

The sarolga: conservation implications of genetic and visual evidence for hybridization between the brolga *Antigone rubicunda* and the Australian sarus crane *Antigone antigone gillae*

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Abstract To investigate the extent of suspected hybridization between the brolga *Antigone rubicunda* and the Australian sarus crane *Antigone antigone gillae*, first noted in the 1970s, we analysed the genetic diversity of 389 feathers collected from breeding and flocking areas in north Queensland, Australia. We compared these with 15 samples from birds of known identity, or that were phenotypically typical. Bayesian clustering based on 10 microsatellite loci identified nine admixed birds, confirming that Australian cranes hybridize in the wild. Four of these were backcrosses, also confirming that wild Australian crane hybrids are fertile. Genetic analyses identified 10 times more hybrids than our accompanying visual field observations. Our analyses also provide the first definitive evidence that both brolgas and sarus cranes migrate between the Gulf Plains, the principal breeding area for sarus cranes, and major non-breeding locations on the Atherton Tablelands. We suggest that genetic analysis of shed feathers could potentially offer a cost-effective means to provide ongoing monitoring of this migration. The first observations of hybrids coincided with significantly increased opportunities for interaction between the two species when foraging on agricultural crops, which have developed significantly in the Atherton Tablelands flocking area since the 1960s. As the sarus crane is declining in much of its Asian range, challenges to the genetic integrity of the Australian sarus crane populations have international conservation significance.

Keywords *Antigone antigone gillae*, *Antigone rubicunda*, Australian sarus crane, brolga, evolution, hybridization, introgression, migration monitoring

Introduction

Hybridization between apparently well-differentiated species is a commonly observed phenomenon and is widely studied in the context of speciation and reproductive isolation among species (Arnold, 1997; Pennisi, 2016). Hybridization has a variety of evolutionary consequences, depending on the fitness of the hybrids, which may range from sterility and reduced fertility, to hybrid vigour or heterosis, whereby hybrid individuals perform better and out-compete parental species (Burke & Arnold, 2001; Pennisi, 2016). As a consequence, if species are rare and locally confined (Rhymer & Simberloff, 1996), genetic mixing can lead to the extinction of one or both parental species (Todesco et al., 2016). Eventually, hybridization can lead to speciation, with two parental species giving rise to a new species (Dowling & Secor, 1997; Soltis & Soltis, 2009), highlighting the relevance of studying hybridization from the perspective of conservation biology. Allendorf et al. (2001) noted that taxa that have arisen through natural hybridization warrant protection. However, they also noted that increased anthropogenically related hybridization is causing extinction of many taxa (species, subspecies and locally adapted populations) by both replacement and genetic mixing, and they argued that hybrid taxa resulting from anthropogenic causes should therefore be protected only in exceptional circumstances. We do not yet know if hybrids of the brolga *Antigone rubicunda* and the Australian sarus crane *Antigone antigone gillae* have arisen as a result of anthropogenic habitat change, nor if agricultural and conservation policy has the potential to curtail this, but it is a matter for careful consideration. The starting point for this is gaining an appreciation of the scale of hybridization. Depending on the interaction of parental alleles, and their dominance patterns as well as epistatic effects, hybrid individuals need not be phenotypically intermediate between their parents (Rieseberg et al., 1999). Also, introgression of alleles from one species into the other need not be symmetrical, as demonstrated in many cases of mitochondrial introgression (Toews & Brelsford, 2012). On the nuclear level, genomic data indicate that hybridization and introgression interacting with recombination and selection result in much more complex scenarios of genetic diversity (Payseur & Rieseberg, 2016).

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PLATE 1 Typical broлга *Antigone rubicunda* (small red skull-cap type comb, dark wattle and grey legs), with unfledged chick, in Gulf Plains, Australia (Fig. 1). (Photo: T. Nevard)



PLATE 2 Typical Australian sarus cranes *Antigone antigone gillae* (pink legs, red comb extending down the neck, whitish crown and darker grey colour), with first-year juvenile, in Atherton Tablelands, Australia (Fig. 1). (Photo: T. Nevard)

Secondary contact of taxa that are well differentiated in allopatry may result in hybridization (Grant & Grant, 2016; Vijay et al., 2016), even among non-sister species (Koch et al., 2017). Such is the situation for the two Australian cranes, the broлга (Plate 1) and the Australian subspecies of the sarus crane (Plate 2). The broлга, which is endemic to Australasia (Australia and New Guinea), probably split from a common ancestor with the sarus crane 3.3–4.9 million years ago (Krajewski et al., 2010). Sarus cranes, whose range includes South and South-east Asia (Fig. 1), were first recorded in Australia in the 1960s (Gill, 1967) but may have been isolated from Asian populations for up to 37,500 years (Wood & Krajewski, 1996).

Hybridization between the broлга and sarus crane was first noted in French aviculture in 1934 (Gray, 1958), when

