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Genetic Diversity of Captive Eastern Sarus Crane in Thailand Inferred from Mitochondrial Control Region Sequence and Microsatellite DNA markers

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ABSTRACT.– The eastern sarus crane, *Grus antigone sharpii*, was reported to be extinct from Thailand, thus a captive breeding is a crucial step to reintroduce the crane population back into the wild. However, genetic diversity in a captive population is also of immense importance as well as the increase number of individuals. This study aimed to assess genetic diversity of captive crane populations at Khao Kheow Open Zoo and Bangpra Water Bird Breeding Station, Chonburi Province, using both mitochondrial and microsatellite markers. Mitochondrial control regions of *G. a. sharpii* were sequenced (1,003 bps.) and 10 microsatellite primer pairs isolated from blue crane were used to screen for polymorphic loci using cross-species amplification. The results revealed that captive *G. a. sharpii* show high haplotype diversity (0.915) and heterozygosity (mean H_E = 0.77 and H_O = 0.85), with low genetic differentiation (F_{ST} = 0.042) and inbreeding (F_{IS} = -0.095). Moreover, Bayesian analysis using STRUCTURE suggested that the two captive populations are genetically similar and shared many common alleles (K = 1), which also supported by haplotype network and phylogenetic analyses. Therefore, these captive crane populations may be suitable for future breeding and reintroduction programs.

KEY WORDS: Conservation breeding, d-loop, haplotype, heterozygosity, polymorphism

INTRODUCTION

The eastern sarus crane (*Grus antigone sharpii*) is a large nonmigratory bird found naturally in Myanmar, Laos, Cambodia and southern Vietnam (Barzen and Seal, 2000; Purchkoon, 2013). It is considered as one of the globally vulnerable crane species by the International Union for Conservation of Nature (BirdLife International, 2016). *Grus a. sharpii* has been extinct in the wild in Thailand about forty years ago, since the last two individuals were recorded in Surin Province near Cambodia border; one of them died in 1984 in captivity while the other was not mentioned the year of death (Purchkoon, 2013). Changing of its natural habitats (wetlands) to agricultural areas and other anthropogenic disturbance are the major causes of crane extinction in Thailand (Purchkoon, 2013; Purchkoon et al., 2015). The eastern sarus crane is thus listed as one of the protected wild animals under the Thai Wildlife Preservation and Protection Act, B.E. 2535 (1992) (Purchkoon, 2013; Purchkoon et al., 2015). Since 1990, Thai government tried to repopulate this bird species in Thailand through captive breeding program by using wild crane individuals from Cambodia as initial breeding stocks under the control of the Department of National Park, Wildlife and Plant Conservation (DNP) and Zoological Park Organization (ZPO) of Thailand under Royal Patronage of H.M. The King (Purchkoon, 2013). This initiative aims to establish an effective breeding facility to increase the numbers and reintroduce *G. a.*

sharpii back into their natural habitats. Until now, three sarus crane breeding facilities have been successfully established: the Nakhon Ratchasima Zoo (NZ), Nakhon Ratchasima Province, the Khao Kheow Open Zoo (KKOZ) and the Bangpra Water Bird Breeding Station (BB), Chonburi Province. NZ is the first breeding facility that received initial breeding stock of 27 cranes from informed contacts near the Thailand-Cambodia border (Purchkoon et al., 2015), while 15 and 17 wild crane were given to KKOZ and BB, respectively. These wild crane populations were used as initial stocks for each breeding facility. In the past few years, some captive bred individuals in NZ were released in Buriram Province, northeastern part of Thailand, once a previous native range of this bird. Several observations involving habitat uses, feeding behavior, survival and reproductive successes, are still an on-going activity to monitor the success of this reintroduction project.

Regardless of the successes of the breeding program, as determined by the number and survivability of newborns, the genetic diversity of the reintroduced crane population might be low due to a smaller number of initial crane founders, thus rendering the possibility of high level of inbreeding and subsequently reduce the ability of populations to adapt to environmental changes. Therefore, to develop long-term and effective strategies for sarus cranes conservation in Thailand, the genetic diversity (i.e., maternal haplotype diversity and heterozygosity) of captive cranes, especially in contributing founders, is also of immense importance.

