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The development of microsatellite loci through next generation sequencing, and a preliminary assessment of population genetic structure for the iconic Australian crane, Brolga (*Antigone rubicunda*)

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Disclaimer

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The lead Nature Glenelg Trust researcher on this project, Dr Adam Miller is also affiliated with the Centre for Integrative Ecology at Deakin University. The majority of the genetic research was undertaken at Deakin University and we acknowledge their generosity in making their facilities available to us.

EXECUTIVE SUMMARY

The Brolga *Antigone rubicunda* is an iconic Australian crane species, with a broad distribution spanning from tropical northern Australia, throughout the south-eastern regions of the country. The species is widespread and abundant in northern Australia, and is recognised as a species of 'least concern' under the IUCN Red List of Threatened Species. However in south-eastern Australia Brolga populations have been in decline since the early twentieth century, primarily due to widespread drainage of wetland habitats. In Victoria it is estimated that only 200-250 nesting pairs of the species remain, and these continue to be under threat. Consequently the species is listed as 'vulnerable' in Victoria, New South Wales and South Australia, and there is an increasing urgency for key biological and ecological information on *A. rubicunda* to help guide future conservation management.

Genetic studies are used widely to guide wildlife management, providing much needed insights into patterns of population connectivity, factors contributing to population fitness and resilience to environmental pressures, and baseline data for monitoring population health across generations. However, at present suitable genetic markers for conducting population genetic analyses are not available for *A. rubicunda*. To address this knowledge gap we used modern genomic sequencing technologies to identify and characterise a large panel of genetic markers (known as microsatellite loci), which will facilitate future population genetic research on *A. rubicunda*. In this report we outline the methodology used to develop these markers and their respective characteristics.

Using limited funds and previously collected Brolga samples made available to us, we then adopted the newly developed microsatellite markers, in combination with available mitochondrial DNA markers, to conduct a preliminary investigation of historical and contemporary population genetic structure among south-eastern and northern Australian Brolga populations.

Mitochondrial DNA analyses revealed no significant genetic differentiation between Brolga populations persisting in the southern and northern extremes of the continent, suggesting historical population connectivity and the sharing of a recent common ancestor. In contrast to these findings, an assessment of microsatellite markers (which evolve more rapidly) indicates potential contemporary genetic structuring across the species' range, with evidence of weak but significant differentiation between south-eastern and northern Australian Brolga populations. This suggests migration and gene exchange might be limited between populations from Victoria and northern Australia.

These findings have potentially important implications for the future management of Victoria's *A. rubicunda* population. If the population is genetically isolated, then its ongoing persistence will be heavily dependent on recruitment from local sources. The population can be expected to respond independently to environmental pressures, and declining local populations are unlikely to be supplemented via the immigration of birds and genes from the northern reaches of the continent. The small and potentially isolated nature of this population suggests it is likely to be particularly prone to loss of genetic diversity and the negative effects of inbreeding.

Estimates of genetic diversity were comparable across the south-eastern and northern Australian populations, but the effects of population decline and inbreeding can take several generations to manifest in the genetic data. Due to the small and potentially isolated nature of the south-eastern Australian Brolga population, conservation efforts in Victoria and South Australia should be geared toward securing and restoring suitable wetland habitats, increasing local population sizes, and preserving genetic diversity.

Our preliminary findings provide some interesting early insights but should be treated with caution due to the limitations of the project; such as small sample sizes, limited geographic extent of samples across the brolga's range, as well as time and funding constraints. It is possible that estimates of genetic differentiation are overstated and simply an artefact of limited sampling. Further investment is needed to sample more intensely across the species range and determine the extent of connectivity and genetic structuring among Brolga populations at the continental

and regional scale. Such comprehensive genetic analyses would provide managers and policy makers with a valuable tool for guiding conservation planning and pro-active environmental initiatives (e.g. wetland restoration), as well as adequately assessing the risks associated with future proposed land-use change in south-eastern Australia.