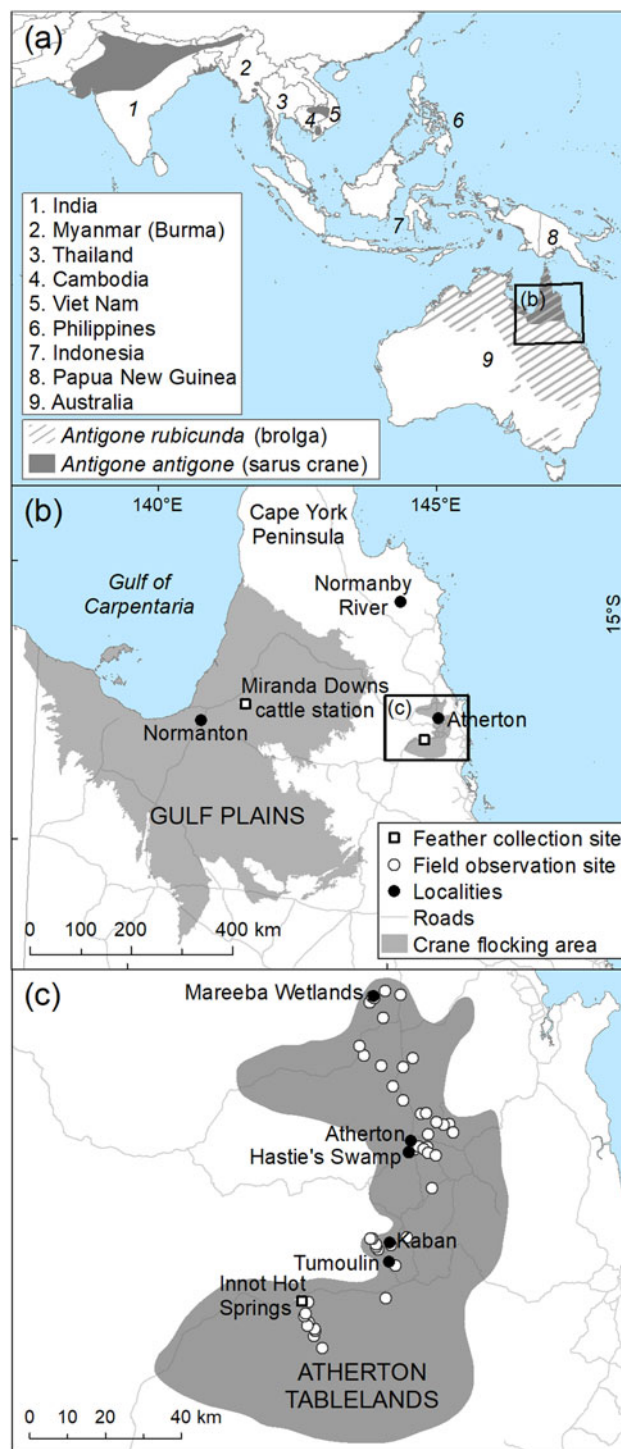


FIG. 1 (a) Global distribution of the broлга *Antigone rubicunda* and the sarus crane *Antigone antigone*, and data collection sites in (b) the Gulf Plains and (c) Atherton Tablelands, Australia. Distribution data derived from BirdLife International and NatureServe Bird Species Distribution Maps of the World (2014) and *The Australian Bird Guide* (Menkhorst et al., 2017). Gulf Plains Interim Biological Regionalization Area derived from Department of Environment & Energy (2012), and state roads from Department of Transport & Main Roads (2016).

fertile hybrids between the brolga and the South-east Asian subspecies of the sarus crane *Antigone antigone sharpii* were produced. In 1972, when observing the flocking behaviour of brolgas and sarus cranes on the Atherton Tablelands in Queensland, Australia, Archibald (1981) identified at least two and possibly six potential hybrids (for which he coined the name sarolga), and speculated that if hybridization were to increase, introgression could be significant in the conservation of cranes in Australia. Similar concerns led Jones et al. (2005) to recommend monitoring of the genetic integrity of Australian sarus cranes. The present research is the first to confirm and quantify the extent of natural hybridization between the brolga and the Australian sarus crane.

Brolga

In Australia the brolga occurs across the north of the country in Queensland, the Northern Territory and Western Australia, and as far south as Victoria, where it is threatened (Marchant & Higgins, 1993; Fig. 1a). In Queensland the species has been recorded throughout the state (Marchant & Higgins, 1993) but is now widespread and abundant only in the north. Although breeding occurs throughout its Queensland range (Marchant & Higgins, 1993; Barrett et al., 2003), it appears to be focused on the grasslands and open savannah woodland of the Gulf Plains, south-east of the Gulf of Carpentaria and in Cape York Peninsula. Scattered breeding occurs elsewhere, with flocking (non-breeding) regions including the Atherton Tablelands and south of Townsville on the north-east coast of Queensland (Lavery & Blackman, 1969).

The brolga population in northern Australia is generally considered to be stable and was estimated to comprise 20,000–100,000 individuals (Meine & Archibald, 1996). A total of 51,969 brolgas were recorded by Kingsford et al. (2012), and all but 135 of these were in northern Australia. The only longitudinal counts are from north-east Queensland (Atherton Tablelands and Townsville regions), with an annual mean of c. 800 individuals recorded by community-based naturalists during 1997–2017, with a peak of c. 2,000 in 2005 (E. Scambler, pers. comm.).

The dispersal patterns and movements of brolgas across their range are poorly understood (Marchant & Higgins, 1993). Brolgas undertake seasonal movements between breeding and non-breeding (flocking) habitats in response to rain and flooding (Marchant & Higgins, 1993). Flocks are formed during the dry season and pairs disperse to breeding sites in the wet season. Although there is an increasing body of work on the species in the southern part of its range, especially in Victoria (Harding, 2001; Herring, 2005, 2007), knowledge of the behaviour and ecology of tropical brolgas remains largely confined to earlier studies, such as those of Hughes & Blackman (1973). Brolgas can use brackish waters, having a unique salt-gland, and when observed with sarus cranes

near Normanton, brolgas favoured deeper, generally coastal marshes (Walkinshaw, 1973). However, they also occur in open forest and woodland, grassland and cultivated land (Lavery & Blackman, 1969).

Australian sarus crane

Although the sarus crane is primarily a South Asian species, it is extinct in the Philippines and declining in much of its Asian range, particularly in Burma, Thailand and Indochina (Meine & Archibald, 1996; Archibald et al., 2003), and is categorized as Vulnerable on the IUCN Red List (BirdLife International, 2016). The Australian sarus crane population is therefore of international conservation significance and hybridization with the brolga could potentially be a threat to the genetic integrity of the Australian subspecies.

