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MITOCHONDRIAL CONTROL REGION VARIATION AMONG THE SUBSPECIES OF SARUS CRANE (GRUS ANTIGONE)

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by

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Approved by

Kamal Ibrahim, co-chair
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Sarus cranes (*Grus antigone*) are the tallest members of the crane family, Gruidae. They are found in four geographically distinct regions: northwest India (Indian Sarus- *Grus antigone antigone*), southeast Asia (Burmese Sarus- *Grus antigone sharpei*) and northern Australia (Australian Sarus- *Grus antigone gillae*). Although the three subspecies are morphologically distinct, their genetic distinctness is unclear. In this study, I focused on control region sequences of mitochondrial DNA (mtDNA) to assess the genetic and phylogeographic distinctness of Sarus subspecies. I used samples from four fragmented populations, seven from India, 16 from southeast Asia, five from Myanmar and eight from Australia. Phylogenetic trees were estimated using two Brolga crane sequences (*G. rubicunda*) as outgroups. All phylogenetic trees had low resolution, but AMOVA showed that all four Sarus populations are differentiated from each other. Nested clade phylogenetic analysis showed that most of the southeast Asian haplotypes are found at the center, suggesting that the Thai population includes the maximum number of ancestral haplotypes. Sarus cranes probably originated in southeast Asia and migrated both north towards India and south towards Australia during the last glacial maximum.

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INTRODUCTION

The Sarus crane (*Grus antigone*) is the tallest member of the crane family, Gruidae. Sarus cranes are found in northwest India, southeast Asia and Australia. Their range includes the plains of northern, northwestern, and western India, and the western half of Nepal's Tarai lowlands. Indian Sarus cranes (*G. a. antigone*) are commonly found in the Indian states of Uttar Pradesh, Rajasthan, Gujarat, Haryana, Bihar and Madhya Pradesh. There are two small populations in southeast Asia, one in central Myanmar and another mostly in Cambodia, both known as Burmese Sarus (*G. a. sharpie*). The Burmese Sarus has also occasionally been reported during the breeding season in northern Myanmar, and a few individuals appear at the beginning of the monsoon season in the eastern Indian states of Tripura and Manipur (Ali 2002). The Australian Sarus (*G. a.* gillae) is currently found in northern Queensland, and in the vicinity of Port Roper, Northern Territory.

The Indian and Burmese Sarus cranes were initially classified as distinct species on the basis of their body size and plumage (Blyth and Tegetmeier 1881). White feathers make a collar between the reddish skin of the upper neck and the gray feathers of the lower neck in the Indian Sarus. The Indian Sarus have red flight feathers and have white tertiary remiges on the wings. The Indian Sarus is distinctly taller than its Burmese counterpart. Tertiary remiges are grey in the Burmese Sarus. Sharpe (1894) and Blanford (1895) gave Indian and Burmese Sarus subspecies status, *G. a. antigone* and *G. a. sharpei*, respectively Sarus cranes were first observed in Australia in 1966. These cranes have grey tertiary remiges, and they are shorter than the Indian Sarus. The Australian

Sarus was initially considered to be *G. a. sharpei* (Gill 1969, Archibald 1981). Later, on the basis of its darker plumage and larger ear patch, it was classified as a distinct subspecies, *G. a. gillae* (Schodde 1988).

Although confidence in the anatomical and geographic distinctness of Sarus crane subspecies has increased in recent years (Meine and Archibald 1996), questions have arisen regarding their genetic distinctness and evolutionary history. Hence, phylogeographic study of these birds is essential. Phylogeography takes a population-genetic and phylogenetic perspective on biogeography.

PREVIOUS STUDIES

Dessauer *et al*. (1992) documented relatively low allozyme heterozygosity (H = 0.024) in a sample of nine Australian Sarus, but did not assay the other subspecies. Krajewski and Wood (1995) studied sequence variation in the mitochondrial cytochrome-b, $tRNA^{Thr}$ and $tRNA^{Pro}$ genes of Sarus cranes. This study revealed 0.7% to 1.5% sequence divergence among three individuals representing each of the subspecies. Wood and Krajewski (1996) added the ND6 (NADH dehydrogenase subunit-6) gene to the previous three genes as well as more Sarus individuals. The Brolga crane (*Grus rubicunda*), which is thought to be the closest relative of Sarus (Archibald 1976), was used as an outgroup. The 1,831-base-pair alignment consisted of nine unique haplotypes. Phylogenetic analysis revealed reciprocal monophyly among the nominal subspecies. Wood and Krajewski (1996) concluded that there may not have been any long-term isolation among subspecies, especially between the Indian and Burmese populations, which had a parapatric distribution until the mid-1900s.

Jones *et al.* (2005) studied the subspecific status of Sarus populations using microsatellite DNA markers. They genotyped 39 samples from India, Myanmar, Thailand and Australia. They reported that the Indian, Myanmar and Thailand (southeast Asian) populations were similar in terms of gene diversity (H_e), heterozygosity (H_o) and allelic richness (ranges 0.30–0.37, 0.27–0.35, and 2.15–2.53, respectively). In the Australian population, gene diversity (H_e), heterozygosity (H_o) and allelic richness were 0.21, 0.17 and 1.78, respectively, and significantly lower than the other three populations. Moreover, Analysis of Molecular Variance (AMOVA) showed an overall F_{st} value of 0.21 ($p<0.05$). Jones *et al.* (2005) showed the estimates of F_{st} were significantly lower between populations that are geographically closer to each other. They suggested that the Myanmar population is an introgression zone between Indian and southeast Asian populations. The Indian population had the highest number of private alleles, while the Australian population had a higher inbreeding coefficient (f=0.18).

CURRENT STUDY

In my study, the control region, a noncoding area of the mitochondrial genome, was sequenced. Light-strand replication of mitochondrial DNA starts in the control region (Fish *et al.* 2004). Although certain domains within the control region are conserved, it has large regions that are highly variable. This makes it useful for the study of recent evolutionary history (Larizza *et al.* 2002). The control region has been extensively used for this purpose in many vertebrate species, including Neotropical cats (Salzano *et al.* 1998) and humans (Saccone *et al.* 1992). Rhymer *et al.* (2001) used mtDNA control region to estimate the intra and inter population divergence of Sndhill

cranes. Later, Morozov *et al.* (2004) used 490 bp of the control region to evaluate inter- and intra-population divergence in Siberian cranes. My study uses a larger sample of individuals (36) than employed in the previous mitochondrial study of Sarus cranes. My goal was to assess the genetic and phylogeographic distinctness of the Sarus populations using highly variable domains in the control region.

Objectives

I analyzed the control region of the Sarus mitochondrial DNA to address the following questions:

- Are the Sarus populations differentiated from each other?
- Is the Australian population the only one that is genetically distinct?
- Is there any discernable (statistically significant) differentiation between the Thailand and Myanmar populations?
- Are genetic and geographic distances correlated among the four Sarus populations?
- Do the control region data yield insight into historical range expansions and / or contractions?

MATERIALS AND METHODS

SAMPLING DESIGN

This study analyzed mitochondrial DNA variation in a sample of 36 Sarus cranes. These same animals were used in the microsatellite study of Jones *et al*. (2005). Five blood samples were collected from Myanmar and 16 were obtained from southeast Asia by International Crane Foundation (ICF) personnel. Other samples were donated by zoos in the United States and by the International Crane Foundation, including eight samples from India and nine samples from Australia (Table 1). Three other DNA samples–one from India (extraction number 262), one sample from Thailand (extraction number 56) and one from Australia (extraction number 73)–could not be amplified by PCR, and so were not used in this study. The Brolga crane (*Grus rubicunda*) was used as the outgroup because it has been shown to be the closest living relative of Sarus cranes (Krajewski *et al.* 2010).

TECHNICAL PROTOCOLS

DNA Extraction and Polymerase Chain Reaction (PCR)

DNA from Sarus cranes was extracted by Wood and Krajewski (1996), who described the laboratory protocol. About one third of the control region (487 bp, including Domain I and part of Domain II) was amplified using PCR with the primer combination L16707 (5'-GTACTGGATTACATTCAG-3') (C. Krajewski unpublished) and H0778 (5'-ACGAATACCATGTATGC-3') (Rhymer *et al.* 2001). PCRs were carried out in 50µl, containing 25µl of 2X PCR Master mix (Promega Corporation, Madison WI), 5µl of 20µM stock solutions of each primers, 4µl (10 ng/µl) DNA template and

16µl of dd H_2O. The 2X PCR Master mix contained 0.05u/µl of *Taq* DNA Polymerase (recombinant), reaction buffer, 4 mM $MgCl_2$, 0.4 mM of each dNTP (dATP, dCTP, dGTP, dTTP). Thermal cycling started with 5 min denaturation at 94°C, followed by 35 cycles of denaturation (94°C, 1 min), annealing (50°C, 1 min) and primer extension (72°C, 1 min); PCR concluded with a terminal extension at 72°C (7 min) and final holding at 4°C. PCRs produced a single band of approximately 487 bp for all 36 Sarus samples.

