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HYBRIDISATION IN THE WILD BETWEEN THE GREAT HORNBILL (*BUCEROS BICORNIS*) AND THE RHINOCEROS HORNBILL (*BUCEROS RHINOCEROS*) IN THAILAND AND ITS GENETIC ASSESSMENT

Siriphatr Chamutpong
Department of Biochemistry, Faculty of Science, Mahidol University, Rama 6 Road, Bangkok, 10400, Thailand

Mathurose Ponglikitmongkol
Department of Biochemistry, Faculty of Science, Mahidol University, Rama 6 Road, Bangkok 10400, Thailand
Email: mathurose.pon@mahidol.ac.th (Corresponding author)

Wutthipong Charoennitikul
Department of Biochemistry, Faculty of Science, Mahidol University, Rama 6 Road, Bangkok 10400, Thailand

Sitthichai Mudsri
National Park, Wildlife and Plant Conservation Department, National Park and
Wildlife Research Division, Natural Resource Conservation Office, Bangkok 10900, Thailand

Pilai Poonswad
Department of Microbiology, Faculty of Science, Mahidol University, Rama 6 Road, Bangkok 10400, Thailand

ABSTRACT. — The great hornbill (*Buceros bicornis*) and the rhinoceros hornbill (*B. rhinoceros*) are among the largest Asian hornbill species and they overlap in parts of their ranges. These two species resemble each other in appearance and breeding habits and are sister taxa. In 2004 and 2008, two occurrences of mating in the wild between a male rhinoceros hornbill and a female great hornbill were discovered in the forests of Budo Mountain, southern Thailand. These mated pairs each successfully raised a chick. Morphological and phenotypic characteristics of the chicks most resemble the great hornbill with a few distinctive features shared between both hornbills. Genetic data confirmed these incidences of hybridisation. Analysis of mitochondrial DNA in the hypervariable control region III of these two chicks showed similar patterns to those of their mother, the great hornbill. An investigation of parentage, using 11 microsatellite loci developed from the great hornbill, indicated that the chicks shared at least one allele with the putative mother at all loci. Three different alleles, specific for rhinoceros hornbill, were also detected in the chicks. This suggests that the female great hornbill and male rhinoceros hornbill were the true parents of these hybrid chicks, and that the chicks shared the same mother. Our results is the first report of hybridisation between the great hornbill and the rhinoceros hornbill in the wild , and has been genetically confirmed.

KEY WORDS. — *Buceros bicornis*, *Buceros rhinoceros*, hornbill, hybridisation, genetic assessment

INTRODUCTION

When two different species interbreed or hybridise, interspecific hybrids are formed (Grant & Grant, 1992; Schwenk et al., 2008). Although such an event is uncommon, it has raised a crucial concern for taxonomic judgments (Short, 1969) and population studies, since the hybrids may become reproductively isolated from both parent species and eventually give rise to a new species (Charlesworth, 1995). Interspecific hybridisation may result from: (1) small population size or low population density, which reduces the chance of females finding a mate (Quader, 2005), and if the range of the two populations overlaps and the unavailability of a conspecific mate means that the individual chooses a heterospecific mate (Randler, 2002; Aliabadian & Nijman, 2007; Lengagne et al., 2008); (2) habitat fragmentation and alteration, which might break down the female mate-choice mechanisms (Randler, 2002); and (3) a failure in female mate recognition (Pierotti & Annett, 1993; Randler, 2002; Lengagne et al., 2008).

In 1992, Grant and Grant showed that hybridisation is prevalent in bird orders, with about 10% known to have interbred in nature and produced interspecific hybrids.

However, no hybridisation between hornbill species, which belong to the order Bucerotiformes, has been reported in the wild. In 2006, McCarthy re-classified some taxonomic subspecies as full species, and reported the incidences of hybridisation in the order Bucerotiformes to be 16% (Aliabadian & Nijman, 2007).

The consequences of hybridisation can be positive or negative. They can lead to an increase in genetic diversity, which is essential for individual adaptability and formation of new combinations of genes that may be beneficial in new environments (Pierotti & Annett, 1993; Grant & Grant, 1996). However, hybrids are commonly supposed to have lower fitness and be mostly sterile, because they are produced from two different parental genetic backgrounds that may not be compatible (Grant & Grant, 1992; Wirtz, 1999; Mallet, 2007).

In this study, the occurrence of two instances of interspecific mating between pairs of sympatric female great hornbills (*Buceros bicornis*) and male rhinoceros hornbills (*Buceros rhinoceros*) were discovered in the forests of Budo Mountain, a part of Budo-Sungai Padi National Park, Thailand. These are sister species in the order Bucerotiformes, resembling each other in appearance, breeding behaviour and genetics (DNA-DNA hybridisation and phylogenetic study of cytochrome *b*) (Kemp, 1995; Viseshakul et al., 2011). Both species have a mainly black plumage, a white tail with black band across the center and a large casque (Poonswad, 1993a). There is an area of overlap in the ranges of the species in southern Thailand and Myanmar, the northern Malay Peninsula and Sumatra, but the great hornbill extends north to India and southern China and the rhinoceros hornbill south to Java and Borneo (Kemp, 1995). Females are generally expected to choose their potential mates from superior courtship displays, which are similar in both species with duet calling and head-raised postures. In both species, the female hornbill has to trust her mate for his care during imprisoning herself inside the nest tree cavity while laying eggs and brooding the young chicks (Tsuji, 1996). The female generally emerges from the nest before the chick fledges and then helps her mate feed their chicks until they are virtually independent, when their casques begin to develop (Kemp & Poonswad, 1993). In Thailand, *Buceros* hornbills have played an important role in the ecological system by acting as large-seed dispersal agents. Great hornbills have been evaluated as Near Threatened, inhabiting several parts of the country, while rhinoceros hornbills are Endangered and inhabit only the southern part (Poonswad, 1993a; Round, 2008).

