Conspecific Brood Parasitism in the White-faced ibis *Plegadis chihi* (Aves: Pelecaniformes) Revealed by Microsatellites' Based Kinship-Reconstruction

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ABSTRACT

The white-faced ibis *Plegadis chihi* Vieillot, 1817 (Pelecaniformes: Threskiornithidae) is a socially monogamous colonially breeding bird in which behavioral and ecological observations suggest the occurrence of conspecific brood parasitism (CBP). We inferred aspects of the genetic mating system of *P. chihi* in nature, using a genetic approach in the absence of parental information. We used five heterologous microsatellite loci and a multiple-step methodological approach to infer kinship patterns among 104 pairs of nestlings sampled inside 80 nests in a breeding colony from southern Brazil. The estimated effective population size was 69 white-faced ibises (95% CI: 50–98), enough to ensure long-term population survival. Kinship patterns were identified for 38% of the analyzed pairs: 60% of the diagnosed pairs were identified as full-siblings, 2.5% as half-siblings and 37.5% as unrelated individuals. CBP could explain the presence of unrelated nestlings within broods, in agreement with available non-genetic evidence. The presence of half-siblings within broods could indicate extra-pair paternity. Results suggest that a non-strictly monogamous genetic mating system may be present in the white-faced ibis. This study is the first molecular approach to better characterize the reproductive behavior of *P. chihi* in the wild. Our findings set the stage for further research to investigate the possible causes and consequences of alternative reproductive strategies in this species. *J. Exp. Zool. 9999A:1–8, 2013.* © 2013 Wiley Periodicals, Inc.

DNA-based methodologies have revolutionized our thinking on reproductive behavior and mating systems in birds, revealing that reproductive strategies alternative to genetic monogamy occur in variable proportions in nature. For example, conspecific brood parasitism (CBP)—a behavior characterized by females laying eggs in the nests of other conspecific females—has been shown to occur in over 150 bird species (Yom-Tov, 2001; Lyon and Eadie, 2008; Eadie and Lyon, 2011). Moreover, modern molecular methods have demonstrated that many species of socially monogamous birds show variable levels of extra-pair paternity (EPP, fertilization of the female by a male other than its social partner; Griffith et al., 2002). CBP has recently been studied using molecular techniques, which allowed researchers to more profoundly explore the role of kin selection in the evolution of this behavior (see references in Eadie and Lyon, 2011). Colonial waterbirds represent

an especially interesting group in which to study reproductive behavior (Miño et al., 2011) because nesting in colonies is one of the breeding habits proposed to promote CBP (Brown, '84; Møller and Birkhead, '93; Lyon and Everding, '96; Brown and Lawes, 2007). Although the literature on mating systems in colonially breeding waterbirds is still notably scarce (Miño and Del Lama, 2009), the few genetic studies available on this group have proposed that both EPP and CBP can occur in natural populations (e.g., Lyon and Everding, '96; Miño et al., 2009, 2011).

The white-faced ibis *Plegadis chihi* Vieillot, 1817 (Pelecaniformes: Threskiornithidae) is a colonially breeding medium-sized bird that inhabits wetlands along the American continent (Ryder, '67; Dark-Smiley and Keinath, 2003); in Brazil, it can be found, for example, in the South region, in Rio Grande do Sul state (Petry and Fonseca, 2005). *P. chihi* is socially monogamous (Ryder and Manry, '94; Dark-Smiley and Keinath, 2003), but observations of the adults' reproductive behavior and the occurrence of occasional supernumerary clutches suggest that CBP can also occur (Kaneko, '72; Capen, '77). Besides, the breeding of this species is highly synchronic—courtship, nest building, incubation, and fledglings can all occur at the same time (Belknap, '57)—a characteristic that might promote CBP.