1 Project background

The Brolga *Antigone rubicunda* is one of Australia's two iconic crane species, measuring up to 1.8 m in height and with a wing span ranging from 1.7 to 2.4 m (Du Guesclin 2003; Marchant & Higgins 1993). The species has a broad distribution spanning from tropical northern Australia, throughout the north-east, east-central, and south-eastern regions of the country (Marchant & Higgins 1993). Brolgas are commonly found in open and shallow wetlands, coastal, subcoastal and volcanic plains, as well as agricultural areas. The species is thought to be largely non-migratory (Meine & Archibald 1996), although they are known to undertake partial migration and dispersive movements, and breeding pairs are suspected to illustrate philopatry, or nest-site fidelity (Marchant & Higgins 1993). The species is widespread and abundant in northern Australia, and is recognised as a species of 'least concern' under the IUCN Red List of Threatened Species.

Despite their abundance in northern Australia, Brolga populations throughout New South Wales, Victoria and South Australia have been in decline since the early twentieth century as a result of widespread drainage of wetland habitats for agriculture. Predation by foxes, and mortality from powerline and fence collisions are recognised as additional threats (Du Guesclin 2003; White 1987), and in Victoria wind farm developments present a potential new threat, with disturbance and collision risk from wind farm infrastructure being a contemporary concern (Hill *et al.* 2011; May *et al.* 2015; McCarthy 2009). In Victoria it is estimated that only 200-250 nesting pairs of the species remain (White 1987), and these continue to be under threat from habitat loss, poor breeding success and recruitment (Du Guesclin 2003). Due to historical declines and on-going threats, including habitat loss, the species is listed as vulnerable in Victoria under the Victorian Flora and Fauna Guarantee Act (1988), in New South Wales under the Threatened Species Conservation Act (1995), and in South Australia under the National Parks and Wildlife Act (1972).

There is an increasing need for the incorporation of key biological and ecological information on *A. rubicunda* to help guide future conservation management, including the restoration of key brolga habitat, and minimising risks associated with

land-use activities in Victoria. Genetic studies are used widely to guide wildlife management, providing insights into patterns of population connectivity, factors contributing to population fitness and resilience to environmental pressures (i.e. levels of genetic diversity and inbreeding), and baseline data for monitoring population health (Berman *et al.* 2016; Miller *et al.* 2014; Miller *et al.* 2016). Estimates of population genetic structure can in turn be used as an effective spatial framework for identifying and prioritising the conservation and restoration of key local habitats (Endo *et al.* 2015; Miller *et al.* 2014). However, at present suitable genetic markers for conducting population genetic analyses are not available for *A. rubicunda*.

In this study we report the development of new microsatellite markers that will facilitate future population genetic research on *A. rubicunda*. We make use of this new resource to perform a preliminary population genetic analysis of the species, providing first insights into historical and contemporary patterns of population connectivity across south-eastern and northern Australian Brolga populations and their respective genetic health. We discuss the potential implications of these findings and the need for further research to validate our findings and provide managers with a reliable and informed framework for guiding future conservation efforts.

2 Materials and Methods

2.1 Sample Collection and DNA extraction

A total of 30 feather samples from individuals representing seven locations across southwestern Victoria were included in our genetic analyses (Table 1). These were opportunistically sampled from flocking areas and dead birds in 2009, 2014, and 2015. Additionally, blood and feather samples were obtained from 17 individual Brolga representing three locations from Northern Australia (Table 1; Figure 1). For mitochondrial and microsatellite genotyping purposes, total genomic DNA was extracted using a modified chelex extraction protocol (Walsh et al. 1991). Using a 0.5 ml Eppendorf tube, the nib of the feather quill was macerated with a scalpel, combined with 150 µl of 5% Chelex (Roche) solution and 3 µl Proteinase K (10 mg/ml), and mixed briefly by vortex. Samples were incubated at 56°C for 2 h with periodic vortexing, followed by further digestion at 95°C for 15 min. Tissue extractions were cooled on ice for 20 min and stored at −20°C until required for analysis. Prior to PCR, Chelex extractions were homogenized by inversion and centrifuged at 13 000 rpm for 2 min. Supernatant was subsequently taken for PCR from the bottom half of the supernatant, above the Chelex resin precipitate.