Hybridisation has been reported for African hornbills in captivity (Kemp, 1995), definitely for at least the Southern red-billed *Tockus erythrorhynchus rufirostris*/Southern yellow-billed *T. leucomelas*, and in the wild for the species/races *T. e. rufirostris/T. e. damarensis*. Mating between female great and male rhinoceros hornbills were recorded previously in captivity at the Neopark Okinawa, Japan (Takaki, 1996). In Thailand, the first two interspecific pairings between these different species in the wild were observed in 2004 and later in 2008, producing a hybrid offspring on each occasion. The first chick was discovered when a female great hornbill left the nest cavity to help a male rhinoceros hornbill feed the chick inside the nest cavity and was flying around the nest. Four years later, the second chick was found at a different nest site 4 km away with a female great hornbill still in the nest and a male rhinoceros hornbill feeding the female and chick inside the cavity. It was observed that the old nest site had already been occupied by a great hornbill pair. We then postulated that these chicks resulted from the hybridisation between these two different hornbill species. We asked the question whether the female great hornbill and the male rhinoceros hornbill, the suspected parents, were the true parents of the chicks or not. Since the interspecific hybridisation in nature was not random, we speculated that two hybrids were formed from the same breeding pair. In this study, we used molecular techniques to clarify these questions using genetic data and reported the first interspecific hybridisation between the great hornbill and the rhinoceros hornbill in the wild.

MATERIAL AND METHODS

Sample collection and DNA extraction. — Blood samples were obtained only from the suspected interspecific chick in 2004 at the forests of Budo Mountain, a part of the Budo-Sungai Padi National Park, and from the chick and the female great hornbill attending the 2008 nest 4 km from the first site and outside the National Park (Fig. 1). No sample was obtained from either parent in 2004 or from the male rhinoceros hornbill in 2008. The blood was maintained in Longmire's lysis buffer (Longmire et al., 1997) and stored at –20°C. Genomic DNA was extracted using QIAamp DNA blood Mini Kit (QIAGEN) according to the manufacturer's protocol. We obtained all samples in compliance with the standards for animal care and use established under the ethical guidelines and policies of Mahidol University, the Office of the National Research Council of Thailand, and the Department of National Park, Wildlife and Plant Conservation (permit reference 0938/14041).

Mitochondrial DNA sequencing. — Amplification of hypervariable control region III (CR III) of the mitochondrial DNA (mtDNA) was performed with DNF1 (5'-CTGATGCACTTTGCTTCGCATTTGGT-3') and tPhe (5'-CCGTCTTGGCATCTTCAGTGCCA-3') as forward and reverse primers respectively. These primers were designed from consensus sequences of the mitochondrial regions of eight hornbill species; *Tockus erythrorhynchus damarensis* (GenBank accession no. AY027932), *T. e. rufirostris* (AY027928), *T. leucomelas* (AY027931), *T. e. kempi* (AY027927), *Bucorvus leadbeateri* (HM640209), *Aceros corrugatus* (HM755883), *A. waldeni* (HQ834450) and *Penelopides panini* (HQ834451). Polymerase chain reaction (PCR) was carried out in the 25 µl reaction mixtures containing 50 ng genomic DNA, 1× PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl), 2 mM $MgCl_2$, 10% betaine (Sigma), 200 µM of each dNTP, 0.4 µM of each primer, and 0.5 U *i-Taq*™ DNA polymerase (iNtRON Biotechnology) in a thermocycler (MJ Research, Watertown, USA). The reaction profile consisted of initial denaturation at 94°C for 5 min followed by 30 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 1 min; and a final extension at 72°C for 10 min.

PCR products were separated on 1% agarose gel in 0.5× TBE, pH 8.0. The excised DNA fragments were purified using QIAquick Gel Extraction kit (QIAGEN) and inserted into the pGEM-T Easy plasmid (Promega), following the manufacturer's instructions. Plasmid ligations were then transformed into *Eschericia coli* cells (DH5α) and grown in LB broth containing 100 µg ml^{-1} ampicillin. Plasmid DNA was then purified using QIAprep Spin Miniprep Kit (QIAGEN) and sequenced by T7 and SP6 primers using the ABI PRISM big dye terminator sequencing chemistry, version 3.1 (Applied Biosystems), and run on an ABI3730XL DNA automated analyser (Applied Biosystems). For confirmation, both strands of inserted DNA fragments from at least 2 recombinant clones were sequenced.

Microsatellite profile analysis. — To verify parental species and reveal the relationship of the two interspecific hybrids, a total of 11 microsatellite markers (*Bbi*2, *Bbi*3, *Bbi*7, *Bbi*13, *Bbi*16, *Bbi*32, *Bbi*38, *Bbi*40, *Bubi*144, *Bubi*294, and *Bubi*342), which were previously isolated from the great hornbill, were used for genotyping analysis (Chamutpong et al., 2009). PCR amplifications were performed with primers of which only reverse primers of each primer pair were fluorescently labelled at the 5' end. The reactions were conducted with an appropriate PCR condition as described previously (Chamutpong et al., 2009). The amplified microsatellite loci were analysed on ABI3730XL sequencer using Genescan 400 HD and 500 LIZ labelled size standards. Genotypes were scored using the Peak Scanner software version 1.0 (Applied Biosystems).

