

# Geographical partitioning of microsatellite variation in the sarus crane

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## Abstract

The sarus crane (*Grus antigone*) ranges across two continents and is the only species of crane (Gruidae) that breeds in India and Southeast Asia. Four subspecies, the Indian sarus (*G. a. antigone*), the eastern sarus (*G. a. sharpii*), the Australian sarus (*G. a. gillae*) and the extinct Philippine sarus (*G. a. luzonica*) were originally described through morphological, plumage, and/or geographical differences. The ranges of the Indian and eastern sarus converge in eastern India and Myanmar, but the Australian sarus has a disjunct Australian distribution. This study assesses population genetic structure of the current sarus populations utilising 13 DNA microsatellite loci. Population structure within this species was investigated utilising traditional  $F_{ST}$  and Bayesian clustering methods. While significant divergence was observed among populations when individuals were assigned to geographical populations, analyses based solely on individual genotypes demonstrated a clinal nature to the variation. The results of this study suggest that the Indian and eastern sarus cranes represent two major breeding groups within Asia and that the birds from Myanmar probably reside in an introgression zone between them. Lastly, because of genetic similarities shown to the eastern sarus, the results support recent theories contending that the Australian sarus was derived from mainland Asian birds.

## INTRODUCTION

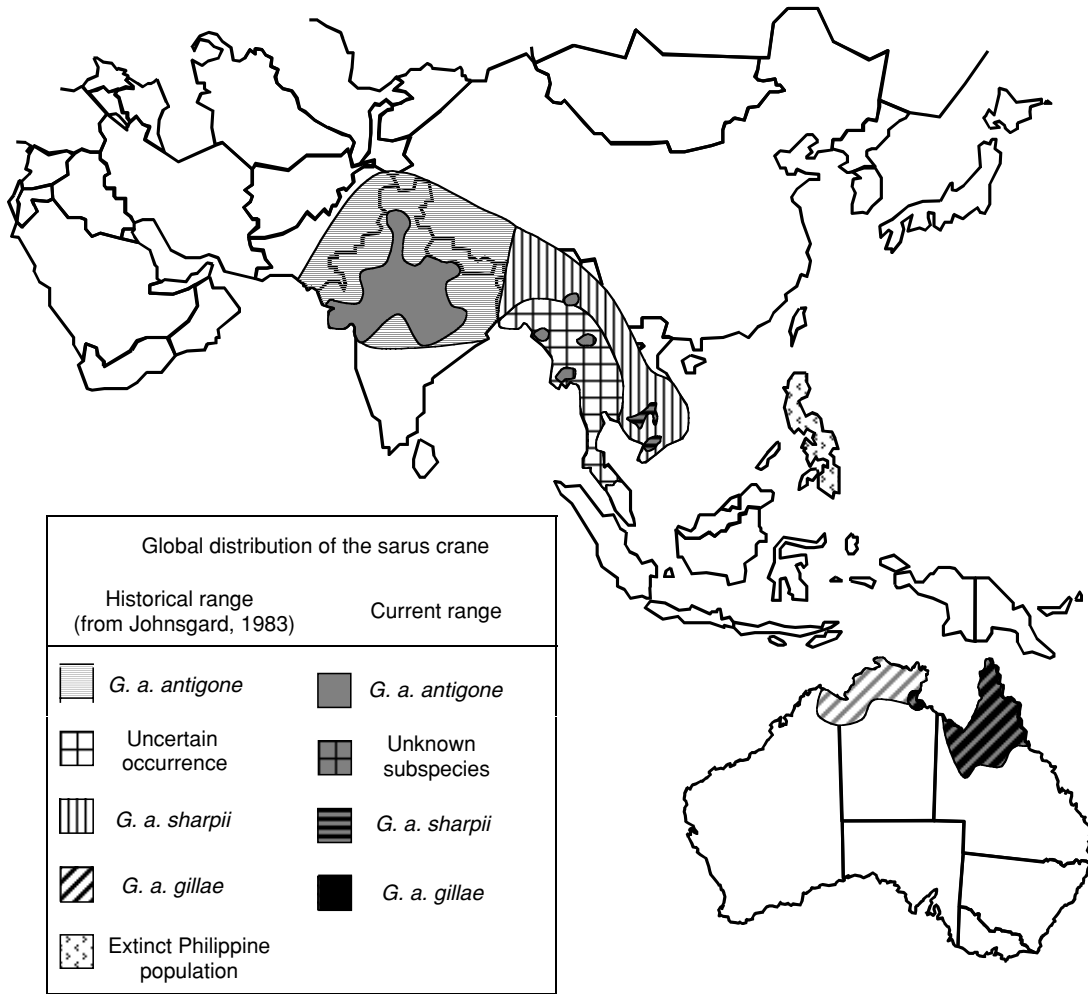
The crane family, Gruidae, comprises 15 extant species of large, graceful birds distributed across five continents. Unfortunately, cranes worldwide are threatened by habitat loss, excessive harvest, and disturbance (Meine & Archibald, 1996), making Gruidae one of the most threatened groups of birds in the world. Within the crane family, 10 species are considered to be globally threatened (Hilton-Taylor & Mittermeier, 2000). The sarus crane (*Grus antigone*), 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 (Fig. 1; Meine & Archibald, 1996). Although this species is considered non-migratory, some populations of sarus cranes will shift their range in response to environmental changes due to fluctuations between wet season and dry seasons (Meine & Archibald, 1996). There are four recognised subspecies of sarus cranes, Indian (*G. a. antigone*), eastern (*G. a. sharpii*), Australian (*G. a. gillae*) and the extinct Philippine sarus (*G. a. luzonica*; Blanford,

