RESEARCH ARTICLE

Identifying Parentage Using Molecular Markers: Improving Accuracy of Studbook Records for a Captive Flock of Marabou Storks (*Leptoptilus crumeniferus*)

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Extra-pair copulations (EPCs) leading to extra-pair fertilization (EPF) are common in avian mating systems, despite the prevalence of observed social monogamy in many species. Colonially breeding birds are interesting species to investigate the prevalence of EPCs and EPF because they show nesting habits including close proximity of nest sites and sexual partners, which are proposed to promote alternative reproductive tactics. Endemic to Africa, the colonial marabou stork (*Leptoptilos crumeniferus*) is one of the most commonly held avian species in North American zoos. The aims of this study were to use genetic information to verify parentage in a population of marabou stork housed at Disney's Animal Kingdom® based on five microsatellite loci and to investigate reproductive behavior. We compared genetic analyses of parents and offspring to studbook data collected through behavioral observations of parental behavior at the nest. Using genetic analyses to reconstruct the pedigree of the marabou stork flock using the program COLONY led to improvement of studbook records by determining parentage of an individual that had previously unknown parentage, and identified one individual that had a sire that differed genetically from studbook records. An important contribution of our analyses was the identification and verification of the most likely parents for offspring hatched in this colony and improving incorrect or undocumented parentage in the studbook. Additionally, the colonial nature of this species makes it difficult to observe and understand reproductive behavior. Gaining better understanding of the mating system of a species is essential for successful breeding and captive management. Zoo Biol. XX:XX–XX, 2013.   © 2013 Wiley Periodicals, Inc.

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INTRODUCTION

Avian mating systems vary widely, with many species practicing extra-pair copulations (EPCs) and extra-pair fertilizations (EPFs) at much higher rates than assumed, particularly those species previously thought to be both socially and reproductively monogamous [Avise, 1996; Miño et al., 2011]. Parentage studies using molecular techniques have improved understanding of the evolution and behavioral ecology of birds, revealing unsolved aspects of their reproductive biology and natural history [Avise, 2004]. With the application of various molecular genetic techniques, extra-pair paternity has been found in 90% of all avian species studied, with over 11% of offspring from socially monogamous species being the result of extra-pair paternity and true genetic monogamy in <25% of socially monogamous species [Griffith et al., 2002]. Using molecular techniques to determine genetic relatedness among individuals and assess kinship is a powerful tool for investigating issues such as mating systems, parental care, dispersal, and other biological parameters in natural and captive populations of birds [Frankham et al., 2002; Avise, 2004].

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Colonially breeding avian species are interesting species in which to investigate the prevalence of EPCs and EPFs because they show nesting habits proposed to promote alternative reproductive tactics such as close proximity of nest sites and sexual partners [Miño et al., 2011]. Indeed, Møller and Birkhead [1992, 1993] reported that extra-pair copulations are found to occur more frequently in colonial than dispersed nesting birds. They interpreted this finding as the result of either increased proximity of individuals or lower intensity of mate guarding in colonial species. Species in the order Ciconiiformes include examples of those that nest colonially and therefore have high competition for nest sites [Burger, 1981]. Their proximity when nesting could easily lead to EPCs, EPFs, and extra-pair paternity (EPP). Studies have documented non-monogamous mating systems in Ciconiiformes such as the roseate spoonbill (*Platalea ajaja*) and the wood stork [*Mycteria americana*; Miño et al., 2009].

One of the hypotheses proposed as to why females accept EPCs is to avoid the potentially larger cost of rejecting a persistent or aggressive male [Kempenaers and Dhondt, 1993]. In this case, females do not necessarily benefit directly from the EPC, but instead avoid the costs associated with refusing the male. In colonial species with a high level of nest-site competition or where nests can easily be destroyed, nest guarding may prevent continuous mate guarding, and females may be left alone and subsequently attacked and forced into extra-pair copulations by males (e.g., white ibis [*Eudocimus albus*; Frederick, 1987b]). It has also been suggested that aggressive male behaviors and forced copulations are more common among colonial than solitary species [Gowaty and Buschhaus, 1998]. Frederick [1987a] reported that white ibis (*Eudocimus albus*) males were frequently observed biting and beating the heads of females, while female were observed to "protest" and "lack coopera-tion" in copulation attempts.

