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Species Delimitation in the Genus *Eudocimus* (Threskiornithidae: Pelecaniformes): First Genetic Approach

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Abstract.—The Scarlet Ibis (*Eudocimus ruber*) and White Ibis (*Eudocimus albus*) are often classified as separate species, but differing scientific opinions exist regarding the taxonomic status of these birds, as they exhibit similar behavior and hybridize in nature. The present study provides genetic data to help clarify this taxonomic issue. DNA was extracted from 10 individuals of each taxon from the states of Florida, USA (White Ibis) and Amapá, Brazil (Scarlet Ibis). The sequences of three mitochondrial and four nuclear markers were obtained from all individuals. The Scarlet Ibis and White Ibis did not share any haplotypes in mitochondrial genes nor in one nuclear marker. Species delimitation using Bayesian analysis, the Knowles-Carstens method and the genealogical sorting index demonstrated the Scarlet Ibis and White Ibis to be two different lineages and revealed a strong signal of speciation despite the polyphyly found in three of the four nuclear markers. *Received 3 April 2014, accepted 31 July 2014.*

Key words.—*Eudocimus albus*, *Eudocimus ruber*, Scarlet Ibis, speciation, species delimitation, Threskiornithidae, White Ibis.

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The genus *Eudocimus* includes two species: the Scarlet Ibis (*Eudocimus ruber*) is typically found in South America and has scarlet plumage (Sick 1997), while the White Ibis (*E. albus*) has white plumage and occurs in southeastern North America, throughout Central America and in northern South America (Heath *et al.* 2009). Their distribution overlaps in the Los Llanos region of Venezuela and Colombia, where they hybridize. However, the majority of pairings in the parapatric zone are between individuals of the same morphotype (Ramo and Busto 1987).

Ridgway (1884) considered Scarlet and White ibises to be a single species, but subsequent interpretations suggest two separate species. Many researchers consider these birds to be different species (American Ornithologists' Union 1998; Remsen *et al.* 2014). Ramo and Busto (1987) proposed that the White Ibis is a subspecies of Scarlet Ibis based on lack of reproductive isolation and the biological species concept. Hancock *et al.* (1992) reported a difference in bill coloration during breeding between Scarlet and White ibises. While there appears to be no differences in breeding behavior or ecological niches, van Wierengen and Brouwer (1990) noted consistent differences in size between the White Ibis of North America and both White and Scarlet ibises of South America. In addition, Hancock *et al.* (1992) and Kushlan and Bildstein (1992) proposed *E. albus* to be the North American species and *E. ruber* to be the South American species, the latter including both the numerically dominant red form and a relatively rare white form. In contrast, Patten (2012) considers the white form from South America to be a subspecies of the White Ibis (*E. albus ramobustorum*), following the current treatment as two different species.

Species delimitation has been proposed through the use of genetic methods (Carstens *et al.* 2013). However, even if the speciation process were immediate, the generation of reciprocal monophyly at multiple loci requires a substantial amount of time after the initial divergence. Species delimita-

tion methods use both mitochondrial DNA (mtDNA) and multiple nuclear loci to obtain a signal of speciation, despite the fact that markers are not monophyletic (Knowles and Carstens 2007).

Morphological, ecological and behavioral data have served as the basis for previous classifications of Scarlet and White ibises as two separate species. The present study provides mitochondrial and nuclear genetic data to contribute to discussions on this issue. We applied recently developed species delimitation methods to evaluate the taxonomic status of Scarlet and White ibises.

METHODS

Samples

Blood samples from Scarlet Ibises ($n = 10$) were collected in the state of Amapá, Brazil (Zelândia Farm ($n = 6$): 01° 09′ N, 50° 23′ W; Se Cria Farm ($n = 4$): 01° 56′ N, 50° 35′ W) and liver samples from White Ibises ($n = 10$) in the state of Florida, USA (Alley North: 26° 12′ N, 80° 31′ W). Buff-necked Ibis (*Theristicus caudatus*) was used as an outgroup. We incubated samples in Tris-EDTA buffer containing proteinase K 20 mg/μL (and 1% Sodium Dodecyl Sulfate for liver samples). We employed the phenol-chloroform method to isolate DNA (Sambrook *et al.* 1989).

Amplification and Sequencing

We amplified three mitochondrial and four nuclear loci. Table 1 displays the experimental conditions and primers. We performed sequencing reactions using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems). We performed automatic sequencing in an ABI3730 Genetic Analyzer (Applied Biosystems). We sequenced 16S rRNA (16S, 591 bp), cytochrome B (CytB, 1017 bp), cytochrome oxidase I (COI, 699 bp), intron 7 of β-fibrinogen (FIB, 749 bp), intron 11 of glyceraldehyde-3-phosphate dehydrogenase (GADPH, 404 bp), intron 4 of the myelin proteolipid protein (MPP, 240 bp) and intron 2 of myoglobin (MB, 657 bp) from all individuals.