Sarus cranes in Australia are almost entirely confined to Queensland (Fig. 1a), with known breeding areas along the Gulf Plains and on the Normanby River floodplains in eastern Cape York Peninsula (Marchant & Higgins, 1993; Barrett et al., 2003; Franklin, 2008). Most breeding records are from the Gulf Plains and concentrated in the Gilbert and Norman River catchments, where cranes make use of both natural and man-made wetlands on cattle properties (Barrett et al., 2003). The only known major dry-season flocking area for sarus cranes is the Atherton Tablelands and its immediate vicinity. Counts of dry-season flocks on the Atherton Tablelands provide the only current estimates of sarus crane recruitment rates (Marchant & Higgins, 1993; Grant, 2005). An unknown proportion of the sarus crane population remains on the Gulf Plains and the adjacent inland area in the dry season, and therefore utilizes different landscapes and food types during this period, as there are no significant cropped areas. Migration routes followed by sarus cranes to the Atherton Tablelands from their breeding areas, and their habitat usage along the way, are currently unknown. The importance of migration routes is becoming increasingly understood, as knowledge of breeding and non-breeding locations alone is insufficient to secure the protection of migratory species (Runge et al., 2014).

Estimates of total numbers of Australian sarus cranes have varied from 5,000 to 10,000 individuals (E. Scambler, pers. comm.), but these have low reliability (Garnett & Crowley, 2000). Annual (unpublished) counts undertaken by local members of BirdLife Australia on the Atherton Tablelands (E. Scambler, pers. comm.) indicate 800–1,500 individuals flocking in dry seasons during 1997–2017 but it is not known what proportion of the total Australian sarus crane population these birds represent. There are no current estimates of population trends in Australia (E. Scambler, pers. comm.), but it is thought that numbers of sarus cranes have increased from perhaps as few as 200 on the Atherton Tablelands in the late 1960s (G. Archibald, pers. obs.).

Aims

Although the presence of putative hybrid cranes on the Atherton Tablelands has been noted by experienced observers for many years, we hypothesized that it is possible that these were non-hybrid birds, exhibiting intrinsic phenotypic variability. We were therefore interested in testing the degree of introgression (if any) in the crane population, as well as providing a benchmark for its future measurement both visually and genetically. To do this we needed to understand whether birds with the visual characteristics that had led observers to believe that they were hybrids (sarolgas) were, in fact, hybrids.

We aimed to (1) establish the extent of hybridization between the Australian sarus crane and the brolga, using molecular methods; (2) seek evidence from these genetic data for the presumed connectivity of breeding and flocking areas in the Gulf Plains and Atherton Tablelands; (3) describe the distribution of individuals with hybrid characteristics on the Atherton Tablelands; and (4) discuss the results primarily with respect to their relevance to biological conservation of the two species in Australia.

Methods

Field observations and sample collection

We observed cranes during July 2013–June 2016 in 51 monthly circuits of 334.7 km that encompassed 46 foraging sites distributed across all known crane flocking areas of the Atherton Tablelands (Figs 1d & 2), which were visible from the road. At each site we counted adult and first-year juvenile brolgas and sarus cranes using 10 × 40 binoculars, and examined potential hybrids (see below and Plate 3) using a 50 × spotting scope. Individuals could not be distinguished and were probably counted on multiple occasions and at multiple sites. However, enough cranes were counted that there is no reason to suspect systematic bias in the estimate of the proportion of visually distinguishable hybrids amongst the wider crane population.

The initial identification of hybrids was based on the presence of at least four readily observable characteristics including at least two of (1), (4) and (6) in Archibald's (1981) typology of sarolgas: (1) larger and heavier than either parent species; (2) an irregular border to the comb; (3) notches in the cap; (4) intermediate or mixed-colour legs; (5) a scalloped mantle; (6) brighter yellow eyes than typical sarus cranes (which are more orange); and (7) a small wattle. Although Archibald's (1981) typology was helpful, in overall appearance the sarolgas he described were more like atypical sarus cranes than atypical brolgas, whereas we also observed putative hybrids that looked more like atypical brolgas (Plate 3b).

In May 2014, freshly shed feathers were collected opportunistically from Miranda Downs cattle station (17°19.755' S, 141°52.770' E, elevation 55 m) on the Gulf Plains, and in September 2015 from Innot Hot Springs (17°43.749' S, 145°16.302' E, 628 m) on the southern edge of the Atherton Tablelands (Fig. 1c). Only feathers with minimal contamination from soil and vegetation were selected and were picked from dry ground, using forceps, placed immediately in sealed plastic bags and refrigerated prior to analysis.

Molecular analysis

A total of 389 feathers were collected and prepared for genotyping: 187 from the Gulf Plains and 202 from the Atherton Tablelands. Fifteen samples (blood, feathers) were taken from reference birds, which were either phenotypically typical or for which a potential history of contact with the other species could be excluded or minimized (3 *A. antigone antigone*, 7 *A. antigone gillae*, 5 *A. rubicunda*; Table 1; note that including the non-Australian *A. antigone antigone* in the reference collection does not affect the veracity of the comparison with brolga). DNA was extracted using the slightly modified sodium dodecyl sulphate/salting-out protocol of Miller et al. (1988). To increase the yield, dithiothreitol and Roti-PinkDNA (Carl Roth, Karlsruhe, Germany) were added. Initially we tested the 22 microsatellite loci that had been used successfully for other species of crane (Hasegawa et al., 2000; Meares et al., 2009) as well as the sarus crane (Jones et al., 2005) and the brolga (Miller, 2016). Of these, 10 could be consistently amplified and scored (Table 2). Polymerase chain reactions (PCRs) were conducted in a volume of 10 µl and contained 1 µl DNA (10–25 ng), 1 µl of 10 × NH₄-based reaction buffer, 1.5–2.25 mM MgCl₂ solution (Table 2), 0.25 mM of each primer, 0.2 mM of dNTP, 0.04 µl of BioTaq DNA polymerase (5 U/µl), 0.6 µl of 1% bovine serum albumin and sterile ddH₂O. For two loci, GR22 and GR25, we used the MyTaq mix (all products from Biorline, London, UK). The PCR profile comprised an initial denaturation at 94 °C, 36 cycles including denaturation at 94 °C, primer specific annealing (Table 2) and extension at 72 °C, each for 30 s, and a final elongation at 72 °C for 10 minutes. Microsatellite alleles were separated on a 3130xl Genetic Analyzer, together with the GeneScan 600 LIZ Size Standard 2.0 (Applied Biosystems, Waltham, USA). Fragment sizes were determined manually to avoid the inconsistencies in automatic calling that result from the arbitrariness of bin width definitions using GeneMapper 4.0 (Applied Biosystems, Waltham, USA). To control the accuracy of size determination we repeated PCRs of samples that initially gave weak signals or had rare variants. In other cases, problematic PCR samples that were initially typed in different runs, and therefore possibly difficult to calibrate, were loaded on the same plate to improve comparability.