RESULTS

Characteristics of the interspecific hybrids. — The hybrid chicks appear to share most of their morphological characteristics with chicks of both the great hornbill and the rhinoceros hornbill (Table 1, Fig. 2). They both had the greyish blue iris, yellow tarsus and undeveloped casque that are common to both great and rhinoceros hornbill chicks. Their face, chin, upper throat and forehead are also black, while their vent and thigh are also white, with a long white tail crossed by a black band similar to hornbill juveniles of both species. However, the top of upper and lower mandibles of the hybrids were stained a yellow colour, unlike those of both great and rhinoceros hornbills that are stained an orange colour. Some phenotypic characters appear to be intermediate (i.e., the creamy white plumage on the head and neck), but others resemble the great hornbill more than the rhinoceros hornbill (i.e., the white feather tips on the upper-wing [primaries, secondaries and tertials] and the white band at base of the under-wing feathers).

Maternal species inferred from hypervariable control region III of mitochondrial DNA. — Analysis of the hypervariable control region III (CR III) of mitochondrial D-loop revealed that the great hornbill could be distinguished from the rhinoceros hornbill at the molecular level by differences in the DNA sequences of tandem repeats in this region (Charoennitikul et al., in prep.). The great hornbill contained perfect arrays of core 11-bp nucleotide sequences whereas the rhinoceros hornbill had a core of 23 (Fig. 3). Based on this dissimilarity, we used CR III sequences to verify the maternal species of the two suspected interspecific hybrid chicks.

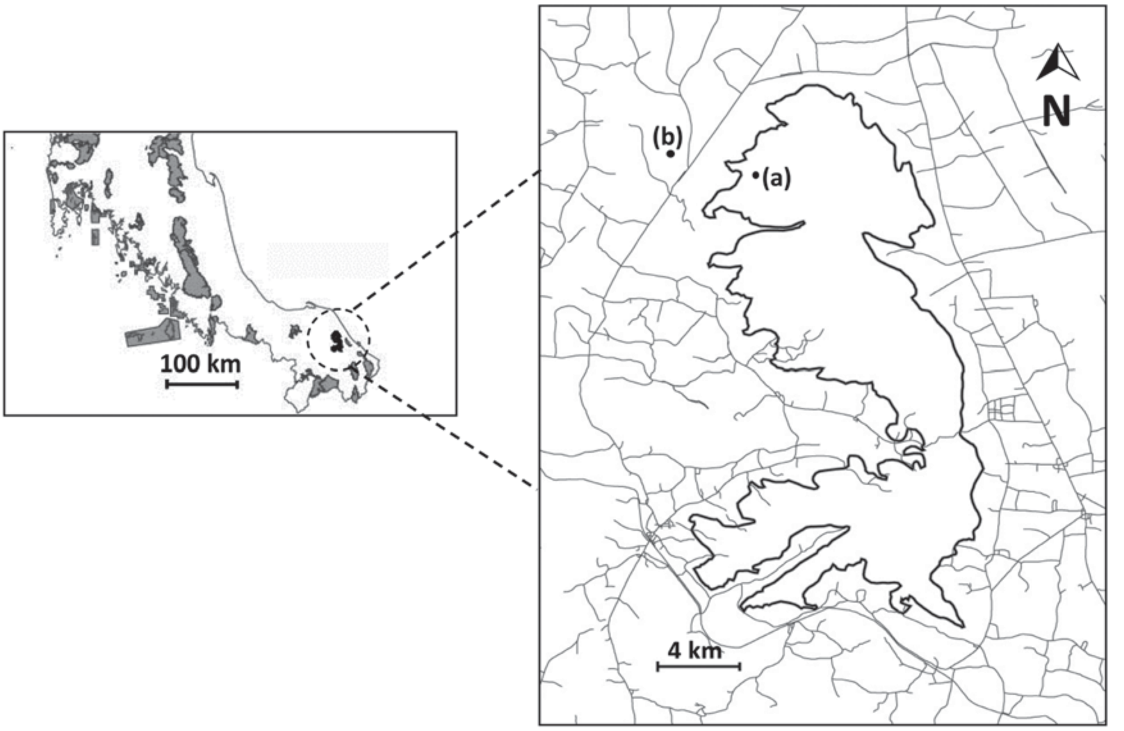

Fig. 1. Capture locations of two interspecific hybrids and a mother great hornbill in 2004 at Budo Mountain within the National Park (a) and in 2008 at 4 km from the National Park (b).

Table 1. Comparative morphological descriptions of the chicks of the great hornbill and the rhinoceros hornbill, and their suspected hybrid chick.

