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Molecular Analysis for Genetic Diversity and Distance of Introduced *Grus antigone sharpii* L. to Thailand

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Abstract: The genetic relationship was examined in a population of *Grus antigone sharpii* L. using DNA markers from the ISSR technique for applying towards breeding purposes for conservation of species. Since their extinction from Thailand, sixteen eastern sarus cranes: *Grus antigone sharpii* L. provided from Cambodia were fed and bred to sixty individuals at Nakhonratchasima Zoo, Northeastern Thailand to re-exist in Thai natural sites. Their genetic diversity and distance were examined to test their possibility to adapt to environmental variation. Blood samples from 27 individuals of *Grus antigone sharpii* L. were collected and DNA was extracted. These DNA samples were amplified using the successful fifteen from twenty four primers inter simple sequences repeat markers. A dendrogram was constructed and shows distance values of the species between 12.1 and 53.5. The samples produced 63.96% polymorphic banding profiles. The genetic diversity (H') in this population was estimated using Shannon's index. The high H' value of 0.501 reflected the somewhat wide range of distribution sites, which would adapt to environmental variations. Genetic evenness is 0.152. This value supports that all the studied samples have a small equal genetic abundance.

Key words: Genetic distance, *Grus antigone sharpii* L., sarus crane, Thailand

INTRODUCTION

The sarus crane: *Grus antigone* L. 1758 is in the crane family, Gruidae, which comprises 15 extant species of large, graceful birds distributed across five continents. Unfortunately, cranes worldwide are threatened by habitat loss, excessive harvest and disturbance (Meine and Archibald, 1996), making Gruidae one of the most threatened groups of birds in the world. The sarus crane, the world's tallest flying bird, has a broad distribution that spans two continents and is the only species of crane that breeds both in India and in Southeast Asia (Meine and Archibald, 1996). There are four recognized subspecies of sarus crane, namely Indian (*Grus a. antigone*), eastern (*Grus a. sharpii*), Australian (*Grus a. gillae*) and the extinct Philippine sarus (*Grus a. luzonica*; Blanford, 1896; Schodde *et al*., 1988; Meine and Archibald, 1996).

The eastern sarus crane, *Grus antigone sharpii* L., became extinct from Thailand because of habitat loss with forest destruction which occurred violently throughout the country. In 1988, Thailand obtained sixteen individuals of eastern sarus cranes originating in Cambodia. They were fed and the population enlarged to sixty individuals at Nakhonratchasima Zoo for releasing them as natural resources in Thailand. There genetic diversity and distance should therefore be examined to test their possibility of adapting to the environmental variations. Genetic diversity allows species to adjust to a changing world, which is caused by both natural and human factors. Understanding genetic diversity within the genus or species is essential for establishing effective and efficient conservation and breeding practices (Chaveerach *et al*., 2006).

Traditionally, morphological characters have been used to characterize levels and patterns of diversity or similarity. Since, these traits represent only a small portion of the plant genome and are influenced by environmental factors, they have limited utility for describing the potentially complex genetic structure which may exist within and between taxa (Avise, 1993). Various molecular approaches have been devised to overcome these constraints (Soltis and Soltis, 1990). A number of PCR-based DNA markers, including Random Amplified Polymorphic DNA (RAPD), Simple Sequence Repeat

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(SSR), Inter-Simple Sequence Repeat (ISSR) and Amplified Fragment Length Polymorphism (AFLP) techniques have been used widely to investigate population genetics. Each of these methods has many advantages and limitations. ISSR markers are extremely variable and have proven to be sensitive enough to differentiate cultivars and natural populations (Wolfe *et al*., 1998; Chaveerach *et al*., 2006). These markers of genetic variation are generally independent of environmental factors and more numerous than phenotypic characters, thereby providing a clearer indication of the underlying variation in the genome (Avise, 1993).

Genetic distance is a measure of the dissimilarity of genetic material between different species or individuals of the same species. It can be used to measure the relatedness of samples by cluster analysis (Nybom and Hall, 1991; Welsh *et al*., 1991). The result is a branching diagram that connects all of Operational Taxonomic Units (OTUs) and OTU clusters at levels corresponding to their degree of distance (Weier *et al*., 1982).

The present study aims to examine genetic relationships consisting of genetic distance and diversity in a population of *Grus antigone sharpii* L. using DNA markers from the ISSR technique.