Data Analyses

We edited sequences using BioEdit (Hall 1999). We deposited all sequences in the GenBank (accession numbers: JF521857-JF521997). We used ClustalX to perform the global alignment (Larkin *et al.* 2007). We phased haplotypes for heterozygous nuclear sequences using Phase (Stephens *et al.* 2001) implemented in DnaSP (Librado and Rozas 2009). We estimated the minimum number of recombination events within each nuclear locus using DnaSP and computed Kimura 2-parmeter (K2P) distances using MEGA (Tamura *et al.* 2013).

We performed several approaches for species delimitation using multilocus data. Bayesian analysis of species delimitation was performed using Bayesian Phylogenetics and Phylogeography BPP (Yang and Rannala 2010). We analyzed mitochondrial and nuclear data separately. We ran 100,000 generations of reversible-jump Markov chain Monte Carlo with a sampling frequency of five and a burn-in of 50,000. We used algorithm 0 with the fine-tuning parameter $\varepsilon = 5.0$ (Yang and Rannala 2010). The *a priori* distributions of the ancestral population size (θ) and root age ($\tau 0$) were (2, 1000) and (2, 2000), respectively, following Yang and Rannala (2010). This analysis allowed us to estimate the posterior probability of each bifurcation in the guide tree (speciation probability). A speciation probability of ≥ 0.95 indicates strong support for a speciation event (Yang and Rannala 2010).

We evaluated the level of genealogical divergence between the Scarlet and White ibises using the genealogical sorting index (*gsi*; Cummings *et al.* 2008) based on nuclear data (with no evidence of recombination), and mitochondrial gene trees obtained using Bayesian inference in BEAST (Drummond and Rambaut 2007). The statistic *gsi* ranges from 1 (monophyly) to 0 (complete lack of genealogical divergence). We calculated *gsi* for 100 trees and obtained an ensemble *gsi* statistic (gsi_T) as a measure of genealogical divergence. We assessed the significance of the statistic using 1,000 permutations.

We used the approach described by Knowles and Carstens (2007) for lineage delimitations. For each locus, we constructed a likelihood gene tree using a HKY + gamma model on a UPGMA topology in PAUP (Swofford 2003). We calculated the likelihood of the gene trees given the species tree using the SpedeSTEM2 (Ence and Carstens 2011). We evaluated two mutually exclusive hypotheses: the Scarlet and White ibises represent different lineages vs. they belong to the same lineage. We used a likelihood ratio test (LRT) with one degree of freedom to test the significance of the difference between the species trees. We evaluated this method separately using mitochondrial data alone, nuclear data alone and the combined data set. The SpedeSTEM2 program assumes a molecular clock, which we tested by LRT in PAUP. Additionally, SpedeSTEM2 assumes that the value $\theta = 4 N e \mu$ is constant across lineages. Thus, we estimated θ using Migrate-n (Beerli and Felsenstein 2001).

RESULTS

Haplotypes were obtained for Scarlet and White ibises (Table 2). Data on three mitochondrial markers revealed no shared haplotypes between these groups. Table 3 displays K2P distances based on mitochondrial data. Three nuclear markers (FIB, GADPH, and MB) revealed shared haplotypes and

Table 1. Marker, length of amplified product in base pairs (bp), primer sequences and their references, and annealing temperatures (Ta) for each marker used in Scarlet and White ibises.

Marker	bp	Primer (5'-3')	Reference	Ta (°C)
16S rRNA (16S)	591	F: CGAGCYRGGTGATAGCTGGTT R: TTACGCTACCTTCGCACGGT	Ramirez *et al.* (2013)	53
Cytochrome B (CytB)	1113	F: AACATCTCWGCHTGATGAAA R: CTTCATTCTTTGGTTTACAAGAC	This study	58
Cytochrome oxidase I (COI)	960	F: AAAGGACTACAGCCTAACGC R: GATGTGAAGTATGCTCGGG	This study	53
Intron 7 of β-Fibrinogen (FIB)	1070	F: GGAGAAAACAGGACAATGACAATTCAC R: TCCCCAGTAGTATCTGCCATTAGGGTT	Prychitko and Moore (1997)	53
Intron 11 of Glyceraldehyde-3-phosphate dehydrogenase (GADPH)	470	F: ACCTTTCATGCGGGTGCTGGCATTGC R: CATCAAGTCCACAACACGGTTGCTGTA	Stone *et al.* (1985)	65-69
Intron 4 of the Myelin proteolipid protein (MPP)	379	F: TACATCTACTTTAACACCTGGACCACCTG R: TTGCAGATGGAGAGCAGGTTGGAGCC	Schliess and Stoffel (1991)	62
Intron 2 of Myoglobin (MB)	758	F: GCCACCAAGCACAAGATCCC R: TTCAGCAAGGACCTTGATAATGACTT	Slade *et al.* (1993) Heslewood *et al.* (1998)	60

Table 2. Haplotypes of three mitochondrial (16S, CytB, and COI) and four nuclear (FIB7, MPP, GADPH, and MB) markers of Scarlet Ibis and White Ibis. Code: sample identification number. '-' indicates that amplification was not obtained. All sequences were deposited in GenBank.