Mitochondrial DNA (mtDNA) control region and the nuclear microsatellite DNA are two of the most powerful markers that have been widely used to quantify intrapopulation and intraspecific genetic variation and population structure of birds (e.g., Hasegawa et al., 1999; Jones et al., 2010; Zou et al., 2010; Henkel et al., 2012). Thus, in the present study, we used both mitochondrial and nuclear markers to assess the level of genetic diversity of captive Thai sarus cranes. Our goals are to screen for polymorphic microsatellite markers using cross-species amplification technique and to investigate the mitochondrial control region sequences in captive eastern sarus crane from KKOZ and BB to assess their genetic diversity. The outcomes from this study will be essential for developing a sustainable breeding process for future reintroduction and effective conservation management strategies of *G. a. sharpii* in the future.

MATERIALS AND METHODS

Samples collection and DNA extraction

We extracted total genomic DNA for PCR amplification from feather specimens that were collected in 2014 from every captive crane individuals in KKOZ (9 founders and 2 progenies of founders; 13°12'54.0"N, 101°03'21.4"E) and BB (17 founders; 13°13'49.7"N, 100°59'46.6"E), using the FavorPrep™ tissue genomic DNA extraction mini kit (Favorgen Biotech Corp., Taiwan) following the manufacturer's protocols.

Mitochondrial DNA amplification and sequencing

For phylogenetic and haplotype network analyses, we amplified and sequenced portions of the mitochondrial control region using primers DLRCF and DLRCR (Miura et al., 2012). This yielded in fragments of 1,003 bps. The amplification reaction was performed in a total volume of 25 µl in a 0.2

ml tube. The reaction mixture contained approximately 30–50 ng of DNA template, 0.5 µM of each primer and 1Xpremix of EmeraldAmp®MAX PCR master mix (Takara, Japan). PCR amplification was conducted under the following conditions: initial denaturation at 95˚C for 3 min, followed by 40 cycles of denaturation at 95˚C for 40 s, annealing at 57˚C for 1.15 min, extension at 72˚C for 1.30 min and a final extension at 72˚C for 10 min. The sizes of the amplicons were subsequently separated by electrophoresis through 0.8% (w/v) agarose gel, stained with SYBR®Safe DNA gel stain solution and visualized under blue light. The desired products were sent to Bioneer Inc., South Korea for purification and sequencing for both directions.

Mitochondrial DNA data analyses

The obtained sequence data were aligned using ClustalW (Thomson et al., 1994) with some base corrections after visual inspection. mtDNA diversity parameters including number of haplotypes (H), number of polymorphic sites (p), mean number of pairwise nucleotide differences (*k*), haplotype diversity (*h*) and nucleotide diversity (π) were calculated in DnaSP v.5.10.1 (Librado and Rozas, 2009). Mean transition/transversion ratio and population genetic distance were calculated using Kimura's two parameter model via MEGA v.6.06 (Tamura et al., 2013). We constructed Neighbor-joining (NJ) and maximum likelihood (ML) trees for phylogenetic analyses. The NJ tree was generated in MEGA using Kimura's two parameter model of sequence divergence (the estimated transition/transversion bias is 3.09). For ML tree, the Akaike information criterion (AIC) algorithm was used to choose the best-fitting model of nucleotide substitution for the data set of control region sequences. In this study, the HKY85 + I model with a proportion of invariant sites of 0.816 was chosen and the ML tree was constructed using PhyML v.3.0 programs (Guindon et al., 2010). One thousand bootstrap resamplings were performed for both trees. The DNA sequence data of two related crane species: *G. vipio* (Genbank accession: LC114481) and *G. rubicunda* (FJ769853) were assigned as outgroups. Haplotype network was generated via NETWORK v.5.0 (Bandelt et al., 1999) to determine the relationships among mtDNA haplotypes based on sequence variations. The default values were used for all parameter options. We also used Arlequin v.3.5.2.2 (Excoffier and Lischer, 2010) to calculate Tajima's *D* and Fu's F_S values for the test of selective neutrality.