2.2 Next-generation sequencing

Genomic DNA was extracted from 10 µg of muscle tissue from a single specimen using the E.Z.N.A. Tissue DNA kit (Omega Bio-tek). The purified genomic DNA was subsequently sheared to 500 bp using a Covaris Sonicator M220 (Covaris, Woburn, MA) and converted into indexed library for next-generation sequencing on the Illumina platform using NEB Next Ultra DNA Library Prep Kit (New England Biolabs, Ipswich, MA). Partial genome sequencing was performed on the Illumina MiSeq desktop sequencer (2 x 250 bp run configuration) located at the Monash University genomics facility in Malaysia.

Table 1. *Details of sample locations and the total number of Antigone rubicunda specimens from each location (n) used in this study. Mitochondrial haplotypes and their frequency per location (provided as number of individuals in parentheses beside haplotype number) also provided.*

Site	Site code	GPS coordinates	*n*	Mitochondrial haplotype (number of individuals)
Victoria				
Darlington	1	38.008, 143.005	1	
Dundonnell	2	37.920, 142.978	1	2 (1), 3 (1)
Salt Lake, Dundonnell	3	37.872, 143.054	1	
Mooramong	4	37.645, 143.262	2	1 (1), 2 (1)
Willaura	5	37.571, 142.599	15	3 (4), 4 (4)
Dunkeld	6	37.758, 142.390	6	2 (1), 3(3)
Penshurt	7	37.843, 142.345	4	2 (2)
Northern Australia				
Nhulunbuy	8	-12.175, 136.776	1	3 (1)
Miranda Downs	9	17.376, 141.926	14	3 (3), Sarus crane (2)
Herbert River	10	17.729; 145.272	2	

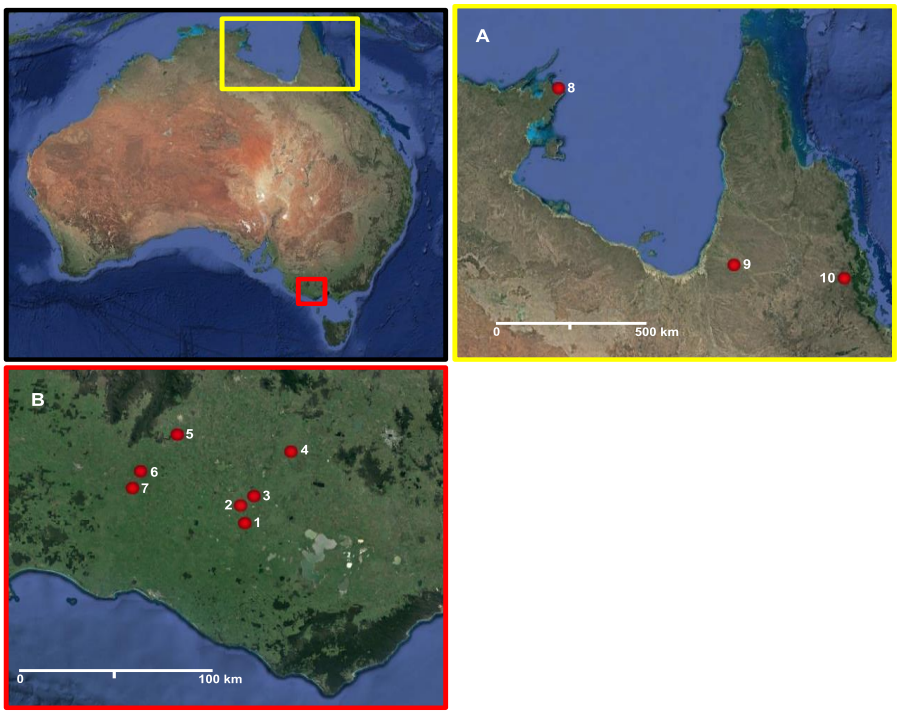

Figure 1. *Map of collection locations for Antigone rubicunda specimens used in the current study, including locations from Northern Australia (A) and Victoria (B). Further location details and the number of specimens per location used for genetic analysis are provided in Table 1.*

2.3 Mitochondrial genetic analysis

Historically there has been some debate about the taxonomic status of the south-eastern and northern Australian Brolga populations and whether they represent different subspecies (Johnsgard 1983; Marchant & Higgins 1993). Consequently we conducted a first pass assessment of genetic variation between these populations using a mitochondrial gene marker to confirm their respective taxonomic status and the degree of historical genetic divergence between them. Given the slow evolving nature of mitochondrial DNA (compared with co-dominant nuclear markers such as microsatellites (Hedrick 2011)) an analysis of genetic differentiation between these populations using mitochondrial markers are regularly used to gauge the degree of historical connectivity between species populations. The cytochrome oxidase subunit 1 gene (COI) in particular is commonly used for such purposes, and is commonly recognised as the reference 'bar-coding gene' for species discrimination analyses (Ratnasingham & Hebert 2007). This first step analysis is critical for downstream population genetic analyses as unresolved deep genetic differentiation can bias estimates of contemporary genetic structure (Miller et al. 2014).