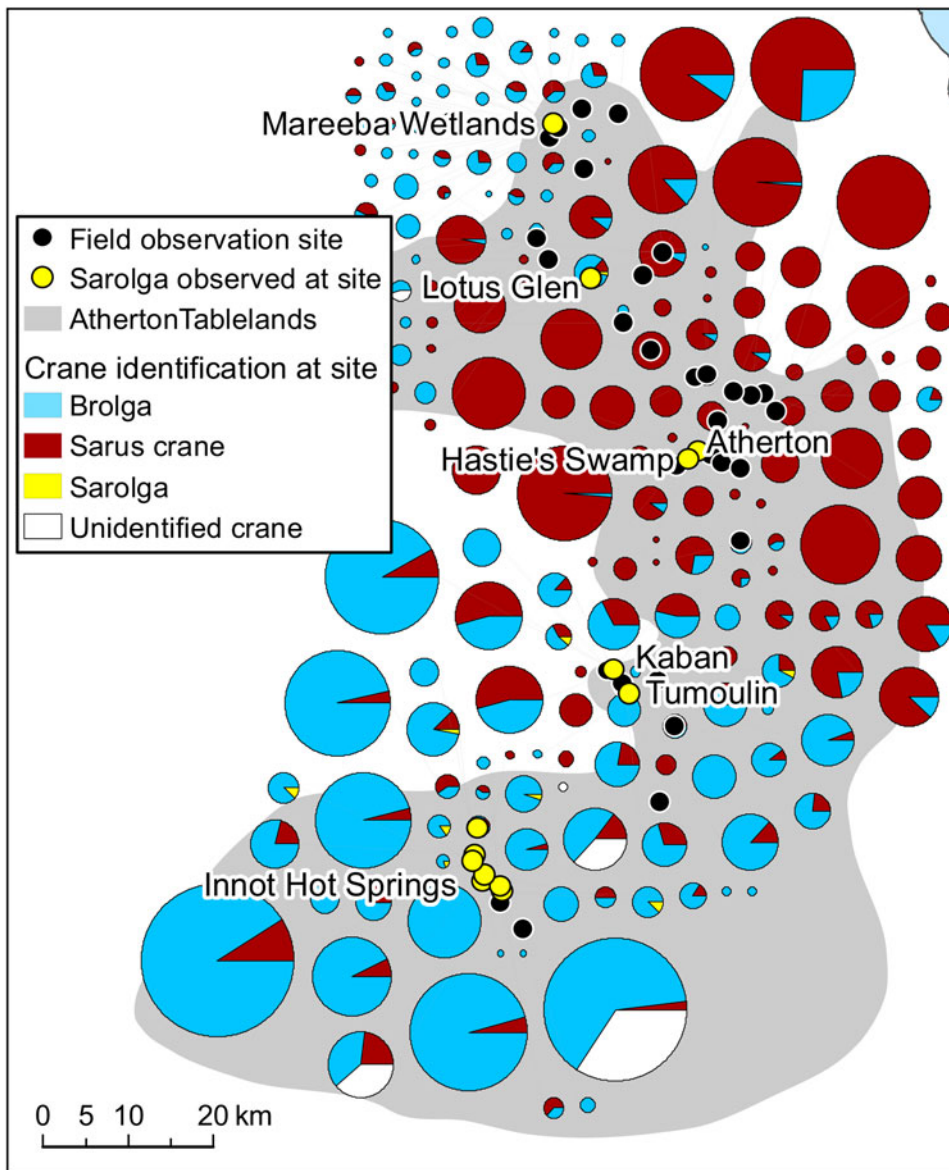


FIG. 2 Distribution and clustering of brolga, sarus crane and sarolga visually identified at 46 sites on the Atherton Tablelands (Fig. 1) during 2013–2016. On some occasions, as a result of poor light and weather conditions, cranes could not be identified.

TABLE 1 Notes on 15 reference individuals of the sarus cranes *Antigone antigone antigone* and *Antigone antigone gillae* and the brolga *Antigone rubicunda* from which samples were taken for molecular analysis. Samples were taken from living or recently deceased wild birds that were phenotypically typical, or from birds whose lineages of descent were known not to have had contact with the other species.

Species	No. of specimens	Locality	Notes
<i>A. antigone antigone</i>	3	Lemgo Avicultural Collection, Germany	Breeding records show no interbreeding with other sarus subspecies since their ancestors were collected from the wild in the 1970s
<i>A. antigone gillae</i>	5	Lemgo Avicultural Collection, Germany	Breeding records show no interbreeding since their ancestors were collected from the wild in the 1970s
<i>A. antigone gillae</i>	2	Kairi, Queensland, Australia	Wild birds; phenotypically typical sarus cranes
<i>A. rubicunda</i>	3	Cairns Tropical Zoo, Queensland, Australia	Breeding records show no interbreeding since collected from a wild population with no history of contact with sarus cranes
<i>A. rubicunda</i>	2	Herbert River, Atherton Tablelands, Queensland, Australia	Wild birds; phenotypically typical brolgas

TABLE 2 PCR specifications, genetic diversity and allele richness of microsatellite loci of brolgas and sarus cranes from the Gulf Plains (Gulf) and Atherton Tablelands (Table), Australia (Fig. 1), with locus/dye (fluorophore at 5'-end of forward primer), MgCl₂ concentration, annealing temperature (T), number of alleles, genetic diversity and allelic richness. The following loci could not be consistently amplified or scored: Gam μ 2, Gam μ 5, Gam μ 6, Gam μ 7, Gam μ 9, Gam μ 12, Gam μ 21, Gam μ 102, GjM11, GjM34, GjM40, GR17 (Hasegawa et al., 2000; Meares et al., 2009; Miller, 2016).