1896; Schodde, Blackman & Haffenden, 1988; Meine & Archibald, 1996). The three extant sarus subspecies were originally described through morphological and plumage characteristics (Meine & Archibald, 1996), but are now known to exhibit a somewhat clinal variation in morphology (J. Barzen, unpublished data). Although these subspecies are all generally grey with a pronounced red head comb, the Indian sarus is differentiated by a distinct white feathering on its throat and tertials (Meine & Archibald, 1996). Differentiation between the eastern and Australian sarus is less obvious, with the Australian sarus having larger and darker ear patches and a more extensively feathered throat (Schodde *et al.*, 1988). In body size, the eastern sarus is intermediate between the larger Indian and smaller Australian sarus.

The ranges of Indian and eastern sarus converge in eastern India and Myanmar (Johnsgard, 1983), whereas the Australian sarus exists only in Australia (Schodde *et al.*, 1988). The more sedentary Indian sarus exists in many subpopulations across India and, with a total population of several thousand birds, it is the least threatened of the sarus populations (Meine & Archibald, 1996; Sundar & Kaur, 2000).

Until the late 1940s the eastern sarus subspecies was distributed throughout Southeast Asia, but has subsequently been extirpated from many of the areas it once occupied (Medway & Wells, 1976; Madsen, 1981;

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**Fig. 1.** Distribution of the sarus crane (adapted from Johnsgard, 1983; Meine & Archibald, 1996; Sundar & Kaur, 2000). The species consists of four subspecies: Indian sarus (*Grus antigone antigone*), eastern sarus (*G. a. sharpii*), Australian sarus (*G. a. gillae*) and the extinct Philippine sarus (*G. a. luzonica*). Intermediate to the Indian and eastern ranges is an area of unknown ancestry.

Yang, 1987, 1991). In Southeast Asia, the current eastern sarus population consists of approximately 1000 birds and is confined mostly to northern Cambodia and southern Vietnam (Duc, 1991; Barzen & Seal, 2000). In the summer of 1996, a small population of less than 100 sarus cranes was re-discovered in Myanmar (Meine & Archibald, 1996). Currently this population is now estimated to be at least 300–400 birds (T. Latt, unpublished data). As the Myanmar population has only recently resurfaced, it is not currently known whether this population originated from India or Southeast Asia, or conversely, whether it represents an autonomous relict population. The Australian sarus, first noted in 1953 (Archibald & Swengel, 1987), was positively identified as eastern sarus by ornithologists in 1964 (Gill, 1969). In 1988, Schodde *et al.* elevated this population to a separate subspecies on the basis of plumage characters. Although its historical range includes portions of the Northern Territory, fewer than 5000 birds now reside primarily in the Cape York Peninsula of Queensland (Blakers, Davies & Reilly, 1984; Marchant & Higgins, 1993; Meine & Archibald, 1996).