PCR Purification and DNA Quantification

PCR products were purified using Qiagen QIAquick PCR Purification Kit (Qiagen Inc.) following the manufacturer's protocol. PCR products were quantified using a fluorometer (Hoefer DyNA Quant 200) following the manufacturer's protocol. PCR products were sent to Functional Biosciences Inc., Madison, Wisconsin, for DNA sequencing using BigDye cycle sequencing chemistry on capillary auto sequencers.

DATA ANALYSIS

Sequence Alignment and Basic Statistics

Sequences were aligned and edited using BioEdit (Ibis Biosciences, Carlsbad, CA 92008). The number of polymorphic sites (S) was counted using MEGA 4.0 (Tamura *et al.* 2007). The diversity parameter θ, which is a product of the mutation rate across the entire DNA sequence and the effective population size was calculated both manually and with the help of Arlequin 3.1 (Excoffier *et al.* 2005). Estimates of the diversity parameter θ were calculated manually from the estimates of number of polymorphic sites (S) and

the estimates of nucleotide mismatches (Π) based on the expectations: E(S) = $\theta(1+{}^{1}/_{2}+{}^{1}/_{3}+\ldots+{}^{1}/_{n-1})$ where n is the number of sequences and, E(Π) = θ. Comparing the S and ∏ estimates of θ is one of the ways to detect departures of the sequences from neutrality and steady state assuming the infinite-sites model (Tajima 1989a). The number of nucleotide mismatches (Π), (defined as the average number of nucleotide differences between any pair of aligned sequences) was calculated in the same way. The nucleotide diversity (π) was also calculated by dividing the nucleotide mismatches (Π) by the total length of sequence. The mean number of pairwise differences was estimated from the equation:

$$\frac{n}{n-1}\sum_{i=1}^{k}\sum_{j=1}^{k} p_i p_j \hat{d}_{ij}$$

Where $\hat{d}_{ij}$ is an estimate of the number of mutations since the divergence of haplotypes i and j, k is total the number of haplotypes, p_i is the frequency of haplotype i, and n is the sample size. Because the sample size is below 50, the sampling bias was reduced by multiplying the estimate by n/(n-1). These pairwise genetic distances were averaged for all comparisons between haplotypes from different populations to obtain estimates of genetic distances between populations. The statistical significance of the genetic distances between populations was tested using permutations (Tajima 1993).

To find out whether the DNA sequences are evolving neutrally or under selection, Tajima's D was calculated and significance was tested using Arlequin 3.1. When demographic parameters are estimated from population genetic data, it is assumed that the genetic marker used is selectively neutral or, for practical purposes, can be considered

neutral. Tajima's D (Tajima 1989a) is a commonly used statistical test to test whether the observed variability in DNA sequences conforms to neutral expectations. Tajima's D is based on the difference (d) between the two estimators of θ, ∏ and S. If the DNA sequences are evolving neutrally, then the two estimates of θ will be fairly close and as a result (d) will be a very small value.

D is calculated by dividing d by the square root of its variance (standard deviation) $\sqrt{\hat{V}(d)}$. So, D = d/ $\sqrt{\hat{V}(d)}$. The significance of Tajima's D is tested by taking the null hypotheses of no selection, i.e., the sequences are evolving neutrally.

The estimates of θ obtained can yield estimates of the effective population size (N_e) using the equation $\theta = 2N_e\mu$, where μ is the mutation rate across the entire sequence. The effective population size is the number of individuals in a theoretically ideal population having the same magnitude of random genetic drift as the actual population. Effective population size is directly proportional to the diversity parameter θ; for a given mutation rate, θ increases with increase in population size.

Based on the effective population size, obtained in the above paragraph, a coalescent tree was drawn using Mesquite 2.72 software package (Maddison and Maddison 2009).

Mismatch distributions (distributions of pair-wise substitution differences between pairs of haplotypes in a population) were analyzed using the demographic expansion model (Rogers and Harpending 1992). If the population expansion is recent or the population is going through a bottleneck, a unimodal distribution will be seen, while long-term stable populations or slowly declining populations will show a multimodal mismatch distribution (Rogers 1995). The distribution of pairwise differences (mismatch

distribution) between pairs of haplotypes was obtained using Arlequin 3.1 and plotted using Excel. The mismatch distribution and population expansion model can be explained from the following equations: suppose, $\theta_0 = 2N_0\mu$; $\theta_1 = 2N_1\mu$; and $\tau = 2\mu t$, where the initial effective population size, N_0, suddenly changes in size to N_1 at τ units of mutational time. τ can be estimated from μ, the mutation rate per generation of the entire nucleotide sequence studied and t, the number of generations since expansion. So, the estimated number of generations (t) before which population expansion took place can be estimated from the equation: $\tau/2\mu$, if μ and τ are known.

Phylogenetic Trees

Minimum evolution and maximum parsimony trees were generated in MEGA 4.0. 1000 bootstrap resamplings were performed for both trees. ModelTest (Posada and Crandall 1998) was used to choose the best-fitting model of nucleotide substitution for the data. The best model was selected on the basis of AIC (Akaike's information criterion) and BIC (Bayesian information criterion) values. After model selection, the maximum likelihood tree was inferred with PhyML 3.0 (Guindon and Gascuel 2003). MrBayes 3.1 (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003) was used for Bayesian analysis. For Bayesian analysis, the nucleotide substitution model option was changed to the best model suggested by the Model Test. The analysis was run for 1,000,000 generations with a sampling frequency of 10 to get at least 1,000 samples from the posterior probability distribution. The analysis was stopped when the standard deviation of split frequencies fell below0.01. The default values were used for all other options. Two runs of the Bayesian analysis were carried out.

Analysis of Molecular Variance (AMOVA)

In order to find out whether the four Sarus populations are genetically distinct, Analysis of Molecular Variance (AMOVA) was implemented by Arlequin v 3.1. The question whether only the Australian population is significantly different and the other three Sarus populations are not differentiated, or whether all four populations are significantly differentiated from each other, was addressed by conducting AMOVA with and without the Australian population, and by comparing the F_{st} The DNA sequences were entered in the form of a text file in Arlequin. In AMOVA settings of Arlequin, the distance option was changed to Nei's pairwise difference. Arlequin calculates and makes a matrix of distances from the entered DNA sequences and yields sums of squares for the various hierarchical levels of the population. It then analyzes the sum of squares using nested ANOVA (Analysis of Variance). The variance components of this nested design are used to calculate F statistics ($F_{st,}$). Arlequin also provided pairwise F_{st} values among the four populations. The statistical significance of these estimates was tested against the null hypothesis of no differentiation among the populations using Arlequin.

Nested Clade Phylogenetic Analysis (NCPA)

Nested clade phylogenetic analysis was performed using the Automated Nested Clade phylogenetic Analysis (ANeCA) software (Panchal 2007) in order to investigate phylogeographic patterns in the dataset. ANeCA uses TCS v 1.21 (Clement *et al.* 2000) to generate a haplotype network following an algorithm recommended in Templeton (1992). This algorithm not only estimates the unrooted haplotype tree but simultaneously

provides a 95% plausible set for all haplotype linkages in the unrooted tree. A nested cladogram is generated in which e individual haplotypes (referred to as "0-step clades") separated by a single mutation are grouped together into "one-step clades" proceeding from the tips to the interior of the network, then one-step clades separated by a single mutation are grouped in "two-step clades", and so on, until all haplotypes are included in the network.

Geodis (Posada *et al.* 2000) was used to examine whether there was any geographical association between the evolutionary history of the haplotypes as represented by the TCS network and the geographical locations of the haplotypes. GeoDis requires geographical information in the form of the latitude and longitude coordinates for each population. Exact geographical information for the Sarus crane samples is unavailable. Therefore, the latitude and longitude of the city or park that lies nearly at the center of each of the four populations were used as an approximation. For the Indian population, this landmark was Bharatpur, Rajasthan; for the Burmese population, it was Bagan, Central Myanmar; for the southeast Asian population, it was Nam Yuan, a city situated within the range of Kulen Prum Tep wildlife sanctuary (Laos); for the Australian population, it was Lakefield National Park, Queensland.