Locus/dye	MgCl ₂ (mM)	T (°C)	No. of alleles		Gene diversity				Allelic richness			
					Brolga		Sarus		Brolga		Sarus	
			Brolga	Sarus	Gulf	Table	Gulf	Table	Gulf	Table	Gulf	Table
Gam μ 3/FAM	1.5	59	7	6	0.618	0.654	0.162	0.229	6.994	6.965	3.183	2.875
Gam μ 18/HEX	1.5	53	3 ¹	1	0.224	0.363	0.000	0.000	2.943	3.000	1.000	1.000
Gam μ 24/HEX	2.25	60	5	5 ¹	0.578	0.628	0.204	0.338	4.943	4.998	3.732	4.000
Gam μ 101b/HEX	2	59	4	3	0.173	0.203	0.349	0.326	3.000	2.566	2.273	2.999
GjM8/FAM	2	59	3	3	0.159	0.265	0.157	0.125	2.996	2.910	2.254	2.000
GjM13/HEX	2	60	5 ¹	3 ¹	0.722	0.557	0.498	0.042	5.000	3.815	2.276	1.875
GjM15/FAM	2	59	9	5	0.794	0.757	0.552	0.539	8.994	8.251	4.422	4.000
GjM48b/HEX	2	56	8 ¹	6 ¹	0.559	0.571	0.160	0.362	6.999	7.853	3.039	3.862
GR22 ² /HEX		60	11 ¹	5	0.625	0.566	0.448	0.434	7.937	8.430	2.575	2.875
GR25 ² /Cyanine3		60	5	3	0.727	0.741	0.512	0.442	5.000	5.000	2.996	2.000

¹Heterozygote deficiency.

²Multiplexed.

TABLE 3 The number of brolgas, sarus cranes and sarolgas observed across 46 sites on the Atherton Tablelands in Queensland, Australia (Fig. 1) during 2013–2016.

Life history stage	No. of brolgas (%)	No. of sarus cranes (%)	No. of sarolgas (%)	Unidentified*	Total no. of cranes (%)
Adult	24,870 (55.8)	12,245 (27.5)	84 (0.2)		37,199 (83.5)
First-year juvenile	2,491 (5.6)	1,136 (2.5)	0 (0)		3,627 (8.1)
Total	27,361 (61.4)	13,381 (30)	84 (0.2)	3,728 (8.4)	44,554 (100)

*Because of poor weather/light conditions.

As there was the possibility that feathers of a particular bird were collected more than once, identical genotypes were identified using *Cervus 3.0.7* (Kalinowski et al., 2007). Consequently, 41 samples (16 Gulf Plains, 24 Atherton Tablelands, 1 captive reference *A. antigone gillae* that was identical to its sibling) were excluded from the following analyses. In 11 of these cases the same genotype was found in both localities. In these cases the sample from the Atherton Tablelands was excluded arbitrarily. Missing data were not considered to be problematic, as only 16.2% of the birds had missing data and only two of the nine potential hybrids lacked data (at one locus each). It was not possible to distinguish siblings from non-siblings because the genetic variance would be too large given the moderate number of markers.

To identify the number of genetic clusters and potential hybrid individuals we conducted Bayesian clustering using *STRUCTURE 2.3.4* (Pritchard et al., 2000; Falush et al., 2007), with *K* (number of clusters) ranging from one to five and 10 replicates, assuming the admixture model and, in different runs, either uncorrelated or correlated allele frequencies. The Markov chains ran for 550,000 generations,

including a burn-in of 50,000. Pilot runs with longer Markov chains yielded identical results. We used *Structure Harvester 0.6.94* (Earl & von Holdt, 2012) to analyse the data, following Evanno et al. (2005), and *STRUCTURE PLOT V2.0* (Ramasamy et al., 2014) and *Adobe Illustrator CS4* (Adobe Systems Inc., San José, USA) to render the graphical output. We defined individuals with admixture *Q* of 0.1–0.9 as hybrids, following Devitt et al. (2011). These thresholds were considerably more conservative in our analysis as we used 10 loci, compared to three used by Devitt et al. (2011). However, this restriction was necessary because the loci used by Devitt et al. (2011) had species-specific alleles whereas the two species of cranes we compared shared some alleles. We did not attempt to identify different classes of hybrids (F₁, F₂ and their backcrosses) because this would have required genotyping at least 50 loci (Fitzpatrick, 2012) and we had only 10 available. The Bayesian clustering approach was validated by *k*-means clustering, which, in contrast to *STRUCTURE*, is free of assumptions about population genetics, using *GenoDive 2.0b23* (Meirmans & van Tienderen, 2004). Individuals were clustered based on their allele frequencies according

to the pseudo F statistic of Calinski & Harabasz (1974; see also Meirmans, 2012).

More detailed population genetic analyses were conducted based on the clusters identified by Bayesian clustering, including descriptive statistics and tests for Hardy–Weinberg equilibrium, linkage disequilibrium, null alleles, and population differentiation, using *FSTAT* 2.9.3.2 (Goudet, 1995), *GenePop* 4.2 (Raymond & Rousset, 1995; Rousset, 2008) and *Micro-Checker* 2.2 (Van Oosterhout et al., 2004). We estimated gene flow using the formula $Nm = [(1/F_{ST}) - 1]/4$ (where Nm is the number of effective migrants) as well as based on private alleles; i.e. alleles occurring only in one subpopulation (Barton & Slatkin, 1986), the latter in *GenePop*. Probabilities that two individuals were identical were calculated using *Cervus*, assuming either that the two birds were unrelated or, more conservatively, that they were full siblings (Waits et al., 2001). In multiple tests, α was Bonferroni corrected.

Lincoln-Petersen index

We applied the Lincoln–Petersen index to our ‘genetically re-trapped’ birds ($n_{\text{Gulf}} \times n_{\text{Tablelands}}/n_{\text{both}}$) based on methodology described by Southwood & Henderson (2000).

