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Perfect and Imperfect Microsatellite Markers in Determination of Genetic Status of Greater Adjutant Stork (*Leptoptilos dubius* Gmelin)

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Abstract

The Greater Adjutant Stork (*Leptoptilos dubius*), the most endangered stork (IUCN Red List criteria under A2bcd+3bcd+4bcd; C2a) losing its number by population has been confined in a small village named Dadra- Pacharia- Singimari in the district of Kamrup, Assam, India. An attempt was made to study the status of genetic variability in Greater Adjutant Stork revealed that though the Greater Adjutant Stork population is highly threatened, yet the group has been appeared as genetically stable as recorded from that of the observed heterozygosity. Therefore, it is of importance to study the distribution enrichment and polymorphism of microsatellites in the genome of the Greater Adjutant Stork. Five microsatellite markers of cross species-specific markers were deployed in this study. All the five microsatellite markers were recorded to be polymorphic with the number of alleles varying between 2 to 9 across all loci used. The locus Ah341 was observed to have 2 alleles whilst the locus Cc07 was with 9 alleles. The heterozygosity of *L. dubius* was observed to be high for the microsatellite markers used, with mean observed heterozygosity (H_o) of 0.752±0.09 and mean expected heterozygosity (H_e) was with 0.677±0.06. The overall mean expected heterozygosity was found to be marginally higher than the observed heterozygosity. The polymorphic interaction count (PIC) was calculated at 0.569±0.03. Iteration value in the consensus sequence recorded for dinucleotide (TG) was dominating at 20 followed by mononucleotide (A) at 12, while the percent (%) of Imperfect iteration stood at the highest of 14.8%. Thus, the result of this investigation has extended a clear genetic polymorphism in favour of the Greater Adjutant stork, perhaps yet to face any genetic threat.

Keywords: Perfect Imperfect Iterations; Microsatellite; Polymorphism; Genetic Status; Greater Adjutant; *Leptoptilos dubius* (Gmelin)

Abbreviations: SSR: Simple Sequence Repeats; PCR: Polymerase Chain Reactions; HWE: Hardy-Weinberg Equilibrium; HI: Heterozygote Instability; PIC: Polymorphic Information Count

Introduction

The Greater Adjutant Stork *Leptoptilos dubius,* an endangered recognized as declining species in number as

per the IUCN Red List criteria under A2bcd+3bcd+4bcd; C2a [1-3] and the species is in the verse of extinction. The species Greater Adjutant stork was once very widely distributed in India, South and South East Asia, but currently available only in Assam and Bihar in India [4-10] and a very few in South East Asian countries [1,11,12]. Of the total estimated global population of about 1000-1200 birds [13], about 800 birds have been found in Assam, considered to be the global stronghold of this stork. According to Luthin [1] South Asia has been endowed with the richest diversity of storks and of the twenty species of storks, eleven are found in South East Asia among which eight species are reported from India [1,14]. Seven species of storks are found in Assam [15,16] of which the Greater Adjutant is an endangered species. The Dadara-Pacharia-Singimari village area in the Kamrup District of Assam, India holds the highest number of Greater Adjutant and is located just 10 km away from the city of Guwahati, Assam, India [17,18]. According to ICBP/IWRB Storks, ibises and spoonbill group including the Greater Adjutant *Leptoptilos dubius* has been adjudged as the systematically disappeared bird species throughout its early distribution ranges and this might be the possible reasons to attract this species as the most threatened one with the extinction possibilities, needs special attention [1,18,19]. Information related to its taxonomy, morphology and to a certain degree of ecological approaches are available [20-25], yet without with any genetic information. Since this species exhibits no migratory behaviour and tends to use the colonial sites over the years like that of wood stork *Mycteria americana* [26], generally response to the seasonal fluctuation like its attempt for pairing and colony formation in the same locality of their nesting sites [27].