Mantel Test

A Mantel test was carried out to assess whether there is any correlation between geographic distances and genetic distances among the four Sarus populations. This procedures tests for an association between two or more independent dissimilarity matrices describe the same groups or entities. The first step in the Mantel test is to

calculate the sample correlation *r* for the two dissimilarity matrices. Then the values of *r* under the null hypothesis (of no association between the matrices) are generated using a randomization procedure, where the rows and columns of the matrices are randomly permuted and the significance is tested. In the current study, the two matrices were the genetic and geographic distance matrices. The geographic distances were calculated as for the GeoDis analysis (see above). Genetic distances were Nei's average pairwise differences (Nei *et al.* 1979).

RESULTS

SEQUENCE ALIGNMENT AND BASIC STATISTICS

A total of 36 mitochondrial DNA sequences, 487 base pairs each, were obtained. The alignment revealed twenty polymorphic sites (S). The nucleotide diversity (Π) was 5.41(Table 2).

The value of θ obtained from S was 4.83, and is fairly close to the Π-based estimate of 5.41, suggesting neutral sequence evolution.

In the current study, Tajima's D was non-significant (overall D = 0.37768, P = 0.67925), suggesting the sequences are evolving neutrally (Table 3).

Using Rhymer *et al.*'s (2001) estimate of mutation rate [3.4×10^{-6} per nucleotide site] and S estimate of θ

$N_e = 4.83/(2 \times 3.4 \times 10^{-6} \times 487) \approx 705882/487 \approx 1449.$

The N_e computed above can be further used to obtain estimates of the total population size (census size). Because mitochondrial sequences are being used, the N_e value computed above estimates the number of breeding females in the Sarus population. In a typical Sarus population, breeding females constitute 30% (Wood and Krajewski 1996). Thus, the total population size is ($N_f \times 100/30$) approximately 5,000 birds. This estimate considers that the generation time of Sarus cranes is 12.5 years but Sarus actually live longer than that. So, the census size is higher (20,000 birds worldwide) than that estimated from N_e.

In the current study, the distribution of pairwise mismatches was unimodal (Figure 1). The graph shows 6 mismatches as the modal class. Using the equations from

mismatch distribution and population expansion model, described in the method section, the following numbers were calculated:

If 3.4×10^{-6} = mutation per nucleotide site per generation (Rhymer *et al*. 2001),

Then, $\mu = 3.4 \times 10^{-6} \times 487 = 1.65 \times 10^{-3}$ mutations in the entire nucleotide sequence studied per generation

$\tau = 2\mu t$, where τ = units of mutational time and t = number of generations since population expansion)

$\tau = 5.37158$ (from Arlequin output)

So, $t = \tau/2\mu = 5.37158/2 \times 1.65 \times 10^{-3} = 1627$ generations

So, 1627 generations back the initial effective population size N_0 suddenly changed to N_1.

PHYLOGENETIC TREES

Minimum evolution (Figure 2) and maximum parsimony (Figure 3) trees showed very little resolution. All nodes have small bootstrap values, particularly in the maximum parsimony tree. The smallest AIC and BIC values (Table 5) were obtained for the TrN+I+G model (Tamura and Nei model with gamma distributed rate variation across sites and a proportion of invariable sites. The maximum likelihood tree (Figure 4) also had extremely small bootstrap values on most nodes. Due to the absence of the TrN+I+G model in MrBayes (Huelsenbeck and Ronquist 2003; Ronquist and Huelsenbeck 2005), I used the next best model, HKY+I+G (Hasegawa, Kishino and Yano model with gamma distributed rate variation and a proportion of invariable sites (Hasegawa *et al*. 1985) with AIC = 2082.6287 and BIC = 2372.3904 (Figure 5).

ANALYSIS OF MOLECULAR VARIANCE (AMOVA)

AMOVA partitioned the total genetic variation in the sequence data into within- and between-population components. The differentiation between the four Sarus crane populations, represented by the overall F_{st} , was 0.22408 ($P < 0.0001$). The within-populations component of the total variance was 77%. AMOVA carried out without the Australian population produced a lower F_{st} of 0.16953 ($P < 0.0001$). Pairwise comparisons (Table 7) of fixation indices revealed that the Myanmar and southeast Asian populations are significantly differentiated from each other ($F_{st} = 0.21$, $P < 0.0001$). F_{st} is lowest for the Indian-southeast Asian pair ($F_{st} = 0.094$, $P = 0.018$) and highest for the Australian-Burmese pair ($F_{st} = 0.389$, $P < 0.0001$).

NESTED CLADE PHYLOGENETIC ANALYSIS

The network of haplotypes produced by NCPA showed that haplotypes from southeast Asia were mostly present at the center, while the haplotypes from the other three populations were commonly found in at or near the tips. One of the southeast Asian haplotypes (extraction number 65) gave rise to three clades and those three clades gave rise to the entire cladogram (Figure 6).

GeoDis revealed that there are two higher-order clades in the nested cladogram with significantly small (293.7016 and 594.6105, respectively) interior D_c (distance between clades) and D_n (distance between nested clades) values (508.128 and 1139.6241, respectively). The chi-square values of those two clades were 7.2727 ($P < 0.01$) and 56.9424 ($P < 0.01$), respectively. The nested cladogram does not show the pattern of

completely non-overlapping ranges within the nested clade series. The GeoDis inference key (Posada *et al*. 2006) suggests that this kind of condition may imply restricted gene flow between populations with isolation-by-distance or allopatric fragmentation.

MANTEL TEST

Pairwise geographic and genetic distances among the four Sarus populations are shown in Table 8 and Table 9, respectively. Mantel test revealed a correlation coefficient of 0.336 (bootstrap 95% confidence interval -0.68, 0.74) between the genetic and geographic distances of the haplotypes. The significance test for this correlation produced a non-significant P value (0.447).

COALESCENT TREE

The coalescent tree, based on the effective population size, drawn using Mesquite 2.72 software package (Maddison and Maddison 2009) revealed that approximately 1650 generations back population expansion took place (Figure 8).

DISCUSSION

CONTROL REGION AS A MOLECULAR MARKER

Studies conducted on the mitochondrial genes *cytochrome b* and *ND6* by Krajewski *et al.* (1995) and Wood and Krajewski (1996), as well as the microsatellite analysis by Jones *et al.* (2005), did not reveal any phylogenetic distinction among the four Sarus populations. In the current study, I used the control region, the most variable portion of the mitochondrial genome, to assess differentiation between populations. While the control region is the most variable part of mitochondrial DNA, it is less variable than microsatellite loci. The main advantage of using mitochondrial DNA for analyses of this nature is its inheritance pattern. Mitochondrial DNA is only inherited maternally. This avoids complications arising from recombination in biparentally inherited genetic markers, including microsatellites. The variations seen in mitochondrial DNA, especially in the noncoding control region, are potentially only due to drift and migration and, as a result, are more suitable for inferring past demographic processes..

DIFFERENTIATION OF SARUS POPULATIONS

All phylogenetic trees obtained in this study showed very low resolution. This is expected because the subspecies represent recent divergences among populations. AMOVA, on the other hand, considers the evolutionary history of the haplotypes and their frequencies, which are affected by demographic history. Drawing phylogenetic trees may not be the best method for finding out variation between haplotypes, which are one or two mutations apart from each other, producing very low level of genetic variations among each other. Methods like AMOVA, which incorporates the demographic histories

of the haplotypes (Excoffier, 2005) worked better in this case showing that all Sarus populations are genetically distinct, which the control region phylogeny did not tell us.

There are, however, some limitations associated with AMOVA. First, AMOVA uses permutation tests to evaluate the null hypotheses of no population structure within and between groups. With few populations per group, between-group structure might be impossible to detect because only a few permutations of the sampled populations are possible (Fitzpatrick 2009). The second limitation arises when the variance within population is very high. As a result, very large sample sizes are required to detect significant differences between populations (Hedrick 1999). High within population variance can be frequently encountered with highly variable genetic markers like microsatellites and the control region. In this study, I had 34 unique haplotypes and two shared haplotypes. There was a high level of variation within populations.The problem of higher within population variance did not affect the current study.