Results

Field observations

Our observations of cranes on the Atherton Tablelands are summarized in Table 3 and Fig. 2. Of 40,826 cranes identifiable to species level (of a total of 44,554 observations), only 84 were classified as morphologically intermediate sarolgas (0.205%). Variation among these intermediate birds meant it was not possible to assess the extent of potential hybridization (Plate 3).

Our observations of identifiable hybrids on the Atherton Tablelands were concentrated in a relatively small number of areas (Fig. 2), including Innot Hot Springs, Tumoulin/Kaban, Hastie’s Swamp and the Mareeba Wetlands. Figure 2 also illustrates that although they occur in mixed flocks, brolgas and sarus cranes are differentially distributed across the Atherton Tablelands, with higher numbers of sarus cranes concentrated on more fertile volcanic soils closer to Atherton itself, and both brolgas and putative hybrids occurring predominantly in areas of lower intrinsic fertility.

At sites with higher counts of either brolgas or sarus cranes, the species with smaller numbers in the flock tended to have proportionally more first-year juveniles (Table 4; χ^2 tests: $P < 0.0001$ in both cases), potentially increasing opportunities for interactions between species while juveniles are at a critical age for pair-bonding.

Population genetics

In all *STRUCTURE* analyses the highest ΔK resulted from assuming two clusters, and this was confirmed by k -means clustering. These clusters corresponded well to the species brolga and sarus crane, as indicated by the reference birds (Fig. 3). As allele frequencies apparently differed considerably between the species, we based the subsequent analyses on the *STRUCTURE* model assuming uncorrelated frequencies. Although the majority of birds were either pure brolga or pure sarus crane, some were admixed (Fig. 3).

According to our criteria, 239 samples belonged to brolgas (90 Gulf Plains, 149 Atherton Tablelands) and 101 to sarus cranes (77 Gulf Plains, 24 Atherton Tablelands). Nine individuals (2.58%; 4 Gulf Plains, 5 Atherton Tablelands) had Q scores of 0.125–0.888 (reference: brolga) and were identified as hybrids (Fig. 3), a proportion c. 10 times greater than in our field observations. Four hybrids had alleles occurring only in brolgas at one or two loci, indicating that hybrids are fertile and can interbreed at least with brolgas or other hybrids.

After exclusion of reference birds and hybrids, we analysed the species separately, dividing both into two subpopulations corresponding to the localities. Genetic diversity is summarized in Table 2. Genetic diversity and allelic richness were both considerably higher in brolgas. Brolgas also exhibited 26 private alleles, 1–6 per locus, whereas sarus cranes had only eight private alleles, and four loci did not have any. This translates into much lower probabilities that two individuals will carry the same genotype in brolgas than in sarus cranes. The probabilities of finding two unrelated individuals with identical genotypes were 7.1×10^{-7} for brolgas and 0.0012 for sarus cranes. Assuming the individuals were full siblings, these probabilities increased to 0.0023 and 0.0395, respectively. In brolgas and sarus cranes, four and three loci, respectively, deviated from the Hardy–Weinberg equilibrium and the same loci had indications of null alleles. However, this was probably because there was a relatively high number of rare alleles (frequency $< 5\%$) in both species, and therefore it was not considered to be problematic for our analyses. In any case, the general results of the *STRUCTURE* analysis did not change after exclusion of these loci. Linkage disequilibrium did not play a role in either species. In both species, subpopulations were hardly differentiated, with $F_{ST} = 0.010$ (95% CI 0.003–0.020) in brolgas and 0.047 (–0.006–0.136) in sarus cranes. The significant result for brolgas is probably attributable to sample size and is not biologically relevant. Estimates of migration based on the private allele method were high in both species, with 6.56 and 7.75 migrants per generation for brolgas and sarus cranes, respectively. Transforming F_{ST} into estimates of migration using the formula $Nm = [(1/F_{ST}) - 1]/4$, we inferred 24.75 and 5.07 migrants, respectively.

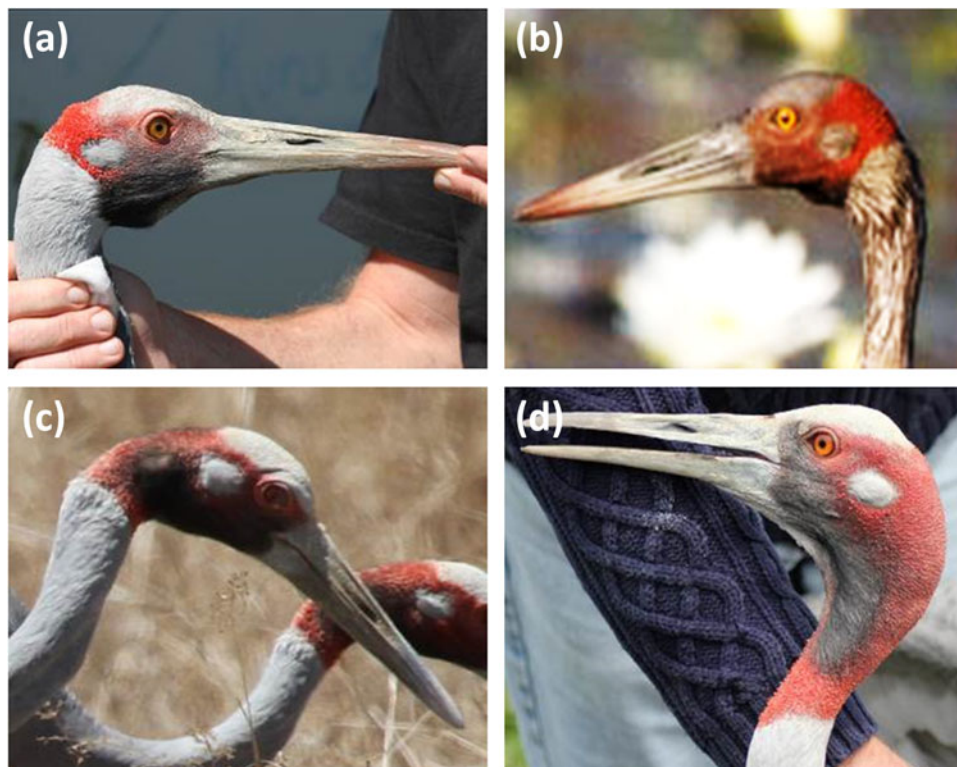


PLATE 3 Heads and necks of typical species phenotypes and presumed hybrids. (a) Typical brolga; (b) sarolga (this bird was congruent with a typical brolga, except that it had pink legs, slightly more red on the nape of the comb than is typical of brolgas, and a very small, almost non-existent wattle; this combination of characters had not hitherto been recorded); (c) typical sarolga, with much shorter neck comb and slight wattle; and (d) typical Australian sarus crane. (Photos a, c & d: T. Nevard; b: B. Johnson)