	Hybrid chick	**Great hornbill (GH) chick**	**Rhinoceros hornbill (RH) chick**
1. Bill & casque	– No casque visible (a, b) – Top of upper mandible stained with yellow* (b) unlike GH, RH; BRIGHT ORANGE (h, n) – Upper and lower mandibles; WHITE but stained with yellow* (b) resemble GH, RH (h, n)	– No casque visible (g, h) – Top of upper mandible; BRIGHT ORANGE (h) – Upper and lower mandibles; WHITE but stained with yellow* (h)	– No casque visible (m, n) – Top of upper mandible; BRIGHT ORANGE (n) – Upper and lower mandibles; WHITE but stained with yellow* (n)
2. Plumage	– Face, chin, upper throat, forehead; BLACK (a, b) with black lined on the crown (b) resemble GH (h) but proportionally larger band (b) – Head, neck; CREAMY WHITE (a, b) resemble GH (g, h) – Under part; BLACK resemble GH, RH – Vent and thigh; WHITE (c) resemble both GH (i) and RH (o) – Tail; WHITE with BLACK band across (f) resemble GH, RH (l, r) – Upperwing; WHITE wing tip (d) resemble GH (j) but partial white tip on upperwing coverts (d) unlike GH (j) – Underwing; WHITE band at basal underwing feather and white wing tip resemble GH (e)	– Face, chin, upper throat, forehead; BLACK (g, h) with black lined on the crown (h) – Head, neck; CREAMY WHITE (g, h) – Under part; BLACK – Vent and thigh; WHITE (i) – Tail; WHITE with BLACK band across (l) – Upperwing; WHITE wing tip (j), tip on upperwing coverts; WHITE (j) – Underwing; WHITE band at basal underwing feather and white wing tip (k)	– Face, chin, upper throat, forehead; ENTIRELY BLACK (m, n) – Head, neck; BLACK (m,n) – Under part; BLACK – Vent and thigh; WHITE (o) – Tail*; WHITE with BLACK band across (r) – Upperwing; ENTIRELY BLACK (p) – Underwing: ENTIRELY BLACK (q)
3. Iris	GREYISH BLUE (b) resemble GH, RH (h, n)	GREYISH BLUE (h)	GREYISH BLUE (n)
4. Tarsus	YELLOW (c) resemble GH, RH (i, o)	YELLOW (i)	YELLOW (o)

*From oil secreted by uropygial gland at tail base

Sequence analysis of the mitochondrial CR III region showed differences in number of core repeats and therefore nucleotide sequences among three individual hornbills, two interspecific hybrids and their suspected mother, the female great hornbill. The two individual hybrids had length variation in the CR III region due to different numbers of tandem repeats. The 2004-hybrid contained 26 repeats of 11 bp core sequences whereas the 2008-hybrid and a female great hornbill showed the same core sequences of 28 repeats. The variation in the number of repeats in the two hybrids might result from PCR slippage (Schlötterer, 2000). However, the sequences of tandem repeats of the two hybrids are identical. Most of the sequences of CR III tandem repeats for both hybrids matched those of the great hornbill sequence with a high degree of similarity, except for some point mutations (C to T) found at the 19 and 21 repeats in the 2004-hybrid and 2008-hybrid respectively. This result suggested that the female great hornbill was the maternal species of both hybrids. We cannot certify if C to T mutations in the hybrids occurred from DNA replication errors as the results were reproducible in our independent clonings and sequencings.

Parentage analysis by microsatellite genotypic profile. — We further explored for the relationship between the two hybrid chicks and their suspected mother using microsatellite genotypic profiles. Analysis of the genotypic profiles using 11 polymorphic microsatellite loci isolated from the great hornbill revealed that each hybrid shared one of its alleles for each locus with the female great hornbill (Table 2). These indicated that both hybrids descended from the same maternal parent, the great hornbill. Together with the CR III sequencing data, it confirmed that the female great hornbill was the genetic mother of these two interspecific hybrids.

Genotyping profiles of these loci in 20 other great hornbills examined confirmed the presence of these alleles in great hornbills, albeit with some variability in lengths of microsatellite repeats (Chamutpong et al., 2009). Examination of the genotypes in five rhinoceros hornbill individuals using these 11 loci revealed that 8 of 11 loci could not be used to differentiate between the two hornbills, since they provided common alleles with similar size ranges. The exceptions were for *Bbi*13, *Bbi*32, and *Bubi*294 that were detected as rhinoceros-specific alleles (Table 1). Given the lack of DNA samples from the male rhinoceros hornbill that was the putative father of the chicks, we cannot confirm whether he was the true father. However, the three specific alleles present in the rhinoceros hornbills but not detected in the great hornbill, an allele of 134 bp at the *Bbi*13 locus in the 2008-hybrid and of 341 bp at the *Bbi*32 locus in the 2004-hybrid, and an allele of 164 bp at the *Bubi*294 locus in both hybrids, were observed. These data indicate that a rhinoceros hornbill was the genetic father of these two interspecific hybrids. The presence of some distinct alleles in the two hybrids indicates that either they received different alleles from their father or that the two fathers of the hybrids were not the same individuals. However, if we assume that hornbills generally pair for life and that hybridisation does not occur randomly, we speculate that these two hybrids probably arose from the same aberrant breeding pair of a female great and male rhinoceros hornbill.

DISCUSSION

Hornbills are as vertebrates in the class Aves, historically in the order Coraciiformes (Wetmore, 1960) and more recently in the Bucerotifomes of the super-class Bucerotes (Kemp, 1995; Mayr, 2011). Based on their morphology, biology, and behaviour, hornbills are divided into two distinct families Bucorvidae and Bucerotidae. The family Bucerotidae consists of eight genera, six (*Aceros*, *Anorrhinus*, *Anthracoceros*, *Buceros*, *Ocyceros*, and *Penelopides*) of which inhabit Asia while two (*Ceratogymna* and *Tockus*) live in Africa (Kemp, 1995, Poonswad, 1993a). The great and rhinoceros hornbills are members of the *Buceros* genus, the species of which are very similar in characters such as the horn-like shape of the casque and its distinct colours (Poonswad, 1993a). The great hornbill has a yellow casque that points straight forward at its two outer points whereas the rhinoceros hornbill has a red casque that is curved upwards at the tip. An additional distinct difference between these two hornbill species is the pattern on their wings: the great hornbill has white greater wing coverts, usually stained yellow, whereas the rhinoceros hornbill has entirely black wings (Poonswad, 1993a). Interestingly, they have overlapping breeding seasons, beginning around the first half of the year, and similar nest cavity characteristics and biparental care of nestlings (Poonswad, 1993b). The great hornbill is adapted to a wider range of habitats compared to the rhinoceros hornbill, since as it has the widest range from the Western India through Nepal, Bhutan, Bangladesh and South China to Southeast Asia (Kemp & Poonswad, 1993). The rhinoceros hornbill, on the other hand, has more restricted habitats from southern peninsular Thailand and Myanmar, Malaysia, Indonesia and Brunei (Birdlife International, 2009).