DNA-based parentage studies, which involve comparing the genotypes of the candidate parents to those of the supposed offspring, have been traditionally used to characterize a species' mating system (Jones et al., 2010). However, those studies are not possible in colonially breeding birds such as the white-faced ibis, due to the extreme difficulty of sampling the candidate parents. In such a situation, genotypes from candidate parents are not available. An appealing approach in those cases is to infer the reproductive behavior of adults by examining the genetic relatedness and kinship patterns among the offspring within the broods (Blouin et al., '96; Avise, 2004), as exemplified in recent studies (Oliehoek et al., 2006; Miño et al., 2009, 2011). Studies of kinship and relatedness in natural populations have preferably used microsatellite markers due to ease of alleles' detection, high levels of polymorphism, random distribution across the genome and selective neutrality (Webster and Reichart, 2005).

The aim of this study was to infer aspects of the genetic mating system of *P. chihi* in nature, using a genetic approach in the absence of parental information. We used data on five microsatellites and applied a multi-steps methodological approach (Miño et al., 2011) to determine the relationship categories among white-faced ibis nestlings that belong to the same broods.

MATERIALS AND METHODS

Sampling, DNA Extraction, and Genotyping
We plucked growing feathers from 179 nestlings from 87 nests (7 nests with only 1 nestling, 68 nests with 2 nestlings, and 12 nests with 3 nestlings) in a breeding colony in Tapes city (Rio Grande do Sul state, Brazil, S 30°41′12 , W 51°23′53). The sampling was carried out when nestlings were 2–3 weeks old, to ensure that they remained inside their own nests. DNA was extracted from samples following Sambrook and Russell (2001). Given that species-specific microsatellite loci are not available in the white-faced ibis, we used heterologous markers. In a previous extensive screening of 44 heterologous microsatellites in this species, 6 primers produced consistent results and were polymorphic (Souza et al., 2012). However, preliminary analyses conducted in this study (data not shown) showed that locus NnF5 (Ji et al., 2004), polymorphic according to Souza et al. (2012), was in linkage disequilibrium with another locus of the set; thus, it was not chosen for our relatedness analyses. All samples were genotyped at five loci (Aaju3, Sawyer and Benjamin, 2006; Eru2, Eru4, Eru5, and Eru6, Santos et al., 2006). Polymerase chain reactions (PCR) were carried out following Souza et al. (2012) in an Eppendorf Mastercycler Gradient® thermal cycler (Eppendorf AG, Hamburg, Germany). Genotyping was carried out in a MegaBACE™ 1000 (GE Healthcare, Piscataway, NY, USA) automatic sequencer and alleles were sized using Genetic Profiler Software Suite v2.2 (GE Healthcare) with ET 550-R as size standard.

Population Genetic Parameters and Evaluation of Performance of Relatedness Index
The occurrence of null alleles, allelic dropout, and stuttering was investigated using the program Micro-Checker v2.2.3 (van Oosterhout et al., 2004). Number of alleles per locus, expected (He) and observed (Ho) heterozygosities, and tests for detecting deviations from Hardy–Weinberg equilibrium (HWE) were computed in GenAlEx v6.4 (Peakall and Smouse, 2006). The program Genepop v1.2 (Raymond and Rousset, '95) was used to test for linkage disequilibrium (LD) between pairs of loci and to compute the inbreeding coefficient (F_{IS}; Weir and Cockerham, '84). The effective size (N_e) of the population was estimated through sibship reconstruction using the program Colony (Jones and Wang, 2010). Estimation of all the above population genetic parameters was based on 87 nestlings, taking one nestling from each nest at random, to support subsequent relatedness analyses.

The Queller and Goodnight's ('89) index (denoted hereafter as Q&Gr) performed better in the context of our analyses and with our set of markers, than any of the other six relatedness estimators evaluated in the program KinInfor (Wang, 2006; data not shown). To further assess the performance of the Q&Gr index, we followed the approach of Russello and Amato (2004), simulated 1,000 pairs of unrelated individuals (UR), full-siblings (FS) and half-siblings (HS) using Kingroup program v2_090501 (Konovalov et al., 2004). The simulated r values were compared to theoretically expected r values for UR ($r = 0$), HS ($r = 0.25$), and FS ($r = 0.5$) using two-tailed t-tests (as in Russello and Amato, 2004) in BioStat v5.0 program (Ayres et al., 2007), adjusting for significance with the Bonferroni correction (Rice, '89). The sampling variance of the Q&Gr index was calculated as the variance of the mean relatedness estimate for each simulated data set (Russello and

Amato, 2004). Expected misclassification rates were computed as the fraction misclassified out of 1,000 simulated pairs of each relationship category, as in Russello and Amato (2004), using the "cut-off" values method of Blouin et al. ('96) (midpoints between the means of the distributions of pairwise relatedness estimates of each simulated relationship category). The program iRel (Gonçalves da Silva and Russelo, 2011) was used to compute both "cut-off" values and misclassification rates.