Analysis of molecular variance with all four Sarus populations revealed a statistically significant F_{st} estimate; AMOVA without including the Australian population also produced a significant F_{st}, suggesting that the three Asian populations are genetically differentiated from each other. Pairwise F_{st} values corroborate this, showing that all four populations have differentiated from each other, including the Myanmar-southeast Asian pair ($F_{st} = 0.21$, $P < 0.0001$). The latter is expected because currently these two populations have a very restricted distribution, one in a small area at Began in northwest Myanmar and the other in southern Thailand near the Thailand-Cambodia border. The aerial distance between these two regions is around 570 km. Being a non-migratory bird, it is unlikely that Sarus disperse across this distance and interbreed. Also,

the rapid loss of wetland breeding areas in Myanmar over the last few centuries may have affected Sarus populations. Extirpation of the southeast Asian Sarus population (Barzen and Seal 2000) would have also caused a reduction of gene flow between the southeast Asian and Myanmar populations. The Indian Sarus population is vastly separated from the Myanmar population (aerial distance between Began and Bharatpur is approximately 2500 km). Sarus cranes are absent in eastern India, with the exception of Tripura and Manipur where a few birds have been reported during the monsoon season, but these cranes are part of the southeast Asian population (Ali 2002). The isolation between Indian and Myanmar populations is probably very recent. These two populations maintained a parapatric distribution across the Yamuna River in Bangladesh until the mid 1900s (Wood and Krajewski 1996). The rapid loss of wetlands and increasing wetland pollution in eastern parts of India is probably responsible for the extirpation Sarus populations there.

A higher number of private alleles at microsatellite loci were seen in the Indian population (Jones *et al.* 2005), possibly because it has maintained a higher effective population size. The Indian population is the only Sarus population in Asia that is increasing in size (Archibald *et al*. 2003) and it is well adapted to humans. My results suggests that the Indian population is genetically closer to the southeast Asian population (pairwise F_{st} =0.094 and mean genetic distance = 0.43) than to the Myanmar population (pairwise F_{st} = 0.295 and mean genetic distance = 2.33). The nested cladogram also supports this, revealing that the Indian birds are one or two mutations away from the southeast Asian birds, but are at least four mutations away from the Myanmar population. India, Myanmar and Cambodia are all parts of the Asian mainland. In contrast, the

Australian population is far from the southeast Asian population (aerial distance approximately 7800 km). Therefore, it is not surprising to find that the Australian population is significantly different from the other three. Sarus cranes are not found in Malaysia, Indonesia or New Guinea, which lie between Thailand and Australia.

DIGGING THE PAST: ORIGIN AND RANGE EXPANSION OF SARUS

The mismatch was unimodal, suggesting a relatively recent population expansion. If we consider the average lifespan of Sarus as 12.5 years (Wood and Krajewski 1996), then the upper limit of the population expansion will be tentatively 20,000 (1627 x 12.5) years ago, when the effective population size suddenly changed from N_0 to N_1 as described above. This falls within the timeframe of Late Pleistocene glacial cycles. This time period is also supported by the coalescent tree (Figure 8), which showed that all four Sarus populations shared a common ancestor nearly 1650 generations back. The haplotype network from NCPA revealed that most of the ancestral (interior) haplotypes belong to the southeast Asian population. The nesting of haplotypes (Figure 7) showed that one-step clades are either exclusively haplotypes from the same population or only include southeast Asian haplotypes. For example, the Indian and Myanmar haplotypes are never seen together in either one-step or two-step clades. This clearly shows that the southeast Asian Sarus are genetically close to all other Sarus populations. The Sarus population probably originated somewhere in southeast Asia and expanded northwards using the huge corridor of the Asian mainland during the Last Glacial Maximum (LGM), 20,000 years ago, when Thailand and Indonesian islands as far east as Borneo and Bali were connected in a landmass called Sundaland

(Sathiamurthy and Voris 2006). The Philippines were also connected to this landmass by the Sibutu Passage and the Mindoro Strait. Sarus populations may have dispersed to Philippines too. The Sarus populations appear to be extirpated in Indonesia, Malaysia and Philippines (Madsen 1981).

During LGM, Australia and New Guinea were connected, forming Sahulland. The width of water gaps between the two continents Sundaland and Sahulland, were considerably smaller than the current locations of Australia and southeast Asia (Sathiamurthy and Voris 2006), due to smaller water gaps in Wallacea (a group of Indonesian islands), which lie between Sundaland and Sahulland. Thus, dispersal of Sarus cranes from Sundaland to Sahulland may have occurred during the LGM. At the end of the LGM, the largely terrestrial connection between southeast Asia and Australia was lost and gene flow between these two populations may have stopped, resulting in their genetic distinction. The results of the GeoDis analysis support this, suggesting isolation among these populations took place due to distance or restricted gene flow (allopatric fragmentation).

COMPARISON OF CONTROL REGION AND MICROSAT STUDIES

Control region results reported here agree with the microsatellite study by Jones *et al*. (2005) in terms of overall F_{st} values (F_{st} = 0.21 in microsat study and F_{st} = 0.224 in this study), suggesting genetic differentiation of the four Sarus populations. However, pairwise F_{st} values were quite different in the current study than those for microsatellites. The microsatellite study found that the pairwise values were lower between populations that are closer geographically (F_{st} for Indian-southeast Asian = 0.25 and Indian-Myanmar

= 0.18), but in the current study the F_{st} of the Indian-southeast Asian pair was significantly smaller (F_{st} = 0.09) than that of the Indian-Myanmar pair (F_{st} = 0.29) (Table 7). Also, Jones *et al.* (2005) found very little micosatellite diversity among the Asian Sarus populations, especially between Myanmar and southeast Asia. However, the control region revealed significant differentiation between the Myanmar and southeast Asian populations (F_{st} = 0.21, P < 0.0001).

CONSERVATION ASPECTS

The effective population size (N_e) estimated in this study is nearly 1500. As mentioned before, the Sarus populations appear to be extirpated in Indonesia, Malaysia and Philippines. Luzon Sarus (*Grus antigone luzonica*) is now extinct. IUCN has already given ”vulnerable” status to the Sarus crane and the population is declining. Its status may change to” critical” in the near future if proper management plans are not implemented. Proper conservation measures should be taken immediately to protect these birds, especially in southeast Asia, where they are suffering from extensive population fragmentation caused by dramatic reduction of habitat. Saving and conserving the wetlands in southeast Asia may be one major management step forward to save these birds. Conservation measures should also be taken to prevent further extirpation of Sarus from Southeast Asia. This study has shown that all four Sarus populations are genetically distinct from one another. Also, there are 34 unique haplotypes out of 36 samples used in this study. So, there is great variation within each Sarus population, Sarus populations should be conserved and managed as separate gene pools. As all four Sarus populations are genetically distinct from each other, the possibility of out-breeding depression exists

if the birds from two different populations are crossed. So, to avoid out-breeding depression, the crossing of birds between the different Sarus populations should be avoided until crossing becomes an absolute necessity to avoid more serious inbreeding depression. (Chalesworth and Charlesworth, 1987; Crnokrak and Roff, 1999; Frankham, 2010).

Table 1. Scientific names and the sources of the samples used in this study.

Species	Extraction No.	Specimen Data
Grus antigone gillae	5	ICF[1] 8-28, UWZM[2] 630
	17	ICF 8-39, UWZM 632
	46	ICF 8-19, UWZM 646
	72	ICF 8-28, UWZM 651
	73	ICF 8-36, UWZM 652
	99	ICF 8-32, UWZM 631
	100	ICF 8-38, UWZM 689
	102	ICF 8-37, UWZM 708
	103	ICF 8-31, UWZM 705
Grus antigone sharpei	55-71	1[3]-17
	312-316	Burma, #1- Burma, #5
Grus antigone antigone	77	ICF 8-45
	78	ICF 8-44
	222	St. Louis Zoo
	252	A00225, Miami Metro Zoo
	253	A03709, Miami Metro Zoo
	254	80-757, Baltimore Zoo
	262	ISIS 374 Sunset Zool Park
	263	ISIS 375 Sunset Zool Park

[1]ICF stands for International Crane Foundation

[2]UWZM stands for University of Wisconsin Zoological Museum

[3]Arbitrary numbers assigned to blood samples from captive birds in Thailand. No voucher data.

Table 2. Nucleotide diversity within and among four Sarus populations.

Population	No. of Samples	No. of Substitutions	Polymorphic Sites	Nucleotide mismatches (∏)	θ from S
Indian	7	17	16	7.286	6.53061
Southeastern	16	15	15	4.467	4.52048
Myanmar	5	4	4	2.000	1.92000
Australian	8	11	10	4.357	3.85675
Overall	36		20	5.41	4.83

Table 3. Tajima's D test.

	Indian	Southeastern	Myanmar	Australian	Overall
Tajima's D	0.64161	-0. 04664	0.27345	0.64229	0.37768
P Value	0.76400	0.52400	0.67200	0.75700	0.67925

Table 4. Population average pair-wise differences.