MATERIALS AND METHODS

Sample collection and DNA extraction: Blood samples from 27 individuals of *Grus antigone sharpii* L. identified by microchip coding (Table 1) and an outgroup of West African crown crane were collected from Nakhonratchasima Zoo, Northeastern Thailand in 2006. Genomic DNA was isolated from blood samples using proteinase K digestion and treatment with phenol/chloroform (Sambrook *et al*., 2001). The quality and quantity of extracted DNA was checked by 0.8% agarose gel electrophoresis stained with ethidium bromide.

ISSR analysis: Amplification reactions were performed using a PCR machine (Gene Amp PCR system 9700, Applied Biosystems). Amplifications were carried out in 20 μL using PCR master mix (Promega) with 1 μM primer and 2 ng of DNA template. Twenty-four ISSR primers were screened and the 15 ISSR primers (Table 2) were successfully amplified clear bands for *Grus antigone sharpii* L. and the outgroup, West African Crown Crane. The reaction mixture was incubated at 94°C for 3 min and the amplification was performed with the following thermal cycles: 35 cycles of denaturation for 1 min at 94°C, 1 min annealing temperature Tm-5, extension for 2 min at 72°C and 7 min final extension at 72°C. Amplification products were detected by agarose gel electrophoresis in TAE buffer (0.4 M Tris, 0.114% acetic acid 1 mM EDTA pH 8.0) and visualized by ethidium bromide staining.

Table 1: Twenty-seven studied individuals and microchip coding of *Grus antigone sharpii* L. from Nakhonratchasima Zoo, Northeastern Thailand

No.	Microchip	No.	Microchip	No.	Microchip	No.	Microchip
1	115232651A	8	123211537A	15	126811095A	22	114822755A
2	114754651A	9	114833192A	16	123179520A	23	115224744A
3	115224744A	10	113556597A	17	116427254A	24	114562111A
4	115229154A	11	122911611A	18	114567283A	25	115311352A
5	115229467A	12	122914754A	19	122913516A	26	114673192A
6	122638531A	13	122917266A	20	116412280A	27	114752571A
7	122652573A	14	122762133A	21	115311146A	28	West African Crown Crane

Table 2: Summary of ISSR primers, number of bands scored, number of polymorphic bands and percentage of polymorphisms for amplification profile of 27 individuals of *Grus antigone sharpii* L.

Primer sequence (5'-3')	No. of bands scored	No. of polymorphic bands	Percentage of polymorphism
$(CT)_8TG$	12	10	83.33
$(CA)_6AC$	8	2	25.00
$(CA)_6GT$	8	3	37.50
$(CA)_6GG$	10	8	80.00
$(GA)_6GG$	10	8	80.00
$(GA)_6CC$	8	4	50.00
$(GT)_6CC$	8	7	87.50
$(CAC)_3GC$	12	9	75.00
$(GAG)_3GC$	8	6	75.00
$(ACTG)_4$	11	6	54.55
$(GT)_8C$	7	3	42.86
$(AC)_8CG$	18	11	61.11
$(CCCT)_4$	10	7	70.00
$CCCC(GT)_6$	8	5	62.50
$(GA)_5$	8	6	75.00
Total	146	95	63.96

ISSR data analysis: The total number of ISSR bands discerned from the agarose gel was documented as diallelic characters: present = 1, absent = 0; as the ISSR markers are considered the dominant markers. The resulting bands were used to construct a dendrogram following the UPGMA with the Fingerprinting II program version 3.0 (Bio Rad). The distance values were obtained from the dendrogram. The percentage of polymorphic loci was calculated with the Shannon's diversity index (H') for each population, $H' = -\Sigma$ pi ln pi, where pi is the frequency of a given ISSR band. Genetic evenness (E) is calculated from genetic diversity/ln individual richness (ln 27 = 3.296) (Nei, 1973, 1978).