Native to tropical Africa, marabou storks (*Leptoptilos crumeniferus*) are the largest storks of the Ciconiidae family, with an average height of 120 cm, wing-span of 2.9 m and pronounced sexual dimorphism [Hancock et al., 1992]. These birds are colonial and have been observed to nest with a few to hundreds of other pairs. In breeding season, male marabou storks establish and aggressively protect a nest-site. Females in breeding condition will repeatedly visit the nest site despite overly aggressive displays from the male that include threat displays and bodily assaults on the female [Kahl, 1966]. Females respond with a submissive display and the male eventually allows her to approach and remain at the nest-site [Kahl, 1966]. Once the pair is formed, they begin work on nest construction or repair. It is during this nest building phase that most copulations occur and eggs are laid soon after the nest is completed [Kahl, 1966]. While many descriptions exist about the behavioral courtship and pairing rituals between males and females in the marabou stork [Kahl, 1966; Pomeroy, 1978] as well as demographic parameters of specific colonies including fecundity [Monadjem, 2005] and juvenile dispersal [Monadjem et al., 2008], little information

is available about the interactions between pairs and non-paired individuals at the colony site.

Marabou storks are one of the most commonly held birds in North American zoos, yet many zoos are rarely successful in breeding this species [Terkel, 1994]. For example, between 1978 and 2002, only eight North American institutions successfully bred marabou storks out of approximately 25 institutions attempting to breed [Hejna, 2002]. Despite the challenges around successfully breeding this species, little research exists on the causes of these reproductive impediments [Kuhar et al., 2004]. Fur-thermore, the captive population faces a significant challenge related to trauma-related mortalities. Between 2006 and 2007, within the captive population held in European institutions, 32% of deaths were attributed to intraspecific aggression, 16% to interspecific aggression (from hoofed stock), and 18% to aggression of an unknown source. Numerous other deaths from conspecifics at zoological institutions throughout the world have been reported [Terkel, 1994]. There have been at least seven deaths from intraspecific aggression in the North American population [Schutz, personal communication] in addition to multiple conflicts resulting in injury requiring veterinary intervention since 2007. It has been observed that females are most often the recipients of this intraspecific aggression leading to a captive population that is heavily skewed towards males [Schutz, 2011]. This skewed sex ratio further contributes to the reproductive challenges facing the captive population and it is possible that the presence of female-targeted aggression in this species could lead to forced copulations, and thus extra-pair fertilizations in this species.

Breeding programs of many species managed by zoos and aquaria rely on a studbook, which is a database comprised of the pedigree information and major life history events for every individual in a defined population [Earnhardt et al., 2005]. This studbook provides data for pedigree analyses, which are the foundation to analyzing and managing the demographic and genetic health of captive populations [Ballou and Foose, 1996]. Accurate pedigrees provide information on inbreeding, kinships among individ-uals, and the distribution of individual founder contributions to a population [Ivy and Lacy, 2010]. However, for pedigree analyses to be effective, the pedigree must be accurate and complete. In the case of pedigrees with missing or incorrect information, molecular genetic tools have the ability to improve breeding programs [see Ivy and Lacy, 2010 for a detailed discussion]. The North American regional marabou stork studbook was first published in 2002 [Hejna, 2002] and the data from the pedigree have been used to analyze the population and publish two breeding plans for this species in North American zoos [Schutz and Christman, 2009; Schutz and Ferrie, 2012].

The aim of this study was to use genetic information to verify parentage in an ex situ population of marabou storks based on five microsatellite loci. Using molecular genetic techniques, we reconstructed the pedigree of a colony of

marabou storks to determine if the genetic pedigree supports the presumed pedigree constructed using parental behaviors observed over multiple breeding seasons. We also used the genetic pedigree to interpret behavior during the breeding season in this colony. Our analyses constitute the first attempt to clarify aspects of the genetic mating system in the marabou stork, which is under-studied both in the wild and in captive populations. Investigating these factors may improve the understanding of this species' reproductive behavior and the management of a colonial species in an ex situ population.

MATERIALS AND METHODS

Study Subjects

Disney's Animal Kingdom® was one of the most successful breeding institutions for marabou stork in North America with the largest flock at one time [Schutz, 2011], and as in other zoological institutions displaying this species, this flock had a history of intraspecific aggression. From 1998 through 2012, Disney's Animal Kingdom® exhibited a flock of marabou storks that varied in number from 6 to 15 birds in an enclosure approximately three acres in size.

