ATA GNN
           ... ... ..C ... ... ... ... ... ... ... ... G.. ..C ..A C.. ..C ... ... N.A
           ... ... ..C ... ... ... ... ... ... ... ... G.. ..C ..A C.. ..C ... ... NCA
           ... ... ..C ... ... ... ... ... ... ... ... ... ..C .NN ... ..C ..A ... N..
           ... ... ..C ... ... ... ... ... ..G ... ... ... ..C ..A C.. ..C ... ... .CA
           ... ... ..C ..G ... ... ..G ... ..G ... ... ... ..C ..G ... ..C ... ... NCA
           ... ... ..C ... ... ... ... ... ... ... ... ... ..C ..A C.. ..C ..A ... .CA
           ..T ... ..C ... ... ... ... ... ... ... ... ... ..C ..A C.. ..C ... ... .CA


           ACC GCC TTT GTG GGC TAT

           NCC GCC TTC GTA GGC TAN
           A.T ..T ... ... ... ..C
           A.T ..T ... ... ... ..C
           N.A ... ... ... ... ..T
           A.. ... ... ..G ... ..T
           A.T ... ... C.. ... ..T
           A.A ... ... ... ... ...
           A.T ... ... ... ... ..T
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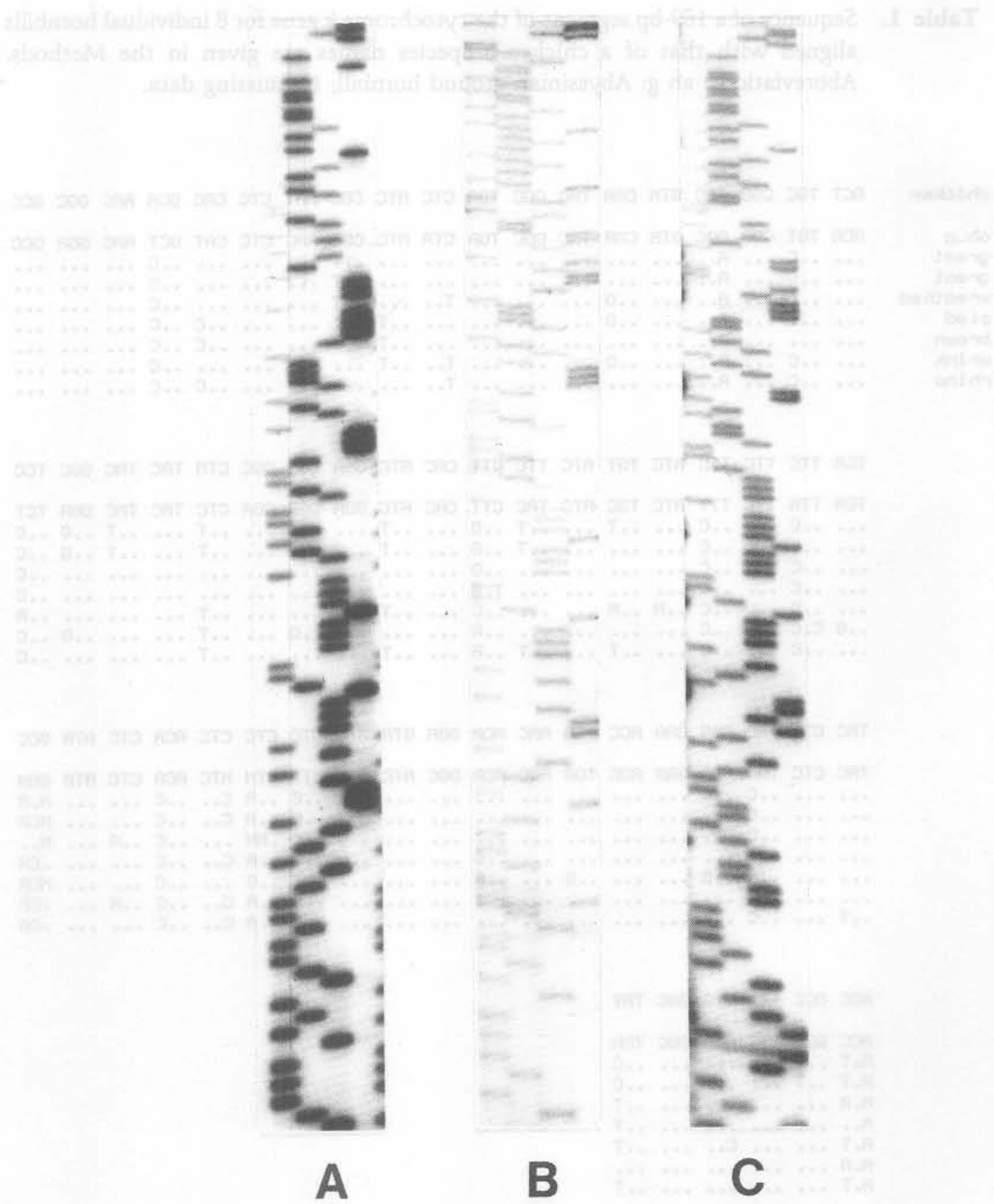