Since the morphological and geographical boundaries separating the sarus subspecies are highly subjective, Wood & Krajewski (1996) attempted to identify genetic boundaries between the three surviving subspecies using mitochondrial DNA sequencing. Their study compared a 1831 base-pair (bp) segment of the mtDNA genome (including the entire cytochrome *b*, tRNA<sup>Thr</sup>, tRNA<sup>Pro</sup> and ND6 genes, as well as three short intergenic spacer regions) and found no distinct haplotypes defining subspecies of sarus. Although little evidence of genetic structure was found within sarus populations, nuclear DNA analysis might offer better discrimination for population distinction (Wood & Krajewski, 1996). Here, we assess population genetic structure, gene flow and subspecific status of the current sarus populations utilising microsatellite DNA markers. Because of their bi-parental nature, presumed neutrality and high levels of polymorphism, microsatellite DNA markers provide high resolution for revealing intraspecific genetic structure and assessing population history (Bruford & Wayne, 1993; Ashley & Dow, 1994; Beaumont & Bruford, 1998), even in species that exhibit little variability at other markers (Schlötterer, 2000).

## METHODS

### DNA sampling

A limited number of DNA samples was available from birds across the major range countries: India (7), Myanmar (5), Southeast Asia (16) and Australia (8). For the 36 samples available, DNA extracted from whole blood was graciously made available from the previous study of Wood & Krajewski (1996).

### Molecular genetic analysis

We genotyped all samples at 13 microsatellite loci (Gam $\mu$ 1, Gam $\mu$ 2, Gam $\mu$ 3, Gam $\mu$ 4, Gam $\mu$ 5, Gam $\mu$ 6, Gam $\mu$ 7, Gam $\mu$ 9, Gam $\mu$ 12, Gam $\mu$ 15, Gam $\mu$ 21, Gam $\mu$ 101, Gam $\mu$ 102; see Glenn *et al.*, 1997; Jones *et al.*, 2002) originally designed from a genomic library of the whooping crane (*Grus americana*) (Glenn, 1997), a congener of sarus cranes (Krajewski & Fetzner, 1994). Microsatellite amplification was carried out following procedures previously published for crane species (Jones *et al.*, 2002; Jones, 2003). Fluorescently labeled polymerase chain reaction (PCR) products were pooled together with an internal size standard (Promega CXR (red) 60–400 bp ladder) and run on an MJ BaseStation automated DNA sequencer (MJ Research, San Francisco, CA). Genotypes were scored with the aid of CARTOGRAPHER software (MJ Research, San Francisco, CA).

### Microsatellite DNA analyses

We investigated genetic diversity by calculating the proportion of polymorphic loci, mean number of alleles per locus, mean number of alleles per polymorphic locus, observed ( $H_o$ ) and expected heterozygosity ( $H_e$ ) and allele frequencies at all loci using the computer program GENETIC DATA ANALYSIS (GDA: Lewis & Zaykin, 2000). Allelic richness, an estimate that compensates for unequal sample sizes (El Mousadik & Petit, 1996; Petit, El Mousadik & Pons, 1998), was calculated and averaged across loci for all populations using FSTAT (Goudet, 2001). We used GENEPOP (Raymond & Rousset, 1995) to analyse populations for deviations from Hardy–Weinberg equilibrium. A sequential Bonferroni test (Rice, 1989) was then used to compensate for multiple comparisons between loci.

We explored population structure using several approaches. Using Weir & Cockerham's (1984) theta ( $\theta$ ) as an estimate of  $F_{ST}$ , overall and pairwise analyses of  $F_{ST}$  were conducted using GDA and tested for significance by bootstrapping. Overall and pairwise values for the number of migrants per generation between populations ( $Nm$ ) were calculated using Slatkin's private allele method (Slatkin, 1985; Barton & Slatkin, 1986). An analysis of molecular variance (AMOVA: Excoffier, Smouse & Quattro, 1992) was performed and tested for significance by permutation using ARLEQUIN (Schneider, Roessli & Excoffier, 2000). Correlations of allele frequencies and

subsequent genotypes between all individuals were investigated using principal components analysis (PCA) using PCA-GEN, a software program written by Jérôme Goudet, Institute of Ecology, University of Lausanne, Lausanne, Switzerland. For this analysis, geographical provenance was not used to assign individual samples to known populations, only individual crane multilocus genotypes were used. Analyses such as this, conducted without prior population information, allow an unbiased test of population structure. From the PCA analysis, a two-dimensional canonical plot was produced which visualises the first two principal components. On this canonical plot, circles encompassing the individuals of each population were drawn post-analysis to illustrate the genetic similarity or, conversely, dissimilarity of the individuals contained within each population.