The performance of our set of microsatellite loci for relatedness analyses was evaluated with a rarefaction procedure in the web-based software RE-RAT (Schwacke et al., 2005). A thousand simulations were ran, randomly drawn a locus without replacement and calculating a pairwise relatedness (r) matrix of Q&Gr. A second locus was selected and another matrix was computed, the resulting r matrix was subtracted from the first r matrix and the average of the differences was calculated. The drawing of loci and calculation of differences was repeated until all five loci have been added.

Genetic Relatedness and Inference of Kinship Patterns Among Nestlings

Kingroup program was used to compute the overall level of relatedness of the population (Q&Gr) based on the 87 supposedly unrelated nestlings, and also, separately, the average r values between nestlings in each nest. To assess kinship patterns for each one of the 104 nestlings-dyads, we applied the multi-step methodological approach described by Miño et al. (2011). Briefly, the approach involves the following: (1) calculating pairwise r values using ML-Relate program (Kalinowski et al., 2006), which adjusts for null alleles in microsatellites; (2) determining the most likely relationship category compatible with the observed r value for each dyad by applying the "cut-off" values method (Blouin et al., '96)–based on the previously computed values in iRel program; (3) choosing the relationship-hypotheses that best suites the data through hypothesis testing using ML-Relate program; and (4) reconstructing kin groups using likelihood approaches with Pedigree v2.2 program (Herbinger et al., 2006) and Colony program (Jones and Wang, 2010) (please refer to Miño et al., 2011 for further additional details on the procedure). A final classification of nestling-pair into a relationship category was achieved if, and only if, there was congruence among results of all the methods used in this approach.

RESULTS

All of the analyzed loci were in HWE, there was no evidence of linkage disequilibrium for any pair of loci, neither of inbreeding (Table S1 in electronic Supplementary Material, ESM). Null alleles were inferred to be present at locus Eru6. The N_e for the Tapes colony was of 69 white-faced ibises (95% CI: 50–98).

The rarefaction analysis indicated that there was little change to the Q&Gr index after four to five loci were added (0.12 of average difference in successive 1,000 simulations; Fig. 1). Simulations
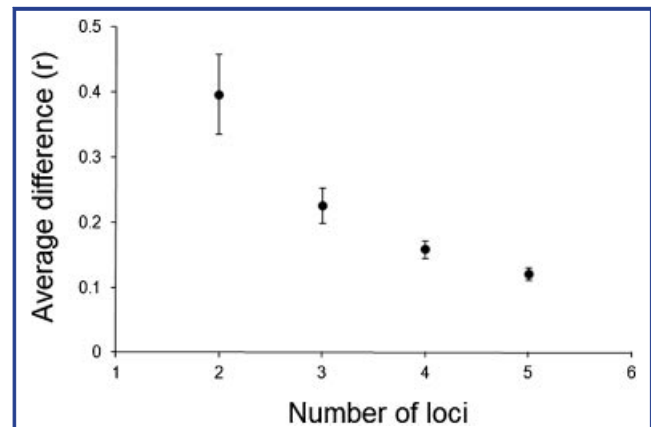

Figure 1. Rarefaction analysis. Result of the rarefaction analysis of Brazilian white-faced ibis population from Tapes colony, Rio Grande do Sul, Brazil, showing the relationship between the number of loci used and the mean difference between consecutive average relatedness (r) estimates (Queller and Goodnight, '89) over 1,000 simulations. Standard deviations are shown as vertical lines. Calculations were performed using the web-based software RE-RAT (Schwacke et al., 2005).

showed that, with our allele frequencies, the estimator of Q&Gr did not depart from the theoretically expected r values and had rather low sampling variances (Supplementary Table S2 in ESM). Misclassification and "cut-off" values for distinguishing between alternative relationships categories ranged from 20% to 36% (Supplementary Table S3 in ESM).