Based on similarities of morphology and behaviour, the great hornbill has always been classified as the species closest to but distinct from the rhinoceros hornbill (Sanft, 1960; Kemp, 1995). However, the phylogenetic analysis of the cytochrome b DNA sequences using either Neighbor Joining (NJ) or Maximum Parsimony (MP) method could not separate these two species since they shared 100% homology of the DNA sequences and were always in the same cluster (Viseshakul, 2011). We report here that the pattern of the repetitive sequences in Domain III of the mitochondrial control region of these two hornbills were similar but not identical. The repeat unit found in the great hornbill seemed to be a subset of that in the rhinoceros hornbill. Moreover, 11 nuclear microsatellite DNA markers isolated from the great hornbills were able to amplify the genomic DNA from

Table 2. Comparison of genotypic profiles among the female great hornbill and two hybrid chicks using 11 microsatellite markers. Bold letters indicate unique alleles of the rhinoceros hornbill.

	Genotype										
	Bbi2	***Bbi3***	***Bbi7***	***Bbi13***	***Bbi16***	***Bbi32***	***Bbi38***	***Bbi40***	***Bubi144***	***Bubi294***	***Bubi342***
Great hornbill F	206/230	127/131	193/193	152/160	168/168	353/353	134/161	270/270	230/230	160/172	151/151
2004–hybrid	206/226	131/135	193/198	148/152	168/174	**341**/353	110/134	255/270	206/230	160/**164**	151/161
2008–hybrid	206/230	131/135	193/198	**134**/152	168/168	353/383	110/134	255/270	206/230	160/**164**	151/151

Fig. 2. Comparative features among the chicks of the hybrid (I and a–f), the great hornbill (II and g–l), the rhinoceros hornbill (III and m–r), and adult males and females of both species (A–C). Descriptions of morphological features of the hybrid chick and chicks of the great hornbill and the rhinoceros hornbill were given in Table 1. Please note that the female great hornbill and the male rhinoceros hornbill in II and III are not the real hybrid parents.

rhinoceros hornbills and we identified rhinoceros hornbill-specific alleles from three microsatellite markers (*Bbi*13, *Bbi*32 and *Bubi*294) (Chamutpong et al., 2009, unpublished data). Therefore, DNA sequences from both the mitochondrial domain III and the nuclear microsatellite regions might be used to genetically differentiate the rhinoceros hornbill from the great hornbill.

The close relationship between great and rhinoceros hornbills in their morphology and genetics has allowed their cross hybridisation to occur successfully in both the wild and the zoo. Although, hybridisation between these two closely related hornbill species was first reported in captive birds in Japan in 1996, the occurrences of interspecific mating between these two different hornbill species in the wild was first discovered in the forests of Budo Mountain, Thailand, in 2004 and 2008. These aberrant matings were suspected to have formed two hybrid chicks, whose specific relationship was of interest.

Our genetic analyses provided the supporting evidence that two unsexed hybrid chicks arose from the same interspecific breeding pair of the female great and male rhinoceros hornbills. Since mtDNA is clonally maternally inherited, mitochondrial CR III sequences were examined for maternal testing. Interestingly, tandem repeat sequences found in each hybrid chick showed an almost perfect match with an 11-bp core sequence of the female great hornbill, their suspected mother, confirming the maternal genetic parent of both hybrids. However, our most surprising result was the finding of mixed type of C and T nucleotides in both hybrids. The 3' terminal units of tandem repeats in the hybrids contained T nucleotides like parts of the repeats in rhinoceros hornbills (Fig. 3) whereas the female great hornbill contained the pure C type of nucleotides at the 10th nucleotide position of the 11-bp repeat units. The cause of C to T mutations in the same repeat units of both hybrids was not fully understood. We have examined the possibility of DNA replication errors during the PCR reaction by sequencing several independent clones from independent PCR reactions using the female's great hornbill DNA as well as those of other great hornbill samples and did not find such mutations.

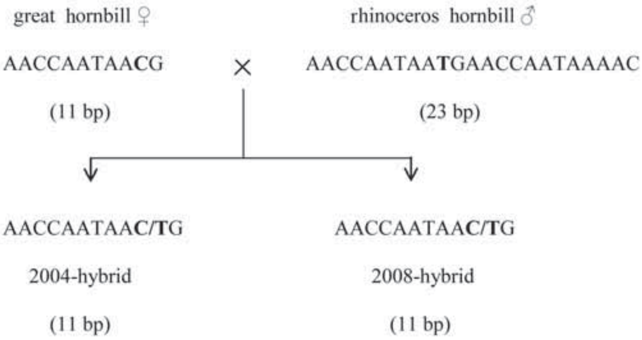

Fig. 3. Nucleotide sequences of core tandem repeats of hypervariable mitochondrial control region III (CR III) in four hornbill individuals; the female great hornbill (GU560189), the male rhinoceros hornbill (GU560192), and two interspecific hybrids (GU560190 for 2004-hybrid and GU560191 for 2008-hybrid). Underlined sequence shows the core sequence of 11 bp in 23 bp tandem repeats in the rhinoceros hornbill that is similar to that of the great hornbill. Bold letters indicate nucleotide sequences at 10th nucleotide position of 11 bp core sequences in the four birds.

