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Impact of genomic leakage on the conservation of the endangered Milky Stork

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ABSTRACT

Endangerment and extinction of threatened populations can often be accelerated by genomic contamination through infiltration with alien alleles. With a growing anthropogenic footprint, many such hybridization events are human-mediated. The Milky Stork (*Mycteria cinerea*) is one such species whose genomic composition is threatened by human-mediated hybridization with its sister taxon, the Painted Stork (*Mycteria leucocephala*). A comprehensive investigation of the stork population in Singapore using three complementary population-genomic approaches revealed a large proportion of hybrids that have undergone several generations of genomic leakage from Painted Storks and fall along a genetic cline that closely mirrors a phenotypic cline from pure Milky to pure Painted. Although originating from a limited number of introduced Painted Storks, these hybrids are now an integral part of both the wild and captive Singaporean and southern peninsular Malaysian stork population. Genetically informed conservation management including the isolation of hybrids in captivity and a strict removal of hybrids from the wild along with a release of genetically pure Milky Storks is imperative for continued survival. Similar approaches must become routine in endangered species conservation as human-mediated hybridization increases in volume.

1. Introduction

The world is currently facing the sixth mass extinction at an accelerated pace (Ceballos et al., 2015), majorly attributed to human impact on the environment (Barnosky et al., 2011). Apart from habitat loss and fragmentation (Turner, 1996; Krauss et al., 2010), extinction through hybridization is one of the major threats to endangered species (Rhymer and Simberloff, 1996; Wolf et al., 2001; Todesco et al., 2016). Due to the dynamic nature of hybridization or inter-specific gene flow (Mallet, 2005; Rheindt and Edwards, 2011), the genomic composition of endangered populations can be compromised by the infiltration of alien alleles into the native gene pool (Rhymer and Simberloff, 1996; Allendorf et al., 2001; Wolf et al., 2001). Facing decline in conspecific mates, individuals of threatened populations become more susceptible to mate with other species, introducing alien alleles into their genome (Pierce, 1996; Pinto et al., 2016; Lawson et al., 2017). Previous studies have documented how hybridization can lead to hybrid swarms (Allendorf et al., 2001), species collapse (Kleindorfer et al., 2014) and eventual extinction (Rhymer and Simberloff, 1996; Wolf et al., 2001). Through this process of introgression, endangered species with very small populations can eventually get absorbed into the genome of the more widespread species.

There are multiple examples of prominent endangered species that have been threatened with extinction through hybridization. Hybridization with coyotes (*Canis latrans*) is the primary threat to the critically endangered red wolf (*Canis rufus*) in North Carolina (Fredrickson and Hedrick, 2006), which itself was shown to be a hybrid lineage from coyote and a declining population of grey wolf (*Canis lupus*) (vonHoldt et al., 2016). The critically endangered Chinese Crested Tern (*Thalasseus bernsteini*), with < 100 individuals, is threatened by hybridization with Greater Crested Terns (*Thalasseus bergii*) in China (Yang et al., 2018). Interbreeding with Blue-winged Warbler (*Vermivora cyanoptera*) threatens Golden-winged Warbler (*Vermivora chrysoptera*) in North America (Moulton et al., 2017). Northern Spotted Owl (*Strix occidentalis caurina*; Kelly and Forsman, 2004), Grevy's zebra (*Equus grevyi*; Cordingley et al., 2009), Black Stilt (*Himantopus novaezelandiae*; Steeves et al., 2010), giant sable antelope (*Hippotragus niger variani*; Pinto et al., 2016), and Mangrove Finch (*Geospiza heliobates*; Lawson et al., 2017) are several other endangered species whose genomic composition is threatened by introgression.

While hybridization is predominantly a natural process, introgression of alien alleles can also be human-mediated. Human impacts such