Sample Collection and DNA Extraction

Blood samples were collected from 11 marabou storks as part of their routine physical examinations. Twelve blood samples were also collected from four additional institutions in the state of Florida to increase sample size as well as to include birds that previously lived in the Disney's Animal Kingdom® flock. Approximately 0.25 g of liver tissue preserved in 95% ETOH were also included from seven samples from deceased birds. Blood samples were preserved by placing three to four drops of blood (approximately 0.25 ml) into 1.5 ml tubes containing 1.0 ml of Longmire buffer [100 mM Tris–HCL pH 8.0, 100 mM EDTA, 10 mM NaCl, 0.5% SDS; Longmire et al., 1988]. Two samples were obtained from each bird. In total, we collected samples from 30 individual birds. Genomic DNA was extracted using a standard phenol–chloroform extraction protocol [Sambrook and Russell, 2001] followed by an ethanol precipitation. Samples were then re-suspended in double-distilled water and the DNA concentration was determined using a Nano-Drop® ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE). Samples were then visualized on a 1% agarose gel to confirm that genomic DNA had not fragmented during extraction. All samples were diluted with double-distilled water to a final concentration of 20 ng/μl.

Microsatellite Development and Genotyping

To isolate microsatellite loci, we generated random DNA fragments (∼200–2,500 bp) using degenerate oligonucleotide-primed polymerase chain reaction (DOP-PCR), using the K6-MW primer and protocol [Macas et al., 1996]. Micro-satellite enrichment of the PCR-amplified genomic fragments employed a 3'-biotinylated (GATA)$_8$ repeat motif

bound to streptavidin-coated particles (Promega Corporation, Madison, WI) enriched via magnetic separation. Enriched DOP-PCR products were made double stranded by a subsequent DOP-PCR and cloned using TOPO TA cloning kit (Invitrogen, Carlsbad, CA). Clones positive for (CA)$_n$ or (GATA)$_n$ microsatellites were identified using the screening procedure of Cabe and Marshall [2001]. In brief, we conducted two PCRs per colony: one PCR included T3 and T7 primers while the second included the (GATA)$_8$ primer in addition to the T3 and T7 primers. We visualized the product of the PCR reactions on a 2.0% agarose gel and positive clones (those containing microsatellites) were identified by a distinctive smear in the (GATA)$_8$ reaction. We then sequenced positive clones and developed PCR primers from flanking regions of DNA surrounding the microsatellite repeats. In total, we developed microsatellite primer sets for 10 loci and genotyped 30 individuals at these loci.

PCRs for all loci were performed in a 20 μl reaction containing 1 μl of template DNA diluted to 20 ng/μl, 2 μl of 10× PCR buffer, 1.25 μl of 25 mM of MgCl$_2$, 200 μM of each dNTP, 0.5 μl of 10 μM M13 labeled [Schuelke, 2000] forward primer and 1 μl of 10 μM reverse primer, 1 μl of 10 μM fluorescently labeled M13 primer, 0.2 μl dimethyl sulfoxide, and 1 unit of Taq polymerase. The fluorescently labeled dyes were ABI DS-30 (6-FAM, HEX, NED; Applied Biosystems, Life Technologies Corporation, Carlsbad, CA). We performed PCRs using a BioRad MyCycler thermal-cycler (Bio-Rad Laboratories, Hercules, CA). The initial denaturing step was 94°C for 4 min, followed by 35 cycles of 30 sec at 94°C, 30 sec at the annealing temperature, and 45 sec at 72°C, then a final extension cycle at 72°C for 7 min, and a hold at 4°C. Annealing temperatures for all loci are listed in Table 1. We visualized PCR products on a 2% agarose gel prior to genotyping.

PCR products were sized using an ABI PRISM® 3730 DNA Analyzer (Applied Biosystems) in 5 μl multiplexed reactions at the University of Arizona Genetics Core (Tucson, AZ). Alleles were sized with respect to size standard ROX (DS-30, Applied Biosystems) using the Peak-Scanner Software (v1.0, Applied Biosystems).

Basic Genetic Parameters

Five of the 10 loci were found to be polymorphic and were used for further analyses (Table 1). We used the program Micro-checker [van Oosterhaut et al., 2004] to check for the presence of null alleles and scoring errors such as peak stuttering or allelic dropout. The software program FSTAT2.9.3 [Goudet, 1995] was used to test for deviation from Hardy–Weinberg equilibrium and for evidence of linkage disequilibrium between pairs of loci, as well as to calculate number of alleles and allelic richness of each locus.