In addition to the traditional population analyses based on  $F_{ST}$ , we used the Bayesian clustering method described by Pritchard, Stephens & Donnelly (2000). In this analysis, the program STRUCTURE was used to infer genetic structure using only genotypic data. Similar to the PCA, this analysis can be applied to groups of individuals without imposing any preconceived biases on population structure. This iterative analysis takes a group of individuals and statistically assigns them to a user-defined number of anonymous genetic clusters using only information from their genotypes. In doing this, the methodology can elucidate cryptic genetic structure that may not be apparent otherwise. For this study, we ran four independent STRUCTURE analyses using simulations of 50 000 burn-in iterations and 100 000 data iterations to classify individuals into one, two, three, or four genetic clusters ( $K$ ), respectively. The probability of the number of  $K$  ( $P(K/X)$ ) was determined using the methods described by Pritchard *et al.* (2000) by fixing  $K = 1-4$  and determining the Ln likelihood ( $\log P(X/K)$ ) of the data. After determination of relevant values for  $K$ , individual admixture proportions (i.e. the proportion of an individual's genetics assigned to each genetic cluster) were sorted and displayed by population.

## RESULTS

Overall, we found the microsatellite data consistent with Hardy–Weinberg expectations and linkage disequilibrium. No test was significant after correction for multiple comparisons. Among populations, with the exception of Australia, sarus populations are not significantly different in gene diversity ( $H_e$ ), heterozygosity ( $H_o$ ) and allelic richness (0.30–0.37, 0.27–0.35, and 2.15–2.53, respectively: Table 1). The slightly higher gene diversity and allelic richness in the Indian sample is consistent with the finding that more private alleles occur in this sample (Table 2). When Australia was compared to the other populations, it was evident that this population has reduced levels of diversity and has a higher coefficient of inbreeding ( $f = 0.18$ : Table 1). Statistical comparisons of gene diversity ( $H_e$ ), heterozygosity ( $H_o$ ) and allelic richness between India and Australia show

**Table 1.** Descriptive statistics for each population derived from multilocus microsatellite DNA genotypes

Population	Average number of genotypes	Proportion of polymorphic loci	Alleles per locus	Alleles per polymorphic locus
India	7.00	0.62	2.85	4.00
Australia	8.00	0.62	1.92	2.50
Southeast Asia	16.00	0.62	2.92	4.13
Myanmar	5.00	0.54	2.15	3.14
Mean	9.00	0.60	2.46	3.44

Population	Allelic richness	$H_e$	$H_o$	$f$
India	2.53	0.37	0.32	0.15
Australia	1.78	0.21	0.17	0.18
Southeast Asia	2.27	0.30	0.27	0.12
Myanmar	2.15	0.32	0.35	-0.14
Mean	2.52	0.30	0.28	0.08

**Table 2.** Alleles found in only one population (i.e. private alleles)

Locus	Allele	Frequency	Found in
Gam $\mu$ 5	182	0.71	India
Gam $\mu$ 9	185	0.43	India
Gam $\mu$ 101	195	0.14	India
Gam $\mu$ 5	188	0.14	India
Gam $\mu$ 101	198	0.07	India
Gam $\mu$ 7	133	0.07	India
Gam $\mu$ 3	111	0.07	India
Gam $\mu$ 3	113	0.07	India
Gam $\mu$ 3	123	0.07	India
Gam $\mu$ 102	127	0.07	India
Gam $\mu$ 101	201	0.10	Myanmar
Gam $\mu$ 4	181	0.13	Southeast Asia
Gam $\mu$ 3	125	0.06	Southeast Asia
Gam $\mu$ 1	138	0.03	Southeast Asia
Gam $\mu$ 101	204	0.03	Southeast Asia
Gam $\mu$ 5	184	0.06	Australia

significantly lower levels of diversity ( $P < 0.05$ ). Other than a significant difference in heterozygosity ( $P < 0.05$ ) between Australia and Myanmar, diversity values also were lower in Australia, compared with Myanmar and Southeast Asia, but not significantly so.

