

## A Simple Method to Extract Mitochondrial DNA in a Non-invasive Phylogenetic Study of Domestic Native Thai Ducks

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### ABSTRACT

A simple low-cost method for extracting mitochondrial DNA (mtDNA) from a single plucked feather for a non-invasive genetic study of ducks is described. The mtDNA was isolated via alkaline lysis, and after neutralization, the crude DNA-containing lysate was used directly for PCR. We have had a 100% (30/30) success in amplification of a 710-bp fragment of the mtDNA control D-loop region. Sequence analysis and phylogenetic relationship of the 667-bp D-loop segments of the two domestic native Thai ducks, Nakorn-Pathom (NP) and Park-Nam (PN), was further investigated. Two breeds exhibited 100% identity over the entire 667-bp products, although several types of mtDNA polymorphism were detected. Comparing the 667-bp D-loop sequence data with those of *Anas* ducks and Muscovy duck used as an outgroup showed that both native Thai ducks and Mallard (*Anas platyrhynchos*) haplotype A clustered together (bootstrap support 91%), indicating that there was common ancestor for those breeds. Here we report a quick, simple, reliable and inexpensive DNA extraction procedure for a duck's feather. Our findings clearly demonstrate that the domestic native Thai ducks derive their origin from the Mallard group A haplotypes.

**Key words:** alkaline extraction, *Anas platyrhynchos*, Mallard, mtDNA, phylogenetics

### INTRODUCTION

Most domestic ducks of the family Anatidae was originated from a common ancestry, Mallard (National Research Council, 1991). The Mallard (*Anas platyrhynchos*) is extraordinarily adaptable and can be found almost anywhere in the world. Hybridization with Mallard has been implicated in the conversion of several native duck species into the Mallard's range since its aggressive nature and ability to hybridize well with other *Anas* species with giving fertile offsprings

(Peters *et al.*, 2005). However, the history of the Mallard in Thailand and its impact on native ducks is little documented.

The Department of Livestock Development (DLD), Ministry of Agriculture and Cooperatives stated that there are two common species of the domestic native Thai ducks, Nakorn-Pathom (NP, breeder population = 275) and Park-Nam (PN, breeder population = 470). It has been assumed that both the native Thai duck species have a close relative of the Mallard. They share similar morphology and behavior with the Mallard,

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but have varying feather coloration that allows them to be distinguished from one another. The color pattern of NP breed is much similar to the Mallard. Female NP ducks have a mottled light brown body, a grey bill and orange legs. Males have a brown and grey body, a grey bill and a dark-brown breast. The head and neck are green and neck ring is white. Legs and feet are orange. Both sexes have dark brown speculum (wing patch) on the wing. The PN duck is considerably smaller in body size than the NP duck. This breed is easily distinguished from the Mallard and NP. Both sexes have a dark-brown to black body, a black bill and legs. Male PN ducks have a bright green head and neck coloration, while females have a black head. Both sexes have blackish-brown wings with no speculum.

During the last 60–70 years, however, numbers of the native Thai ducks have been reduced by the introduction of exotic duck breeds for commercial farming. Currently, it appears that PN and NP ducks are found nowhere else in Thailand, except those conserved by the Animal Husbandry Division, DLD, and one farm in Nakorn-Pathom. A little information is available for the history and biogeography of the ducks, and there is no record regarding the origin and the genetic relationships between those two breeds.

The recent advent of molecular techniques, such as PCR and automatic sequencing, has revolutionized the studies of avian ecology and evolution (Morin *et al.*, 1994). One approach to identifying genetic relationships between birds is by comparing their mitochondrial DNA (mtDNA) (Hebert *et al.*, 2004). Constructing phylogenetic trees from the control region or displacement loop (D-loop) in duck mtDNA have been published (Donne-Gousse *et al.*, 2002; Peters *et al.*, 2005).

For bird species, feathers are common tissue samples for DNA isolation since collection of feathers is easy, causes less pain to animals and one feather is enough for the isolation of mtDNA

used in PCR (Taberlet and Bouvet, 1991). To obtain a success of phylogenetic studies based on DNA sequence data, it is necessary to isolate good quality DNA. Although, simple and efficient DNA extraction protocols are currently available; most of the methods are time-consuming, require a high level of skill and expensive. Recently, a success in using an alkaline digestion for isolating mtDNA from feathers for large-scale sex typing of ostriches was reported (Malagó *et al.*, 2002). It appeared that an alkaline extraction is a fast, safe, accurate and inexpensive procedure for DNA extraction from feathers.