We also estimated probability of identity (PID) in the five polymorphic loci using the program Gimlet [v1.33,

TABLE 1. Characterization of five microsatellite loci used in marabou stork (*Leptoptilus crumeniferus*) identity and parentage analysis

Locus	Primer sequence (5'–3')	T_a (°C)	Repeat motif	Size (bp)	Number of alleles	Allelic richness	H_{exp}	H_{obs}	PI$_{unbiased}$	PI$_{SIB}$
Lcru105	F: *TTATAAAACGTGGCGGGAAG	54	(CA)$_{14}$	164–192	11	10.583	0.80	0.67	0.04870	0.3673
	R: CCATTTAGCACAAACAAATTCC									
Lcru108	F: *CTTGGTGGGCACCTAGCAG	54	$^+$(GATA)$_{13}$	311–331	7	6.862	0.75	0.77	0.004070	0.1470
	R: CATGGCACCATGAAAAAGAG									
Lcru101	F: *CCCAAAAGGCAAATGCATAC	52	$^+$(CA)$_{41}$	184–207	9	9.000	0.72	0.50	0.0003558	0.02482
	R: GTTGAGGGGGAAGAAAATGC									
Lcru109	F: *TGCAGGAGCACAAGTAGATG	54	$^+$(GATA)$_{10}$	214–234	6	5.900	0.74	0.90	0.00003207	0.05972
	R: AGGGGTAAAAAGCGAAGCTG									
Lcru107	F: *ACTGAGAACGGGATTTGTCC	54	$^+$(CATA)$_4$(GATA)$_{15}$							
	R: TTCTGGAAAAACCGAAGACC		(CATA)$_4$	246–266	5	4.999	0.70	0.60	0.000003977	0.01086
Mean							0.74	0.69		

Loci were amplified using PCR conditions described in the text. Loci are sorted by rank of unbiased probability of identity (PI), which was calculated using Gimlet [v1.33; Valière, 2002]. Number of alleles and allelic richness (based on minimum sample size of 27 diploid individuals) were calculated with FSTAT [v2.9.3; Goudet, 1995]. Expected and observed heterozygosity were calculated in Gimlet. The asterisk (*) in the primer sequence denotes a 5' tail (TGTAAAACGACGGCCAGT) attached to the primer sequence; $+$ in the repeat motif represents an imperfect repeat in the sequence; T_a, annealing temperature; H_{exp}, expected heterozygosity; H_{obs}, observed heterozygosity; PI$_{unbiased}$, unbiased probability of identity; PI$_{SIB}$, probability of identity in a population of all siblings. Note that values for PI$_{unbiased}$ and PI$_{SIB}$ are cumulative, such that the value listed for Lcru107 is the cumulative effect of all loci.

Valière, 2002]. PID estimates the likelihood of sampling the same genotype by chance given the diversity of the loci used in the analysis, and in general, should be <0.001 for a randomly sampled population and <0.05 in a population comprised of siblings [Schwartz and Monfort, 2008]. Gimlet was also used to calculate the observed and expected heterozygosities for the microsatellite loci used in this study. Basic genetic parameters were calculated with all individual birds ($N = 30$).

Parentage Analysis

Of the 30 samples collected, 18 were used specifically for parentage analyses, as these were birds that had lived in the Disney's Animal Kingdom® flock and were either offspring hatched in this flock or were potential parents. The remaining 12 individuals were excluded from parentage analyses as they were never a part of the study flock, or lived in the flock after the study period. Between August 2002 to March 2007, 12 males and 15 females were present in this population of marabou storks. An individual was determined to be a potential sire or dam if they were sexually mature (>5 years for males, and four years for females), and were present in the flock during a portion of the timeline of this study and if chicks hatched during that time. Institutional animal records were examined to determine each bird's timeline of presence in the flock. Comparing the timeline of each individual's presence in the flock, as well as when they became sexually mature, to the dates that the egg of each offspring was laid allowed us to reduce the number of candidate sires and candidate dams in the flock to eight for both sexes. We genetically sampled nine offspring (individuals hatched at Disney's Animal Kingdom®), six candidate sires and four candidate dams. Therefore, the probabilities that the sire and dam were included in the sampled dataset were 75% and 50%, respectively. We used these a priori probabilities in both

CERVUS [v3.0.3; Kalinowski et al., 2007] and COLONY [v2.0.3.0; Wang and Santure, 2009] for parentage screening. By examining dates of hatch and each individual's timeline of presence in the flock, we were able to exclude some candidate males from paternity analysis for three offspring, and exclude some candidate females from maternity analysis for all nine offspring.