The occurrence of mitochondrial recombination between maternal and paternal mtDNA could result in mitochondrial heteroplasmy in the hybrids. Mitochondrial heteroplasmy is widely recognised in plants (maize: Yamato & Newton, 1999; wheat: Hattori et al., 2002; Gynodioecious: Pearl et al., 2009) and animals (mice: Meirelles & Smith, 1997; bird: Crochet & Desmarais, 2000; Kvist et al., 2003; fish: Hoarau et al., 2002; Guo et al., 2005). It could be generated by mutation, recombination, or paternal leakage (Kmiec et al., 2006; White et al., 2008). Paternal leakage occasionally generated heteroplasmy when the paternal mtDNA recognition and elimination failed during fertilisation. The loss of discrimination of paternal mtDNA followed by recombination of homologous sequences of the maternal mtDNA (Morris & Lightowlers, 2000; Rokas et al., 2003) could give rise to mitochondrial recombination. However, it was difficult to understand that recombination alone would cause the mixed C/T array since full core repeats (23 bp) of CR III of rhinoceros hornbills were not observed in these hybrids. Another possible cause of these mixed types of repeats in individual hybrids reported here might be from point mutations. Defective DNA-repair was also shown to cause point mutations, deletions, and variation in tandem repeats (Kmiec et al. 2006). Yet, it was less likely that point mutations could repeatedly generate identical types and positions of mutations from C to T nucleotides in the hybrid offspring. Analyses of the mtDNA control region in a flatfish population demonstrated the existence of a variable number of tandem repeats and high level of heteroplasmy in *Platichthys flesus* (Hoarau et al., 2002). Most flatfish individuals carried a pure C or T array, but one showed two C to T point mutations in core repeats resulting in a compound C/T array. It was suggested to be the effect of a recombination mechanism that was supported by the C/T array, as discovered in each of three independent DNA extraction, PCR, and sequencing reactions from individual recombinants. However, if recombination occurred in the hybrid hornbills as observed in flatfish, a T type of mtDNA molecule might be present in the great hornbill, at undetectable levels, or, less likely, obtained from the rhinoceros hornbill. Contamination between samples is unlikely to occur since the microsatellite patterns obtained could still differentiate the two hybrids.

The variation in number of tandem repeats in CRIII, which was observed in the 2008-hybrid and its suspected mother, was believed to result from slipped strand mispairing during mtDNA replication or homologous recombination within individuals, as has been widely suggested (Rokas et al. 2003).

Codominant inheritance of microsatellite markers, which are widely used in pedigree analysis, were chosen to clarify the family relationships. Genotyping data obtained from 11 polymorphic microsatellite loci showed that each hybrid shared one of its alleles at all loci with the same suspected maternal trait, the great hornbill. This finding verified that both hybrids originated from the same maternal parent. Moreover, three unique alleles which presented in rhinoceros hornbills were detected in both hybrids, confirming that a rhinoceros hornbill was their paternal genetic parent.

Lacking the data of the male rhinoceros hornbill made it impossible to definitely identify their paternal parent. However, considering that hornbills usually pair for life (Tsuji, 1996; Kinnaird & O'Brien, 2007), we suspected that the hybrids might have belonged to the same father.

Hybridisation in bird species arises from many causes. One commonly accepted cause is failure in mate choice recognition of females, which are commonly recognised as the choosy sex in birds (Randler, 2002; Quader, 2005). In general, most females prefer to mate with males of particular phenotypic traits (Jennions & Petrie, 1997; Hebets, 2003; Godin et al., 2005). The great and the rhinoceros hornbills resemble one another in their overall body size, appearance, bill and plumage, with similar sexually dimorphic eye colours and black markings on the bill and casque, but with lesser differences in casque shape, colour and size, and in the extent of white on the wing feathers. This similarity may be one explanation for an error by the female great hornbill in mate choice decisions.

The pristine tropical rainforest of Budo Mountain has been reduced in area and quality, and currently encounters serious problems of heavy encroachment and conversions to fruit and Para rubber plantations. The change in hornbill habitat patterns could subsequently have affected the ranging of populations of these two sympatric species, so that spatial limitation of habitat may restrict mobility and force them to live closer together and share flocking sites. Chaisuriyanan (2005) found that Great and rhinoceros hornbills in Budo-Sungai Padi National Park have similar breeding cycles, with great hornbills generally starting only one week earlier than rhinoceros hornbills. These overlapping breeding seasons, territories and food resources would give them more chances to meet one another. It is also possible that a female unable to locate a mate of its own species would decide to mate heterospecifically with a male rhinoceros hornbill, due to his similar courtship displays. Furthermore, great hornbills have been observed to react strongly to play-back of tape recording of rhinoceros hornbills during a study on the effect of vocal/auditory contact at Rotterdam Zoo (Galama et al., 2002). Therefore, all these characters combined suggest that their reproductive biology was compatible and might explain the interspecific mating between these two different bird species on Budo Mountain.

Genetic compatibility or genetic similarity of the potential mates between different species is considered to be another important factor for determination of successful hybridisation (Gill, 1998; Sherman et al., 2008). A phylogeny of Asian hornbills proposed by Kemp (1993) using combination data of feather lice (Elbel, 1976), cladistic analysis (Kemp, 1988), and DNA-DNA hybridisation (Sibley & Monroe, 1990) supported a single clustering node of Great and rhinoceros hornbills as sister species. Moreover, our genetic data on CR III of the mitochondrial DNA of these two birds revealed similar sizes and DNA sequences. This information implies that Great and rhinoceros hornbills might have a common ancestor and coevolved with genetic similarity to support successful hybridisation. We concluded that the intrinsic genetic similarity, together with the decreasing number of the hornbills and the extrinsic pressure from habitat changes surrounding the Budo Mountain, may affect hornbill mating behaviour and allow interbreeding between these two *Buceros* species to occur.