Here, we reassessed using the alkaline strategies of DNA extraction that we were able to isolate the mtDNA from a single duck's feather and used it directly as template in PCR reactions. We also examined sequences of the mtDNA control region from individuals representing the two domestic native Thai ducks, and conducted sequence comparisons of these DNA fragments for a preliminary evaluation of phylogenetic relationships within the genus *Anas* (dabbling ducks).

## MATERIALS AND METHODS

### Specimens and DNA extractions

Ten animals (five for each breed; Nakorn-Pathom, NP and Park-Nam, PN) representing the two domestic native Thai ducks from 2 locations (Prachin Buri province and Nakhon Pathom province) in Thailand were used for mtDNA analysis. A single feather was plucked from the breast of alive ducks using sterile forceps, cut about 3–5 mm segment from the root end (bulb) of individual feathers, placed into a 1.5 ml microcentrifuge tube containing 70% ethanol and stored at 4°C until DNA extractions were conducted. Full precautions were taken to prevent contaminating between the samples. Specifically, DNA extractions and PCR amplifications were conducted in a laboratory where pre- and post-

PCR works are physically isolated and the use of aerosol-resistant pipette tips in all pre-PCR steps. A feather bulb was removed from 70% ethanol, washed 2 times with 1X PBS pH 7.4, then DNA was extracted from the pulp cells inside each of the feather quill in three ways. Protocol 1: DNA was extracted using a commercial kit (QIAamp® DNA Mini Kit, Qiagen) according to the manufacturer's protocols. Protocol 2: the calamus was placed in 1.5 ml microcentrifuge tube with 300 µl digestion buffer (20 mg/ml proteinase K, 10% sodium dodecyl sulfate (SDS) and 3M sodium acetate (pH 5.2) and heated at 56°C for 1 h. Then, the samples were extracted twice to standard phenol/chloroform/isoamyl alcohol (25:24:1) extraction and ethanol precipitation and dissolved in 100 µl distilled water. Protocol 3: DNA isolation was performed according to Malagó *et al.* (2002). A feather bulb was submerged in 20 µl of 0.2 N NaOH at 75°C for 20 min in a water bath. To neutralize the solution, 180 µl of a 0.04 M Tris-HCl pH 7.5 was added. Then, the DNA was either extracted twice with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1), precipitated with ethanol and dissolved in 50 µl DNase-free water, or the supernatant (n = 30) was used directly as the DNA template in the PCR reaction.

### PCR amplification, cloning and sequencing

The target region (5' portion of the mtDNA control region: positions 79-773 in the chicken (*Gallus gallus*) mtDNA (Desjardins and Morais, 1990)) was amplified from the total DNA extracts using the polymerase chain reaction (PCR) with the primers L78: 5'-GTTATTTGGTTATGC ATATCGTG-3' (Sorenson and Fleischer, 1996) and H774 5'-CCATATACGCCAACCCTCTC-3' (Sorenson *et al.*, 1999). PCR was carried out in a GeneAmp 9700 PCR System thermocycler (Applied Biosystems) using 50 µl reaction mixture containing 1 µl template DNA, 1X PCR buffer, minus Mg<sup>2+</sup> (Invitrogen), 1.5 mM MgCl<sub>2</sub>, 2mM

each dNTP, 0.2 µM each primer, and 1U platinum taq DNA polymerase (Invitrogen). Thermal cycling was as follows: 7 min preheat at 94°C, followed by 45 cycles of 30 sec at 94°C, 30 sec at 52°C, 1 min at 72°C, and final extension of 7 min at 72°C. The PCR products were electrophoresed on 0.8% low-melting agarose gels, stained with ethidium bromide and the image was acquired on the KODAK Electrophoresis Documentation and Analysis System 290 (EDAS 290, Eastman Kodak).