Microsatellite DNA amplification and genotyping

Ten primer pairs originally designed from a genomic library of the blue crane (*Anthropoides paradisea*) (Meares et al., 2008) were used to amplify and screen for candidate polymorphic microsatellite markers. The amplification reactions were carried out in a total volume of 25 µl containing EmeraldAmp®MAX PCR master mix, 0.5 µM solutions of each forward and reverse primers and approximately 30–50 ng of DNA template. The PCR process was performed on the Bio-Rad iCycler as described by Meares et al. (2008). The polymorphisms of the amplicons were checked on 3% Nusieve™ 3:1 agarose gel (Lonza Rockland Inc., USA) staining with SYBR® Safe DNA gel stain and visualized under blue light transilluminator. To confirm whether the putative polymorphic bands are truly polymorphic and useful in the evaluation of genetic diversity, we

TABLE 1. Genetic diversity in the mtDNA control region of captive eastern sarus crane in Thailand

Population	n	p	*k*	H	*h* ± S.D.	*π* ± S.D.	Tajima's *D* (*p*-value)	Fu's F_S (*p*-value)
KKOZ	11	12	4.73	6	0.800 ± 0.114	0.0047 ± 0.00064	0.668 (0.768)	0.676 (0.666)
BB	16	16	5.35	8	0.883 ± 0.052	0.0053 ± 0.00052	0.432 (0.705)	0.242 (0.548)
Overall	27	17	5.54	12	0.915 ± 0.028	0.0055 ± 0.00027	0.894 (0.834)	–0.876 (0.383)

k; the average number of pairwise nucleotide differences, *h*; haplotype diversity, π; nucleotide diversity

genotyped the amplified bands to determine the exact allelic size of all microsatellite loci by conducting the amplification procedures as described above with 6-FAM fluorescent-labeled primers (Macrogen Inc., South Korea). The fluorescent-labeled PCR products were then sent to Macrogen Inc. for genotyping service. Fragment lengths were checked in comparison to an internal standard size using the GeneMarker® software v.2.6.4 (SoftGenetics, LLC).

Microsatellite data analyses

We used GENEPOP v.4.2 (Raymond and Rousset, 1995; Rousset, 2008) to estimate the number of alleles per locus (N_A), observed (H_O) and expected (H_E) heterozygosities, as well as a test for deviations from the Hardy–Weinberg equilibrium (HWE *p*-value) for each microsatellite locus. FSTAT v.2.9.3 (Goudet, 2001) was used to calculate the allelic richness (A_R) and linkage disequilibrium (LD) between all pairs of loci following multiple testing for statistic significances using Bonferroni correction ($p < 0.01$). The occurrence of null alleles was investigated using MICRO-CHECKER v.2.2.3 (van Oosterhout et al., 2004) and ML-NullFreq (Kalinowski and Taper, 2006). We also used GENEPOP for assessing the degree of population differentiation or pairwise F_{ST} values for all polymorphic loci and the level of inbreeding within a population or inbreeding coefficient (F_{IS}) at each locus. In order to examine the population genetic structure, the number of genetic cluster (*K*) of all samples was identified, using Bayesian admixture model with correlated allele frequencies option implemented in STRUCTURE v.2.3.4 (Pritchard et al., 2000). We conducted one million Markov chain Monte Carlo (MCMC) repetitions and a burn-in of 100,000 replications with five independent runs for $K = 1– 5$. The most likely number of *K* was determined on the basis of the highest values of log likelihood (Ln P(D)) across five runs.

RESULTS

Genetic diversity of mitochondrial control region sequence

Of 28 crane individuals, we failed to obtain a DNA sample from one individual in BB population due to insufficient feather specimen for DNA extraction; we therefore used 27 individuals for mtDNA analyses. The aligned control region sequences comprised of 1,003 nucleotides which were A+T rich (58.9%), with nucleotide composition of: T (30.9%), C (25.9%), A (28.0%), G (15.2%). A single indel (insertion or deletion) was detected at the nucleotide position of 21. Kimura-2-parameter genetic distances between