So far, no approach has been made to investigate on the genetic variability in Greater adjutant, which could perhaps be able to determine its position with the help of molecular markers like microsatellites. Microsatellites or simple sequence repeats (SSR) are the nucleotide tandem repeats of short sequence motif of 1-6 base pairs [28], distributed both in coding and non-coding regions of DNA [29,30]. Microsatellites are the regions of DNA that contain di, tri or tetra nucleotide repeats. They are randomly distributed across the genome of most species [31,32]. Because of their high degree of polymorphic nature, they are extensively used in studies related to evolution of genetic status of species [33]. Increase or decrease of repeat base number in microsatellite in the coding region often changes the protein product [29,34], and in the noncoding regions leading to the mutations [35]. They are concomitant and highly polymorphic markers [36] carries high increases the mutation rate like 10^{-2} to 10^{-6} per meiotic cycle [37]. Because of their high polymorphism, they are extensively used in studies related to evolution of genetic status of a species [33]. Due to its higher rate of mutability the microsatellite has been placed on record as the source of genetic diversity of a species [38]. The categories of microsatellites, the imperfect and perfect repeats states that the former one is more stable compared to the later [39,40]. And the application of bioinformatics tool gives the opportunity to identify the perfect and imperfect mutation [41].

It has already been accepted that except for repeat copy number variation, a microsatellite (e.g., ATATATATAT) also suffers from nucleotide substitutions and insertion/deletion mutations, attains the status of imperfect (e.g., ATATATCATAT: AT repeat with an insertion of C and the genomes possess a relatively small but significant number of imperfect microsatellites [42]. Imperfect microsatellites is critical for their maintenance in the genome [43] due to mismatch variation being less prone to slippage mutation, allows the imperfect microsatellites to be more stable compared to perfect microsatellite [40]. Yet, the level of understanding of motif mismatches in imperfect microsatellites is still extremely limited and their correlation with life aspects demands critical evaluation.

Lack of species-specific microsatellite markers, perhaps be one of the reason for no genetic variability investigation, yet certain attempts were made on the genetic variability using microsatellite markers in other birds [44,45] like oriental population of white stork [46], European white stork [47], white faced Ibis [48], Painted stork [49], Wood stork [50] and in Asian woolly necked stork [51]. Thus, the rapid decline of the Greater adjutant has drawn wider attention to this background. Hence, the aim of the present study was to analyse perfect and imperfect microsatellite markers for determination of genetic status of Greater Adjutant Stork (*Leptoptilos dubius* Gmelin). The objectives were to

- Examine the levels of genetic diversity,
- Inbreeding status, and
- Population differentiation in breeding colonies of *Leptoptilos dubius*.

This study reports on the five new microsatellite markers developed for L.dubius that have been compiled in a series of PCR with six markers previously described for white stork *Ciconia ciconia* and white heron *Ardea herodias* [47,52]. Hence the goal of the present study was to examine the levels of genetic diversity, inbreeding and population differentiation in breeding colonies, which could perhaps be able to suggest a benchmark information to formulate a conservation action plan.

Materials and Methods

Sampling

Samples for the present work had been collected from

the dead specimens of Greater adjutant either from adult or from the chickens at certain wetlands and from the lone garbage site of the capital city of Guwahati in the Kamrup District of Assam situated between 25°43/ and 26°51/ N Latitudes and 90°12/ and 90°36/ E Longitudes (Table 1). A total of 23 tissue samples were opportunistically collected following the procedure of Huang and Zhou [53]. In brief 0.2 g of dead tissues (for each sample) was placed in 2mL of centrifuge tube to which 500 µL of the following solution was added comprising of 10 mmol-L^{-1}, Tris-HCl, 100 mmol-L^{-1} EDTA, 150 mmol-L^{-1} NaCl and 0.8% SDS followed by the addition of K 40 µg.mL proteases. A veterinarian from the College of Veterinary Science, Guwahati, India collected the tissue samples, kept in 95% ethanol and stored at -20°C till the extraction of DNA.

Sl. No	Wetlands and other foraging sites	GPS point
1	Digheli Beel, Dadara	26°14′17.87′′N/91°39′17.92′′E
2	Bhoka Beel, Dadara	26°13′26.51′′N/91°38′31.06′′E
3	Pondoba Beel, Dadara	26°13′25.55′′N/91°38′41.05′′E
4	Singimari, Dadara	26°13′11.69′′N/91°38′36.36′′E
5	Deepar Beel, Guwahati	26°06′53.01′′N/09°40′49.62′′E
6	Jeng Beel,North Guwahati	26°16′45.30′′N /91°46′33.40′′E
7	Garbage Dump, Boragaon	26°06′53.01′′N/91°40′49.62′′E

Table 1: GPS coordinates of foraging wetlands and sample collection sites for Greater Adjutant Stork.