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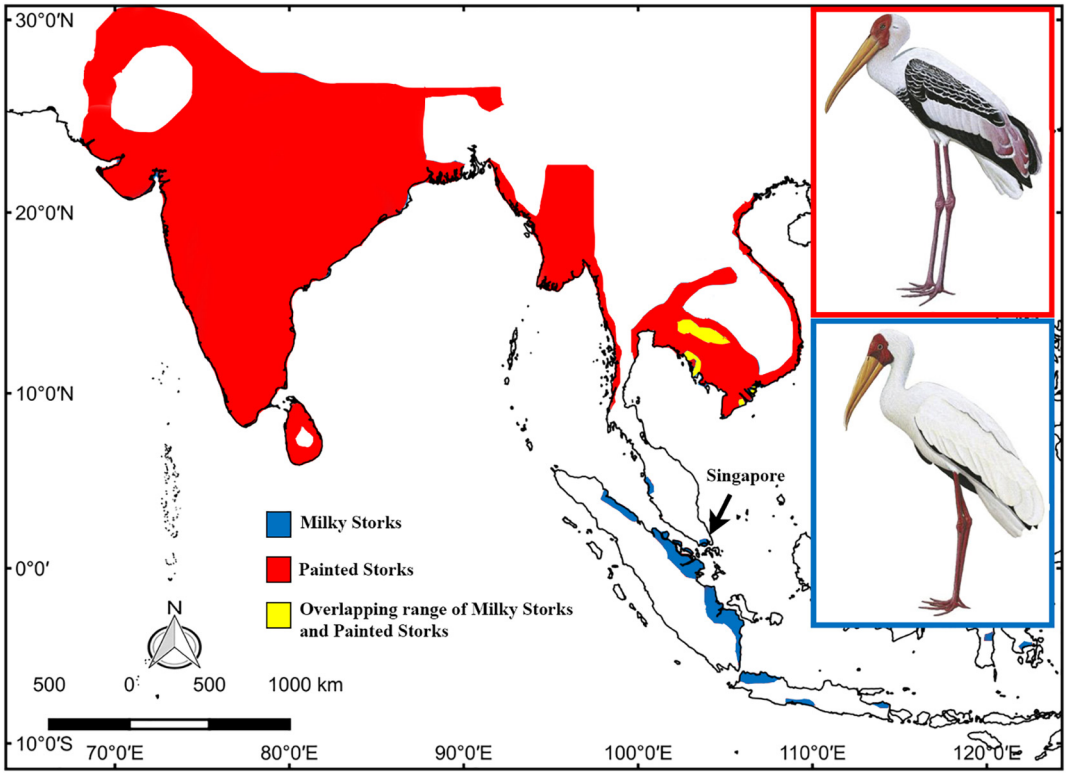
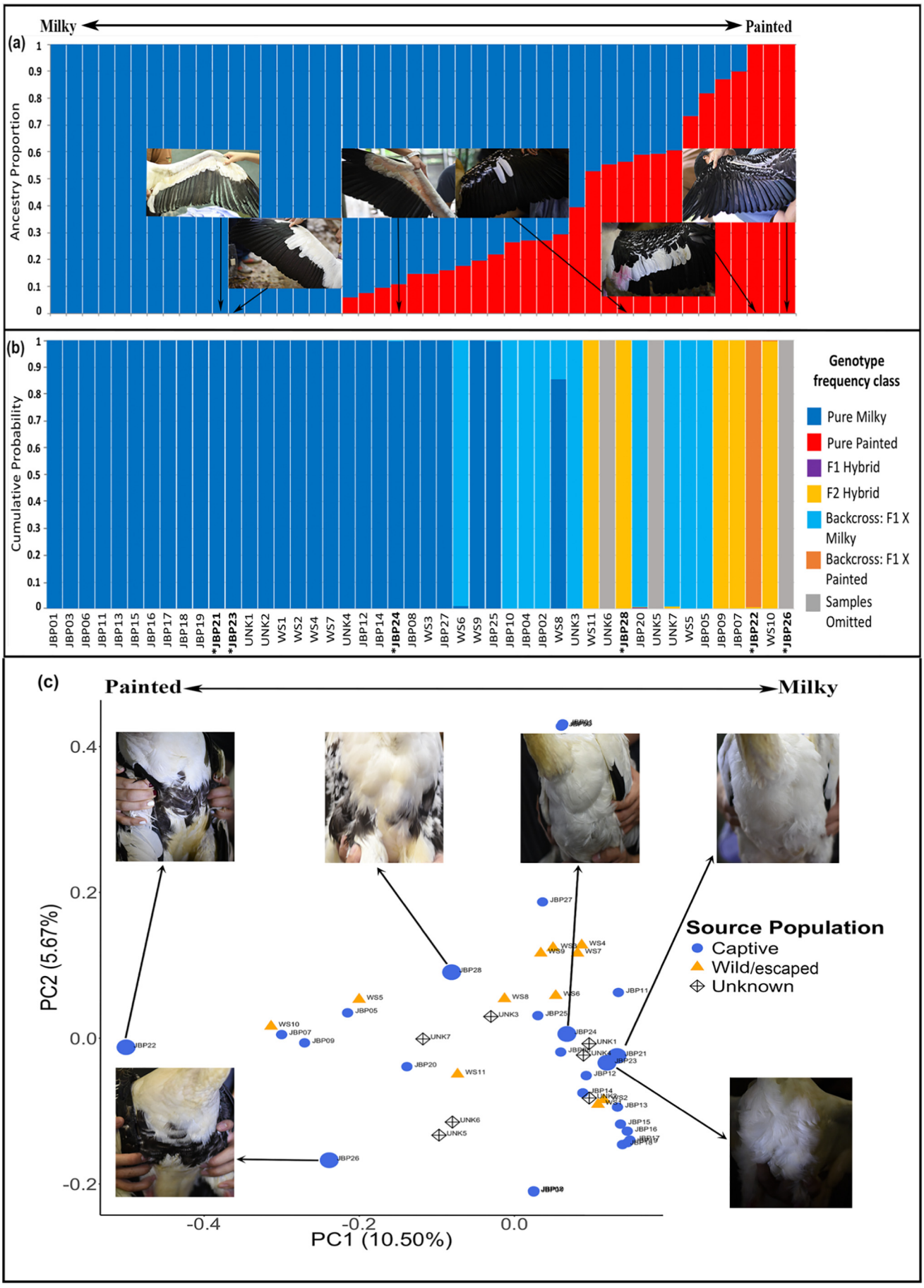