	Indian	Southeastern	Myanmar	Australian
Indian	7.28			
Southeastern	0.43 (P = 0.003)	4.47		
Myanmar	2.33 (P = 0.03)	1.22 (P = 0.34)	2.00	1.62 (P < 0.01)
Australian	1.61 (P <0.01)	1.62 (P < 0.01)	2.32 (P < 0.01)	4.36

Table 5. AIC and BIC scores for different ML models.

Model	-lnL	K	AIC	BIC
TrN+I+G	944.9846	9	2073.3383	2366.4654
TIM2+I+G	944.0125	8	2074.2309	2370.7096
TrN+I	949.1183	6	2078.7829	2368.5446
HKY+I+G	951.0412	6	2082.6287	2372.3904
GTR+I+G	948.0543	8	2088.0297	2391.1697
HKY+I	955.5336	5	2088.8044	2375.1870
TVM+I+G	950.1051	9	2089.2667	2389.0831
GTR+I	951.2605	9	2091.5776	2391.3940
K80+I+G	962.8741	3	2097.9080	2377.4915
K80+I	966.0745	2	2101.5403	2377.7041

Table 6. AMOVA Results.

AMOVA Design	Variance components		Percentage of variation				Population specific F_{st} values			
	Among populations	Within populations	Among populations	Within populations	F_{st}	P-value	Indian	S.east	Myanmar	Australian
1. With Australian Population	0.67	2.33	22.41	77.59	0.224	<0.0001	0.177	0.223	0.281	0.231
2. Without Aus--tralian Population	0.48	2.37	16.95	83.05	0.169	<0.0001	0.121	0.171	0.233	NA

Table 7. Pair-wise F_{st}.

	Indian	Southeastern	Myanmar	Australian
Indian				
Southeastern	$F_{st} = 0.09$ (P = 0.02)			
Myanmar	$F_{st} = 0.29$ (P = 0.01)	$F_{st} = 0.21$ (P<0.01)		
Australian	$F_{st} = 0.22$ (P <0.01)	$F_{st} = 0.27$ (P< 0.01)	$F_{st} = 0.39$ (P<0.01)	

Table 8. Pair-wise geographical distances among four Sarus populations in km.

	Southeastern	Australian	Indian	Myanmar
Southeastern				
Australian	7274.1			
Indian	2493.9	9677.8		
Myanmar	559.2	7808.1	1934.8	

Table 9. Pair-wise genetic distances (Nei's) among four Sarus populations.

	Southeastern	Australian	Indian	Myanmar
Southeastern				
Australian	1.61935			
Indian	0.42738	1.610714		
Myanmar	1.21667	2.32143	2.32857	

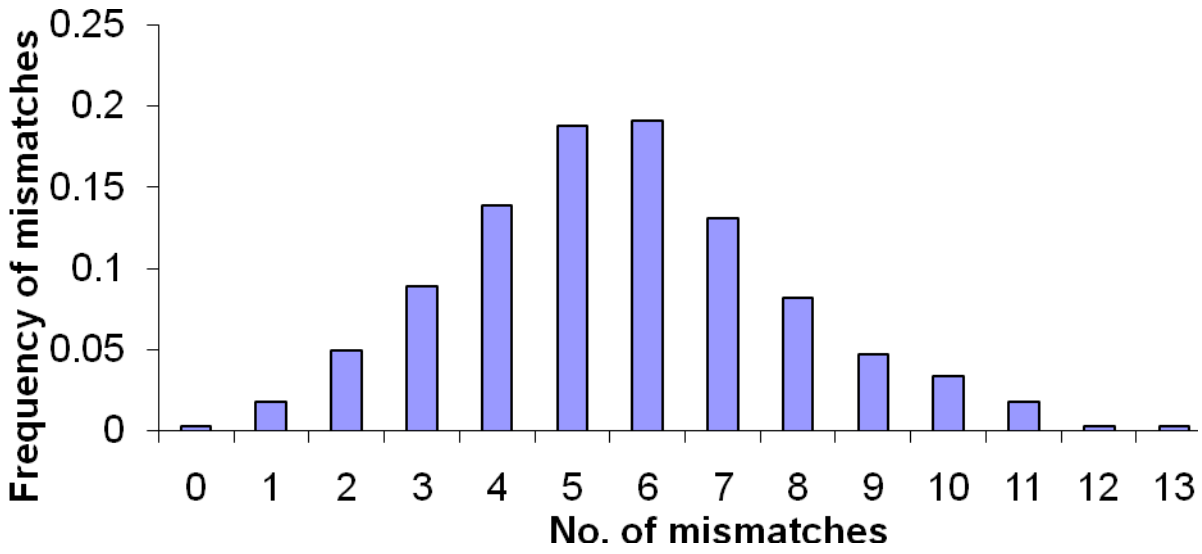

Figure 1: Pair-wise nucleotide mismatch distribution, comparing the number of mismatches between a pair of sequences with the frequency of that mismatch in the population.

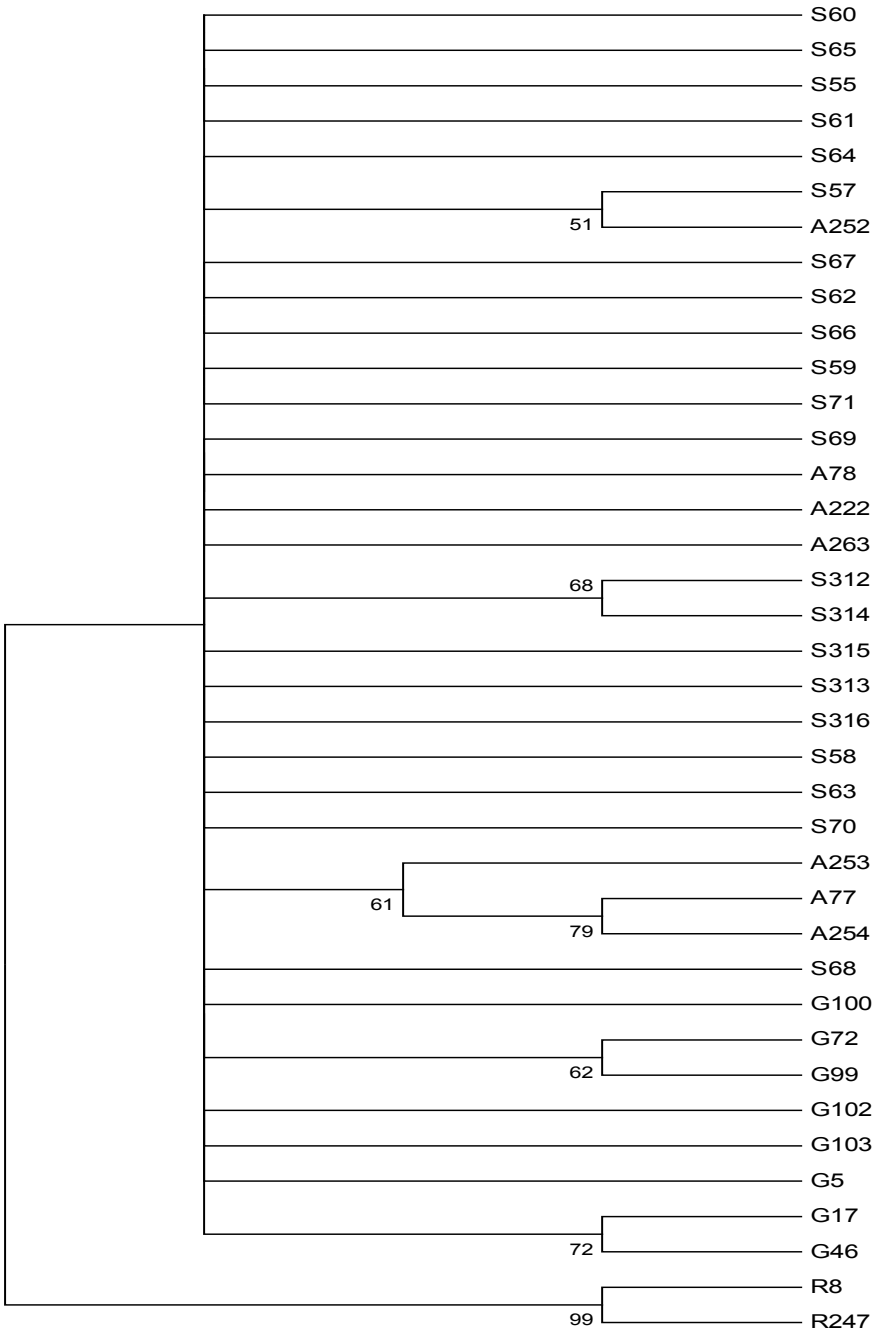

Figure 2: Minimum Evolution tree with bootstrap values. The nodes with less than 50% bootstrap support have been collapsed. S designates *Grus antigone sharpei,* G designates *Grus antigone gillae*, A designates *Grus antigone antigone* and R designates *Grus rubicunda.* The numbers associated with the haplotypes suggest the extraction numbers of those samples.