Extraction of DNA and Selection of Marker

DNA isolation was carried out using modified Phenol-Chloroform method [54]. The Genetic status of *L. dubius* was attempted to be evaluated by using microsatellite markers and no microsatellite markers have been developed from *L. dubius* so far. Development of microsatellite markers requires substantial input of time as well as expertise.

For this reason, considerable effort was given in general on the use of existing microsatellite markers on species for which they were not originally designed [55]. Therefore, in the present study cross-species amplification of six highly polymorphic microsatellite markers comprising of three markers originally developed from White stork [47] and other 3 markers originally developed from Great blue heron [52] were initially tested to assess the possibility of their use to ascertain the genetic marker for *L. dubius*. The details of the markers used are given in the Table 2.

Sl.No.	Locus	Primer Sequence (5'-3')	TA (^{0}C)	Developed From
1	Cc01	F: TTCTTGCATTTGCTCCAGTG	55	*Ciconia ciconia*
		R: CACAAACATCAGCAAGGACAG		
2	Cc06	F: CTCGCTGTCTCCTCTGCTCT	55	*Ciconia ciconia*
		R: GAACAGCAATATCGCATCTACA		
3	Cc07	F: GCATGAAAATGCATAGAGCAGA	55	*Ciconia ciconia*
		R: CCACCGTTATGATCCTTTGG		
4	Ah211	F: GCTCATCAGGAGTTGAATCTGGC	55	*Ardea herodias*
		R: TCTGTCATTCAGCAATGGACC		
5	Ah341	F: GGTAATGATTCTGATTTACCACTGAGGG	55	*Ardea herodias*
		R: ATGTGTTATCATATCTGGTCTTCACAGC		
6	Ah343	F: CATTGCTTAACTTCTGAAGAAAC	55	*Ardea herodias*
		R: CTTGACCCAGCATTTGTGAATAAAACTG		

Table 2: PCR primers used for amplification of microsatellite loci of Greater adjutant Stork.

Amplification of Microsatellite Markers

All the Polymerase Chain Reactions (PCR) were set for a reaction volume of 25µl composed of 100ng of a DNA template, 0.4 µmol. L^{-1} per primer, 1.5 m.mol. L^{-1} $MgCl_{2,}$ 0.2mmol. L^{-1} DNTP and 0.8 U *Taq Pol* [53]. The PCR was allowed to proceed on a Bio-Rad thermal cycler. The settings were as follows that the products were pre- degenerated at 95°C for 15 min, denatured at 94°C for 30 s, annealed at 50 -55°C for 80 sec, extended at 72°C for 60s and after 40 cycles the products were extended at 72°C for 10 min. The reaction was allowed to terminate at 4°C. The PCR product had been detected by 1% agarose Ethidium bromide (EB) gel electrophoresis and the effective primers were selected. The PCR products were gel purified and sequenced for both forward and reverse direction using an ABI PRISM® 3100 Genetic Analyser and the allele sizes were determined using GENEMAPPER version 4.0 (Applied Biosystems) and 500-LIZ as size standard. The sequences generated were then compared with NCBI GenBank database using programme BLASTN [56] and were deposited in the GenBank.

Verification of Amplified PCR Products and Genetic Analysis

The genetic status of *L. dubius* was evaluated in terms of observed heterozygosity (H_o) and expected heterozygosity (H_e).The estimates of H_o and H_e were obtained using population genetics software package Excel Microsatellite Toolkit 3.1.1 (http://animalgenomics.ucd.ie/sdepark/mstoolkit/). And this tool generates perfect and imperfect repeats [57] as well as the differential distribution of repeats [58]. Deviations from the Hardy-Weinberg Equilibrium were tested using Chi-square test. The nucleotide counts, A-T and G-C content as well as individual nucleotide frequencies for all the sequences generated were further estimated.

In Silico Microsatellite Analysis

The microsatellites were obtained using the bioinformatics software tool IMEx (Imperfect Microsatellite Extractor) followed after Mudunuri and Nagarajaram [59]. IMEx uses simple string-matching algorithm with sliding window approach to screen DNA sequences for microsatellites and reports the motif, copy number, genomic location, nearby genes, mutational events and many other features useful for in-depth studies. The percentage of mutation was calculated as:

Percent (%) = number of point mutation in the observed tract X100 / Total number of bases in the equivalent perfect tract.