Fig. 1. Distribution range of the Milky Stork (*Mycteria cinerea*) and the Painted Stork (*Mycteria leucocephala*) in South and Southeast Asia. Species range modified from del Hoyo et al. (2018) with updates of recent records. Map was generated using QGIS v2.18.2.
Data were sourced from www.naturalearthdata.com for the coastline of landmasses. Illustrations from del Hoyo et al. (2018).

as landscape changes (vonHoldt et al., 2016; Moulton et al., 2017), introduction of exotic species (Huxel, 1999; Vilà et al., 2000), climate change (Garroway et al., 2010; Canestrelli et al., 2017), wildlife trade (Fong and Chen, 2010), and pollution (Seehausen et al., 1997) can bring two species into fresh contact and lead to hybridization. This could happen through habitat changes and resulting range shifts, breakdown of isolating mechanisms due to environmental changes, direct transportation of species outside their natural range, to name a few. A growing human footprint on our planet leads to increased instances of secondary contact between previously allopatric species that can hybridize (Moulton et al., 2017; Grabenstein and Taylor, 2018). Hence, studies to characterize genomic contamination and design effective solutions for conservation management have become increasingly important.

Here, we present one of the first studies to use genome-wide data to shed light on the genomic introgression in a threatened species, the endangered Milky Stork (*Mycteria cinerea*). The Milky Stork is found in coastal mangroves, mudflats, and estuaries across Southeast Asia (Fig. 1; Hancock et al., 2010; Eaton et al., 2016; Birdlife International, 2018) and is currently considered endangered on the International Union for the Conservation of Nature (IUCN) red list with about 1500 individuals left in the wild (Birdlife International, 2018). The population trend of Milky Storks is rapidly declining (Li et al., 2006; Li and Ounsted, 2007) due to widespread habitat destruction and hunting (Verheugt, 1987; Iqbal et al., 2008). The Milky Stork and its sister taxon, the Painted Stork (*Mycteria leucocephala*), have been reported to undergo frequent hybridization to produce reproductively viable offspring in captivity (Li et al., 2006). Genetically, the two species exhibit 0.9% divergence in mitochondrial cytochrome *b* indicating recent divergence (Slikas, 1997). Even though Milky Storks have historically overlapped with Painted Storks in a few regions (Fig. 1; Campbell et al., 2006), reports of hybridization in the wild have only started appearing since the recent drastic decline of the population of Milky Storks (e.g., Eames, 2007). These recent hybridization events are presumably due to limited mate choice in mixed nesting colonies (Li et al., 2006; Hancock et al., 2010), hence further putting the Milky Stork in peril. Morphologically, the Painted Stork is differentiated from the Milky Stork by the presence of a black pectoral band and a pink flush in the inner wing coverts and tertials (Fig. 1; Robson, 2015; Elliott et al., 2018a, 2018b). Reported hybrids display a mix of these morphological characteristics (Li et al., 2006). This admixture may compromise the genomic composition of the Milky Stork, as has been observed in other threatened birds (Barilani et al., 2007; Lawson et al., 2017). Such admixture can also counteract the efforts of ongoing captive breeding and reintroduction programs (Yaacob, 1994; Ismail et al., 2011; Faiq et al., 2016) by contaminating the Milky Storks' gene pool with Painted Stork alleles (Urfi, 2011).

In the southernmost Malay Peninsula, Milky Storks have been held in captivity since the late 1980s, with inadvertent cross-breeding events with Painted Stork whenever the two were held in the same enclosure (Li et al., 2006). A few of these hybridized storks have escaped into the wild. Although some of these hybrid escapees have been recaptured, others continue to roam freely, posing a major threat to the genomic composition of the Milky Stork population in this area (Yaacob, 1994). It is speculated that the stork population in Singapore has suffered heavily from introgressive hybridization, with many individuals bearing intermediate traits at variable proportions (Tsang, 2007; Wilton-Jones, 2016) in an area that is within the native historical range of the Milky Stork (Fig. 1; Gibson-Hill, 1949; Medway and Wells, 1976; Keng and Hails, 2007; Clements et al., 2017). In this study, we used a double digest RAD sequencing (ddRADseq) approach (Peterson et al., 2012) to study the genetic makeup of the stork population in Singapore. One of the primary aims was to characterize the patterns of introgressive hybridization and genomic leakage from Painted into native Milky Storks.