We used a mitochondrial genome sequence representing *A. rubicunda* on Genbank (Accession: NC_020580.1) as a reference to develop species-specific primers to target approximately 700 base pairs (bp) of the COI locus (primers Brolga COIF: CTA ACC ACA AAG ATA TCGG and Brolga COIR: CTG GGT GGC CGA AGA ATC AG). Polymerase chain reactions were performed in 30 µl reaction volumes containing 3 µl of template DNA, 9.6 µl deionized H_20, and 15 µl of MyTaq Red mastermix (Bioline). PCR conditions were as follows: 94 °C for 2 min, followed by 35 cycles of 94 ^{o}C for 30 s, 55 ^{o}C for 30 s, 72 ^{o}C for 45 s, and a final extension of 72 ^{o}C for 5 min. PCR products were purified and sequenced in both directions on an ABI3730XL DNA sequencer. Consensus gene sequences were aligned and edited for all individuals using Geneious version 5.6.5 (Biomatters 2012). After editing, the mitochondrial COI alignment was 607 bp in length. Shared haplotypes were identified and uncorrected pairwise genetic distance (%) were calculated using Geneious. This analysis was performed on 22 birds in total, including 18 from Victoria and six from Northern Australia (Table 1).

2.4 Microsatellite isolation and characterisation

Partial genome assembly involving sequence reads of nuclear origin were performed using IDBA-UD (Peng et al. 2012) with the modified setting of –mink 31 and –maxk 251. The assembled partial genome was subsequently scanned for unique sequence contigs possessing microsatellite motifs using the software QDD version 3.1(Meglecz et al. 2010). Primer 3 (Rozen & Skaletsky 2000) was subsequently used to design optimal primer sets for each unique contig where possible.

A selection of 40 contigs including di-, tri-, tetra, and penta-nucleotide repeats, were used for subsequent analysis. Loci were screened for polymorphism using template DNA from eight individuals, representing four individuals from south-western Victoria and Northern Australia (Table 1). Loci were pooled into groups of four, labeled with unique fluorophores (FAM, NED, VIC, PET) and coamplified by multiplex PCR using a Qiagen multiplex kit (Qiagen) and an Eppendorf Mastercycler S gradient PCR machine following the protocol described by Blacket et al. (Blacket et al. 2012). Genotyping was subsequently performed using an Applied Biosystems 3730 capillary analyzer (http://www.agrf.org.au) and product lengths were scored manually and assessed for polymorphisms using GeneMapper version 4.0 (Applied Biosystems). Polymorphic loci were selected, pooled into three groups for multiplexing based on observed locus specific allele size ranges, and further characterized using DNA from 47 individuals comprising 30 and 17 individuals from the south-eastern and Northern Australian Brolga populations, respectively (Table 1). Microsatellite profiles were again examined using GeneMapper version 4.0 and alleles scored manually.

The Excel Microsatellite Toolkit (Park 2001) was used to estimate locus specific descriptive statistics including expected (H_E) and observed (H_O) heterozygosities, and number of alleles (N_A), while examination of conformation to Hardy–Weinberg equilibrium (HWE), the inbreeding coefficient (F_{IS}), and linkage disequilibrium estimates between all pairs of loci was conducted using GENEPOP version 4 (Raymond & Rousset 1995). Where necessary, significance values were adjusted for multiple comparisons using Bonferroni corrections (Rice 1989). Finally, all loci were assessed using MICRO-CHECKER to check for null alleles and scoring errors (Van

Oosterhout et al. 2004). The frequency of null alleles per locus was obtained using the 'Brookfield 1' formula, as evidence of null homozygotes across loci was not observed (Brookfield 1996).