TABLE 4 Total number of cranes (and % juveniles) at sites dominated by either brolgas or sarus cranes.

	No. of brolgas (% juveniles)	No. of sarus cranes (% juveniles)
Brolga-dominated sites	25,510 (8.8)	2,273 (14.5)
Sarus-dominated sites	1,851 (13.3)	11,308 (8.9)
<i>Total</i>	27,361 (9.1)	13,581 (9.8)

Lincoln–Petersen index

In 11 cases the same genotypes were collected at both Miranda Downs in the Gulf Plains and at Innot Hot Springs in the southern Atherton Tablelands. Of these, nine were attributable to brolgas and two to sarus cranes. According to the probabilities of encountering two individuals carrying identical genotypes by chance with our sample sizes, only the two sarus cranes could be cases of chance identity, provided the birds were siblings (see above). Applying the Lincoln–Petersen index ($n_{\text{Gulf}} \times n_{\text{Tablelands}} / n_{\text{both}}$; Southwood & Henderson, 2000) to these results implies that at least 1,473 brolgas and 923 sarus cranes could have moved between the Gulf Plains and Atherton Tablelands.

Discussion

Since Archibald's first observation of brolga–sarus crane hybrids in 1972 (Archibald, 1981), local observers have

continued to note the presence of apparent hybrids amongst dry season, non-breeding crane flocks on the Atherton Tablelands (Matthiessen, 2002) and elsewhere. We have shown that natural hybridization between brolgas and sarus cranes is happening and occurs more widely than is visually detectable. As hybridization is still relatively rare and brolgas and sarus cranes are differentially distributed on the Atherton Tablelands (Fig. 2), its evolutionary influence could potentially be relatively limited. However, a closer look at the allele combinations in the hybrids suggests that introgression could become more widespread. Four of the birds had alleles that occur only in brolgas at one or two loci. Although one of these birds had a membership index of 0.5, suggestive of being an F₁-hybrid, these pure brolga combinations indicate that the birds are hybrids of a later generation with a hybrid parent that back-crossed with a brolga or another hybrid. Sarolgas therefore appear to be fertile at several generational recombinations.

Longitudinal research on Darwin's finches *Geospiza* spp. has shown that they evolved from a common ancestor in the Galápagos archipelago in the last 2 million years (Grant & Grant, 2008), with both natural selection and introgressive hybridization being key processes in their evolution. At this stage it is not possible to predict the trajectory of brolga/sarus crane introgression but Lamichhaney et al. (2016) demonstrated that introgression at only a single locus may have important evolutionary consequences. Interaction of introgression and selection may be complex and locality dependent, as shown for the European crow *Corvus corone*

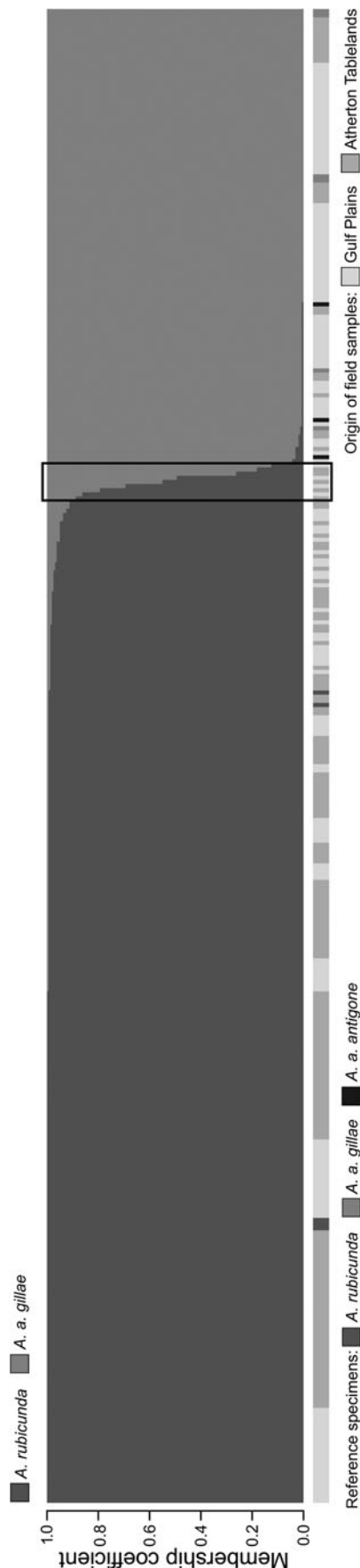


Fig. 3. STRUCTURE plot with $K = 2$ showing admixture (Q) among a total of 363 brolgas and sarus cranes, ordered horizontally by decreasing Q, with brolga as reference. The rectangle includes nine hybrids, with Q = 0.9–0.1. The bar underneath indicates the origin of birds and reference specimens.

(Vijay et al., 2016), in which genetic and phenotypic differentiation varies significantly across its range, with some boundaries being firm and others mobile. Ongoing brolga/sarus crane introgression may provide alleles to either species that could facilitate their adaptation to environmental changes brought about by such stresses as climate change or agricultural intensification; however, whether the larger body size of hybrids identified by Archibald (1981) confers a selective advantage over the parental species, potentially leading to its displacement, cannot yet be determined.