In order to perform the sequencing reactions the 710-bp PCR band in the agarose gel was purified by QIAquick gel extraction kit (Qiagen). The purified product was ligated into pCR®4-TOPO® plasmid (Invitrogen). The plasmid was transformed into the competent *E.Coli* TOP10 cells (Invitrogen) and the transformants were screened for ampicillin resistance. Twenty antibiotic resistance colonies (two colonies from each animal, n = 10) were picked and amplified in LB media at 37°C overnight. The recombinant plasmid containing a 710 bp fragment of the duck control region DNA was obtained by alkaline plasmid miniprep kit (Qiagen). After verifying by EcoRI restriction enzyme and agarose gel electrophoresis, the plasmid was submitted for sequencing in both directions at the KU-VECTOR custom DNA synthesis service, Kasetsart University. Both ends were sequenced on an ABI 377 automated sequencer using a dideoxy chain terminator method (Applied Biosystems). The sequences have been deposited in GenBank (accession numbers: EU013948-EU013957).

### Data analysis

The 667-bp fragment of the resulting sequences (710-bp PCR products subtracting the size of the forward L78 (23-bp) and reverse H774 (20-bp) primer, respectively) were compared and aligned using ClustalW program (Thompson *et al.*, 1994). DnaSP 4.0 (Rozas *et al.*, 2003; <http://www.ub.es/dnasp>) was used to estimate population

haplotype diversity (h), nucleotide diversity ( $\pi$ ), and mean number of pairwise differences (K). Tests of neutral evolution: Tajima's D (Tajima, 1989), were also computed using the same program. Genetic variation within and among population of the 2 duck breeds was evaluated by the Analysis of Molecular Variance in Arlequin 3.11 (Excoffier *et al.*, 2005; <http://cmpg.unibe.ch/software/arlequin3>). The Tamura-Nei model was selected as the best-fit model using Findmodel (Posada and Crandall, 2001; <http://hcv.lanl.gov/content/hcv-db/findmodel/findmodel.html>). Since there was no significant genetic difference among the 2 breeds, all 10 sequences were assembled using CAP3 (Huang and Madan, 1999); <http://bioweb.pasteur.fr/seqanal/interfaces/cap3.html>). The consensus outcome, a representative of the native Thai ducks, and other ducks in genus *Anas* were used to build a phylogenetic tree. The tree was constructed based on the 667-bp mtDNA control (D-loop) region and using neighbor-joining analysis in MEGA 3.1 (Kumar *et al.*, 2004). The Tamura-Nei distance was applied (Tamura and Nei, 1993). Bootstrap support for internal nodes was calculated using 1,000 replicates. GenBank accession numbers for the 667-bp mtDNA D-loop control region sequences are as follows: *Anas platyrhynchos* haplotype A (AY928897) and haplotype B (AY928900), *Anas zonorhyncha* haplotype A (AY506952) and haplotype SB (AY506953), *Anas fulvigula* (AF382649), *Anas rubripes* (AF382426), *Anas strepera* (DQ449422), *Anas acuta* (AY112939), *Anas bahamensis* (AY112940), *Anas clypeata* (AY112941), *Anas crecca* (AY112942), *Anas sibilatrix* (AY112943),

*Anas americana* (AY881739), *Anas penelope* (AY881775), and *Anas falcata* (EF537675). Muscovy duck (*Cairina moschata*) (AY112952) was used as an outgroup.

## RESULTS

### Evaluation of DNA yields

For each method tested, the quality of genomic DNA from a single plucked feather in the extracts was analyzed on an electrophoresis gel and visualized with ethidium bromide (data not shown). The quality of DNA obtained by a commercial kit (QIAamp<sup>®</sup> DNA Mini Kit, Qiagen) was greater than the recoveries obtained using the proteinase K/SDS and the alkaline method. Although a comparison of DNA yield from each method was not achieved due to the different numbers of the pulp and blood cells in each feather sample, a photometer was used to measure the quantity/purity of DNA yield according to the standard A260/280 protocol, and the results are shown in Table 1.