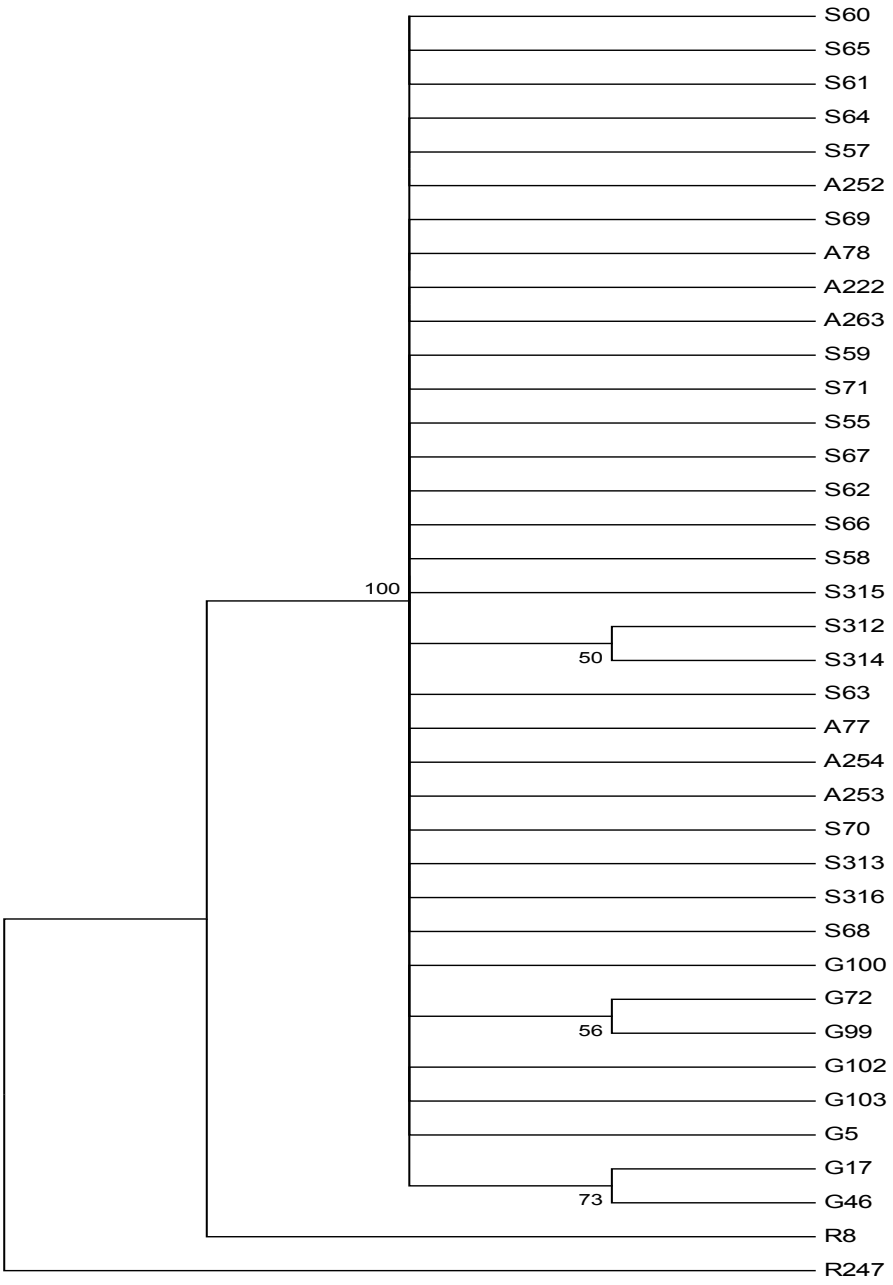

Figure 3: Maximum parsimony tree with bootstrap values. The nodes with less than 50% bootstrap support have been collapsed. S designates *Grus antigone sharpei,* G designates *Grus antigone gillae*, A designates *Grus antigone antigone* and R designates *Grus rubicunda.* The numbers associated with the haplotypes suggest the extraction numbers of those samples.

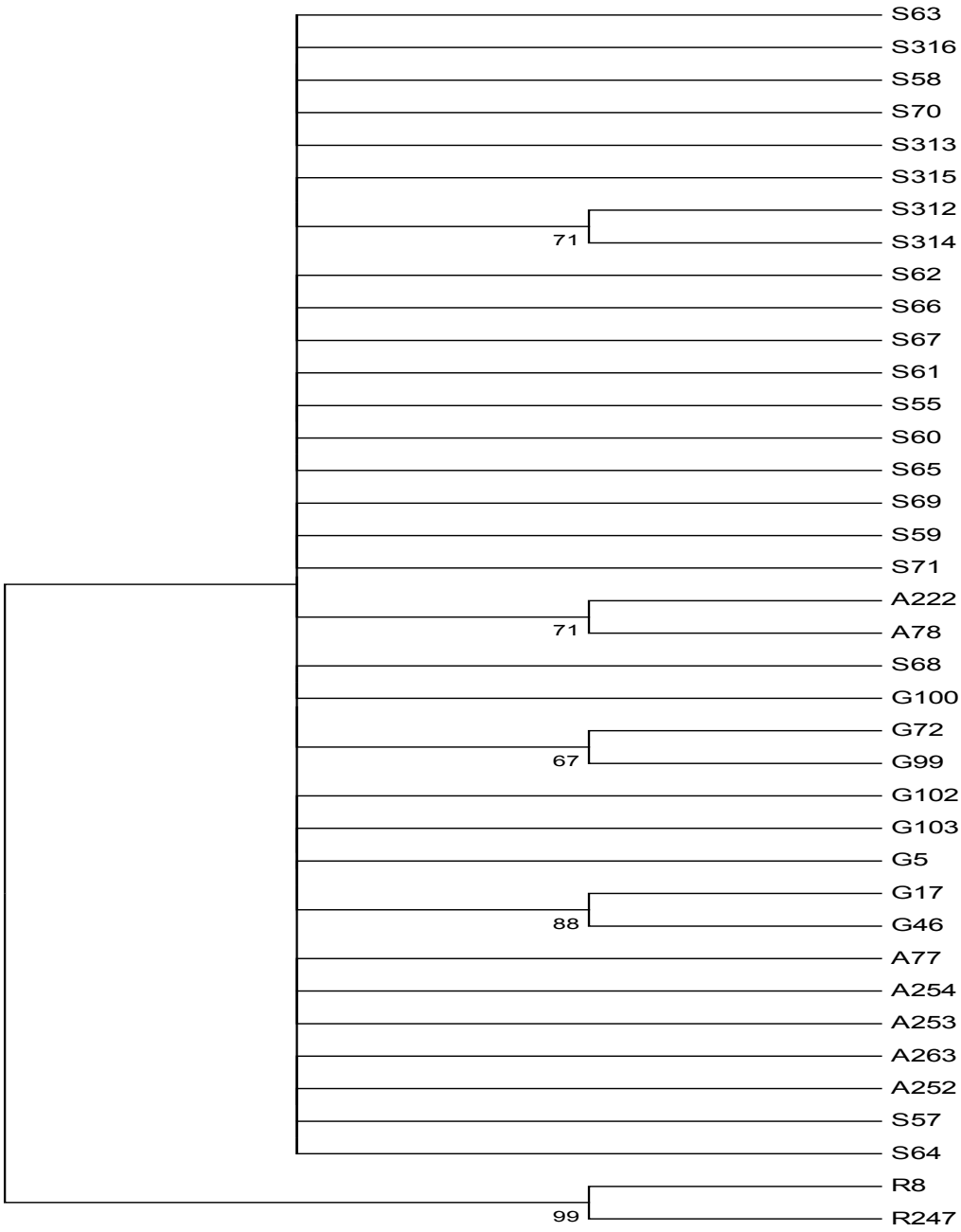

Figure 4: Maximum likelihood tree, using TrN+I+G model with bootstrap values. The nodes with less than 50% bootstrap support have been collapsed. S designates *Grus antigone sharpei,* G designates *Grus antigone gillae*, A designates *Grus antigone antigone* and R designates *Grus rubicunda.* The numbers associated with the haplotypes suggest the extraction numbers of those samples.