Using the program CERVUS [v3.0.3; Kalinowski et al., 2007], which uses a pair-wise likelihood comparison based approach to assigning parentage, we first ran an allele frequency analysis on the five loci discussed above, which we used to calculate multilocus parental exclusion probabilities [Selvin, 1980]. We then ran a parentage analysis simulation to determine the parent pair with known sexes. We included the probabilities that the candidate parents were sampled. This simulation calculates the critical delta of each assignment, which is a derivative of the likelihood score used in the parentage analysis and provides a threshold for assigning parentage with varying levels of confidence [Kalinowski et al., 2007]. Finally, we conducted a parentage analysis of the parent pair with known sexes including a separate file for each list of candidate sires and dams.

We also used COLONY [v2.0.3.0; Wang and Santure, 2009], which implements a full-pedigree maximum likelihood method to assign parentage and sibship among individuals with multilocus genotypes, to run a parentage analysis and examine family relationships. For the COLONY analysis, we chose a polygamous mating system which allows for maternal-only and paternal-only sibships (half sibs) and no inbreeding. We ran a full likelihood analysis with a long run length and no sibship prior. We input the five microsatellite marker types, and included an error rate of 0.02, an error rate for sibship reconstruction suggested by Wang [2004]. The genotypes of the nine offspring, six candidate sires, and four candidate dams were input as

separate files, as well as the sire and dam exclusion for each offspring.

Finally, we ran a kinship identification test to assign the parent pair in Gimlet [v1.33; Valière, 2002], which uses a pair-wise comparison of the parent–offspring genotypes using the alleles at each locus and a threshold for number of allelic incompatibilities. We accepted kinship with no incompatibilities per locus and input demographic information including sex, dates of birth and death, and age at first reproduction of 4 years for females and 5 years for males. We also limited kinship to the potential sires and dams by timeline of presence in the flock as described above.

To compare parentage recorded from the studbook to the genetic analyses, we first recorded each sire and dam listed in the North American regional marabou stork studbook [Schutz, 2011] for each offspring hatched in population. These putative parents were recorded in the studbook based on animal keepers' observations of nesting, incubation, and chick-rearing behaviors. We then used the results of the CERVUS, COLONY, and Gimlet parentage analysis to assign sires and dams based on strict confidence (95%) and relaxed confidence (80%). Last, we built a pedigree of the flock based on the parentage results, as well as the best configuration of families provided by COLONY.

RESULTS

Using FSTAT [v2.9.3; Goudet, 1995] the five polymorphic loci were found to be in Hardy–Weinberg equilibrium using the Bonferroni corrected significance value of $\alpha = 0.01$, and there was no evidence of linkage disequilibrium between the loci based on the Bonferroni corrected significance value of $\alpha = 0.005$. Locus Lcru101

shows signs of null alleles when analyzing with Micro-checker; however, there was no evidence of scoring error due to stuttering or of allelic dropout. Population mean expected heterozygosity was 0.74 and mean observed heterozygosity was 0.69 (Table 1). The cumulative unbiased probability of identity (over all loci) was 0.000003977, suggesting that approximately one in 250,000 genotypes will match by chance alone. The cumulative probability of identity in a population comprised of full siblings was 0.01086, suggesting that approximately one in 100 genotypes would match by chance if all individuals were full siblings. These five loci therefore show sufficient discrimination ability for this study and were used in parentage screening of the captive-hatched individuals in the marabou stork population.

The parentage of each offspring hatched in the flock obtained from the studbook and the three methods of assignment based on molecular markers (CERVUS, COLONY, and Gimlet) are shown in Table 2. Of the three programs, analyses from COLONY resulted in more assignments (sires: 6 with strict, and 1 with relaxed confidence; dams: 3 with strict confidence), which were made with higher confidence than CERVUS (sires: 3 with strict, and 3 with relaxed confidence; dams: 1 with relaxed, and 2 with low confidence) and Gimlet (Table 2). The results of assignment tests showed that five offspring had sires match the studbook parentage and that two offspring had dams match the studbook with strict or relaxed confidence. Two additional offspring were assigned sires that matched the studbook with low confidence (<80%); studbook (SB) 428 was assigned sire SB 375, with low confidence (77%, COLONY) but there were no mismatching alleles between sire and offspring genotypes. SB 410 was assigned sire SB 368 with almost no confidence (4%, COLONY). There was