### PCR amplification after DNA extraction

The quality of mtDNA yields was evaluated using the PCR amplification. It was evaluated first whether the mtDNA extract obtained by each of the three methods was clean enough to yield PCR amplification. Each DNA extract was used as a template for PCR amplification, using the primer pair, L78/ H774, that targeted the duck mtDNA control region. Ten  $\mu$ l of each PCR sample was electrophoresed on a 0.8% agarose gel and stained with ethidium

**Table 1** Comparison of DNA yields and purity obtained by three extraction methods.

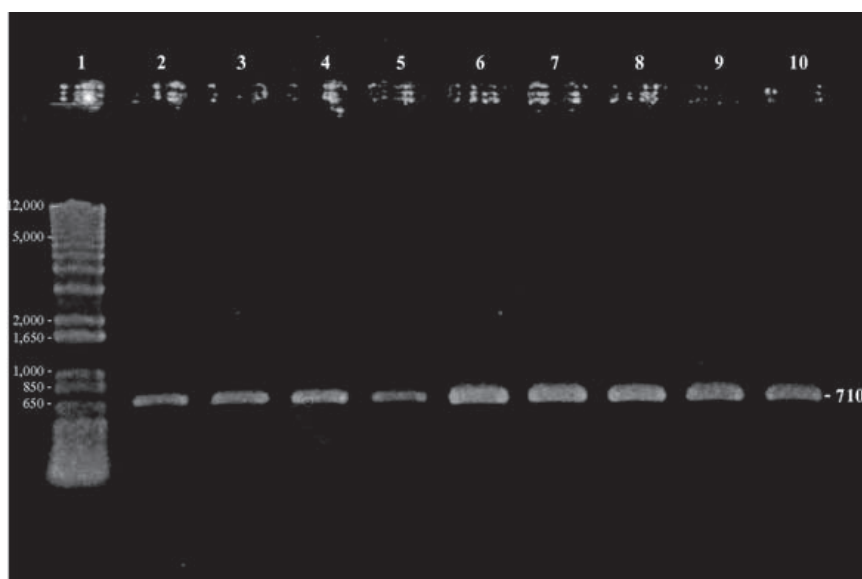
Extraction method	Purity of DNA <sup>a</sup> (A260/A280)	DNA concentration <sup>a</sup> ( $\mu$ l/ml)
QIAamp DNA Mini kit (n=5)	2.03 $\pm$ 0.74	198 $\pm$ 78.23
Proteinase K/SDS <sup>b</sup> (n=10)	1.56 $\pm$ 0.26	300 $\pm$ 204.45
Simple alkaline extraction <sup>b</sup> (n=10)	1.42 $\pm$ 0.30	132 $\pm$ 62.21

<sup>a</sup> mean  $\pm$  SD

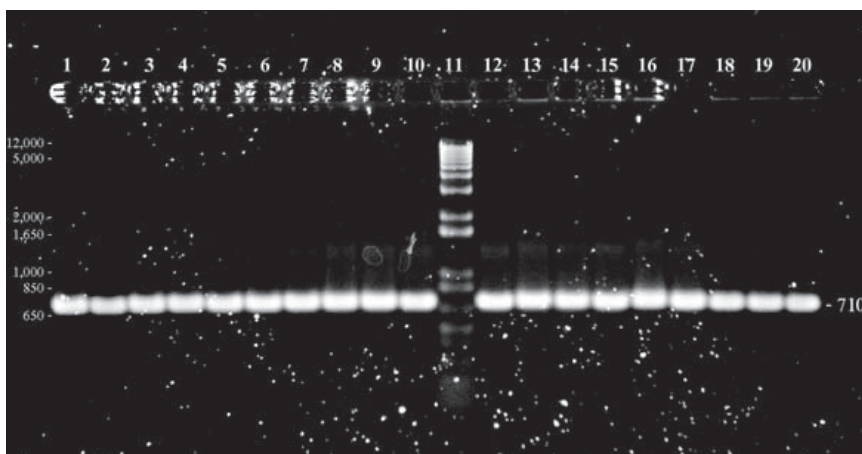
<sup>b</sup> followed by phenol/chloroform/isoamyl alcohol (25:24:1) extraction

bromide. The results revealed that all three extraction techniques exhibited a correct size of PCR product at the same length (710 bp), indicating that the alkaline method used in the experiment yielded adequate quantity and quality of mtDNA templates for PCR analysis (Figure 1).