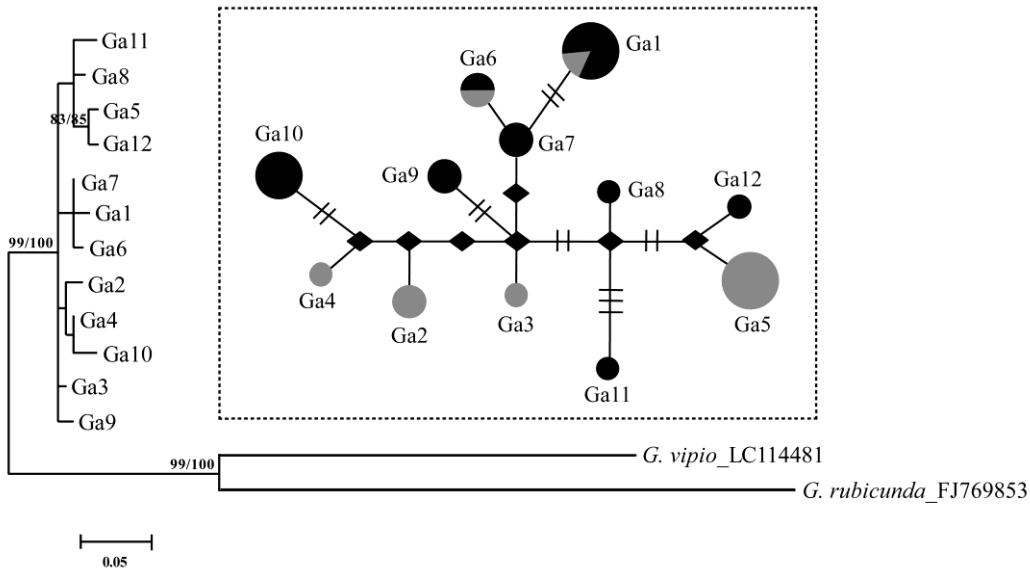

FIGURE 1. ML tree of 12 haplotypes constructed based on control region sequences of the captive eastern sarus crane. Numbers above nodes represent the ML/NJ bootstrap values when ≥ 50%. A dashed square besides the tree represents a median-joining haplotype network constructing based on 17 polymorphic sites. The sizes of the circles are proportional to the corresponding haplotypes to their frequencies. The black small squares represent missing haplotypes. Haplotypes found in KKOZ and BB populations are shaded in light and dark grey, respectively. Numbers of base substitutions (>1) are indicated by slashes on the network.

populations ranged from 0 to 0.008. This value is similar to values calculated for within KKOZ (0–0.008) and BB (0–0.009) populations. Haplotype genetic diversity indices were calculated for each and overall captive populations and shown in Table 1. Both the haplotype (*h*) and nucleotide (π) diversities were slightly higher in BB than in KKOZ, with the average number of nucleotide differences of 5.54. The overall estimate for *h* and π were 0.915 and 0.0055, respectively, indicating high genetic diversity in the captive sarus crane population. Moreover, no significant Tajima's *D* or Fu's F_S was observed for each and overall populations (Table 1), suggesting that the variations in control region sequences was selectively neutral.

The NJ and ML trees resulted in similar topologies; we therefore only showed the ML tree with the support values of both analyses (Fig. 1). In ML tree, the captive cranes examined were not grouped by locations, though individuals from KKOZ and BB were nested within the same clade, suggesting low genetic differentiation between populations; a resulted haplotype network also supports this. On a median-joining network of mtDNA haplotypes, a total of 12 haplotypes were identified based on 17 (1.69%) polymorphic sites detected from multiple alignments, in which the haplotypes from KKOZ did not form a distinct cluster from those of BB (Fig. 1; Table 2). The two populations exhibited very low level of genetic variations as one or two mutational changes were commonly detected between haplotypes. Two haplotypes (Ga1 and Ga6) were found in both populations, and Ga1 is the most

TABLE 2. Polymorphic sites of twelve haplotypes in the control region sequences. The number of individuals and frequency of each haplotype are shown in the three columns on the right. Periods within the nucleotide position indicate the same nucleotides as those given in Ga1.