The average relatedness value observed among individuals from different nests was -0.008 ± 0.002 which did not deviate from the expected for unrelated individuals. The mean relatedness observed inside broods was 0.334. Pairwise relatedness values for co-nesting nestlings ranged from -0.685 to 1 (Fig. 2). Kinship patterns were determined for 40 nestling-pairs (38% of all the analyzed pairs) from 28 broods (35% of the sampled nests; Table 1). Most of the diagnosed nestling-pairs (60%) were classified as full-siblings (24 pairs), whereas 37.5% (15 pairs) were considered unrelated nestlings and one pair (2.5%) was diagnosed as half-siblings (Table 1). Sixty-four pairs of nestlings remained undiagnosed for kinship (Supplementary Table S4 in ESM). Some of the nests with three nestlings had all the pairs diagnosed for relationship: for example, nests #55 and #78 had only FS (Table 1), whereas both UR and FS were identified at nests #64 and #77 (Table 1). Nests with only part of their pairs classified were excluded from the computation of the overall proportion of CBP in the population.

DISCUSSION

We performed microsatellite-based kinship reconstruction analyses to clarify the genetic mating system of white-faced ibises
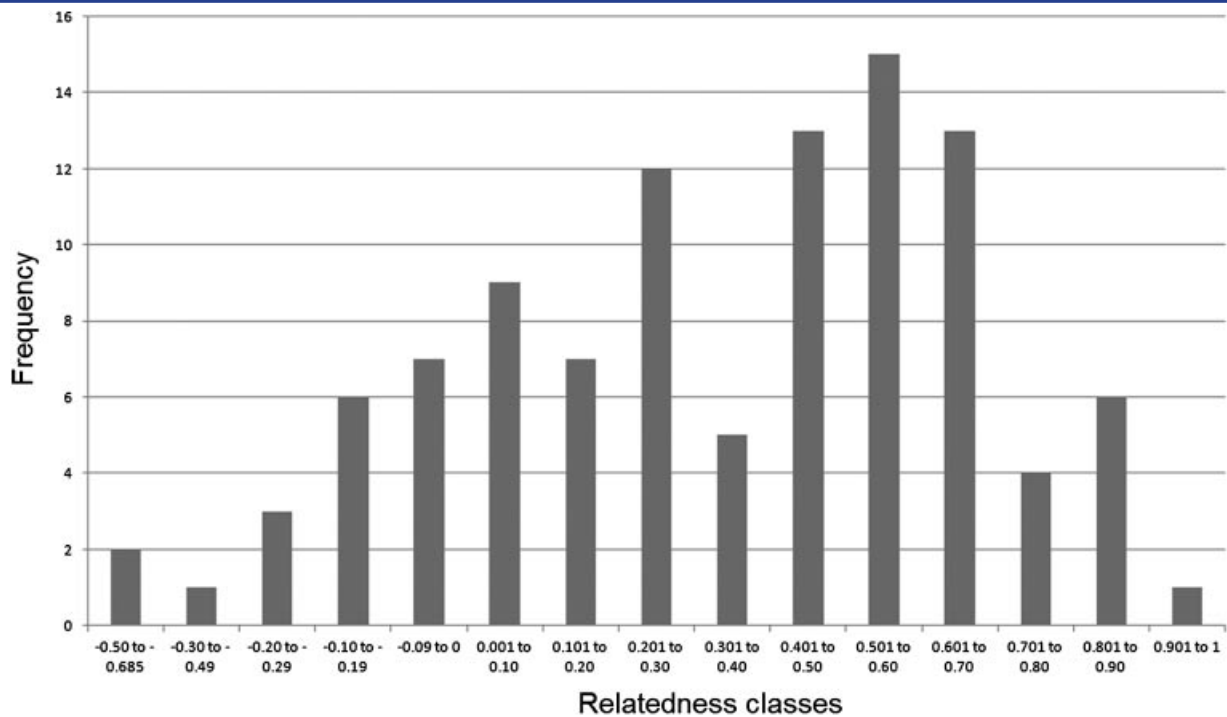
Figure 2. Relatedness values observed among nestmates. Histogram showing the distribution of Queller and Goodnight ('89) pairwise relatedness values observed between 104 white-faced ibis nestling-pairs sampled within broods. Please see Table 1 and Table S4 of the electronic Supplementary Material for all the pairwise relatedness values for each one of the analyzed pairs.