2.5 Population genetic analysis

To demonstrate the utility of the microsatellite loci, a preliminary population genetic analysis was subsequently conducted to determine the likely extent of gene flow between the south-eastern Australian and northern Australia Brolga populations. Descriptive statistics described above were included in this analysis. FSTAT version 2.9.3 (Goudet 1995) was used to calculate a global estimate of population differentiation (F_{ST}) with 95% confidence limits (Weir & Cockerham 1984), and a factorial correspondence analysis (FCA), implemented in GENETIX version 4.05 (Belkhir et al. 2004), was used to summarize patterns of genetic differentiation between sample sites. The first two underlying factors that explained the majority of variation in multi-locus genotypes across loci were plotted.

3 Results

3.1 Mitochondrial genetic analysis

A total of five mitochondrial haplotypes (unique DNA sequences) were identified from the 24 Brolga samples genotyped from Victoria and northern Australia. Two birds from the Miranda Downs sample location carried a highly divergent haplotype (average genetic distance between this and the remaining haplotypes = 2.3%), however BLAST searches against the NCBI Genbank archives determined that this sequence probably represents the closely-related Sarus crane (*Antigone antigone*). In contrast, the remaining four haplotypes were weakly differentiated (average genetic distance between haplotypes = 0.2%), with all four described in Victoria, and only a single haplotype found in northern Australia (also recorded in Victoria; haplotype 3) (Table 1; Figure 2). These findings indicate that the south-eastern and northern Australian Brolga populations cannot be differentiated using mitochondrial markers, suggesting that the populations share a recent common ancestor and are unlikely to represent different subspecies.

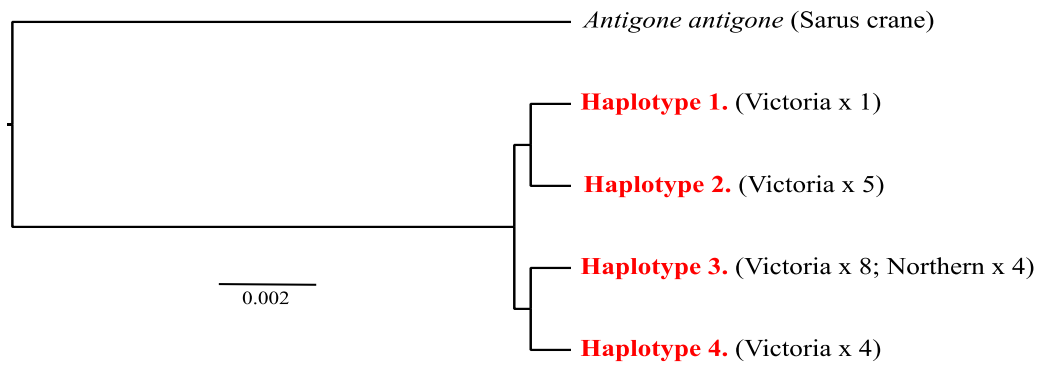

Figure 2. *Genealogical relationships among mitochondrial haplotypes derived from south-eastern and northern Australian Brolga populations. Details concerning the total number of individuals per population characterised by each haplotype are also provided. Haplotype IDs correspond with those provided in Table 1.*

3.2 Microsatellite isolation and characterisation

A total of 339 unique sequence contigs possessing microsatellite motifs were identified by QDD analysis with optimal priming sites. Initially, 40 contigs were screened for polymorphism, with 27 containing di-nucleotide repeats, eight containing trinucleotide repeats, one containing a tetra-nucleotide repeat, and two containing penta-nucleotide repeat motifs. The screening analysis found 26 loci to be polymorphic, three were monomorphic and 11 failed to amplify.

Of the 26 polymorphic loci we selected 18 loci and grouped them into three multiplexes for further characterisation (Table 3). A majority of these 18 loci were characterized by low to moderate genetic variation, with an average of 4 alleles per locus (N_A range = 2–7 alleles) and heterozygosity estimates ranging between 0.03 and 1.00 (mean H_E= 0.44) (Table 4). Linkage disequilibrium analyses confirmed marker independence (indicating no evidence of significant linkage between loci), while MICRO-CHECKER analyses revealed no evidence of null alleles or scoring issues across loci. Only a single locus was found to deviate significantly from HWE expectations, with corresponding F_{IS} estimates indicating a significant excess of heterozygotes in both cases. This locus is likely to be an unreliable marker for population genetic analysis and was consequently removed from further analyses.