Haplotype	Variable sites in sequences																	No. of individuals		% freq.
	6 4	8 9	1 1 6	1 2 4	1 2 8	1 6 9	1 8 1	1 8 4	3 8 9	4 2 9	5 7 0	6 3 0	7 1 4	8 0 9	9 2 6	9 3 9	9 8 3	KKOZ	BB	
Ga1	G	C	C	G	G	C	A	T	C	T	G	A	A	C	A	C	A	1	4	18.5
Ga2	.	.	.	A	.	T	G	.	T	C	.	G	G	.	.	.	.	2		7.4
Ga3	.	T	.	A	.	T	G	.	T	.	.	.	.	.	.	.	.	1		3.7
Ga4	.	.	.	.	.	T	G	.	T	.	.	.	G	.	.	.	.	1		3.7
Ga5	.	.	T	A	.	T	G	C	T	.	.	.	G	.	.	T	C	5		18.5
Ga6	.	.	.	A	.	.	.	.	T	.	.	G	.	.	.	.	.	1	1	7.4
Ga7	.	.	.	A	.	.	.	.	T	.	.	.	.	.	.	.	.		2	7.4
Ga8	.	.	.	A	.	T	.	.	T	.	.	.	.	.	.	T	C		1	3.7
Ga9	A	.	.	A	A	T	G	.	T	.	.	.	.	.	.	.	.		2	7.4
Ga10	.	.	.	.	.	T	G	.	T	C	.	.	G	T	G	.	.		4	14.8
Ga11	A	.	.	.	.	T	G	.	T	C	.	.	.	.	.	T	C		1	3.7
Ga12	.	.	T	A	.	T	G	C	T	.	A	.	.	.	.	T	C		1	3.7

frequent shared haplotype by five individuals (18.5%) of the two populations, whereas Ga5 is only found in KKOZ population (Fig. 1; Table 2). Nucleotide sequences of this study were deposited in GenBank under accession number MG799170-MG799181 for Ga1 to Ga12 haplotypes, respectively.

Genetic diversity of microsatellite markers

All 10 microsatellite primer pairs used in this study were successfully amplified for 28 eastern sarus cranes with no individuals failed to produce PCR products; however, two loci (Gpa01 and Gpa24) were monomorphic (Table 3). Of 8 polymorphic loci analyzed, the mean number of alleles per locus was 12, in which locus Gpa37 was highly polymorphic with 25 alleles while Gpa38 exhibited the lowest polymorphism with 4 alleles per locus. Significant deviations from Hardy–Weinberg equilibrium (HWE) were observed at locus Gpa38 (p = 0.003) and Gpa41 (p = 0.007) (Table 4a). There was no evidence of a high level of null allele frequency (NAF) and large allele dropout. One locus, Gpa36, showed linkage disequilibrium (LD) whereas seven loci

TABLE 3. Ten microsatellite loci isolated from *A. paradisea* (Meares et al., 2008) were used in the genetic analyses of the captive eastern sarus crane. Allelic sizes and annealing temperatures (T_a) of each locus are provided.

Loci	Repeats	Allele size (bp)	T_a (°C)
Gpa11	$(TTTC)_{21}$	180, 184, 188, 192, 196, 200, 204, 208	60
Gpa14	$(TTTC)_{15}(TC)_3(TTTC)_3$	332, 356, 364, 368, 372, 376, 380, 384, 388, 392, 408,412, 428, 440, 444, 452, 460	57
Gpa32	$(GT)_{14}$	170, 176, 178, 184, 186	57
Gpa36	$(GATA)_{13}$	212, 216, 220, 224, 228, 232, 236, 240	60
Gpa37	$(TTTC)_{31}$	268, 292, 312, 316, 320, 324, 328, 332, 336, 340, 344, 348, 352, 364, 372, 376, 380, 384, 388, 392, 396, 400, 404, 416, 420	60
Gpa38	$(CTAT)_{12}$	184, 188, 192, 196	60
Gpa39	$(GATA)_{13}$	98, 102, 106, 110, 114, 118	57
Gpa41	$(TTTC)_{13}(TC)_2(TTTC)_2(TC)_2(TTTC)_2$	294, 298, 370, 382, 386, 390, 394, 402, 408, 412, 416, 420, 424, 428, 436, 444, 456, 464, 468, 476, 480, 484	54
Gpa01			63
Gpa24			57

Note: Gpa01 and Gpa24 were monomorphic loci.

were considered as independent from each other (Table 4a). We therefore excluded Gpa36 locus to avoid the errors for the genetic analyses.