Significant estimates of theta ( $F_{ST}$ ) ( $P < 0.05$ ) were found for overall and pairwise combinations of populations (Table 3). With the exception of comparisons of Southeast Asia to Myanmar and Australia, estimates of  $F_{ST}$  were lower between populations that are geographically closer. Gene flow estimates ( $Nm$ ) based on the number and frequency of private alleles indicated that a low level of gene flow occurs between populations (Table 3). As with estimates of  $F_{ST}$ ,  $Nm$  also shows a geographical bias, with generally higher levels of gene flow estimated between adjacent populations. Similarly, the AMOVA (Table 4) demonstrated that even though most of the genetic variability lies within populations,

**Table 3.** Overall and pair-wise estimates of  $F_{ST}$  (lower) and  $Nm$  (upper) values based on microsatellite DNA genotypes

	India	Myanmar	Southeast Asia	Australia
India	–	0.59	0.44	0.35
Myanmar	0.18	–	0.78	0.26
Southeast Asia	0.25	0.14	–	0.89
Australia	0.34	0.29	0.13	–
Overall $F_{ST}$	0.21			
Overall $Nm$	0.69			

All estimates of  $F_{ST}$  showed significant deviations at  $P < 0.05$ .

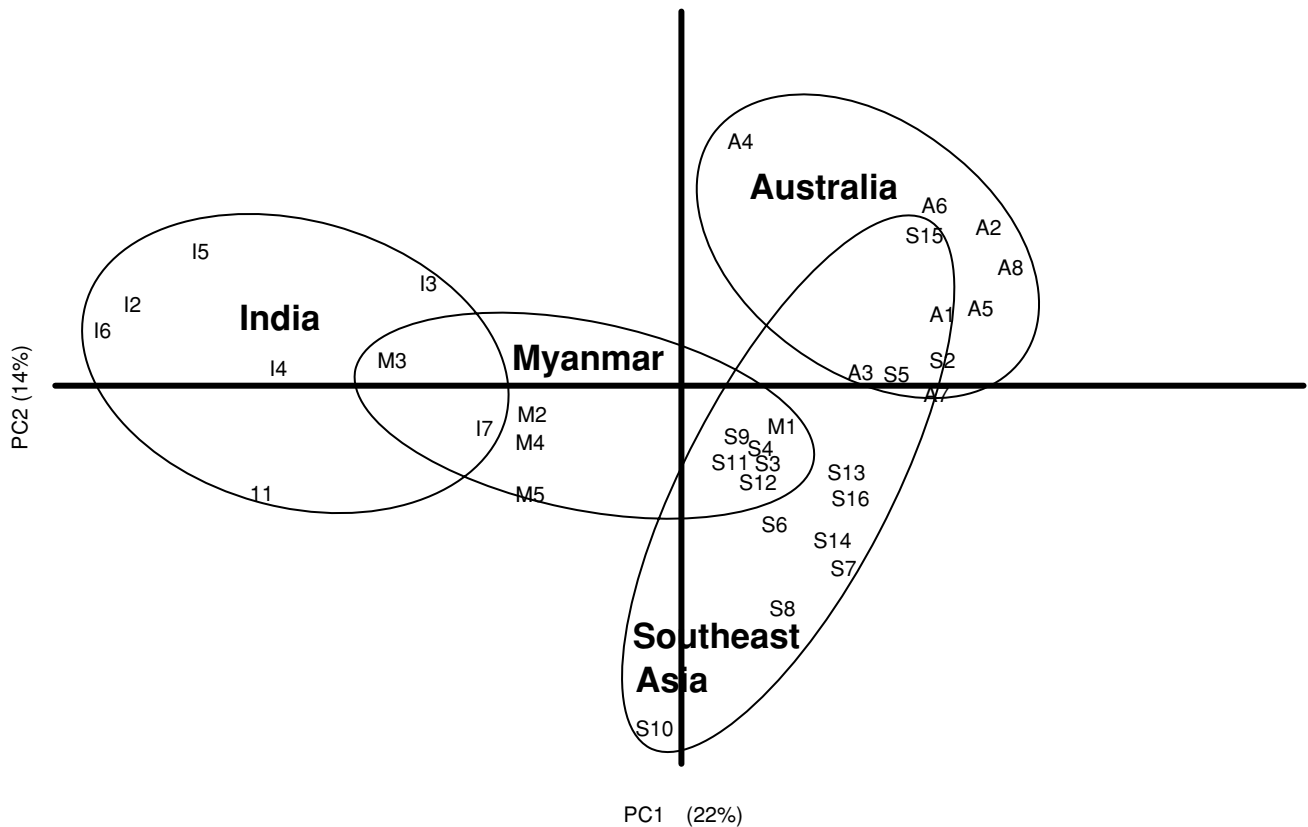
**Table 4.** Results of analysis of molecular variance (AMOVA) performed on microsatellite genotype data