(caption on next page)

Fig. 2. Population structure of storks in Singapore using ADMIXTURE, NEWHYBRIDS, and principal component analysis (PCA). (a) Each stork is represented by a stacked column of ancestral genetic components shown in color for K = 2 based on ADMIXTURE. The two colors represent two different ancestral populations. Pictures of wing feathers of six of these storks are shown. (b) Genotype frequency class assignment of storks in NEWHYBRIDS using 400 diagnostic SNPs filtered for high F_{ST} and low linkage. Each stork is represented by a stacked column of probability of belonging to one of the six genotype frequency classes: pure Milky Storks, pure Painted Storks, first generation hybrids (F1), second generation hybrids (F2), and backcrosses of F1 to pure Milky Stork or pure Painted Stork. Sample names are indicated on the x-axis. Asterisk (*) indicates samples with known morphological identity. (c) Principal components 1 and 2 (PC1 and PC2) accounted for a combined > 16% of observed variability in the analysis. The enlarged blue circles indicate samples with known morphological identity. Pictures of breast plumage of these storks are shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2. Methods

2.1. Sample collection and sequencing

We obtained a total of 46 tissue samples of Singaporean stork individuals of a wild/escaped (11), captive (28), and unknown origin (7) through Jurong Bird Park (Appendix B, Table B.1). The birds of unknown origin had an incomplete record but were likely captive. The individuals were caught using drop net traps. We followed the Wildlife Reserves Singapore (WRS) Animal Welfare Code approved by WRS Animal Welfare and Ethics Committee during sample collection. The morphological identity of six individuals among the 46 samples was recorded with photographic documentation, but unfortunately this was not available for the remaining 40 samples. These six individuals were classified as “pure”-looking for either species or as hybridized on the basis of (1) the presence or absence of a black breast band, (2) the presence or absence of a pink flush on the tertials, inner secondaries and wing coverts, and (3) the white versus black coloration of the upperwing coverts (Fig. 1; Robson, 2015; Elliott et al., 2018a, 2018b). These traits are well known to be exclusive to one or the other species and must all simultaneously be present for an individual to classify as pure. Intermediacy in these characters is widely observed in hybrids (Li et al., 2006). Intra-specific morphological variation within both of these stork species is limited and does not relate to the specified characters (Urfi and Kalam, 2006; Ong et al., 2012).

We used Qiagen DNeasy Blood and Tissue kits (Qiagen, Germany) for DNA extraction and a Qubit® 2.0 High Sensitivity DNA Assay (Invitrogen, USA) for DNA quantification. We adopted the ddRADseq library preparation protocol from Peterson et al. (2012) with slight modifications.

In our modified approach, genomic DNA was digested with the restriction enzymes *Eco*RI and *Msp*I, and the restricted samples were ligated to a unique P1E adapter using T4 DNA ligase. After ligation, samples of the same concentration band were pooled into two pools. Adapter-ligated DNA fragments were size selected with Pippin Prep (Sage Science, USA, setting: 350 bp “tight”) using 2% agarose. Size selected fragments were amplified through polymerase chain reaction (PCR) for 10 cycles and a unique PCR 2 primer was used for each pool. Cleanup after restriction, ligation, and size selection was performed with AMPure XP beads (Beckman Coulter, USA). We used a Qubit® dsDNA High Sensitivity Assay Kit (Invitrogen, USA) for library quantification, and an Agilent High Sensitivity DNA kit (Agilent Technologies, USA) to check the quality of the pooled libraries. The pooled libraries were sequenced on an Illumina HiSeq 4000 lane at Novogene (Singapore). We obtained 150 base pair (bp) paired end reads after sequencing.

2.2. Quality filtering and SNP calling

The quality of the raw sequence data was checked using FastQC v0.11.7 (Babraham Bioinformatics, UK). Reads with uncalled bases and/or low quality (Phred score < 20) were removed. We demultiplexed and filtered raw sequence data to obtain reads for each individual sample using the *process_radtags* command installed in Stacks v1.46 (Catchen et al., 2013). We aligned the reads to the reference genome of the crested ibis *Nipponia nippon* (Li et al., 2014) using the software package BWA 0.7.12 (Li, 2013). Aligned reads of low mapping quality (MAPQ score < 20) were removed to ensure unique mapping using SAMtools v0.1.19 (Li et al., 2009).

SNPs were called with a minimum stack depth of 10 using the *ref_map* pipeline in Stacks v1.46 (Catchen et al., 2013). SNPs present in < 90% of all samples were filtered using the *populations* module in Stacks v1.46. All samples were defined as one population and only the first SNP of every locus was retained to avoid linkage issues.