TABLE 2. Sires and dams of offspring hatched in the Disney's Animal Kingdom® marabou stork (*Leptoptilus crumeniferus*) flock determined from the studbook data [Schutz, 2011], and three methods of parentage assignment

Offspring sampled	Hatch year	Sex	Sire				Dam			
			Studbook	CERVUS	COLONY	Gimlet	Studbook	CERVUS	COLONY	Gimlet
406	2002	F	296[a]		296[d]	296/375	369[b]			
415	2003	F	296[a]	296[d]	296[c]	296	369[b]			
427	2004	M	296[a]	296[d]	296[c]	296/368[e]	369[b]			
428	2004	M	375[a]		375	383	371[b]			406
431	2005	F	296[a]	296[c]	296[c]	296/368[e]	369[b]			
432	2005	M	296[a]	368[c]	368[e]	368[e]	369[b]			
433	2007	M	368[a]	352[c]	368[c]	368	364[a]	364	364[c]	364
410	2002	U	368[a]		368		364[a]	364[d]	364[c]	364
n242	2002	U	UNK	375[d]	368[c]	368[e]/375	UNK	381	364[c]	364

Methods of parentage assignment include CERVUS v3.0.3 [Kalinowski et al., 2007], COLONY v2.0.3.0 [Wang and Santure, 2009], and Gimlet v1.22 [Valière, 2002]. Light gray shaded boxes are assigned parents that match the putative sire and dam with >80% confidence. No allelic mismatches were present in assignments made with >95% or >80% confidence from CERVUS and COLONY results.
[a]Putative parent included in genetic sampling.
[b]Putative parent NOT included in genetic sampling.
[c]Assignment confidence >95%.
[d]Assignment confidence >80%.
[e]Missing allele in Gimlet analysis.

one mismatching allele between these two individuals, and a missing genotype in SB 368.

Reconstructing the pedigree based on COLONY best family configuration results, paternity, maternity, full sibship, and half sibship assignments resulted in three discrepancies from the recorded studbook data (Fig. 1). First, we found one case of incorrect assignment of a sire that was assigned differently from the studbook (Star 1, Fig. 1). Studbook (SB) 296 was listed as the sire of offspring SB 432 with dam SB 369. The daily observation notes of reproductive behavior list one clutch of four eggs for this pair (SB 296 and SB 369) in 2005. In all three analyses (CERVUS, COLONY, and Gimlet), SB 368 was assigned as the sire to SB 432, with >95% confidence (CERVUS and COLONY; Table 2). Offspring SB 432 was laid as an egg in early May 2005, and at this time male SB 368 had just had a clutch fail (April 2005) and was observed in breeding color and copulating with his mate (SB 364). Thus it appears that offspring SB 432 is a result of an extra-pair copulation between male SB 368, and female SB 369, who was not reproductively monogamous in 2005 (Fig. 1). Second, we were also able to determine parentage of an individual that had previously unknown parentage (no parents listed in the studbook; Table 2). Following assignment tests, offspring SB n242 assigned to sire SB 368 (97%, COLONY; Table 2) and dam SB 364 (95%, COLONY; Table 2; Star 2, Fig. 1). Third, offspring SB 410, which according to the studbook would have been a clutch-mate to SB n242 with the recorded sire SB 368 and dam SB 364 did not assign to any sire in the other assignment tests (Table 1; Star 3, Fig. 1). SB 410 did assign to dam SB 364 with strict confidence in both CERVUS and COLONY (95%). However, the presence of a null allele in the putative sire (SB 368), could explain this discrepancy. SB 368 was scored as a homozygote at locus Lcru105, but if it was scored such that it was a heterozygote exhibiting a null allele, it would yield a result that is consistent with a single family

group; therefore, the putative sire (SB 368) could have given the null allele to offspring SB 410.

DISCUSSION

This study compared studbook data to genetic analyses of parents and offspring from a captive population of marabou storks. Reconstructing the pedigree from genetic analyses has shown that one pair in the flock housed at Disney's Animal Kingdom® was not reproductively monogamous, and, like many other avian species, this species may make use of a mating system other than strict monogamy. Pairs change mates between years, and our evidence suggests that even within one clutch there is evidence for extra-pair fertilization and multiple-paternity. Despite the small sample size in this study, the identification of extra-pair fertilization in one captive flock of marabou storks suggests that the rates of this behavior may be quite high in this species in the appropriate environment. Evaluation of the studbook records suggest that data in the studbook may be missing, with no known parents recorded for some offspring, or may be incorrect, with a sire or dam listed that is not the genetic parent. While we did find one individual that did not assign with any confidence to a sire (SB 410, Star 3, Fig. 1), it is likely due to presence of a null allele, and provides a cautionary tale of adhering strictly to genetic data, particularly in that the genetic data can have scoring or sequencing errors. More microsatellite loci may have helped in resolving the parentage of this individual by reducing effects of potential error. Overall, these genetic analyses led us to two major findings in our pedigree that differed from studbook records. Both inaccuracies in the studbook can have implications on future genetic and demographic analyses or management of the population.