To determine whether the hazardous chemicals could be reduced from the alkaline extraction method, the phenol/chloroform/isoamylalcohol steps were excluded prior to PCR. As shown in Figure 2, mtDNA prepared according to the alkaline extraction technique with or without the



**Figure 1** Ethidium bromide-stained 1% agarose gels showing PCR amplification of a 710-bp fragment of mtDNA control region. Templates for PCR assays were extracted by means of three methods: QIAamp<sup>®</sup> DNA Mini kit (lanes 2-3), protenase K/ SDS extraction (lanes 4-5) and alkaline lysis (lanes 6-10). Lane 1 was 1Kb Plus Ladder (Invitrogen).



**Figure 2** Ethidium bromide-stained 1% agarose gels of the PCR products. DNA extracted from a single duck feather using an alkaline extraction method with (lanes 1-10) or without (lanes 12-20) the phenol/chloroform step. Lane 11 is 1Kb Plus Ladder (Invitrogen).

phenol/chloroform ( $n = 30$ , each) were sufficient in purity for the PCR amplification. A comparison of costs, times and safety from phenol/chloroform for each DNA extraction method are also shown in Table 2.

### Mitochondrial DNA sequencing

The primers L78/ H774 amplified a sequence comprising part of the 5' end of the D-loop control region (710 bp) of the mtDNA. Of a total of 667 bp considered for the analysis, 663 were constant, 3 variable characters were parsimony-uninformative, and 1 variable character was parsimony-informative. Four base transition substitutions were found at the nucleotide positions 157, 362, 649 and 655, while 5 different haplotypes were obtained from 10 individuals, regardless of locations, breeds or sex (Table 3). The most common haplotypes were 1 and 2 with 5 and 2

representatives, respectively.

Table 4 summarized the estimates of DNA variation at the mtDNA control region of the whole Thai native duck samples. Although haplotype diversity ( $h$ ) and nucleotide diversity ( $\pi$ ) were highest in the Park-Nam ducks, no difference was observed between and within breeds, as indicated by Tajima's D statistic (Tajima 1989) ( $P > 0.10$ ; Table 4). The mean base composition was 26% A, 26% T, 33% C, and 15% G. The average percentage of GC content (48.7%) was similar to that of other birds (Kulikova *et al.*, 2004).

Likewise, the  $F_{st}$  values calculated by the method of Tamura and Nei (1993) showed no significant patterns of differentiation between those 2 breeds,  $F_{st} = 0.10077$ ;  $P > 0.15$ ). The AMOVA analysis indicated that 89.92 % of the genetic variability occurred within each population

**Table 2** Comparison of DNA extraction methods based on costs, times, and additional reagents required.

Extraction method	Phenol/ chloroform	Cost/test (baht)	Processing time (min)
QIAamp DNA Mini kit	No	150	20
Proteinase K/SDS	Yes	80	200
Simple alkaline extraction	Yes	34	120
	No	0.06	20

**Table 3** Haplotype variation at 4 nucleotide sites among Thai native ducks.

Haplotype	157	362	649	655	Nakorn-Pathom	Park-Nam	Total
A1	A	T	G	A	3	2	5
A2	G	.	.	.	-	2	2
A3	.	C	.	.	-	1	1
A4	.	.	.	G	1	-	1
A5	.	.	A	.	1	-	1

**Table 4** Genetic diversity of mtDNA control region sequences in Thai native ducks: number of haplotypes per breed ( $n_h$ ), number of individual sequenced ( $n_t$ ), haplotype ( $h$ ) and nucleotide ( $\pi$ ) diversity ( $\pm$  standard deviation); mean number of pairwise differences ( $K$ ).

Breed/haplotype	$n_h$	$n_t$	$h$	$p$	$K$	Tajima's* D value
NakhonPathom	3	5	$0.7000 \pm 0.218$	$0.0012 \pm 0.0005$	0.80000	- 0.97256 ns
ParkNam	3	5	$0.8000 \pm 0.164$	$0.0015 \pm 0.0004$	1.00000	0.24314 ns
Total	5	10	$0.7556 \pm 0.130$	$0.0014 \pm 0.0004$	0.95556	-1.24468 ns