We removed any loci and individuals that contained > 10% missing data using PLINK v1.9 (Chang et al., 2015). We also filtered linked loci using PLINK v1.9 (Chang et al., 2015) with the following parameters: sliding window of 25 SNPs, step size of 10 and pairwise linkage disequilibrium < 0.95, obtaining a final set of 9465 loci for 46 individuals.

2.3. Population genomic analysis

To visually illustrate the genetic relationship among all individuals, we performed principal component analysis (PCA) using the R package *SNPRelate* (Zheng, 2013) in RStudio v.1.0.143 (RStudio Team, 2015).

We estimated individual ancestries with a maximum likelihood algorithm using ADMIXTURE v1.3.0 (Alexander et al., 2009; Alexander and Lange, 2011). In the ADMIXTURE analysis, we explored a number of ancestral populations (K = 1, 2, 3; Appendix A, Fig. A.1, Fig. 2a) and employed cross-validation values (CV) to identify the best K value with the lowest CV error (K = 2) for an appropriate modelling choice (Alexander and Lange, 2011). We further explored individual-based pairwise coancestry using the Markov chain Monte Carlo (MCMC) coalescence algorithm as implemented in fineRADstructure v0.3.1 (Malinsky et al., 2018) using haplotype linkage information. While preparing the fineRADstructure input file (using the STACKS output file), we removed samples with > 20% missing data and more than five SNPs at each locus. We also explored > 10% missing data as a cutoff, but results were similar, prompting us to continue only with results from the > 20% missing data analysis (Appendix A, Fig. A.2). In the fineRADstructure pipeline, we used RADpainter to calculate the coancestry matrix followed by assigning individuals to populations at default parameters, including a burn-in period of 100,000 and 100,000 MCMC iterations. Pairwise kinship relationships among individuals were investigated in *SNPRelate* (Zheng, 2013).

In addition, we performed Bayesian population clustering using fastSTRUCTURE (Raj et al., 2014). We ran fastSTRUCTURE for a variable number of clusters (K = 1, 2, 3) using both simple and logistic prior models.

To identify genotype frequency classes, we used *HYBRIDDETECTIVE* (Wringe et al., 2017a), an R package workflow that implements hybrid detection using the Bayesian model based program NEWHYBRIDS v.1.1 (Anderson and Thompson, 2002). We obtained different panel sizes of unlinked informative SNP loci (50, 100, 200, 400) based on high F_{ST} values using the *getTopLoc* function. We used genotype data of individuals with a high probability of being pure Milky Storks or Painted Storks based on the ancestry fraction threshold (Q values > 0.999 from ADMIXTURE analysis) as the input in this step. We created three replicates of three sets of simulated data with six different genotype frequency classes (two pure populations, first and second generation hybrids [F1 & F2], and backcross of F1 with each of the pure populations) for each panel size using the *freqbasedsim_AlleleSample* function.

We analyzed the simulated data sets using NEWHYBRIDS as implemented in the R package *parallelnewhybrids* (Wringe et al., 2017b) (burnin period: 50,000; MCMC sweeps: 300,000). We checked the results for convergence of MCMC chains followed by evaluation of accuracy, efficiency, and power of class assignment of each genotype frequency class for different panel sizes. We found the panel size of 400 loci to be most successful for class assignment at all posterior probability thresholds (Appendix A, Figs. A.3–A.5) and hence used it for investigation of experimental data. We then evaluated the 46 storks in Singapore with *parallelnewhybrids* (Wringe et al., 2017b) (burnin period: 50,000; MCMC sweeps: 300,000), checking the results for convergence of MCMC chains. Simulated pure individuals were included in this analysis to improve efficacy.

3. Results

ADMIXTURE and PCA analysis along with the available morphological data (Fig. 2) revealed a genomic cline in the composition of the stork population in Singapore, ranging from individuals with a relatively high Milky Stork genomic composition to those with a relatively high Painted Stork composition in their genome, with individuals of intermediate ancestral genetic proportions of the two species in between. ADMIXTURE identified 18 "pure" Milky Storks and three "pure" Painted Storks (Q values > 0.99, Appendix B, Table B.2). The remaining 25 individuals have different levels of genetic proportions from both species (Fig. 2a). In PCA analysis, the stork genome is differentiated along principal component 1 based on Painted or Milky Stork contribution, as indicated by their morphology (Fig. 2c). Two individuals, JBP22 and JBP26, were identified as relatively pure Painted Storks based on ADMIXTURE analysis (Fig. 2a) and showed the distinctive black breast band (Fig. 2c) and black wing coverts of that species (Fig. 2a). Another two individuals, JBP21 and JBP23, were identified as relatively pure Milky Storks according to ADMIXTURE (Fig. 2a) and lacked the breast band (Fig. 2c) while exhibiting clean white wing coverts (Fig. 2a) typical of that species. One individual, JBP28, was a genetic intermediate characterized by a predominantly Painted Stork phenotype (Fig. 2a) yet with a diffuse breast band (Fig. 2c). Another admixed sample, JBP24, with a predominantly Milky Stork genome (Fig. 2a) showed diffuse pink in the plumage (Fig. 2a) but an otherwise Milky Stork phenotype (Fig. 2c). The population structure inferred through fastSTRUCTURE indicated a greater number of "pure" Painted and Milky Storks as compared to ADMIXTURE (Appendix A, Fig. A.6).