A common challenge in genetic parentage studies is that not all potential parents are sampled. In our study only
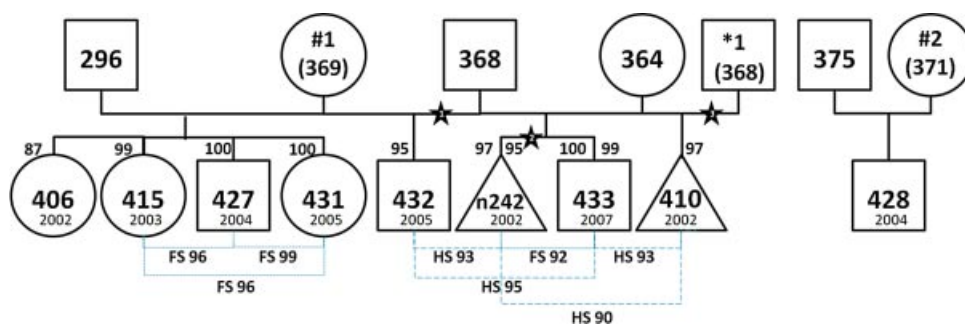

Figure 1. Pedigree of Disney's Animal Kingdom® marabou stork (*Leptoptilus crumeniferus*) flock based on COLONY [v2.0.3.0; Wang and Santure, 2009] best configuration results, paternity, maternity, full sibship and half sibship assignments. Individuals sampled for genetic analyses are labeled within squares (males), circles (females), and triangles (unknown sex). Year of hatch is listed below individual number. Probabilities of assignment for sires are to the left of the offspring node, and for dams to the right of the offspring node. Probability of assignment for full-siblings (FS) and half-siblings (HS) are below pedigree. Stars (★) represent relationships in the genetic pedigree that do not match the studbook records. These differences are numbered, and are discussed in the text in numerical order. * represents either unsampled putative sire or relationship unsubstantiated by genetic analyses (see text), # represents unsampled putative dams or relationship unsubstantiated by genetic analyses. In the case where a putative sire or dam is listed in the studbook, these are included under the * or #.

75% of potential sires and 50% of potential dams were included. In this study, we were not able to compare studbook data to genetic data for most dam assignments, and two individuals assigned to sires with little to no confidence (Table 2). SB 428 did not assign to a sire with confidence, and it is possible in this case that the studbook could be incorrect and we did not sample the true genetic parent. Alternatively, this may be a case that our data did not have the ability to discern the parent. Using genetic data for parentage assignment, there is always the potential to assign a parent incorrectly, particularly based on a limited sampling of the potential parents, as in this study. However, there is also the ability to exclude a parent if molecular analyses do not support that the parent listed in the studbook is the genetic parent. We believe that we avoided potential errors of incorrect parent assignments by carefully reviewing animal records and timelines to determine which exact individuals were potential parents, and by adhering to assignments with high confidence and no allelic mismatches. Future studies can avoid these challenges by including more molecular markers (i.e., more microsatellites) to strengthen the parentage assignment analysis methods. Opportunistic sampling and storage of genetic material of more individuals and species in general can also help future studies reduce issues of missing samples.

One of the most important contributions of our genetic analyses to pedigree building was the identification and verification of the most likely parents for offspring hatched in this colony. This study revealed that one female reproduced with a male who was not her behavioral mate, resulting in one clutch in 2005 with multiple-paternity and observations demonstrating that the chick resulting from this copulation was reared by an individual other than its genetic parent. In this colony of marabou storks, sex ratio was male biased leading to the presence of unpaired, potentially aggressive males, and nest sites were all constructed less than a meter from each other simulating a situation of high density nesting. Using the pedigree and observations of parental behavior that led to determination of parents for the studbook allows us to examine the factors that support an environment that promotes EPCs and the reproductive behavior observed in this colony.