The following parameters show the average of seven loci for each population after reanalyzing the data: the expected (H_E) and observed (H_O) heterozygosities in KKOZ were similar to those found in BB population (H_E: 0.76 vs. 0.78; H_O: 0.85 vs. 0.85) (Table 4b). F_{IS} were low in both populations (-0.105 and -0.089 in KKOZ and BB, respectively). These results indicated that both KKOZ and BB populations exhibited high level of genetic diversity with no homozygous excess. In addition, number of allele per locus (N_A) and allelic richness (A_R) in BB was higher than KKOZ. Only BB population showed a signal of deviation from HWE in three loci (Gpa37, Gpa38 and Gpa41) with the absence of NAF (Table 4b).

TABLE 4. Genetic diversity from eight polymorphic loci of 28 captive eastern sarus cranes. Two polymorphic loci significantly deviated from Hardy-Weinberg equilibrium (HWE) are denoted with *. The averages were calculated from 8 loci (denoted with †) and 7 loci (excluding Gpa36; denoted with ††). (a) Data were analyzed combining results from both KKOZ and BB populations. (b) Data were analyzed for each population from two breeding facilities.

(a)

Locus	N_A	A_R	H_E	H_O	F_{ST}	F_{IS}	HWE *p*-value	NAF
Gpa11	8	7.2	0.80	0.93	0.120	-0.147	0.935	0.000
Gpa14	17	11.5	0.87	0.86	0.087	-0.013	0.472	0.000
Gpa32	5	4.2	0.52	0.63	-0.000	-0.183	0.847	0.000
Gpa36	8	7.2	0.84	0.85	0.029	-0.016	0.204	0.007
Gpa37	25	15.5	0.95	1.00	0.028	-0.051	0.029	0.000
Gpa38	4	3.2	0.58	0.85	0.008	-0.515	0.003*	0.000
Gpa39	6	5.4	0.75	0.72	-0.032	0.055	0.286	0.000
Gpa41	22	14.2	0.94	0.94	0.037	0.010	0.007*	0.000
Average†	11.9	8.6	0.78	0.85	0.040	-0.084		
Average††	12.4	8.7	0.77	0.85	0.042	-0.095		

(b)

	KKOZ								BB							
Locus	*N*	N_A	A_R	H_E	H_O	F_{IS}	HWE *p*-value	NAF	*N*	N_A	A_R	H_E	H_O	F_{IS}	HWE *p*-value	NAF
Gpa11	11	5.0	5.0	0.75	0.91	-0.212	0.890	0.000	17	8.0	7.3	0.85	0.94	-0.111	0.759	0.000
Gpa14	11	9.0	9.0	0.80	0.73	0.091	0.204	0.000	17	16.0	12.7	0.93	1.00	-0.071	0.926	0.000
Gpa32	11	4.0	4.0	0.55	0.73	-0.322	0.666	0.000	17	5.0	4.5	0.49	0.53	-0.083	0.726	0.000
Gpa36	11	7.0	7.0	0.84	0.82	0.022	0.310	0.007	17	8.0	7.4	0.85	0.88	-0.039	0.115	0.000
Gpa37	11	14.0	14.0	0.94	1.00	-0.063	0.579	0.000	17	18.0	14.4	0.96	1.00	-0.044	0.011*	0.000
Gpa38	11	4.0	4.0	0.64	0.82	-0.286	0.081	0.000	17	3.0	2.6	0.52	0.88	-0.690	0.004*	0.000
Gpa39	11	5.0	5.0	0.74	0.73	0.018	0.664	0.000	17	6.0	5.8	0.76	0.71	0.077	0.116	0.000
Gpa41	11	12.0	12.0	0.93	1.00	-0.073	1.000	0.000	17	16.0	12.8	0.94	0.88	0.063	0.045*	0.000
Average†	11	7.5	7.5	0.77	0.84	-0.087			17	10.0	8.4	0.79	0.85	-0.082		
Average††	11	7.6	7.6	0.76	0.85	-0.105			17	10.3	8.6	0.78	0.85	-0.089		