breeding in Rio Grande do Sul state, Brazil. Our results suggest that CBP and, to a low extent, EPP can be present in this species. We highlight the necessity of further research to disentangle the possible causes and consequences of these alternative reproductive strategies. The molecular markers and methodological approach applied in this study can be useful to investigate genetic mating system in the endangered and understudied puna ibis (*P. ridgwayi*).

Our genetic estimate of the effective population size constitutes valuable information on the approximate number of reproductive white-faced ibises in Tapes city colony, given that ecological data is not available to date. Considering that the ratio of N_e to the census population size (*Nc*) in waders is approximately 0.10 (Buehler and Baker, 2005), we estimated an *Nc* in the range of 500–980 birds in this breeding colony. In theory, >500 individuals would be enough for ensuring long-term population persistence (Whitlock, 2000). A good survival capacity could be important to counterbalance the relatively high rate of nest-loss reported for this species in Rio Grande do Sul (Petry and Fonseca, 2005). Reproductive failure can be promoted by widespread habitat loss due to conversion for farming (Azambuja et al., '96), poor habitat conditions—particularly water level and quality—and exposure to pesticides in agricultural fields, that cause eggshell thinning (King

et al., 2003; Ivey et al., 2004). N_e can be influenced by many factors, including genetic mating system, and is an important parameter to be considered in conservation measures.

Although based on a limited number of microsatellite markers, the Q&G*r* index performed well in the context of our analyses (Supplementary Table S2). The moderately high misclassification rates observed in simulation analyses (Supplementary Table S3) suggest that if we had only relied on the pairwise *r* values, we would have erroneously deducted the relationships among nestlings, with important consequences for mating system's inference. This emphasizes the importance of applying a multi-steps approach when inferring relationships, particularly if limited genetic data are available, as already suggested by other studies (van Horn et al., 2008). In order to be more conservative in kinship diagnosis, given the limitations imposed by lack of parental samples and by the small number of microsatellite loci used, we applied the stringent multi-steps analytical approach of Miño et al. (2011). This method uses several different and complementary tools to identify relationships between individuals and was aimed specifically for situations such as the one of the present study (Miño et al., 2011). Even though locus Eru06 showed evidence of null alleles, we included this marker in our analyses because it was one of the most informative loci (seven alleles).