The fineRADstructure coancestry matrix revealed that many of the Milky Storks identified as "pure" by ADMIXTURE are genetically more similar to one another as compared to the three Painted Storks identified as "pure" by ADMIXTURE (Fig. 3). These three "pure" Painted Storks clustered with hybrid stork individuals with a ≥50% Painted Stork ancestral proportion (Fig. 3). The majority of the hybrids are neither closely related to one another nor to any of the putatively pure individuals (Fig. 3). A few closely related "pure" Milky Storks (JBP01, JBP03, JBP06; as identified by ADMIXTURE) seem to be close kin to each other, as do a few of the hybrids (JBP02, JBP04, JBP10) (kinship coefficient > 0.25, Appendix B, Table B.3).

We used NEWHYBRIDS to detect genotype frequency classes in the Singaporean stork population based on 400 diagnostic SNPs (Fig. 2b). Simulated data showed that this panel of 400 SNPs is able to assign individuals to the six genotype frequency classes with accuracy, efficiency and a power of 100% at a probability threshold of 90% (Appendix A, Figs. A.3–A.5). Neither "pure" Painted Stork nor any first generation hybrid (F1) between pure parents of each species was identified. The results reveal a greater proportion of "pure" Milky Storks as compared to the ADMIXTURE analysis (Fig. 2a, b). JBP24, identified as a hybrid in ADMIXTURE, is identified as "pure" Milky in NEWHYBRIDS class assignment (Fig. 2a, b). Nine storks were identified as backcrosses between F1 and pure Milky Storks, and another five as second generation hybrids (F2). WS10, identified as "pure" Painted in ADMIXTURE, emerged as F2, while JBP22, another "pure" Painted Stork in ADMIXTURE, is revealed as a backcross between F1 and pure Painted Storks (Fig. 2a, b). Three samples dropped out during NEWHYBRIDS analysis due to computational underflow (UNK5, UNK6, JBP26).

4. Discussion

Our study provides the first estimation of the population genomic status of the endangered Milky Stork in Singapore, with an evaluation of genetic infiltration from the Painted Stork. Our results indicate the existence of storks of a relatively high proportion of Milky ancestry in the population, which may putatively be pure, even though a majority of sampled individuals carried the signature of different degrees of introgression from Painted Storks. Moreover, a set of morphological traits seemed to closely correspond to levels of hybridization as detected through genomic approaches.

4.1. Hybridization endangers the Milky Stork

Based on a genome-wide dataset of 9465 SNPs, storks in Singapore display a genomic cline ranging from a high Painted Stork component to a high Milky Stork component that closely tracks a similar morphological cline (Fig. 2). In this population, ADMIXTURE identified > 50% (n = 25) from among a panel of 46 storks as hybrids (Fig. 2a). An alternative genotype class assignment approach using a select panel of 400 diagnostic SNPs identified ~35% (n = 15) of samples as hybrids, with a large overlap with ADMIXTURE. These results attest to a high incidence of hybridization in a Milky Stork population that has been affected by infiltration of a limited number of Painted Storks or their hybrid offspring for only ~2 decades. The population of Milky Storks roaming in the wild in southern peninsular Malaysia and Singapore may number ~100–150 individuals (pers. obs.; Li et al., 2006; Ismail et al., 2011; Ismail and Rahman, 2016), which comprises approximately 7–10% of the global Milky Stork population. This high incidence of hybrids is alarming for a species that only has 1500 individuals left in the wild (Birdlife International, 2018).

NEWHYBRIDS did not identify any pure Painted Storks or any first-generation hybrids between pure parents of either species in the study population (Fig. 2b). This result is expected, given that the Painted Stork is non-native to Singapore (Fig. 1) and presumably in the minority. The initial source of Painted Stork DNA would have been from only a few escapees from collection-based institutions. Over the course of ~2 decades, these Painted Storks would have quickly lost their genetic purity and backcrossed into the native Milky Stork population. Given the methodological difference from NEWHYBRIDS (use of a diagnostic panel of loci vs all loci), ADMIXTURE provides a hybrid assignment acknowledging the existence of pure individuals for both species. However, NEWHYBRID yields a hybrid assignment acknowledging the existence of unsampled pure individuals through simulation. Therefore, the "pure" Painted Storks identified by ADMIXTURE are unlikely to be pure, but merely comprise those storks with the highest Painted Stork genetic component among our panel of 46 storks. In a similar vein, the coancestry matrix reveals that the majority of hybrids as identified by ADMIXTURE are neither closely related to one another nor to any of the putatively pure individuals, indicating recent hybridization (Fig. 3, Barrera-Guzmán et al., 2017). This pattern illustrates the pernicious nature of accidental releases of exotic congeners or hybrids for the well-being of threatened species, even if restricted in scope. Our results underscore the importance for immediate conservation action for the Milky Stork, and for adding the threat of genomic contamination to the list of factors that put the Milky Stork at risk (e.g., habitat degradation, poaching etc.).