Parameters Used For Repeat Types

- **Imperfect:** Imperfection was counted at 10% for Mono, Di, Tri, Tetra, Penta and Hexa repeats. Mismatches allowed in Pattern are for Mono: 1, Di: 1, Tri: 1, Tetra: 1, Penta: 2, Hexa: 2;
- **Minimum No. of Repeat Units:** Mono: 5, Di: 3, Tri: 2, Tetra: 2, Penta: 2, Hexa: 2

Results and Discussion

Evaluation of Genetic Status: All the five microsatellite markers were recorded to be polymorphic with the number of alleles varying between 2 to 9 across all the loci used (Table 3). The locus Ah341 was observed to have 2 alleles, while the locus Cc07 presented 9 alleles. The mean observed Heterozygosity (Ho) 0.752±0.09 and the mean expected Heterozygosity (He) 0.677±0.06 has been depicted in the Table 3. The test of Hardy-Weinberg Equilibrium (HWE) presented 3 out of the 5 microsatellite loci namely Cc01, Cc06, Cc07 and Ah341 deviated from the HWE.

Locus	Repeat Motif	Allele Size	Na	H_e	H_o	PIC	P	%
Cc01	$(TTCT)_{10}$	161-259	6	0.842	0.761	0.534	0.0467*	55
Cc06	$(TG)_{13}$	201-262	5	0.638	0.854	0.653	0.0312*	60
Cc07	$(AAAG)_{10}$	273-290	9	0.613	0.347	0.456	0.0212*	75
Ah211	$(CA)_{13}$	100-114	4	0.845	0.792	0.574	0.1217**	21
Ah341	$(AC)_{12}$	182-207	2	0.813	0.631	0.655	0.0342*	61
Mean			5.2	0.752±0.09	0.677±0.06	0.574±0.03		

Table 3: Characterization of polymorphism in five Microsatellite loci of Greater Adjutant Stork.
Na= Number of alleles; He = Expected heterozygosity; Ho= Observed heterozygosity; PIC = Polymorphism information count; P= probabilities of Hardy-Weinberg Equilibrium Test (HWE); P=<0.05 indicates significant deviation from that of HWE test.
'*' = Significantly different.

Microsatellites amplified with specific primers (12 sequences out of 23 samples) have been depicted in the Table 4, with iteration and probable mutation (P %). The sequence KY 5252(67, 68, 69, 70, 71, 72 and 78) showed for imperfect iteration (P %) in between 3-15%. The total number of base pairs was 2918 (Table 4).

The present analysis on the status of the genetic polymorphism in Greater Adjutant Stork revealed that the population has been qualified to be threatened despite of being genetically stable in terms of heterozygosity. All the microsatellite markers had well been recorded as polymorphic with the number of alleles varying in between 2-9 in all the loci used so far. The loci Ah341 had been observed to have the lowest number of alleles, whilst the locus Cc07 was recorded for 9 alleles (Table 3). The heterozygosity of the *L. dubius* might be high for the microsatellite markers as used in the present study with the mean value recorded against observed heterozygosity (Ho) at 0.752 ±0. 09. The sequences have been found to be identical in nature with that of *Ciconia boyciana* [53]. The expected heterozygosity (He) is the probability that two alleles chosen at random from a population are different [60] and proportionate representation of a sample is the observed Heterozygosity (Ho) [61]. Therefore, these two definitions of mean gene diversity could well be regarded as a measure of genetic variability which might be significantly informative in nature. The use of microsatellite in establishing the polymorphic character and genetic variability has already been well accepted [62] and favours the present observations on *L dubius.*