Table 1. Inferred kinship for white-faced ibis nestling-pairs

Nest	Pair	Q&Gr	ML–R	$P(H_P/H_A)$[a]	Pedigree[b]	Colony[c]	Inferred kinship
5	Tp10–Tp11	0.611	FS	0.0084**	FS = 12.285	0.962	FS
8	Tp16–Tp17	0.723	FS	0.0003***	FS = 88.515	0.074	FS
10	Tp20–Tp21	0.761	FS	0.0000***	FS = 282.526	1.000	FS
12	Tp24–Tp25	0.692	FS	0.0050***	FS = 19.143	0.858	FS
19	Tp38–Tp39	0.134	HS	0.0500*	HS = 2.043	–	HS
21	Tp42–Tp43	−0.259	UR	0.0161*	–	–	UR
26	Tp52–Tp53	0.827	FS	0.0000***	FS = 4229.365	1.000	FS
29	Tp60–Tp61	0.609	FS	0.0095**	FS = 12.972	0.757	FS
32	Tp66–Tp67	0.441	FS	0.0433*	FS = 4.025	0.974	FS
35	Tp73–Tp74	0.654	FS	0.0262*	FS = 6.078	1.000	FS
39	Tp82–Tp83	0.696	FS	0.0010***	FS = 64.479	1.000	FS
43	Tp90–Tp91	−0.023	UR	0.0146*	–	–	UR
45	Tp97–Tp98	0.365	FS	0.0062**	FS = 19.972	–	FS
48	Tp104–Tp105	0.113	UR	0.0310*	–	–	UR
49	Tp106–Tp107	−0.075	UR	0.0164*	–	–	UR
50	Tp108–Tp109	0.669	FS	0.0369*	FS = 4.340	0.987	FS
53	Tp114–Tp115	0.595	FS	0.0210*	FS = 7.422	0.008	FS
53	Tp115–Tp116	−0.039	UR	0.0397*	–	–	UR
55	Tp119–Tp120	0.614	FS	0.0089**	FS = 12.007	0.048	FS
55	Tp119–Tp121	0.443	FS	0.0500*	FS = 2.705	0.048	FS
55	Tp120–Tp121	0.610	FS	0.0008***	FS = 50.487	1.000	FS
58	Tp127–Tp129	−0.304	UR	0.0066**	–	–	UR
58	Tp128–Tp129	−0.504	UR	0.0091**	–	–	UR
64	Tp141–Tp142	1.000	FS	0.0128*	FS = 10.377	0.955	FS
64	Tp141–Tp143	0.084	UR	0.0000***	–	–	UR
64	Tp142–Tp143	−0.283	UR	0.0310*	–	–	UR
66	Tp146–Tp148	0.547	FS	0.0251*	FS = 5.870	0.070	FS
67	Tp149–Tp150	−0.685	UR	0.0171*	–	–	UR
70	Tp155–Tp156	0.647	FS	0.0049***	FS = 22.196	1.000	FS
71	Tp157–Tp158	0.512	FS	0.0021***	FS = 44.538	1.000	FS
75	Tp167–Tp168	0.604	FS	0.0050***	FS = 19.738	1.000	FS
75	Tp168–Tp169	−0.004	UR	0.0429*	–	–	UR
77	Tp173–Tp174	0.846	FS	0.0100**	FS = 8.154	1.000	FS
77	Tp173–Tp175	−0.121	UR	0.0140*	–	–	UR
77	Tp174–Tp175	−0.071	UR	0.0102*	–	–	UR
78	Tp176–Tp177	0.801	FS	0.0024***	FS = 31.675	0.167	FS
78	Tp176–Tp178	0.820	FS	0.0017***	FS = 34.752	0.835	FS
78	Tp177–Tp178	0.567	FS	0.0114*	FS = 12.003	0.002	FS
79	Tp179–Tp180	0.019	UR	0.0370*	–	–	UR
79	Tp180–Tp181	0.004	UR	0.0270*	–	–	UR

Kinship patterns inferred for 40 co-nesting nestling-pairs of the white-faced ibis (*Plegadis chihi*) from Tapes colony, Rio Grande do Sul, Brazil. Q&Gr Queller and Goodnight's relatedness index ('89) estimated in ML-Relate program (Kalinowski et al., 2006); *ML-R*: most likely relationship indicated by ML-Relate; $P(H_P/H_A)$: probability value of the hypothesis testing in ML-Relate. Asterisks indicate significance at 5% (*), 1% (**), and 0.5% (***) levels; *Pedigree*: probability value for the relationship reconstructed in the program Pedigree v2.2 (Herbinger et al., 2006); *Colony*: probability value for full-siblings successfully recovered by the program Colony (Jones and Wang, 2010). UR, unrelated; HS, half-siblings; FS, full-siblings. Results for the non-diagnosed nestling-pairs can be found in Table S4 on the electronic Supplementary Material.

[a]H_P putative hypothesis, most-likely relationship indicated by ML-Relate, H_A: alternative hypothesis (if the most-likely relationship was FS, H_A = UR, if the most-likely relationship was either UR or HS, then H_A = FS). A small *P*-value indicates that H_P agrees better with genetic data than H_A (tests were carried out in ML-Relate program with 10,000 simulations).