Notes: *N*, number of individual; N_A, number of allele per locus; A_R allelic richness; H_E, expected heterozygosity; H_O, observed heterozygosity; F_{ST}, fixation index; F_{IS}, inbreeding coefficient; HWE *p*-value, HWE analysis; NAF, null allele frequency

For the overall populations, A_R ranged from 3.2 to 15.5 alleles per locus with 8.7 on average. The H_O and H_E range from 0.63 (Gpa32) to 1.00 (Gpa37) and 0.52 (Gpa32) to 0.95 (Gpa37), respectively, and the mean H_O (0.85) was higher than the mean H_E (0.77), indicating high heterozygosity in captive sarus cranes (Table 4a). The overall

TABLE 5. The inferred number of genetic clusters (*K*) of two captive cranes from KKOZ and BB

Repeat	Ln P(D)				
	***K* = 1**	***K* = 2**	***K* = 3**	***K* = 4**	***K* = 5**
1	-826.7	-827.5	-1137.2	-996.5	-985.7
2	-827.7	-836.0	-1011.8	-1586.0	-940.1
3	-828.3	-828.1	-1103.6	-1796.6	-1567.7
4	-826.8	-827.9	-1345.6	-1030.2	-980.4
5	-829.0	-830.8	-919.7	-1121.9	-1861.4
Mean	-827.7	-830.1	-1103.6	-1306.2	-1267.1

F_{ST} is 0.042 with no significance genetic subdivision between the two groups of captive sarus crane populations. Moreover, low inbreeding coefficient was also detected within both populations (F_{IS} = -0.095). The Bayesian clustering analysis from STRUCTURE suggested K = 1 with the highest likelihood values (Ln P(D) = -827.7; Table 5).

DISCUSSION

Genetic diversity is one of the most crucial parameters for assessing the long-term persistence of animal populations in fluctuating environment, it is therefore necessary to monitor the species genetic diversity and inbreeding levels before conducting long-term conservation management programs (Frankham et al., 2002). In the present study, we used both mtDNA and nuclear markers to assess the level of genetic diversity of captive Thai sarus cranes, especially in founding populations. The control region is the most variable portion of mtDNA genome in which the 5' fragment portion (approximately base position 1-360 bps) is considered to be the most hypervariable region (Desjardins and Morais, 1990; Randi and Lucchini, 1998); this is consistent with our results. Nucleotide variations detected in this study is likely a result from random genetic drift, the values of Tajima's *D* or Fu's F_S also support this. The average observed level of haplotype diversity (0.92) in sarus cranes from two facilities in Thailand was high comparatively to other Gruiformes species, such as red-crowned crane (0.25) (Hasegawa et al., 1999) and whooping crane (0.50) (Glenn et al., 1999), despite similar nucleotide diversity indices. Our mtDNA investigations indicate that sarus crane populations from KKOZ and BB showed high level of genetic diversity. This finding is consistent with a previous study of mtDNA variation (including cytochrome *b*, *ND6* and control region) found in crane founders collected from KKOZ, BB and NZ, in which 46 haplotypes based on the analysis of 2,670-bp sequences derived from 51 individuals were recognized (Insee, 2013). However, identification and phylogenetic relationships of Thai crane species and subspecies were the two primary objectives in that study. The level of genetic diversity was therefore demonstrated only on the basis of degree of nucleotide variation and number of haplotypes detected within each and overall populations, leaving other parameters (e.g., *h*, π, *k*, and genetic distance) as well as the

relationships among mtDNA haplotypes undetermined thus far. In addition, our phylogenetic and haplotype network analyses clearly showed that the KKOZ and BB populations are genetically closely related to each other with a low genetic differentiation between populations. Moreover, the results of STRUCTURE analysis and the low F_{ST} values also support this, suggesting that these two populations are considered to be genetically homogeneous.