RESULTS

Twenty-four primers were screened and fifteen informative ISSR primers produced a total of 146 band locations with sizes ranging from 100-2,500 and an average of 9.733 bands per primer. Of these bands, 63.96% (95 bands) were polymorphic. Percentages of Polymorphic

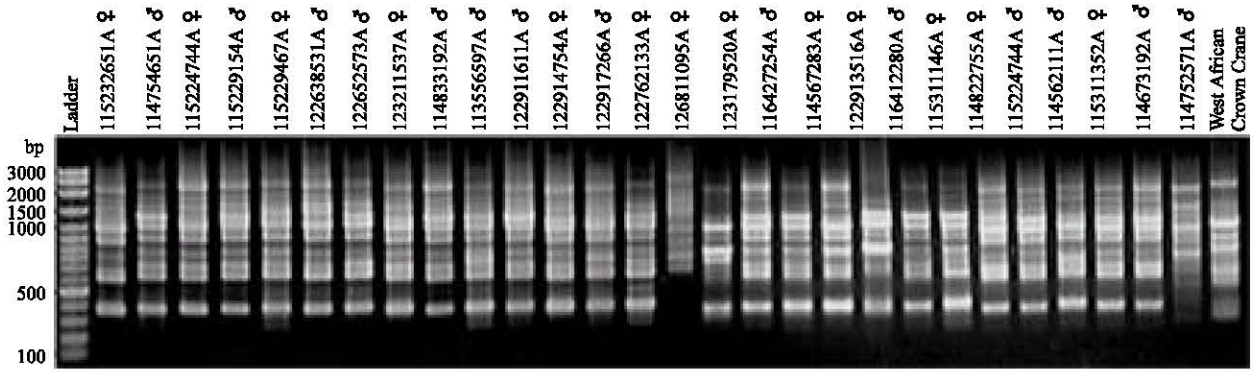

Fig. 1: ISSR profile of *Grus antigone sharpii* L. and an outgroup, West African Crown Crane with primer $(CAC)_3GC$

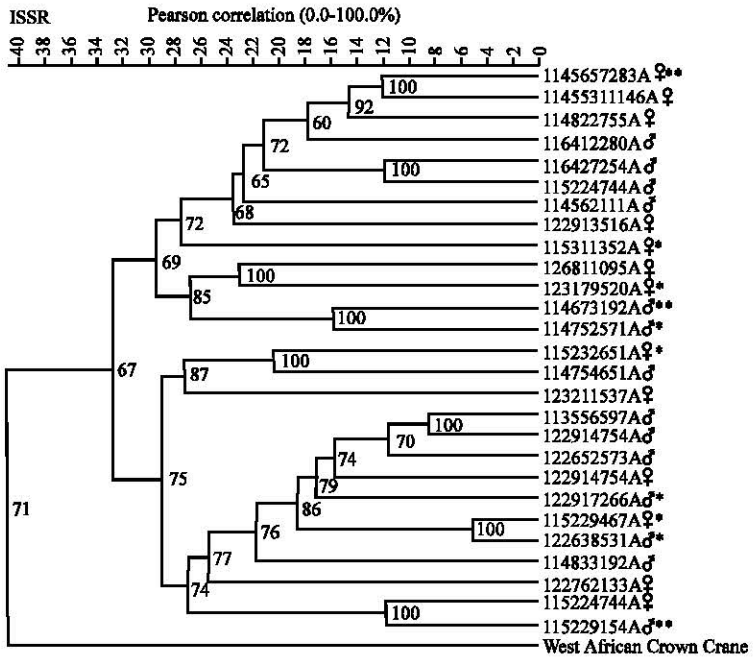

Fig. 2: A dendrogram constructed from 15 ISSR primers determined by UPGMA (Fingerprinting II, Bio Rad) to clarify the genetic relationships of *Grus antigone sharpii* L. and West African Crown Crane. ♀ indicates female and ♂ indicates male individuals. Individuals with * are parents of natural reproduction and individuals with ** are of insemination

Bands (PPB) for each primer ranged from 25 to 87.50%. Primer $(AC)_8CG$ generated the highest number of bands (18 bands). The minimum number of bands (7) was produced by primer $(GT)_8C$ (Table 2). Figure 1 shows the banding pattern using the primer $(CAC)_3GC$, one of the 15 informative primers which demonstrate the differences of banding pattern between individuals in a population of *Grus antigone sharpii* L.

The dendrogram (Fig. 2) clearly distinguishes the studied samples into three groups without consideration of male and female or artificial breeding (insemination) or natural reproduction. The first group comprises 13 individuals, namely 114567283A♀, 115311146A♀, 114822755A♀, 116412280A♂, 116427254A♂, 115224744A♂, 114562111A♂, 122913516A♀, 115311352A♀, 126811095A♀, 123179520A♀, 114673192A♂ and 114752571A♂. The second includes 115232651A♀, 114754651A♂, 123211537A♀, 113556597A♂, 122911611A♂, 122652573A♂, 122914754A♀, 122917266A♂, 115229467A♀, 122638531A♂, 114833192A♂, 122762133A♀, 115224744A♀ and 115229154A♂. The third is an outgroup, West African Crown Crane.