Although the interbreeding between two sympatric *Buceros* species has formerly occurred in captive hornbills (Takaki, 1996), this is the first report for hybridisation in wild hornbills. Interestingly, the occurrences took place twice in four years and produced two survived hybrids in the vicinity of Budo Mountain. It is worth noting that the cross-species pairing of hornbills may last for several years and the production of hybrid offspring from these pairings could have unexpected consequences. It is not known if the hybrid birds will have altered behaviour patterns that could affect their ability to survive and subsequently to mate and produce offspring. In addition, the feeding behaviour of the hybrid birds may change, which may have adverse impacts on the ecosystem. Therefore it is of special interest to understand the biological concepts of interbreeding and to follow the survival and evolution of the hybrids. The fertility and continuation of these emerged hybrids would imply that the Great and the rhinoceros hornbills originated from a common ancestor and diverged along with the accumulation of mutations. However, if these hybrids are infertile or suffer reduced viability, and so cannot continue mating, it is most likely that these two hornbills are truely different species.

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LITERATURE CITED

Aliabadian, M. & V. Nijman, 2007. Avian hybrids: Incidence and geographic distribution of hybridization in birds. *Contributions to Zoology*, **76**: 59–61.

Birdlife International, 2009. *IUCN Red List for Birds.* http://www.birdlife.org.

Chaisuriyanun, S., 2005. *A Comparative Study of Diets of Great Hornbill (*Buceros bicornis*) and Rhinoceros Hornbill (*Buceros rhinoceros*) During the Breeding Season in Budo Su-Ngai Padi*

National Park, Southern Thailand. MSc thesis, King Mongkut's University of Technology Thonburi, Bangkok. 89 pp.

Chamutpong, S., D. S. Saito, N. Viseshakul, I. Nishiumi, P. Poonswad & M. Ponglikitmongkol, 2009. Isolation and characterization of microsatellite markers from the Great Hornbill, *Buceros bicornis*. *Molecular Ecology Resources*, **9**: 591–593.

Charlesworth, D., 1995. Hybrid speciation: Evolution under the microscope. *Current Biology*, **5**: 835–836.

Crochet, P. A. & E. Desmarais, 2000. Slow rate of evolution in the mitochondrial control region of gulls (Aves: Laridae). *Molecular Biology and Evolution*, **17**: 1797–1806.

Elbel, R. E., 1976. *Bucerocophorus*, a new genus of ischnoceran Mallophaga from African hornbills (Bucerotidae). *Proceedings of the Biological Society of Washington*, **89**: 313–323.

Galama, W., C. King & K. Brouwer, 2002. *EAZA Hornbill Management and Husbandry: Guidelines***.** The EAZA Hornbill TAG, Amsterdam. 94 pp.

Gill, F. B., 1998. Hybridization in birds. *Auk*, **115**: 281–283.

Godin, J. J., E. J. Herdman & L. A. Dugatkin, 2005. Social influences on female mate choice in the guppy, *Poecilia reticulata*: Generalized and repeatable trait-copying behavior. *Animal Behavior*, **69**: 999–1005.

Grant, P. R. & R. B. Grant, 1992. Hybridization of bird species. *Science*, **256**: 193–197.

Grant, P. R. & R. B. Grant, 1996. Speciation and hybridization in island birds. *Philosophical Transactions of the Royal Society London B*, **351**: 765–772.

Guo, X., S. Liu & Y. Liu, 2005. Evidence for recombination of mitochondrial DNA in triploid Crucian carp. *Genetics*, **172**: 1745–1749.

Hattori, N., K. Kitagawa, S. Takumi & C. Nakamura, 2002. Mitochondrial DNA heteroplasmy in wheat, *Aegilops* and their nucleus-cytoplasm hybrids. *Genetics*, **160**: 1619–1630.

Hebets, E. A., 2003. Subadult experience influences adult mate choice in an arthropod: Exposed female wolf spiders prefer males of a familiar phenotype. *Proceedings of the National Academy of Sciences of the USA*, **100**: 13390–13395.

Hoarau, G., S. Holla, R. Lescasse, W. T. Stam & J. L. Olsen, 2002. Heteroplasmy and evidence for recombination in the mitochondrial control region of the flatfish *Platichthys flesus*. *Molecular Biology and Evolution*, **19**: 2261–2264.

Jennions, M. D. & M. Petrie, 1997. Variation in mate choice and mating preferences: A review of causes and consequences. *Biological Reviews of the Cambridge Philosophical Society*, **72**: 283–327.

Kemp, A. C., 1993. Conservation of Asian hornbills and their habitats: An introduction. In: Poonswad, P. & A. C. Kemp (eds.), *Manual to the Conservation of Asian Hornbills*. Hornbill Project, Bangkok. Pp. 4–24.

Kemp, A. C., 1995. *The Hornbills*. Oxford University Press, New York. 302 pp.

Kemp, A. C., 1988. The systematics and zoogeography of Oriental and Australasian hornbills (Aves: Bucerotidae). *Bonner Zoologische. Beiträge*, **39**: 315–345.

Kemp, A. C. & P. Poonswad, 1993. Life history of great hornbill *Buceros bicornis*. In: Poonswad, P. & A. C Kemp (eds.), *Manual to the Conservation of Asian Hornbills*. Hornbill Project, Bangkok. Pp. 100–114.