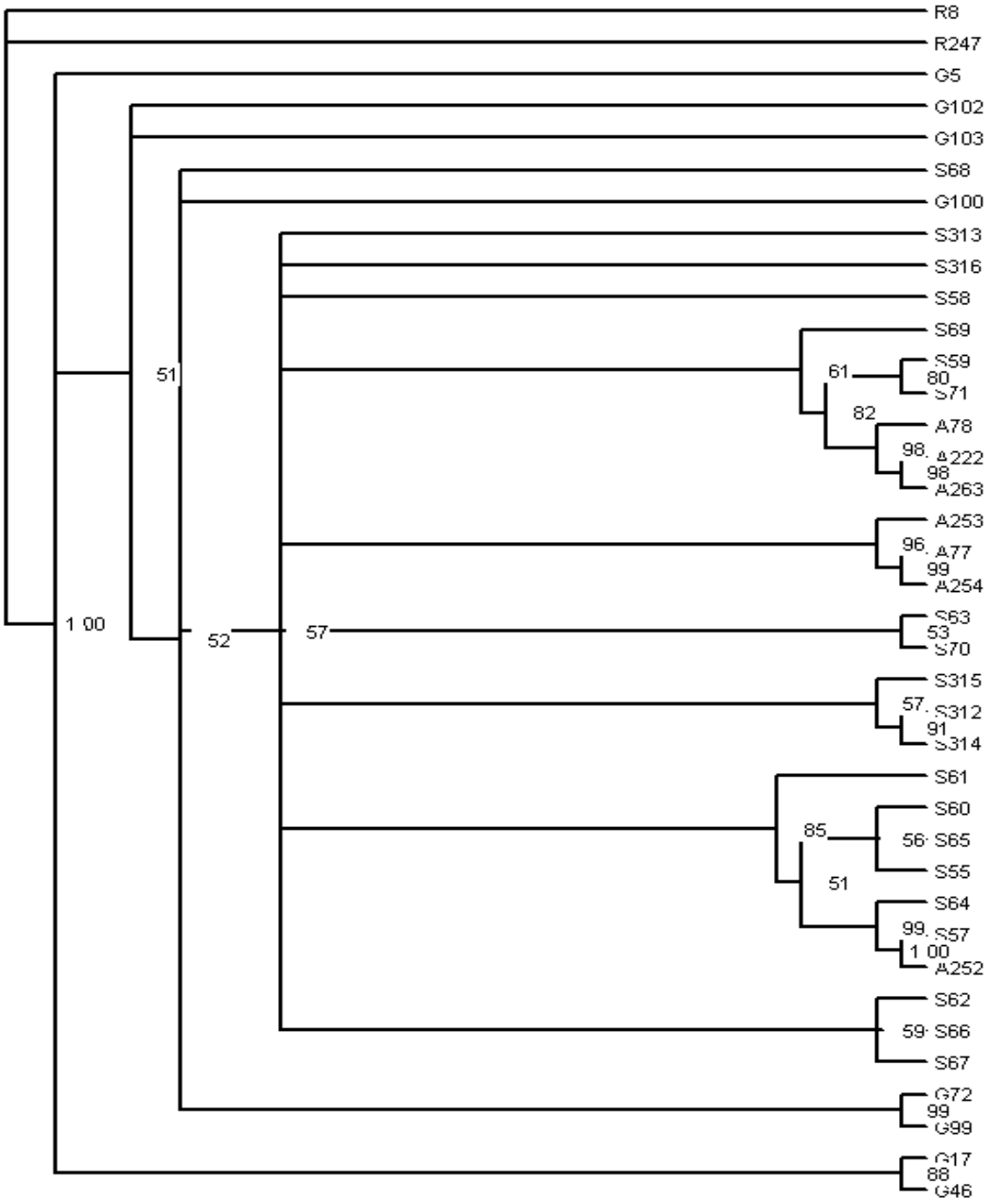

Figure 5: Bayesian phylogenetic tree, using HKY+I+G model with bootstrap values. The nodes with less than 50% bootstrap support have been collapsed. S designates *Grus antigone sharpei,* G designates *Grus antigone gillae*, A designates *Grus antigone antigone* and R designates *Grus rubicunda.* The numbers associated with the haplotypes suggest the extraction numbers of those samples.

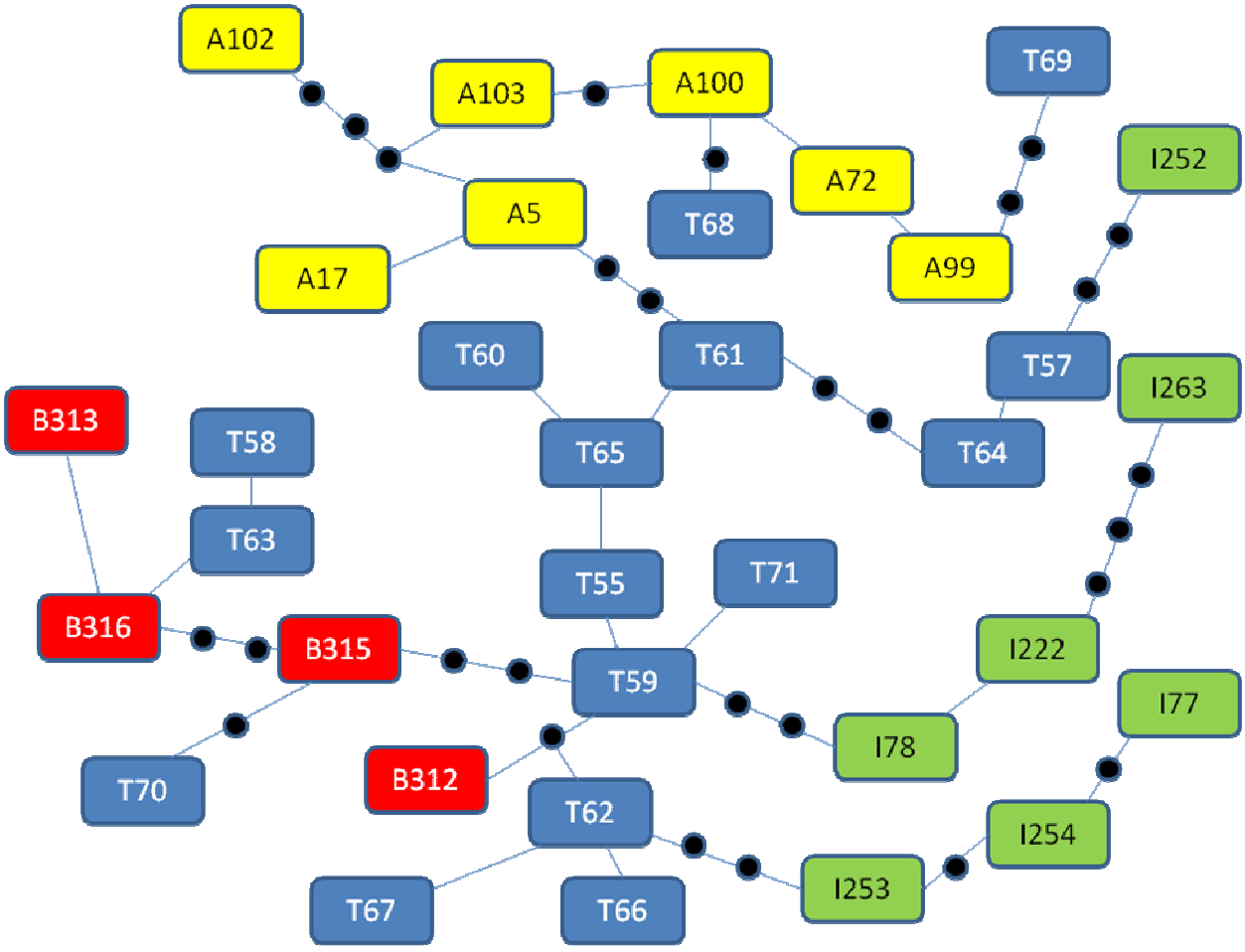

Figure 6: Haplotype network of Sarus haplotypes. "A" designates Australian Sarus, "T" designates southeast Asian Sarus, "I" designates Indian Sarus and "B" designates Myanmar Sarus. The black balls designate the mutation steps. The numbers associated with the haplotypes are their extraction numbers. The haplotypes are given four different colors on the basis of the population, they belong to: Indian Sarus green, Australian Sarus yellow, Burmese Sarus red and southeast Asian Sarus have blue color.