[b]Probability values correspond to FS or HS, depending on the pair being inspected. Simulation-derived minimum values for most likely unrelated individuals were: FS = 0.00097, HS = 0.03125; maximum values for most likely related individuals were FS = 4.229,365, for HS = 157,464.

[c]Probability values are available only for successfully reconstructed full-siblings.

Wagner et al. (2006) suggested that loci with null alleles can be included in relatedness analyses if they have a good level of polymorphism.

Forty white-faced ibis nestlings-pairs from the same broods had their kinship patterns inferred in the absence of parental information (Table 1), most of which were full-siblings, in agreement with the hypothesis of genetic monogamy, the expected pattern derived from the observation of social monogamy in this species (Ryder and Manry, '94; Dark-Smiley and Keinath, 2003). However, unrelated individuals were also found inside broods in a relatively high proportion, and half-siblings were also detected, although at a low rate (Table 1). In addition, the average overall relatedness value observed within broods ($r = 0.334$) was in the range expected for half-siblings. The unrelated nestlings found within broods could be explained assuming that they are product of CBP, whereas half-siblings could be result of EPP.

Although our findings may be interpreted with caution, they are not unexpected since CBP has already been reported in natural populations of the white-faced ibis. Field observations of adult females laying eggs in other females' nests and of nests with supernumerary clutches of six to eight eggs (Kotter, '70; Kaneko, '72) were interpreted as evidence of CBP in the wild. Colonial breeders in general have been shown to be more prone to CBP than solitary nesters (Brown and Brown, 2001). The greater availability of suitable host nests and the ease of their discovery within colonies (Geffen and Yom-Tov, 2001; Lyon and Eadie, 2008; Paillisson et al., 2008), as well as the lack of territorial defense (Brown, '84), have been proposed as possible mechanisms leading to greater CBP in colonial nesters. Moreover, defending against parasitism is significantly challenging for colonially breeding species, given substantially higher densities of nests (Brown and Brown, '96). CBP has already been reported in other waterbirds, including some of the same family as the white-faced ibis (listed by Yom-Tov, 2001), and also in seabirds such as the wiskered tern (Paillisson et al., 2008). The few studies based on genetic data available for waterbirds also reported deviation from monogamy within broods in natural populations of the roseate spoonbill, the wood stork, and the great egret (Miño et al., 2009, 2011). In addition, genetic evidence supported the occurrence of CBP in other birds, such as the northern lapwing (Grønstøl et al., 2006), the black-headed gull (Ležalová-Piálková, 2011), and the black-capped chickadee (Otter et al., 2011). CBP is an interesting behavior that should be seen as a complex interplay between population dynamics and evolutionary forces (Valpine and Eadie, 2008), and that might not have a single or a few causes.

Our results also suggest that EPP might be present in the white-faced ibis, as for the low proportion of pairs diagnosed as HS (2.5%). However, given that most of the analyzed nestling-pairs remained without a final kinship classification, we cannot rule out the possibility that EPP could be even more frequent in this species. Although EPP has not been previously reported in the white-faced ibis, detailed behavioral field observations are still lacking. Exhaustive observations on the reproductive behavior can be hampered by the colonial breeding habit and by the absence of secondary sexual dimorphism. Nonetheless, EPP in the white-faced ibis is not totally unexpected, since this behavior has been proposed to occur more frequently in synchronous (Stutchbury and Morton, '95) and colonial breeders (Møller and Birkhead, '93; Lyon and Everding, '96; Westneat and Sherman, '97; Yom-Tov, 2001; Brown and Lawes, 2007). Ecological observations have already shown the occurrence of EPP in other waterbirds of the same family (European shags, Graves et al., '93; scarlet ibises, Elbin and Burger, 2005; grey herons, Ramo, '93; cattle egrets, McKilligan, '90, Krebs et al., 2004; and Chinese egrets, Guo-An et al., 2005).

In summary, we present genetic evidence of the occurrence of unrelated nestlings and, to a less extent, of half-siblings in broods of the white-faced ibis. We suggest that CBP and EPP can be present in the studied colony. Our findings help to better characterize the reproductive behavior of this species in the wild and suggest that a non-strictly monogamous genetic mating system might be present.

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