	114567283A♀	115311146A♀	114822755A♀	116412280A♂	116427254A♂	115224744A♂	114562111A♂	122913516A♀	115311352A♀	126811095A♀	123179520A♀	114673192A♂	114752571A♂	115232651A♀	114754651A♂	123211537A♀	113556597A♂	122911611A♂	122652573A♂	122914754A♀	122917266A♂	115229467A♀	122638531A♂	114833192A♂	122762133A♀	115224744A♀	115229154A♂	West African-Crown Crane
114567283A♀	0.0																										
115311146A♀	12.1	0.0																									
114822755A♀	15.3	14.0	0.0																								
116412280A♂	21.3	12.1	20.2	0.0																							
116427254A♂	25.3	18.9	25.6	24.6	0.0																						
115224744A♂	21.6	14.7	17.8	21.4	12.0	0.0																					
114562111A♂	25.3	24.0	20.3	20.8	31.6	13.4	0.0																				
122913516A♀	24.4	24.7	18.7	26.1	25.8	22.2	23.4	0.0																			
115311352A♀	34.7	21.6	27.8	25.0	33.3	25.0	25.1	36.2	0.0																		
126811095A♀	34.6	31.3	36.9	30.4	41.0	37.7	35.0	40.4	34.5																		
123179520A♀	26.2	20.1	28.7	20.9	21.1	21.9	27.5	35.8	24.3	0.0	0.0																
114673192A♂	32.6	27.3	32.1	17.0	32.4	25.7	19.9	29.3	32.4	23.0	26.3	0.0															
114752571A♂	32.7	29.7	32.1	25.5	26.2	23.9	28.5	32.7	30.8	32.0	25.2	15.8	0.0														
115232651A♀	32.3	31.9	44.6	31.9	34.1	36.5	40.7	41.2	37.7	23.9	26.8	33.0	33.2	0.0													
114754651A♂	39.5	38.7	41.5	38.7	47.3	41.2	39.7	42.0	46.9	25.8	29.9	32.7	35.9	20.4	0.0												
123211537A♀	32.9	27.7	37.9	34.8	45.0	40.5	43.0	39.1	37.8	30.0	27.2	39.7	43.8	29.5	25.0	0.0											
113556597A♂	29.9	23.1	32.4	30.2	35.9	27.9	33.8	40.9	42.0	24.3	26.6	30.7	28.5	36.0	21.6	25.4	0.0										
122911611A♂	26.6	17.6	30.3	24.2	29.2	21.9	27.7	35.4	31.5	24.8	24.5	24.4	26.1	25.8	27.2	25.0	8.5	0.0									
122652573A♂	28.6	23.5	36.7	26.0	36.0	29.5	33.0	43.1	35.8	26.4	24.3	27.7	32.2	20.6	19.0	24.1	9.8	13.5	0.0								
122914754A♀	34.3	26.0	39.1	31.8	33.0	30.4	38.0	42.4	41.8	30.2	33.3	33.9	35.0	30.1	26.9	31.1	16.3	11.5	19.3	0.0							
122917266A♂	32.5	25.1	33.1	22.3	32.9	26.4	25.9	39.6	36.9	25.0	29.5	25.8	29.3	29.0	32.6	40.1	16.1	14.1	18.8	17.5	0.0						
115229467A♀	28.9	19.5	32.5	24.7	35.6	27.6	29.4	32.2	32.2	32.9	25.8	33.7	39.1	31.2	30.4	32.5	21.2	16.5	17.6	20.8	20.3	0.0					
122638531A♂	28.1	17.5	29.1	24.1	29.7	24.4	27.7	30.5	30.5	33.1	28.9	31.4	35.9	29.8	31.3	31.8	18.1	15.5	15.2	21.3	19.5	5.2	0.0				
114833192A♂	34.9	29.1	35.9	36.8	43.3	38.0	42.8	43.3	53.3	31.3	34.4	42.7	41.0	33.5	27.3	27.3	9.6	20.2	19.6	23.1	23.6	28.9	26.8	0.0			
122762133A♀	42.1	32.9	42.3	33.8	43.3	31.7	36.6	41.0	41.0	30.8	31.6	31.1	31.2	36.4	31.6	36.1	22.5	19.8	23.6	30.1	18.6	25.5	27.5	36.3	0.0		
115224744A♀	25.1	24.9	29.0	34.7	29.0	23.8	25.9	35.7	35.7	34.9	31.9	33.5	37.3	29.2	36.3	27.8	27.6	22.7	26.2	31.5	33.3	24.2	22.3	33.1	39.1	0.0	
115229154A♂	23.6	22.4	32.0	27.3	31.3	27.5	27.9	34.0	34.0	30.7	29.8	28.4	34.1	24.8	27.7	31.0	24.7	17.4	19.6	27.4	28.0	18.2	17.4	34.6	38.6	11.8	0.0
West African-Crown Crane	26.3	32.1	42.2	30.3	39.2	44.2	49.7	37.0	37.0	39.7	37.6	34.5	36.7	33.9	47.2	44.8	46.0	40.0	36.7	41.7	41.1	44.8	41.8	54.7	52.0	48.9	38.5	0.0