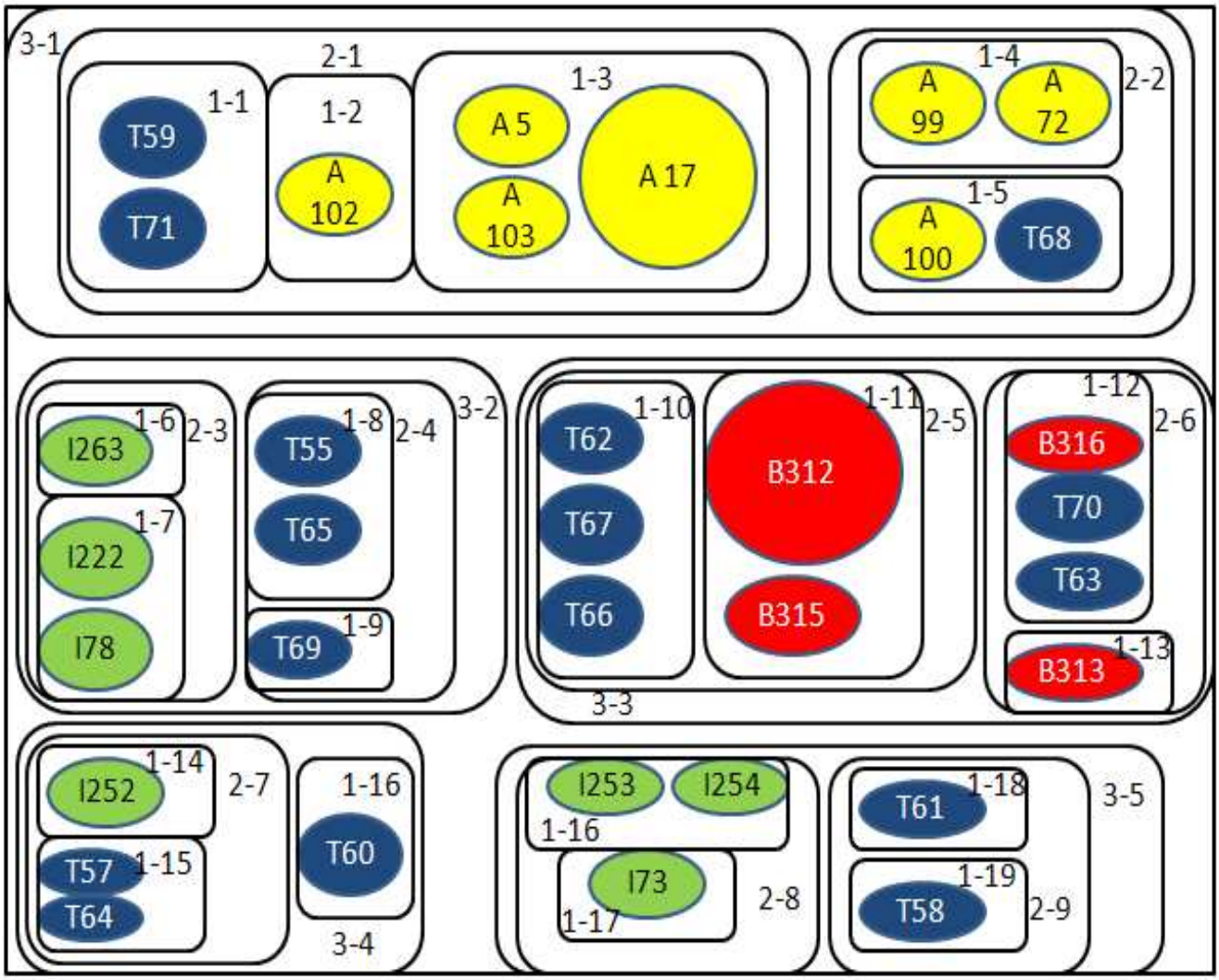

Figure 7: The nesting of Sarus haplotypes. “A” designates Australian Sarus, “T” designates southeast Asian Sarus, “I” designates Indian Sarus and “B” designates Myanmar Sarus. The numbers associated with the haplotypes are their extraction numbers. The one-step, two step and three-step clades are separated by boxes. The haplotypes are given four different colors on the basis of the population, they belong to: Indian Sarus green, Australian Sarus yellow, Burmese Sarus red and southeast Asian Sarus have blue color.

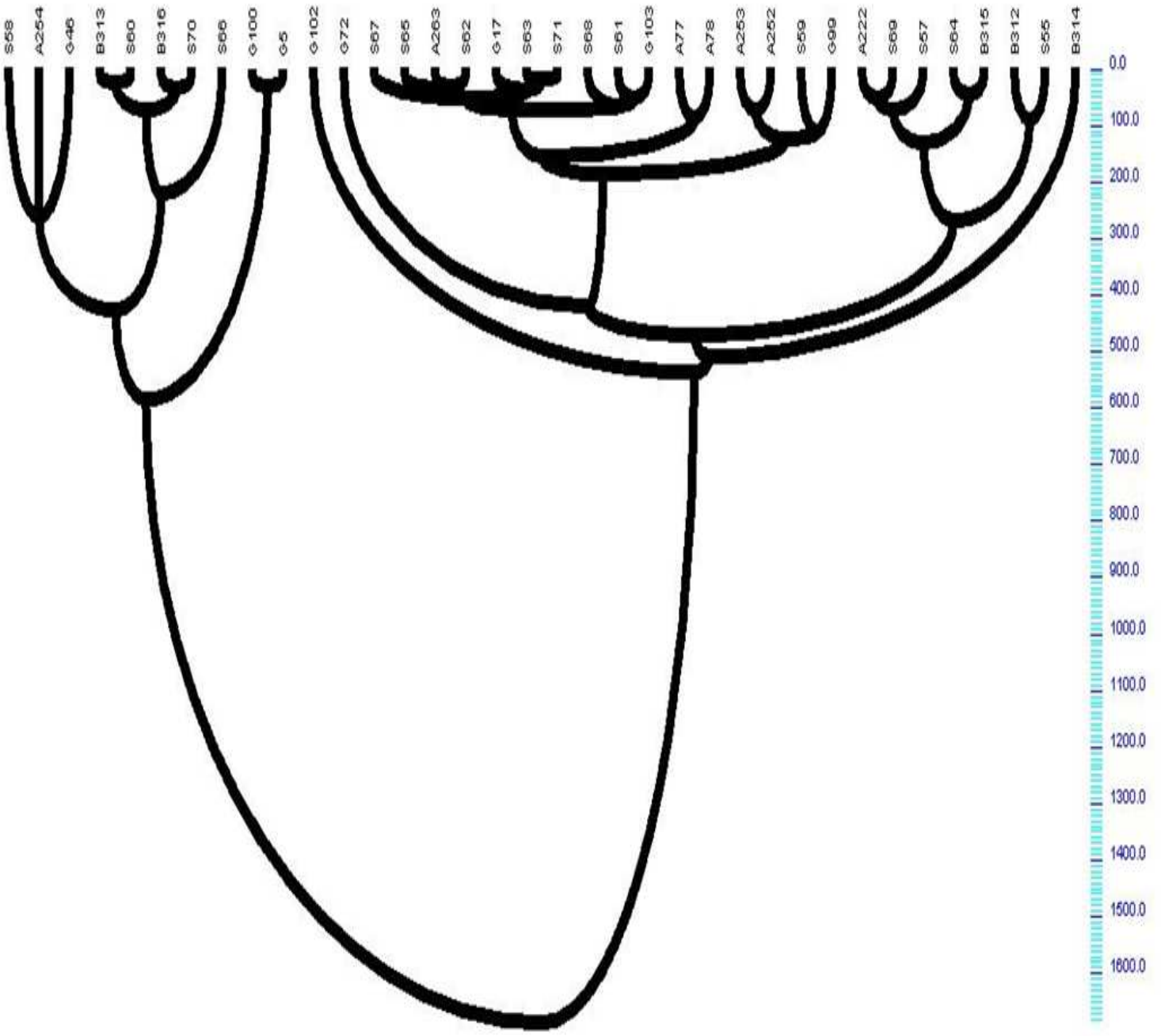

Figure 8: Coalescent tree for the four Sarus populations. “G” designates Australian Sarus, “S” designates southeast Asian Sarus, “A” designates Indian Sarus and “B” designates Myanmar Sarus. The numbers associated with the haplotypes are their extraction numbers. The side bar designates the generation time.

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Major Professors: Kamal M. Ibrahim and Carey Krajewski