Kinnaird, M. F. & T. G. O'Brien, 2007. *The Ecology and Conservation of Asian Hornbills: Farmers of the Forest***.** The University of Chicago Press, Chicago. 352 pp.

Kmiec, B., M. Woloszynska & H. Janska, 2006. Heteroplasmy as a common state of mitochondrial genetic information in plants and animals. *Current Genetics*, **50**: 149–159.

Kvist, L., J. Martens, A. A. Nazarenko & M. Orell, 2003. Paternal leakage of mitochondrial DNA in the great tit (*Parus major*). *Molecular Biology and Evolution*, **20**: 243–247.

Lengagne, T., S. Plenet & P. Joly, 2008. Breeding behaviour and hybridization: Variation in male chorusing behaviour promotes mating among taxa in waterfrogs. *Animal Behavior*, **75**: 443–450.

Longmire, J. L., M. Maltbie & R. J. Baker, 1997. Use of "lysis buffer" in DNA isolation and its implication for museum collections. *Occasional Papers, Museum of Texas Tech University*, **163**: 1–4.

Mallet, J., 2007. Hybrid speciation. *Nature*, **446**: 279–283.

Meirelles, F. V. & L. C. Smith, 1997. Mitochondrial genotype segregation in a mouse heteroplasmic lineage produced by embryonic karyoplast transplantation. *Genetics*, **145**: 445–451.

Morris, A. A. & R. N. Lightowlers, 2000. Can paternal mtDNA be inherited? *Lancet*, **355**: 1290–1291.

Mayr, G., 2011. Metaves, Mirandornithes, Strisores and other novelties: A critical review of the higher-level phylogeny of neornithine birds. *Journal of Zoological Systematics and Evolutionary Research*, **49**: 58–76.

Pearl, S. A., M. E. Welch & D. E. McCauley, 2009. Mitochondrial heteroplasmy and paternal leakage in natural populations of *Silene vulgaris*, a gynodioecious plant. *Molecular Biology and Evolution*, **26**: 537–545.

Pierotti, R. & C. A. Annett, 1993. Hybridization and male parental investment in birds. *Condor*, **95**: 670–679.

Poonswad, P., 1993a. Identification of Asian hornbills. In: Poonswad, P. & A. C. Kemp (eds.), *Manual to the Conservation of Asian Hornbills*. Hornbill Project Thailand, Bangkok. Pp. 26–75.

Poonswad, P., 1993b. Aspect of the biology and ecology of some Asian hornbills. In: Poonswad, P. & A. C. Kemp (eds.), *Manual to the Conservation of Asian Hornbills*. Hornbill Project Thailand, Bangkok. Pp. 76–97.

Quader, S., 2005. Mate choice and its implications for conservation and management. *Current Science***, 89**: 1220–1229.

Randler, C., 2002. Avian hybridization, mixed pairing and female choice. *Animal Behavior*, **63**: 103–119.

Rokas, A., E. Ladoukakis & E. Zouros, 2003. Animal mitochondrial DNA recombination revisited. *Trends in Ecology and Evolution*, **18**: 411–417.

Round, P. D., 2008**.** *Check-list of Thai Birds up to Nov.2006*. Bird Conservation Society of Thailand, Bangkok. http://www.bcst.or.th/data/checklist2006.xls.

Sanft, K., 1960. Bucerotidae (Aves/Upupae). *Das Tierreich*, **76**: 1–176.

Schlötterer, C., 2000. Evolutionary dynamics of microsatellite DNA. *Chromosoma*, **109**: 365–371.

Schwenk, K., N. Brede & B. Streit, 2008. Introduction: Extent, processes and evolutionary impact of interspecific hybridization in animals. *Philosophical Transactions of the Royal Society B Biological Sciences*, **363**: 2805–2811.

Sherman, C. D., E. Wapstra, T. Uller & M. Olsson, 2008. Males with high genetic similarity to females sire more offspring in sperm competition in Peron's tree frog *Litoria peronii*. *Proceedings of the Royal Society B*, **275**: 971–978.

Short, L. L., 1969. Taxonomic aspects of avian hybridization. *Auk*, **86**: 84–105.

Sibley, C. G. & B. L. Monroe, 1990. *Distribution and Taxonomy of Birds of the World*. Yale University Press, New Haven & London. 1,111 pp.

Takaki, Y., 1996. Hornbills in Japan. *EEP Hornbill Taxon Advisory Hornbill Newsletter*, **2**: 34–35.

Tsuji, A., 1996. *Hornbills: Master of Tropical Forests*. Sarakadee Press, Bangkok. 94 pp.

Viseshakul, N., W. Charoennitikul, S. Kitamura, A. Kemp, S. Thong-Aree, Y. Surapanpitak, P. Poonswad & M. Ponglikitmongkol, 2011. A phylogeny of frugivorus hornbills links to the evolution of Indian plants with Asian rainforests. *Journal of Evolutionary Biology*, **24**: 1533–1545.

Wetmore, A., 1960. *A Classification for the Birds of the World*. Smithsonian Institution. 37 pp.

Wirtz, P., 1999. Mother species-father species: Unidirectional hybridization in animals with female choice. *Animal Behavior*, **58**: 1–12.

White, D. J., J. N. Wolff, M. Pierson & N. J. Gemmell, 2008. Revealing the hidden complexities of mtDNA inheritance. *Molecular Ecology*, **17**: 4925–4942.

Yamato, K. T. & K. J. Newton, 1999. Heteroplasmy and homoplasmy for maize mitochondrial mutants: A rare homoplasmic *nad4* deletion mutant plant. *Journal of Heredity*, **90**: 369–37.