Both paired as well as unpaired males in colonial species have been observed attempting to aggressively copulate with females besides their social mates [Gowaty and Buschhaus, 1998]. Unpaired males direct aggressive "copulations" at unmated or unguarded females or females moving unescorted in the environment [Gowaty and Buschhaus, 1998]. Males may themselves create the dangerous environment for the advantage they will accrue from completing copulation, especially those who might otherwise have little or no opportunity to mate due to subordinate position or inability to gain a mate [Gowaty and Buschhaus, 1998]. We have frequently observed increased aggression between males and females at the nest site, even between birds assumed to be monogamous and paired for multiple breeding seasons (not just in newly established pairings). This female-targeted aggression often leads to visible injury in the females (small lacerations to head and gular sac, and limping) and has at times escalated to death of the female. At this point, we do not understand males' motives for this aggression, but it could be due to two situations. First, males may require females to "trade" copulations for protection [Gowaty and Buschhaus, 1998] from other males and the aggression could be a result of the female's refusal. Second, the aggression could be retribution for the female's solicitation of an EPC. In social animals, there is evidence that retaliatory aggression is common, and individuals often punish group members to maintain dominance relationships, discourage cheats, and discipline sexual partners [Clutton-Brock and Parker, 1995]. More information is needed to examine these hypotheses.

High densities of breeding adults and nest sites can increase the chances of encounters between fertile males and females, reducing the energetic costs for individuals searching for extra-pair mates and favoring increased rates of extra-pair copulations [Westneat and Sherman, 1997; Thusius et al., 2001; Miño et al., 2011]. Although extra-pair encounters might increase with increasing density of individuals in the colony, at higher densities social mates are forced into greater proximity, which should make it easier for males to see and repel intruders, to control female movements, or to witness an EPC and perform compensatory within-pair copulation [Westneat and Sherman, 1997]. Alternatively, increasing density may severely impair a male's abilities to successfully guard his social mate, because either there are too many males to chase away or nests are clustered at sites distant from other resources important to males. In addition, habitat complexity allows females more opportunity to escape from male surveillance and increasing density makes it more valuable for them to do so [Westneat and Sherman, 1997]. In this colony of marabou storks, we observe that nests are, in fact, clustered closely together, males are observed interacting aggressively at the nest site, and the exhibit size and configuration of the environment may allow females to remove themselves from being guarded by their mate. Additionally, as a part of husbandry management in this colony, birds are often required to enter a feeding pen to receive daily feedings, leading to a situation in which males may choose to leave their females unguarded. Under these conditions, the skewed sex ratio and unguarded females leads to a favorable environment for EPCs to occur.

This study further supports the fact that molecular data can be useful in verifying recorded parentage and missing data in studbooks of captive populations [Jones et al., 2002; Ivy et al., 2009] which are essential for pedigree analyses of the population's genetic composition and demographic history. Accurate studbook information can provide information on mating system and behavior. Understanding the mating system of a species is essential for successful breeding, captive management, and conservation programs [Miño et al., 2009].

This study contributes to better understanding of the mating system of a zoo-living colony of marabou storks by helping to disentangle the relationships among the chicks hatched in this breeding colony and supplementing or correcting records in this species' studbook. At this point, little information exists regarding the actual mating systems of wild marabou stork beyond behavior of individuals during breeding season [Kahl, 1966]. Future work should investigate the female's role in accepting EPCs and EPFs and how these behaviors may increase the genetic quality of offspring [Neff and Pitcher, 2005]. Other work may examine the complex social and reproductive behaviors between individuals in a colony of wild marabou stork, as well as to seek a greater understanding of the social structure and aggressive interactions in these birds both in zoos and in the wild in order to assist in management of captive populations of marabou storks and other colonial nesting birds [Miño et al., 2009]. Finally, understanding how frequently extra-pair paternity occurs in colonially nesting species will have implications for the genetic management of captive avian populations.

CONCLUSIONS

1. Using genetic analyses to reconstruct the pedigree of the marabou stork flock led to improvement of studbook records by determining parentage of an individual that had previously unknown parentage, and identified one individual that had a sire that differed genetically from studbook records, confirming one case of extra-pair fertilization and extra-pair paternity in this species.
2. Our analyses contributed to the identification and verification of the most likely parents for offspring hatched in one ex situ marabou stork colony and improved some studbook records.
3. Using molecular genetic analyses can lead to a better understanding of the mating system of species with cryptic or difficult to study reproductive behavior. Understanding these behaviors can be essential for developing a successful breeding and captive management program.

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