Fig. 1. DNA sequence of a portion of the mitochondrial cytochrome *b* gene. A) DNA obtained using a standard method for extraction from frozen muscle tissue of a wrinkled hornbill (*Rhyticeros corrugatus*). B) DNA extracted from a feather of a great hornbill (*Buceros bicornis*) using the original extraction technique (see text). C) DNA extracted from a yellow-browed leaf warbler (*Phylloscopus inornatus*) feather using the Chelex method.

Table 2. Pairwise comparisons of sequence divergence. Transitional substitutions below the diagonal, transversional substitutions above.

	1	2	3	4	5	6	7	8
1-great	-	0	1	0	4	2	0	3
2-great	1	-	2	0	4	2	0	3
3-wreated	11	11	-	2	5	0	2	5
4-pied	18	17	9	-	3	2	0	3
5-brown	12	11	11	10	-	5	4	4
6-wrinkled	16	15	7	10	13	-	2	5
7-rhino	10	11	10	12	10	10	-	3
8-ab. g.	16	15	10	11	14	15	14	-

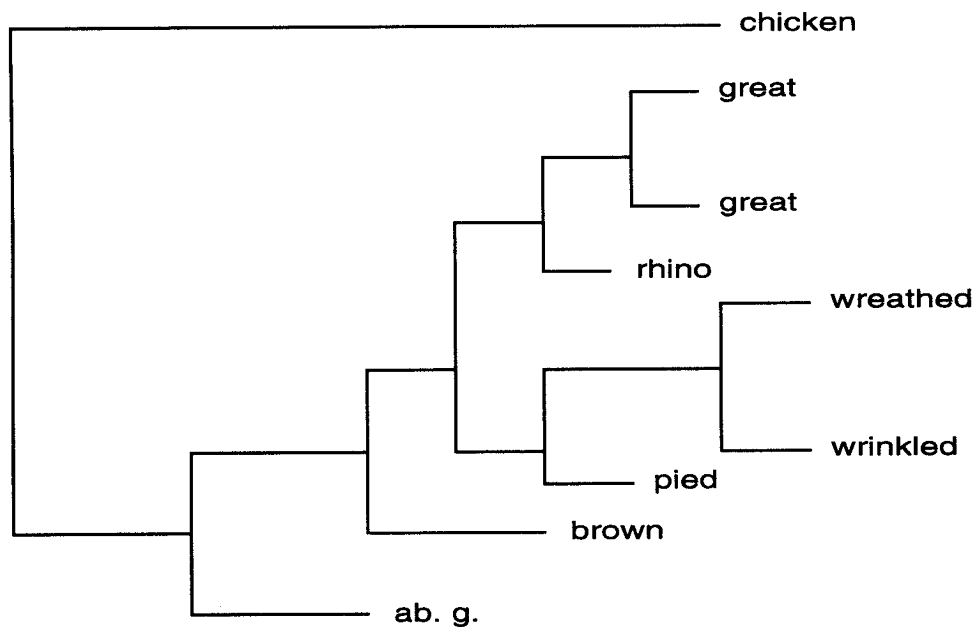

Fig. 2. Most parsimonious tree relating 8 individual hornbills based on the cytochrome *b* sequences in Table 1. Branch lengths are proportional to the inferred number of base substitutions.

DNA using traditional RFLP methods.[32] Furthermore, the sequences constitute a database which can be expanded as more individuals are studied in this and other laboratories anywhere in the world. This gives sequence data a significant advantage over multilocus DNA fingerprints as the latter are difficult to interpret genetically, especially when individuals are not run side-by-side on the same gel.