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Between populations	3	32.35	0.53	21.44
Within populations	68	131.90	1.94	78.56
Overall $F_{ST}$	0.21 ( $P < 0.05$ )			

Significance testing was performed via 16 000 permutations.

a great amount of the variation (21%) is due to the differences seen between populations. With PCA, the first two principal components explained 36% of the variation when only information from individual genotypes was used. The resulting canonical plot (Fig. 2) illustrates a slight geographical bias to the similarities between individuals.

Using a different approach, Bayesian analysis implemented using the program STRUCTURE was used to evaluate the underlying genetic structure of the microsatellite variation. Although there are four geographical populations, the analyses suggest that only three genetic



**Fig. 2.** Canonical plot of the first two principal components. Ellipses were drawn to encompass individuals from each of the four geographical populations.

**Table 5.** Inferred number of genetic clusters ( $K$ )

$K$	$\log P(X/K)$	$P(K/X)$
1	-702.7	$\sim 0$
2	-646.9	0.05
3	-643.9	0.95
4	-653.3	$\sim 0$

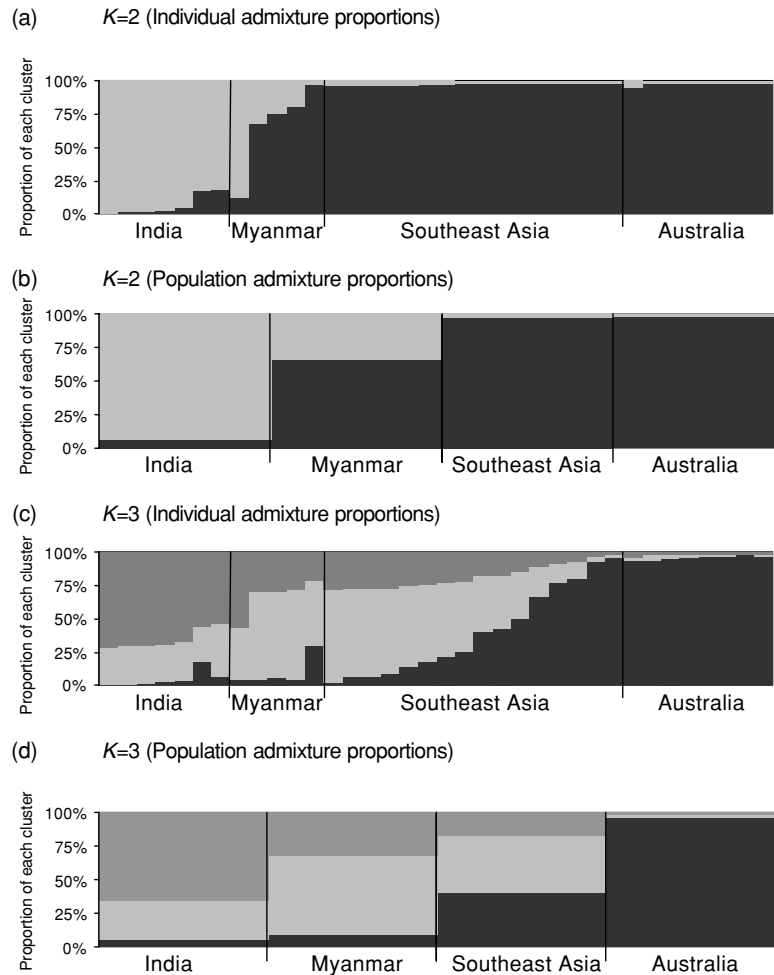
clusters exist ( $P(K/X) = 0.95$ : Table 5). To explore the geographical placement of these genetic clusters, we sorted and graphed the individual admixture proportions by population (Fig. 3). Where two genetic clusters were specified ( $K = 2$ ), the clustering of the genetic variation suggests that two genetic populations of sarus cranes occur, Indian and eastern. With genetic variance constrained to two groups, cranes in Myanmar were depicted as a mix of the Indian and eastern birds, while Australian cranes were firmly grouped with the eastern sarus. When genetic variation was properly sub-divided into three groups ( $K = 3$ ), a new grouping was delineated (light grey bars), which is consistent with an admixture between the two genetic types. The primary geographical placement of this admixture in Myanmar and Southeast Asia suggests clinal variation in the microsatellite DNA. Since the admixture proportions when  $K = 1$  or  $K = 4$  provided no additional insight to genetic structure, they are not presented here.