Table 3. *Primers sequences and characteristics of 15 polymorphic microsatellite loci isolated from A. rubicunda*

Locus	Primer sequences (5'-3')	Repeat motif	N_A	Size range (bp)
Multiplex 1				
GR3	GGGCTCTAAGACTCACCGC	AG	4	130-136
	CCCTAGCCAGGACTATGCAG			
GR8	CATCTGATTAGGCCACAGGG	AC	2	232-234
	CAGAGTAGGGAGGCATGGAG			
GR13	TTTGGTGCAGCATGTAAGATTC	AC	2	235-237
	TCCTTTCTAATTGCTCAGCCTC			
GR28	ACACCCGATCAGTCCAAATC	AC	7	212-224
	AAGGCTTGTGAATCCTGGG			
GR30	CAGCATTTGAATGGTGATGC	AC	2	285-287
	AAAGGGATGTTCTGGCAATG			
GR32	TCAAGCAACTTGAATTAGCAGC	AT	5	145-156
	TTCAGCATGCCACAGTTCTC			
GR34	ACTCTCCTGGCAAGAAGCTG	AT	7	224-240
	TGCCAAGTTTAATGTGCTGC			
Multiplex 2				
GR5	ATTCCCTACACCTTGCCCTC	AC	6	214-224
	TGCGGTTATGCTGAGTATGG			
GR10	TCCCTTCCCATAGTGTCAGG	AG	2	173-175
	CACTGCATTCCCTTCTGAGG			
GR15	ACTCGTGGGTGGATCTATCG	AAT	3	231-238
	TTCCTTGAAGTTGGATCATGG			
GR23	AAGATGCTGAATCCCACCAG	AC	2	209-211
	GGCAATATTATAGATGGCCAGG			
GR36	TCCACCAAAGAAAGCATTCAG	AAAC	5	243-258
	GCACATGATTCAGTTTCACCC			
GR40	AGAGCCCTGGTTGCTGTG	AGC	2	329-332
	AGCCACTTTCTAATGTGTCCG			
Multiplex 3				
GR17	TCACTCAGTCTTCAAGGTGGC	AC	6	216-224
	TCTTCACGTGTGTTTAGCGG			
GR20	TGCAATGCAGAAGAGACCAC	AG	2	230-234
	GGCTGTGCTCAGGTATGAGG			
GR22	AAGGGCTCGGCTATGCTAAC	AC	5	160-172
	ATGCATCTCGGTTCAGGC			
GR25	GCACTTTGGCATGACTTGAG	ATC	5	211-226
	ATTAGGGCTTTCCATTTCCC			
GR38	AAGAGTGCCCTAATAGCAGGC	AG	2	324-326
	GTTAGAGCCGTGGTGCTCTC			

Table 4. *Characteristics of 18 microsatellite loci isolated from A. rubicunda based on 30 individuals from Victoria and 13 individuals from northern Australia. Number of alleles (N_A), observed (H_O) and expected (H_E) heterozygosities, Hardy–Weinberg equilibrium P values (HWE) and inbreeding coefficient (F_{IS}).*