As there is no published work available for species-specific microsatellite markers for eastern sarus crane, cross-species amplification technique can be considered as a suitable alternative. Currently, there are numbers of available primers for cross-species amplification in many crane species (Jones et al., 2005; Meares et al., 2008; Jones et al., 2010; Zou et al., 2010). However, the polymorphic loci obtained from this method should be sufficiently high enough for the analyses, i.e., at least 10 polymorphic loci should be implemented (Witzenberger and Hochkirch, 2011). In this study, 10 primer pairs developed for blue crane, a relatively distant species from *G. a. sharpii* (Krajewski et al., 2010), were amplified successfully. However, the genotypes of one polymorphic locus (Gpa36) exhibited significant LD without the presence of NAF. We therefore excluded this locus from further analysis. We found three loci deviated from HWE, in which locus Gpa41 shows excess of homozygosity and loci Gpa37 and Gpa38 show heterozygosity excess (Table 4a, 4b). Heterozygote deficiency in locus Gpa41 can be explained through the inbreeding coefficient obtained (F_{IS} = 0.010 for overall populations (Table 4a); F_{IS} = 0.063 for BB population (Table 4b)). On the other hand, heterozygote excess in loci Gpa37 and Gpa38 (F_{IS} = -0.515 for overall populations (Table 4a); F_{IS} = -0.044 and -0.690 for BB population (Table 4b)) may have caused by allele frequencies that are differed from the parents (Rousset and Raymond, 1995). Despite that Gpa38 and Gpa41 were deviated from HWE, we did not remove these loci from the overall population analyses because the average values of H_O and H_E did not change significantly after excluding Gpa38 and Gpa41 (H_O = 0.83 and H_E = 0.78). In addition, we also test whether the detected LD at Gpa36 is a result from sex linkage by repeating the analysis after discriminating male and female individuals in the overall population. The results showed that H_O and H_E for males (homogametic; ZZ) were 0.85 and 0.89, respectively whereas these values for females (heterogametic; ZW) were 0.87 and 0.83, respectively. This result therefore denies the above hypothesis.

After the re-analysis by excluding Gpa36, the F_{IS} is even lower, though the values of heterozygosities are still high (mean H_O = 0.85 and H_E = 0.77). By using similar microsatellite primers, the results revealed that captive eastern sarus cranes exhibited high heterozygosity (H_O ranged from 0.63-1.00) when compared with other crane species such as blue crane (H_O: 0.50–0.95), grey-crowned crane (H_O: 0.30–0.80) and wattled crane (H_O: 0.00–0.90) (Meares et al., 2008). Moreover, we argue that such high heterozygosity detected in this study is due to highly polymorphic alleles at loci Gpa37 (25 alleles) and Gpa41 (22 alleles). This is because the mean heterozygosity indices remain unchanged when we excluded these two loci (H_O = 0.80 and H_E = 0.70). On the other hand, as they exhibit extreme high polymorphism, we suggest that these two loci are suitable for using as genetic markers for individual identification

when non-invasive sarus crane specimens, such as feathers and feces, are applied.

Although there is no available information on the genetic background and origin of captive crane founders in Thailand, the results of K and F_{ST} values suggest that the two captive Thai populations might have originated from the same founder population in Cambodia. Further study in wild ancestral Cambodian populations using these polymorphic primers is needed in order to search for the historical origin of Thai captive and reintroduced populations.

In conclusion, our results of mtDNA variation agree with the microsatellite polymorphism suggesting that founder cranes in KKOZ and BB still showed high level of genetic diversity, both in terms of maternal haplotype and heterozygosity, with low genetic differentiation and inbreeding. These suggest that the breeding stocks may be suitable for future breeding and reintroduction programs. We additionally suggest that the two populations should be conserved and managed as a single gene pool, thus exchange individuals between populations to increase diversity and avoid a risk of inbreeding within a population is possible. In the case of small population size in captivity, transferring genes or crane individuals from wild population (may be Cambodian population, an original group of crane population in Thailand) could be an important option to manage the efficient captive breeding management (Ebenhard, 1995). Moreover, genetic monitoring for reintroduced eastern sarus crane population in the wetland areas at Buriram Province is especially important to compare with captive crane population; the compared data is essential for long-term conservation management plan of both captive and reintroduced crane populations. Population genetics studies in endangered species is crucial knowledge to reduce inbreeding and loss genetic diversity for minimizing species from extinction.

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