## DISCUSSION

Although diversity estimates were low across the species, the three mainland populations of sarus cranes differed little in their levels of microsatellite diversity, whereas Australian birds had notably less variability (Table 1). Along with a significant reduction of allelic richness, Australia's observed and expected heterozygosities were significantly lower when compared to Indian birds.

With regard to allelic differentiation between populations, there were microsatellite alleles that were unique to each population. However, the higher numbers of private alleles found in the Indian population is probably due to a higher long term effective population size ( $N_e$ ), which would have maintained the higher level of genetic diversity seen. Even though sample sizes in this study are small, the observation of two high frequency private alleles in India (71% and 43%) indicates reduced gene flow between India and the other populations (Table 2). These allelic data are supported by the  $F_{ST}$ ,  $Nm$  and AMOVA analyses, where significant differences are seen between all populations. Although a divergence was shown between the populations when birds are assigned to geographical populations, analyses based on individual genotypes (Figs 2 & 3) showed a more clinal nature to the variation, where geographically adjacent populations were genetically more similar.

Given the considerable nuclear, morphological and geographical divergence between sarus populations, our data suggest that the Indian and eastern sarus cranes



**Fig. 3.** Bayesian analysis of the microsatellite DNA genetic structure. Individual genotypes were iteratively assigned to  $K$  number of genetic groupings. The stacked bar chart shows the proportion of the variation assigned to each of the  $K$  subgroups. The proportions assigned to each cluster were plotted for each population by individuals ((a) and (c)), as well as by the mean of the population ((b) and (d)).

represent the two major breeding groups within the mainland Asian population. Within this region, however, the question still remains of whether birds in Myanmar compose an isolated breeding population or represent a segment of a cline between the Indian and Southeast Asian populations. The geographical and genetic intermediacy of the Myanmar birds suggests that the Myanmar population, although slightly more similar to the eastern sarus, probably represents an introgression zone between the Indian and eastern populations. Gene flow between current crane populations in Myanmar and Southeast Asia, however, are probably reduced due to the relatively recent extirpation of sarus from Thailand (Barzen & Seal, 2000). In future studies, a more complete sampling regime would better determine whether a truly clinal population remains in Myanmar.

The evolutionary history of the Australian sarus population is less obvious than those of mainland Asia. Given that the genetic similarity between the Southeast Asian and Australian sarus is the closest of any other comparison among the four populations sampled and this population shares all but one of its alleles with the

mainland, the current results corroborate the hypothesis that the Australian population was derived from mainland populations (Schodde *et al.*, 1988; Wood & Krajewski, 1996; Archibald, Sundar & Barzen, 2003). Currently the vast geographical separation between Australia and mainland Asia severely restricts the possibility of future gene flow to and from this population. This reproductive isolation, along with potential genetic introgression with its close relative the brolga (*Grus rubicunda*) (Archibald, 1981), will probably result in a genetically distinct Australian sarus. The Australian population should, therefore, continue to be managed as a separate wild population. With the fate of the Australian sarus uncertain, future research in Australia should include field and genetic monitoring of the sarus and brolga populations, including assessments of current rates of introgression.

The cumulative results of this study support a hypothesis of limited, but evolutionarily important, gene flow between all populations. The clinal variation shown in the nuclear DNA is consistent with previously published data from mitochondrial DNA, where no phylogeographic partitioning of mitochondrial haplotypes was found

(Wood & Krajewski, 1996). It is likely, given the mitochondrial and nuclear evidence, that limited gene flow occurred across all Asian sarus populations. Thus, the morphotypes of Indian sarus (*G. a. antigone*) and eastern sarus (*G. a. sharpii*) are more representative of two ends of a cline than of distinct subspecies. Even so, due to extensive fragmentation caused by the dramatic reduction and widespread extirpation among Asian subpopulations, these morphotypes now have reduced and non-overlapping ranges which, for Asia, are typified by very small population sizes. Since this species is threatened in all but a few areas of India (Archibald *et al.*, 2003), conservation strategies should focus upon preventing further extirpation of remnant populations, regardless of their taxonomic origin.

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