The Greater adjutant occupies a significant role in the wetland ecosystem and being carnivorous, occupies the top position [63]. The loci Cc01, Cc06, Cc07 and Ah 341 have been able to present a significant mean value of (0.677) in terms of the observed heterozygocity (Table 3). Thus, all the values obtained in terms of the characterization of the polymorphism in L. *dubious* have been able to substantiate that the species still out of danger regarding its genetic status viability. Thus, in a given species, microsatellite markers could well be isolated and developed from scratch using methods such as magnetic beads [64] or by applying cross-species amplification using existing markers from closely related species [65,66]. This small group of Greater Adjutants usually might have a high degree of relatedness which showed even a marginal deviation from that of HWE. Therefore, it is speculated that the number of loci deviating from the HWE might be evident that these individuals are not the single breeding population group, possibly represent for many from different range within their distributional jurisdiction. The mean observed heterozygosity of the present study might be within the similar range with that of another stork group like Painted stork *Mycteria leucocephala* [49]. Recent studies on the genetic diversity of Asian woolly necked stork (*Ciconia episcopus)* in 66 individuals of population structure in captivity (Nakhon Ratchima Zoo, NRZ) using 13 microsatellite loci, allowed to infer that the deleterious genetic issues had been resulted out of captivity [51], though such experimentation and evaluation had not been possible for the Greater adjutant. However, the present study for the first time extended the idea of genetic polymorphism and favours the proposition that the Greater adjutant is yet to face any genetic threat. Further, it has well been argued for the heterozygote instability (HI) hypothesis, that it locally increases the mutation rate and there has been the occurrences of rare allele AC sequence of microsatellite [67], since the tandem repeats [68] suggest the HI potentially increases the mutation rate of heterozygous site itself.

The polymorphic information count (PIC) values encountered to the microsatellite loci studied from 0.455 to 0.655 with a mean value of 0.577 (Table 3) might be suggestive of allele medium number carries a considerable degree of genetic variability could well be supported by the work of Fonteque and colleagues [62]. It was of the opinion that when the PIC of the gene locus is greater than >0.5 has been considered as the high degree of polymorphism, while the PIC in between 0.25 to 0.5 the gene locus is moderate against the low value while it is at <0.25. In the present experiment the PIC stands at its lowest level at 0.455 for Cc07 while the highest had been obtained against Cc06 at 0.653 (Table 4) favoured high degree of genetic variability as mentioned in earlier works [53,69].

Tandem repeats recorded in this study for 12 microsatellite sequences are found to its maximum for the GenBank sequence Ky515273.1 at 14.8% for GAAA with imperfect mutation (Table 4), has been able to obtain the support of certain workers [68]. The repeats of perfect and imperfect might be used to support the present findings in the form of polymorphism (Table 3). It has been categorically mentioned that the bulk of sample repeats are embedded in noncoding DNA sequence and it is assumed that such markers like microsatellites generally evolved naturally [58], however, demands further evaluation. The dominance of dinucleotide followed by others might be explained to the level of only microsatellite evolution [57]. The abundance of dinucleotide might also be evident in L. *dubius* (Table 4) and the existence of repeats having little or no dependence on the population size [67].

GenBank Accession number	Consensuses sequences with iterations and the (%) value of imperfect mutation within the parenthesis, if any. The total number of base pairs were 2918									
KY515267	TCT (2)	TCTG (3.8%)	TCTT (4.6%)	TCA (2)	GAAA (5.9%)	GA (3)				
KY515268	TCT (2)	TCTG (4.6%)	TCA (2)	GTTTT (2)	GACG (3.8%)	GGGA (3.8%)	AG (4)			
KY515269	GCAT (2)	TCT (2)	TCTG (4, 6%)	TCA (2)	GAAA (3.8%)	GACA (5.8%)	AGA (2)	TCG (2)		
KY515270	CAGGG (2)	TG (8.6%)	AT (3)	TA (3)	TC (3)	CAG (2)				
KY515271	CAGGG (2)	TG (9.5%)	AT (3)	TA (3)	TC (3)	CCTT (2)	TG (3)			
KY515272	TG (9.5%)	AT (3)	TA (3)	TC (3)	CCTT (2)	TG (3)	GTG (2)	AGA (2)		
KY515273	TTG (2)	ATC (2)	AT (3)	GTAT (2)	AGAA (13.9%)	GAAA (14.8%)	AAGA (2)	ATG (2)	GAAA (9.5%)	AAGA (2)
KY515274	AG (3)	TAC (2)	CAA (2)	TGC (2)	A (5)	G (5)				
KY515275	GCT (2)	ACAGC (2)	A (5)	TG (3)	GT (3)	TA (3)				
KY515276	A (7)	AT (3)	GAACGC (1)							
KY515278	CA (4)	CTCA (2)	TAC (2)	GT (3)	ATG (2)	AGTG (3.1%)				
KY515279	CA (4)	CTCA (2)	TAC (2)	AG (4)	AGCCTT (2)					

Table 4: Microsatellite repeat numbers for perfect and imperfect iterations are shown with repeat numbers for Mono (7), Di (9.5) Tri (2), Tetra (14.8), Penta (2) and Hexa at (2). Category wise dinucleotide numbers are TG=20, AT=15, AG=11, CA=8, TC=3, GT=3, CA=3, while the mononucleotides are A=12 and G=5 for the microsatellite representations. (% =Imperfection).
'p %' = Imperfection %.