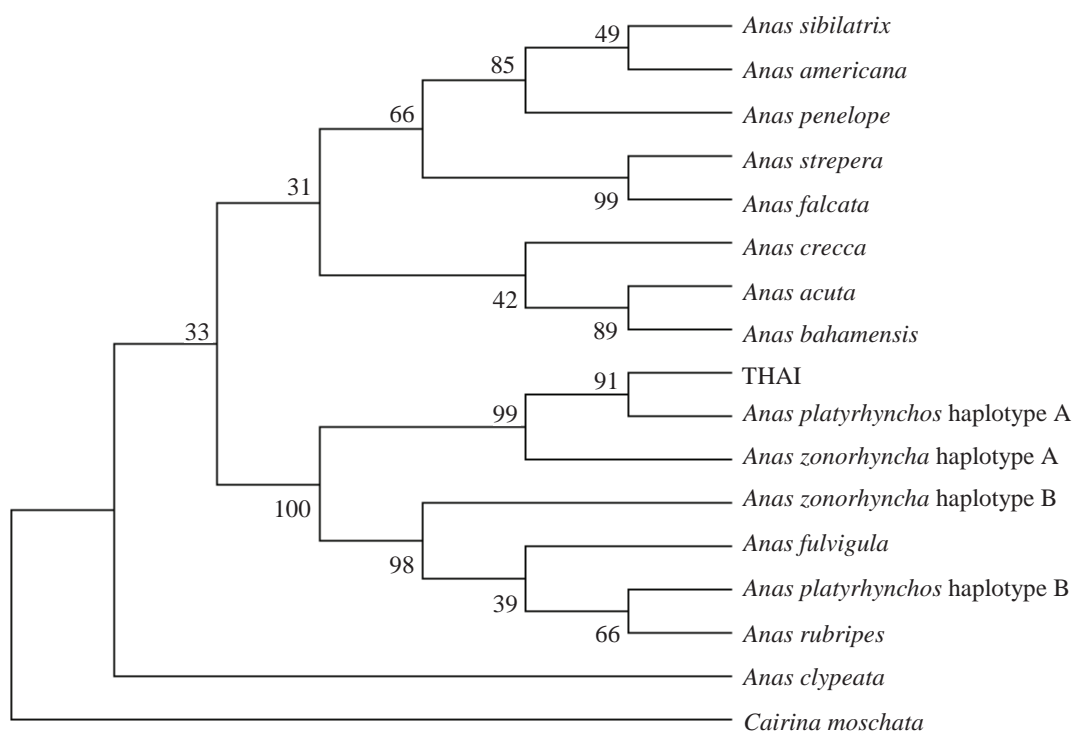
of duck breeds, and only 10.08 % was contributed from those breeds. This suggested that there is very little genetic differentiation between the 2 Thai native duck breeds. Pairwise comparisons of nucleotide sequences showed 0-0.003% differences in the mtDNA control region, indicating high sequence similarity among the ducks. The average pairwise distance calculated among the NP (n=5) and PN (n=5) breeds was  $0.01 \pm 0.01\%$  and  $0.02 \pm 0.01\%$ , respectively, and the overall mean pairwise distance was  $0.001 \pm 0.001\%$  among the ducks examined.

The 667-bp mtDNA control D-loop sequence was used as a phylogenetic tool to assess the relationship of the native Thai ducks. In the rooted neighbour-joining trees based on the D-loop region, the native Thai duck mtDNA clustered with Mallard duck (*Anas platyrhynchos*) haplotype A and Eastern spot-billed duck (*Anas zonorhyncha*)

haplotype A with 100% robust bootstrap support (Figure 3). Compared with the Eastern spot-billed duck haplotype A, the position of the native Thai duck mtDNA sequence in the same clade with the Mallard group A haplotypes is supported by 99% bootstrap values (Figure 3).

## DISCUSSION

In birds, feathers are common tissue samples for DNA isolation. Horrath *et al.* (2005) reported that the amount of DNA extracted from mature moulted feather is lower than those of freshly plucked non-growing feathers since the mesenchymal pulp, containing feather follicle cells along with an axial artery, is completely reabsorbed from the calamus when the feather is completely developed. The present work, therefore, chose a single plucked non-growing feather as a



**Figure 3** The phylogenetic relationship between mtDNA control D-loop region from Thai native domestic duck, Mallard (*A. platyrhynchos*), and other *Anas*. The number at each node represents bootstrap proportions based on 1,000 replications.

noninvasive source of DNA for studying molecular genetic in the domestic native Thai ducks.