The current Australian population of sarus cranes appears to have increased relatively rapidly from what is thought to have been small numbers in northern Australia since its formal identification in the 1960s (Gill, 1967). Archibald (1981) estimated the sarus crane population was c. 200 on the Atherton Tablelands in 1972, when he identified at least two and possibly six potential brolga–sarus crane hybrids (Archibald, 1981).

Crops have been grown in northern Australia since the late 19th century and agriculture expanded substantially on the Atherton Tablelands in the 1960s, soon after Gill's first observation of sarus cranes. Templeton et al. (1990) found that changes in landscapes can be associated with genetic consequences, including introgression, which threatens species integrity (e.g. kākārīki *Cyanorhamphus* spp., Triggs & Daugherty, 1996, and black-eared miners *Manorina melanotis*, Clarke et al., 2001). Landscape features and heterogeneity can also be reflected in population genetic structure within and across species (Miller & Haig, 2010; Safner et al., 2011), and often changes in landscape are associated with habitat loss, contributing to population decline and possibly resulting in changes in the genetic diversity of remaining populations (Templeton et al., 1990; Keyghobadi, 2007).

That the species with fewer individuals in a mixed-species flock has proportionally more first-year juveniles means that young brolgas and sarus cranes are more exposed to one another other at a time when imprinting is important (Horwich, 1996). This may make pairing with the other species more likely. The enduring pair bond and longevity in both species (Johnsgard, 1983) suggests that, once formed, hybrid pairs could contribute numerous hybrid offspring to the population. Although we cannot yet predict the trajectory of crane introgression in northern Australia, there are clear conservation implications. The sarus crane is thought to be declining in much of its Asian range, particularly in Burma, Thailand and Indochina (Meine & Archibald, 1996; Archibald et al., 2003). The maintenance of Australian sarus crane populations and their genetic integrity may therefore provide security for the species should populations continue to decline in Asia. However, there seems to be no practical way of eliminating Australian crane introgression in the wild, as (1) hybrids are not necessarily visually distinguishable in the field, (2) Australian

cranes occur in remote and inaccessible areas, and (3) the underlying behavioural stimuli are not yet understood. If related to anthropogenically driven land-use change, brolgas and sarus cranes may be brought into ever-closer and more frequent foraging and flocking proximity, potentially leading to more opportunities for hybridization. Therefore, should the maintenance of genetically pure populations of Australian sarus cranes and northern Australian brolgas be considered desirable for conservation purposes, one or more well-managed extralimital populations may need to be established. As far as we know, the only captive flock of Australian sarus cranes is in Lemgo, Germany, whose ancestry dates from a wild capture in the 1970s. This could potentially form a nucleus for a captive metapopulation, spread across several institutions. Establishing a flock managed to maintain its genetic variability at this stage could also serve as insurance against climate change impacts, with some models predicting a potential 91% decline in climatic suitability for the Australian sarus crane by 2085 (Garnett et al., 2013).

As we have collected feathers from what appear to be the same birds in both the Gulf Plains and Atherton Tablelands, considering the low probabilities of encountering genetically identical individuals, we have been able to confirm what has hitherto only been assumed by birdwatchers in North Queensland, that cranes migrate between breeding areas in the Gulf Plains and the Atherton Tablelands, a distance of c. 500 km. There appears to be no material genetic difference between Gulf Plains and Atherton Tablelands cranes, indicated by estimates of population differentiation and migration after exclusion of the duplicate genotypes. The conservation implications of this are significant, as both areas will need to be targeted with appropriate conservation measures to ensure the survival of north Queensland's brolgas and sarus cranes. There are currently no laws protecting species that migrate within Australia (Runge et al., 2017).

The identification of identical birds in both the Gulf Plains and Atherton Tablelands based on shed feathers highlights the potential of genetic analyses to explore migration patterns and potential trends, not only in Australian cranes but also in other species. Although our collection of feathers was opportunistic rather than deliberately random and our estimates using the Lincoln–Petersen index are therefore necessarily indicative, they are of the same order as other estimates of crane populations on the Atherton Tablelands (Brolga 800–2,000 counted, 1,473 calculated; sarus crane 800–1,500 counted, 973 calculated). Similarly to the use of non-invasive genetic monitoring techniques elsewhere, such as for large carnivores in Europe (Kaczensky et al., 2012), feather genetics could contribute to the assessment of population trends and movements of cranes within the Atherton Tablelands and between the Atherton Tablelands and other locations where either species occurs.

Although feathers have the advantage of being a non-invasive means of gathering genetic data on individuals, as noted by Hou et al. (2018), the probability of detecting hybrids was > 10 times greater using genetic analysis rather than visual observation. However, the boundaries between pure and hybridized birds are unclear and are probably indefinable given that hybrids are fertile, meaning that introgression may have reached the point where it has no clear thresholds. A subject of future research could therefore be the definition of the relationship between genetics and phenological expression, using the feathers of known birds with hybrid phenotypes, and using data from more than the 10 loci we had available (Fitzpatrick, 2012). In addition, genomic approaches targeting coding loci (Payseur & Rieseberg, 2016) would provide a deeper insight into the evolutionary consequences of the hybridization, as the example of Darwin's finches has shown (Lamichhaney et al., 2016). However, to obtain DNA of sufficient quality and in the necessary quantities would necessitate catching many more cranes to obtain tissue and blood samples.

Whilst acknowledging that the proportion of apparent hybrids identifiable by observers is likely to be an order of magnitude lower than are actually present in the crane population, both visual observation and analysis of shed feathers could provide valuable collateral data to monitor the trajectory of Australian crane introgression.

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Conflicts of interest None.

Ethical standards The research was carried out in accordance with Charles Darwin University Animal Ethics Committee's approval A13019 and Queensland Department of Environment and Heritage Protection Scientific Purposes Permit WISP13984714. Export of samples to Greifswald University was undertaken under Australian Customs permit ACLTEM47 K and CITES permit PWS2014-AU-001240. The authors abided by the *Oryx* Code of Conduct for authors.

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