	GR10	**GR15**	**GR23**	**GR36**	**GR40**	**GR5**	**GR17**	**GR20**	**GR22**	**GR25**	**GR38**	**GR13**	**GR28**	**GR30**	**GR32**	**GR34**	**GR3**	**GR8**
Victorian																		
N_A	2	3	2	4	2	6	5	2	4	5	2	2	6	2	5	7	4	2
H_E	0.03	0.63	0.41	0.68	0.49	0.72	0.55	0.14	0.44	0.54	0.51	0.49	0.70	0.31	0.78	0.83	0.52	0.33
H_O	0.03	0.64	0.33	0.50	0.54	0.68	0.52	0.07	0.14	0.48	1.00	0.19	0.54	0.13	0.83	0.56	0.47	0.33
HWE	0.12	0.91	0.36	0.22	0.71	0.07	0.94	0.11	**<0.01**	0.77	**<0.01**	0.01	0.01	0.02	0.61	0.01	0.79	1.00
FIS	0.00	-0.03	0.19	0.27	-0.09	0.05	0.05	0.48	**0.68**	0.11	-1.00	**0.61**	0.23	0.60	-0.06	**0.33**	0.10	-0.03
Northern																		
N_A	2	3	2	4	2	4	6	2	3	4	2	2	4	2	5	5	3	2
H_E	0.08	0.65	0.15	0.74	0.27	0.75	0.65	0.08	0.53	0.70	0.52	0.08	0.68	0.09	0.74	0.74	0.34	0.37
H_O	0.08	0.77	0.15	0.77	0.31	0.50	0.85	0.08	0.50	0.69	1.00	0.08	0.69	0.09	0.77	0.36	0.38	0.46
HWE	0.10	0.71	1.00	1.00	1.00	0.19	0.91	0.10	1.00	1.00	**<0.01**	0.10	0.36	0.10	0.52	0.02	1.00	1.00
FIS	0.00	-0.19	-0.04	-0.05	-0.14	0.34	-0.33	0.00	0.06	0.02	-1.00	0.00	-0.02	0.00	-0.04	**0.52**	-0.15	-0.26

3.3 Population genetic analysis

Estimates of genetic diversity were comparable across the south-eastern and northern Australian Brolga populations, with average numbers of alleles per locus being 3.61 and 3.16, and observed heterozygosities being 0.41 and 0.44, respectively. Estimates of F_{ST} across all loci were weak but significantly different from zero (F_{ST} = 0.04; 95% CI = 0.01–0.07) indicating potential gene flow limitations between the south-eastern and northern Australia populations. This relationship between populations is best depicted by the 2-dimensional FCA of the microsatellite variation (Figure 3). When the two factors explaining the highest percentage of the microsatellite variation (factor 1 = 12.52%, factor 2 = 11.30%) are plotted against each other, the Victorian and northern Brolga samples are separated with minimal overlap. Initial FCA analyses identified a total of four highly divergent individuals from Northern Australia clustering separately, two of which were identified previously as probable Sarus crane based on mitochondrial analysis (discussed above). Our microsatellite data identified all four specimens as pure Sarus cranes based on the presence of 7 unique alleles. Consequently, these samples were removed from all analyses.

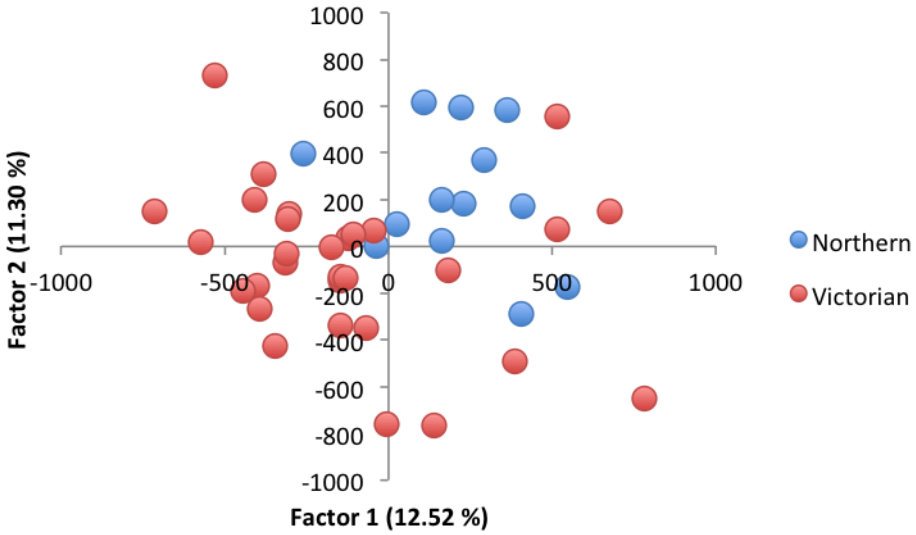

Figure 3. *Two-dimensional scatter plot showing the relationships among Antigone rubicunda sample locales based on a factorial correspondence analysis of 17 microsatellite loci. The first factor (x axis) explains 12.52% of the variance, whilst the second factor (y axis) explains 11.30%.*

4 Discussion

While birds are thought to have inherently low numbers of microsatellite loci (Primmer et al. 1997), the Illumina MiSeq sequencing platform was used successfully to develop 18 polymorphic microsatellite markers, 17 of which are expected to be a valuable resource for future population genetic research on *A. rubicunda*. These new genetic markers will be an invaluable resource for investigating the evolutionary histories and trajectories of Brolga populations, in turn providing a framework for establishing effective conservation strategies for the species.