Primers are now available for numerous other mitochondrial gene sequences of birds including the control region. As different mitochondrial regions evolve at different rates, investigators can select sequences whose variability is sufficient to answer the questions of concern. Primers are also available for some avian nuclear genes[33,34] and others can be developed by traditional methods: cloning and sequencing of fragments from genomic libraries.[32] One group of nuclear loci that offer the greatest promise for resolving questions regarding pedigrees, population structure and gene flow are the hypervariable simple sequence repeats (SSRûs, sometimes referred to as microsatellites). These loci comprise mono-, di-, tri-, or tetranucleotide repeats and, because they are dispersed randomly throughout the chromosomes, play a pivotal role in various genome mapping projects. Ellegren[35] has recently shown that SSR's occur in birds and they are expected to be quickly employed in numerous avian sociobiological studies. As several mitochondrial and nuclear loci can be amplified from the same feather it is now possible to acquire multilocus genetic data that can be used to test hypotheses at the population, regional and species levels. The power of this hierarchical approach is well illustrated by recent studies of free-ranging chimpanzees based on DNA derived non-invasively from hair.[36-38]

Our experience does not put a lower limit on the size of the feather required. In an on-going study of loggerhead shrikes, *Lanius ludovicianus,* we are finding plucked 15 mm body feathers contain enough DNA for most analyses. In many cases it is therefore now possible to use feathers recovered without handling birds at all. Certainly, plucking and storing 2-4 feathers should become a routine part of bird-banding programs and will eventually permit longitudinal studies. In addition, museum specimens may be useful sources of DNA for studies of historical population genetic variation and rare and/or endangered species.[18,40]

These technical developments permit rapid multilocus avian genotyping using feathers as the source of DNA. In 1994, U.S. laboratories equipped for DNA extraction, amplification and sequencing are typically spending about U.S. $30 for chemicals and expendable supplies per 200-300 bp determination, after protocol optimization. It is important to note, however, that the optimization phase of an investigation of a previously unstudied species/locus is still very expensive and can take many months. A number of books are now available for the reader seeking additional information on the use of the PCR and the analysis of molecular genetic data.[32,40-43]

Of the animal phyla in Thailand, birds are probably the best known; their taxonomy and geographic distributions are reasonably well documented[44] and some groups like the hornbills have been the subject of intensive ecological and behavioral research.[21,45,46] Globally, studies of birds have resulted in major contributions to many areas of biology including our understanding of the evolution of species, behavioral traits, mating systems and other social behaviors including altruism.[47] The Thai avifauna of over 900 species presents many opportunities for basic or fundamental research and the non-invasive genotyping methods

described above will make it easier for Thai scientists to contribute to the next great advance in avian biology, which will be based on studying the genetics of free-flying birds. It will also permit wildlife managers to monitor and conserve the genetic variability remaining in recently isolated and now threatened populations that are restricted to forest fragments.[48-50]

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บทคัดย่อ

การวิจัยนี้ได้บรรยายถึงวิธีสกัด DNA จากขนนก เพื่อใช้ในการศึกษา non-invasive multilocus genotyping ของนกไว้สองวิธี DNA ที่สกัดได้แล้วนั้น จะถูกนำมาผ่านปฏิกิริยาที่เรียกว่า PCR (Polymerase Chain Reaction) เพื่อเพิ่มจำนวนของ double-strand DNA ซึ่งจะนำไปศึกษาโครงสร้างลำดับนิวคลีโอไทด์ โดยวิธี direct sequencing โดยมิต้องผ่านขั้นตอนการเลี้ยงเซลล์ของวิธีการ cloning การใช้ Chelex เป็นสารสกัด DNA เป็นหนึ่งในสองวิธีการที่ใช้ในการสกัด DNA จากขนนก วิธีการนี้ช่วยลดโอกาสของการเกิดการปนเปื้อน (contamination) และช่วยตัดขั้นตอนของการใช้สารอินทรีย์ และเอนไซม์โปรตีเอส จึงนับได้ว่าวิธีการนี้เป็นหนึ่งในวิธีที่ง่าย รวดเร็ว และค่อนข้างประหยัดวิธีหนึ่งในการศึกษาระดับ DNA genotyping การวิจัยนี้ได้รายงานโครงสร้างลำดับนิวคลีโอไทด์ของ cytochrome b ซึ่งเป็นส่วนหนึ่งของ DNA ในไมโตรคอนเดรีย ของนกในวงศ์นกเงือก (Family Bucerotidae) 7 สปีชีส์ และของนกในวงศ์นกกระจิ๊ด (Genus Phylloscopus) ไว้ 1 สปีชีส์ รวมทั้งได้แสดงวิธีการใช้ข้อมูลนี้ในการศึกษาความสัมพันธ์ทางวิวัฒนาการในระดับสปีชีส์ (phylogeny) และความเป็นไปได้ในการศึกษาความสัมพันธ์ทางวิวัฒนาการในระดับครอบครัว (genealogy) ของนกทั้งที่มีชีวิตและที่มีตัวอย่างอยู่ในพิพิธภัณฑ์