Our results attest to hybridization between both storks in Singapore for several generations in the wild as well as in captivity. Storks with a

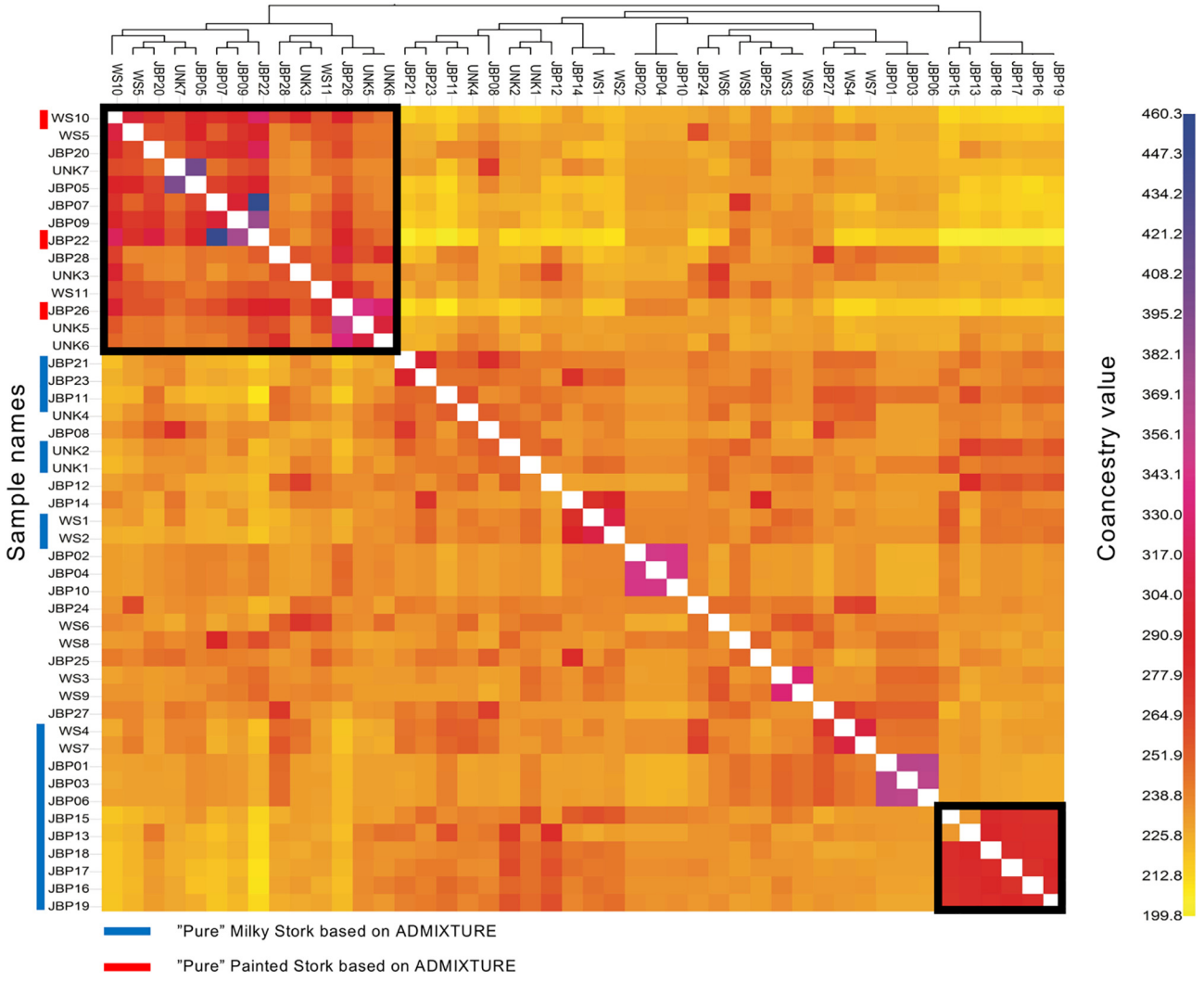


Fig. 3. Clustered fineRADstructure coancestry matrix of storks in Singapore. The highest coancestry value is indicated by colors in blue. The lowest value of coancestry is indicated by shades of yellow. Sample names are indicated on the axes. The distinct red block on the bottom right corner denoted by a black border consists of relatively pure Milky Storks according to ADMIXTURE. The "pure" Painted Storks identified by ADMIXTURE clustered together with hybrid stork individuals with a ≥50% Painted Stork ancestral proportion as indicated by the red block on the top left corner demarcated by a black border. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