The present analysis showed the dominance of dinucleotide followed by mono, di, tri, tetra, penta and hexa at only 2. And among the dinucleotide TG (20), AT (15), AG (11), CA (8) and GA, TA, TC, GT are at 3, while the mono A is at 12 as well as G is noted at 5. However, it is a matter fact, how microsatellites are defined, since among the repeats there are atleast 12 bp long mononucleotides repeats which outnumber dinucleotide repeats; the reverse situation is not valid until a higher threshold is used [67]. Among dinucleotide (TG)n repeats are the most frequent, followed by (AT)n, (CA)n and (GC)n, the last type of repeat being rare. Note that there are only four possible types of dinucleotide repeat, because CA = AC = GT = TG, GA = AG = CT = TC, AT = TA, and GC=CG [57]. Data, not to speak of stork, but also from other birds are least available till date. However, mouse genome has confirmed an impressive number of microsatellites (57,70]. A comparative analysis on the patterns of 3 avian microsatellites loci, chosen for three different class of microsatellites which are possibly in line with the present like perfect $(GA)_n$ repeat and other complex compound $(GT)_{n,}$ and $(GA)_n$ repeats both shows only moderate level of polymorphism, yet we demand further study over their aspects [70]. The recorded dinucleotide repeats were/are (Table 4) most prevalent followed by tri, mono, tetra, penta and hexa nucleotide motif respectively. The dinucleotide repeats have been known to be associated with copy number variations strand slippage and polymorphic, accounting for genome evolution and adaptation [71]. Further, the dinucleotide contribution towards SSR motifs extends a platform like repeat and another complete compound (GT). However, the microsatellite density has often been attempted to correlate with the genome size, yet the present investigation is data deficient in this direction. A model based on the distribution of microsatellite repeat length, which might be at equilibrium perhaps due to a balance between length and point mutation [72,73], could not be extended to the present work. However, the tandem repeats [68] as presented in the Table 4 might be able to extend an explanation in the form of iterations of repeat

units of a single nucleotide to hexanucleotide leading to the polymorphic chair, demands further detailed investigation.

It is an undeniable fact that there has been no genetic status report on the Greater Adjutant, which has been systematically disappearing. Yet, Zan and colleagues [74] analysed the sequences of 66 storks of Oriental white stork (*Ciconia boyciana)* and 17 storks from a Japanese population. An analysis of molecular variance showed a significant population subdivision between the two populations, where the Chinese population had a relatively higher genetic diversity with a haplotype diversity and nucleotide diversity [53]. However, the closer genetic relationship is obtainable in cross-specific amplification as evident in this study (Table 3). It has also been favoured that the observed low genetic variation in *Mycteria Americana* of Cuba with small colonies suffering from anthropogenic interferences [50] might be considered as valuable information for the conservation management of stork population in a given habitat. It may also be well argued that these sets of microsatellites used in this study proved to be useful for the Greater adjutant stork genetics and conservation implications. Earlier it has been advocated that they could be used for genetic linkage mapping in the common pheasants and its closely related pheasants [45,64].

Conclusion

This research used five microsatellite markers that were cross-species specific. All five microsatellite markers were found to be polymorphic, with the number of alleles ranging from 2 to 9 in each locus. The locus Ah341 was found to have two alleles, while the locus Cc07 had nine. For the microsatellite markers used, the heterozygosity of *L. dubius* was found to be high, with a mean observed heterozygosity (H0) of 0.0.753 +/- 0.09 and a mean predicted heterozygocity (He) 0.677±06. The predicted heterozygosity was found to be slightly higher than the observed heterozygosity. It can be inferred that the microsatellites used in this analysis were beneficial to the genetics and conservation consequences of the Greater adjutant stork.

Authors Contributions

DKS and PDB were involved in planning, designing, and realizing the present work. PD performed the experiments and CB performed the data analysis. DKS and CB wrote the manuscript. All authors read, revised, and approved the final manuscript.

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