Fig. 3: Genetic distance (D) values of *Grus antigone sharpii* L. population and West African Crown Crane

Table 3: Genetic diversity (H') and evenness (E) values of *Grus antigone sharpii* L. population and an outgroup

Primer sequence 5'-3'	H'	E
$(CT)_8TG$	0.574	0.174
$(CA)_6AC$	0.514	0.156
$(CA)_6GT$	0.507	0.154
$(CA)_6GG$	0.353	0.107
$(GA)_6GG$	0.512	0.155
$(GA)_6CC$	0.479	0.145
$(GT)_6CC$	0.504	0.153
$(CAC)_3GC$	0.478	0.145
$(GAG)_3GC$	0.604	0.183
$(ACTG)_4$	0.522	0.158
$(GT)_8C$	0.423	0.128
$(AC)_8CG$	0.493	0.150
$(CCCT)_4$	0.500	0.152
$CCCC(GT)_6$	0.423	0.128
$(GA)_5$	0.626	0.190
Average	0.501	0.152

The genetic distance (D) values of a population range display from 12.1 between 115311146A♀ and 114567283A♀ to 53.1 between 114833192A♀ and 115311352A♀ (Fig. 3).

The genetic diversity of the population of the 27 studied samples in 60 individuals enlarged from 16 original individuals is 0.501 (Table 3). The value reflects the fairly good range of distribution sites of the species. Genetic richness is 27, ln genetic richness (ln 27) is 3.296, so the genetic evenness is 0.152.

DISCUSSION

The high genetic distance values of 12.1-53.3 in a species indicate that they possess several different genetic variations and the population can be further enlarged toward the goal of existing naturally in Thailand.

Genetic diversity is an important factor for genetic variation measurement. The genetic diversity in the population of the introduced eastern sarus crane is 0.501. It is not at satisfactory level. However, it reveals a fairly good value to support the broad range of distribution sites. This shows that the genetic diversity of *Grus antigone sharpii* L. may be able to fit environmental variation (Fu *et al*., 2003). High genetic diversity is important, allowing *Grus antigone sharpii* L. to adjust to the ever-changing environment in Thailand, whether the changes are due to the natural or human factors (Chamberlain and Hubert, 2001).

The genetic evenness value is very low, 0.152, showing that the studied samples have little equal genetic abundance. The genetic evenness is the measure of whether the number of alleles at a locus occurs equally frequently. For example, if three alleles

are available at a locus, do they occur equally frequently (1:1:1) or not, such as one prominently and the other two rarely (100:2:1). When using genetic diversity data, genetic richness is defined as the number of alleles potentially found at a locus and genetic evenness is a measure of whether the number of alleles at a locus occurs equally frequently (Brown and Weir, 1983).

The dendrogram cannot show the result position of natural reproduction of recent parents, 122638531A♂* x 115311352A♀* and offsprings, 122917266A♂*, 115232651A♀* and 115229467A♀* and similarly cannot show the recent position of artificial insemination, namely microchip coding 114673192A♂** x 114567283A♀** and offspring, 115229154A♀**. Since for a small population in a limited area, human factors for enlarging the population have varied methods including natural and artificial reproduction. The *Grus antigone sharpii* L. in this area may change their behavior from living in lone male/female pairs. As shown in the dendrogram, the sixteen original parents from Cambodia, males and females parents were not indicated.

The measurements of richness, evenness, genetic distance and diversity show that the population of *Grus antigone sharpii* L. introduced to Thailand are rather suitable to be maintained in a Thai natural habitat in the form of traditional variety.

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