We used the simple alkaline extraction method and compared its efficiency with other DNA extraction procedures. Despite the DNA content obtained by the alkaline extraction from a single feather is typically low compared to other extraction procedures, our results on the overall successfulness of PCR amplification of duck's mtDNA D-loop control region indicated that mtDNA obtained from the alkaline extraction without phenol/chloroform method is enough for the amplification in PCR. In this report, we have shown that the simple alkaline extraction method offers several advantages. Firstly, only a single feather is required for mtDNA preparations. Secondly, the method employs reagents and equipment regularly available in common laboratories, and the procedure is simple, fast, accurate and inexpensive. Finally, the method dispenses with the phenol/chloroform, and thus, it is safe as no hazardous extraction step for preparing mtDNA from feather samples.

The two domestic native Thai ducks, Park-Nam and Nakhon-Pathom breed ducks, share similar morphology and behavior, but have varying feather coloration that allow them to be distinguished from one another. Although sample size in the present study is insufficient for a proper population genetic analysis, sufficient information exists to build up a rational explanation of their evolution. The mean value of pairwise sequence diversity within these birds was very low (0-0.003%) when assayed across 667-bp mtDNA D-loop sequence. Both breeds had low levels of genetic diversity. Tajima's test resulted in non-significant P values ( $P > 0.10$ ) in both breeds leading to the acceptance of the null-hypothesis of neutrality for the D-loop control region (Table 4). In addition, the overall lack of genetic subdivision among samples detected by analysis of molecular variance, pairwise  $K_{st}$  values, and the exact test of population differentiation

indicated that there is no genetic differentiation between the two domestic native Thai breeds. This analysis revealed that only 10.08 % of the total variation was distributed between the two breeds. A very little differentiation might reflect a lack of resolution by the technique used in the study, as well as small sample sizes originated from a single source (PN ducks in Nakhon-Pathom province were originally taken from Prachin Buri). Alternatively, it is possible that we failed to detect the DNA variation due to the conserved sequence characteristics in the D-loop control region of mtDNA. As described by Peter *et al.* (2005), there may have been an insufficient amount of time for mtDNA to have sorted to reciprocal monophyly for several closely related species. This suggested that the Nakorn-Pathom and Park-Nam breeds are extremely closed relatives.

Neighbor-joining phylogenetic analyses with *Cairina moschata* as an outgroup supported the hypothesis that the domestic native Thai ducks could probably be domesticated from Mallard ducks (*Anas platyrhynchos*) and it constituted a monophyletic group with A haplotype (Figure 3). Although the tree also included Eastern spot-billed ducks haplotype A in a same clade with the Mallard duck haplotype A and native Thai ducks, the divergence of Eastern spot-billed ducks predated the divergence of the other two species. Kulikova *et al.* (2004) suggested that this may be the result of hybridization between Mallards and those other ducks. Mallard have widespread geographical distributions that extend across multiple continents, and they are able to hybridize and produce viable off spring (Peters *et al.*, 2005). Their hybrids with Eastern spot-billed ducks have been identified in Hong Kong and China (Melville, 1999), and also in Japan (Kanouchi *et al.*, 1998). In addition, Kulikova *et al.* (2004) indicated that that Eastern spot-billed ducks are more closely related to North America's Mottled, American Black, and Mexican ducks than they are to Mallards. Our data were thus consistent with the

hypothesis that the domestic native Thai ducks could probably be domesticated from Mallard ducks and/or the product of the hybridization between them. We also compared the Thai duck mtDNA sequence with the other Mallards' mtDNA sequence available in the GenBank, the result indicated that the native Thai duck is the group A haplotypes of the Mallard as described by Kulikova *et al.* (2005) (data not shown).

In summary, we report a fast, safe, accurate, and inexpensive method for extracting mtDNA from a feather as a source for genetic study of ducks. Although our genetic analyses could not differentiate between Park-Nam and Nakhon-Pathom breed ducks, but it is conclusive that they share a common ancestor, Mallard. To the best of our knowledge, this is the first document that showed an genetic evidence that the domestic native Thai ducks derive their origin from the Mallard group A haplotypes.

#### ACKNOWLEDGEMENTS

This study was supported by Thesis and Dissertaion Support Fund, the Graduate School, Kasetsart University. We are grateful to the Kabinburi Livestock Breeding and Research Center, Department of Livestock Development, Kabinburi district, Prachin Buri province, Thailand, for providing duck sample feathers.

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