high Painted Stork genetic component are present in both captive and wild populations, posing a threat to the remaining pure Milky Storks in Singapore. We recommend the use of both our analytical approaches to identify hybrids for management action: ADMIXTURE may over-estimate the purity for the minority species (i.e., Painted Stork JBP22, WS10, and JBP26) while *HYBRIDDETECTIVE* may overlook smaller amounts of introgression (i.e., hybrid JBP24).

4.2. Phenotypic admixture is a reliable first indicator of hybridization

Though our study demonstrates a correlation between the genomic and morphological cline among the two storks in Singapore (Fig. 2), it is based on a small morphological dataset of six individuals.

Even so, interesting phenotypic patterns arise: One hybrid, JBP24 (as identified based on ADMIXTURE) is predominantly Milky in phenotype but exhibits traces of pink in its wing coverts, consistent with Painted admixture but *contra* the NEWHYBRIDS class assignment as "pure" Milky (Fig. 2). Pink in stork plumage is generated by carotenoid-derived pigments (Thomas et al., 2014), precursors of which are derived from the diet (Negro and Garrido-Fernandez, 2000). However, recent studies have identified the role of different genes in carotenoid processing and transport (Toews et al., 2016; Toomey et al., 2017). Little is known about the exact nature of the genes involved in carotenoid color production. However, the functional loci responsible for the generation of pink color would form a minute percentage of the entire genome and are easy to fall outside the top 400 locus panel with high F_{ST} used in the NEWHYBRIDS assignment of JBP24 as a "pure" Milky Stork. The inclusion of stork individuals of a confirmed pure Milky ancestry, perhaps from native parts of the Indonesian range, would make this analysis more robust.

4.3. The remaining wild population

The wild population of Singaporean storks, as represented by 11 individuals in our dataset (WS1 to WS11), contained one individual with a very high Painted Stork genetic component (WS10), four Milky Storks identified as "pure" by both ADMIXTURE and NEWHYBRIDS, as well as six hybrids with varying proportions of mixed genotypes. As all individuals were sampled on the same day at the same location, Milky Storks at different points along the introgressive spectrum seem to form flocks in the wild. Because these storks nest colonially, they probably breed in mixed nesting colonies (Kahl, 1987; Hancock et al., 2010;

Elliott et al., 2018a) promoting hybridization. Based on our results, the endangered Milky Stork in Singapore has already had several generations of genetic leakage from Painted Storks. Moreover, Singapore is situated at the tip of the Malay Peninsula, directly adjacent to the last stronghold of viable breeding Milky Stork populations in the mangroves of eastern Sumatra, i.e. Kumpai Lake, Kuala Puntian, and Banyuasin peninsula (Iqbal and Hasudungan, 2008; Iqbal et al., 2008; Iqbal et al., 2012). If allowed to increase in population size, hybrid storks from the Malay Peninsula may well disperse and infiltrate these core regions, compromising the wild gene pool of Milky Storks, similar to what happened when the hybrid escapees infiltrated the Singaporean population.

4.4. Future conservation action

The genomic composition of Milky Storks in Singapore is highly compromised by hybridization, and immediate conservation action is warranted. Future conservation action should be based on conservation genetic data to avoid an exacerbation of the problem if genetically admixed individuals are used for conservation breeding or reintroduction.

We recommend that hybrid storks in Jurong Bird Park (Singapore), Zoo Negara (Kuala Lumpur), and Dusit Zoo (Bangkok) should be identified and isolated from pure Milky Storks to prevent crossbreeding, and that a thorough genetic analysis should ensure the purity of any planned breeding programs and/or releases. Finally, we recommend a strict removal of hybrids from the wild. Humane removal of hybrids can be carried out the same way that tissue samples were obtained (via drop net traps) and supplemented with the release of pure Milky Storks (confirmed by genomic analysis). Caught hybrid individuals can then be kept in isolated enclosures. These conservation management guidelines could be applied to other endangered species threatened by genomic contamination through hybridization.

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Appendix A. Supplementary data

Supplementary figures showing ADMIXTURE results (K = 1 and K = 3), accuracy, efficiency, and power of genotype frequency class assignment, and results of fastSTRUCTURE analysis (Appendix A) and supplementary tables showing detailed sample information, individual ancestry proportions based on ADMIXTURE and pairwise kinship coefficient for individuals with kinship coefficient > 0.25 (Appendix B) can be found online. Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocon.2018.11.009.

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