Currently there is uncertainty surrounding the connectivity of the south-eastern and northern Australian Brolga populations, with previous studies indicating that they are potentially geographically isolated (DuGuesclin 2003). Additionally, although they are currently considered to belong to a single species (Marchant and Higgins 1993), their respective taxonomic status has not been investigated in detail prior to the current study. Our mitochondrial analysis indicates a lack of genetic differentiation between the south-eastern and northern Australian populations at the COI locus, suggesting that these populations share a recent common ancestor and do not represent different subspecies (Johnsgard 1983). However these results do not reflect current day patterns of Brolga population connectivity as the mitochondrial genome is a relatively slowly evolving locus and is used primarily for resolving historical evolutionary patterns (Endo et al. 2015). Consequently, estimates of genetic differentiation from more rapidly evolving genetic markers, such as microsatellites, are needed to determine contemporary patterns of genetics structure.

Our preliminary analysis of contemporary population genetic structure, inferred from our newly developed panel of microsatellite loci, indicated weak yet significant genetic differentiation between the south-eastern Australian and northern Brolga populations. More intensive sampling across the Brolga's range is needed to confirm these findings, as the number of sample locations and replicate samples per site were limited in the current study. However our results indicate that gene exchange between south-eastern and northern Australian populations is potentially limited. These findings are consistent with previous studies suggesting a lack of interaction

between these populations (Du Guesclin 2003), and may have significant management implications for the future evolutionary potential and hence conservation management of the species.

If the connectivity between south-eastern Australian and northern Australian Brolga populations is in fact limited, then Victoria's declining population is unlikely to be supplemented via the immigration of birds and genes from the northern reaches of the continent. The potential self-recruiting nature of the Victorian population also emphasises its vulnerability to negative demographic factors and stochastic processes. Small isolated populations (less than 500 breeding individuals) are particularly prone to rapid losses of genetic diversity and the negative effects of inbreeding (Frankham et al. 2009; Weeks et al. 2011). This can have severe impacts on population fitness and environmental resilience. Given the small census size of the Victorian population (200-250 breeding pairs) this is a major concern.

While estimates of genetic diversity are comparable across the Victorian and northern populations, the effects of population decline and inbreeding can take several generations to manifest in the genetic data (i.e. a compounding effect of ongoing inbreeding (Frankham et al. 2009)). If the Victorian Brolga population is an isolated gene pool then the threat of inbreeding is unlikely to be alleviated by natural gene flow from northern populations. Consequently, conservation efforts should be geared toward securing wetland habitats, increasing local population sizes, and preserving genetic diversity.

Since the mid twentieth century there has been speculation about potential interbreeding between Brolga and Sarus cranes in northern Australia (Archibald 1981), however molecular studies have provided no supporting data to date. Collaborators Nevard and Haase are currently conducting a research program to quantify the extent and drivers of hybridization across northern Australia. We await the outcome of their work with interest, but point out that the conservation of the south-eastern Brolga populations might become increasingly important in the future for preserving the genetic integrity of the species.

The genetic markers characterised in this study will be vital for future population genetic research on *A. rubicunda*. Our preliminary findings provide some interesting early insights but should be treated with caution due to the limitations of our sampling, a direct result of sample availability as well as time and funding constraints. We recommend that further genetic sampling be undertaken across the entire species range to determine the true extent of genetic connectivity and structuring among Brolga populations. Until then, it will not be possible to determine if the results presented here are a true reflection of contemporary genetic structure, or if our estimates are simply an artifact of our limited sampling. Further investment and sampling effort is needed to clarify these findings and to provide conservation and land managers and policy makers with a resource for guiding conservation planning, and adequately gauging the risks associated with future proposed land-use change in south-eastern Australia.

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