Code	Species	16S	CytB	COI	FIB7	MPP	GADPH	MB
M129	*Eudocimus ruber*	Hap 1	Hap 1	Hap 1	Hap 1/1	Hap 1/1	Hap 1/2	Hap 1/1
M143	*Eudocimus ruber*	Hap 2	Hap 1	Hap 1	Hap 1/1	Hap 1/1	Hap 1/3	Hap 1/1
M159	*Eudocimus ruber*	Hap 3	Hap 2	Hap 1	Hap 1/2	Hap 1/1	Hap 3/9	Hap 1/1
M193	*Eudocimus ruber*	Hap 1	Hap 1	Hap 1	Hap 3/3	Hap 1/1	Hap 4/5	Hap 1/1
M226	*Eudocimus ruber*	Hap 1	Hap 1	Hap 1	Hap 4/4	Hap 1/1	Hap 6/7	Hap 1/2
M241	*Eudocimus ruber*	Hap 1	Hap 1	Hap 1	Hap 1/1	Hap 1/1	Hap 2/8	Hap 1/3
M272	*Eudocimus ruber*	Hap 1	Hap 3	Hap 1	Hap 1/1	Hap 1/1	Hap 7/10	Hap 1/1
M337	*Eudocimus ruber*	Hap 1	Hap 4	Hap 1	Hap 1/1	Hap 1/1	Hap 2/9	Hap 4/4
M376	*Eudocimus ruber*	Hap 1	Hap 1	Hap 1	Hap 1/1	Hap 1/1	Hap 1/9	—
M425	*Eudocimus ruber*	Hap 4	Hap 1	Hap 1	Hap 1/1	—	—	Hap 5/5
EA172	*Eudocimus albus*	Hap 5	Hap 5	Hap 2	Hap 4/5	Hap 2/2	Hap 11/12	Hap 1/5
EA175	*Eudocimus albus*	Hap 5	Hap 6	Hap 2	Hap 1/4	Hap 2/2	Hap 13/13	Hap 1/2
EA177	*Eudocimus albus*	Hap 5	Hap 6	—	Hap 4/4	Hap 2/2	Hap 14/15	Hap 6/6
EA180	*Eudocimus albus*	Hap 5	Hap 5	Hap 2	Hap 4/6	Hap 2/2	Hap 16/17	—
EA189	*Eudocimus albus*	Hap 5	Hap 5	Hap 2	Hap 4/4	Hap 3/3	Hap 18/19	Hap 3/5
EA200	*Eudocimus albus*	Hap 5	Hap 5	Hap 2	Hap 7/7	Hap 3/3	Hap 20/21	Hap 1/6
EA206	*Eudocimus albus*	Hap 6	Hap 6	Hap 2	Hap 8/8	Hap 2/2	Hap 5/22	Hap 2/6
EA210	*Eudocimus albus*	Hap 5	Hap 7	Hap 2	Hap 4/6	Hap 2/2	—	Hap 1/6
EA225	*Eudocimus albus*	Hap 5	Hap 8	Hap 2	Hap 3/4	Hap 2/3	Hap 17/17	Hap 1/6
EA227	*Eudocimus albus*	Hap 5	Hap 8	Hap 2	Hap 1/4	Hap 2/3	Hap 23/23	Hap 1/7

evidence of recombination. In contrast, the nuclear marker MPP exhibited no shared haplotypes or evidence of recombination.

Bayesian species delimitation analysis based on the mitochondrial and nuclear data resulted in a speciation probability of 1.0. For the mitochondrial markers, the gsi_T statistic showed a significant value of 1.0 for trees obtained for both ibises. The gsi_T values for MPP data were 0.937 for the White Ibis and 0.994 for the Scarlet Ibis, both of which were significant.

The LRT test did not reject the molecular clock hypothesis for any marker. The average estimate of θ was 0.002. Lineage delimitation using the Knowles-Carstens method (Knowles and Carstens 2007) based on mitochondrial genes revealed a significantly higher lnL value for the hypothesis that the Scarlet and White ibises are two different lineages. Nuclear genes and combined data also revealed highest values for the hypothesis of two different lineages (Table 4), but they were not significant.

DISCUSSION

This study applies a multilocus genetic approach to evaluate species delimitation of Scarlet and White ibises. The absence of genetic differentiation among populations

Table 3. Comparison of K2P genetic distances between pairs of species based on three mitochondrial (16S, CytB, and COI) and one nuclear (MPP) markers. In parentheses are the numbers of species.

Species	16S rRNA	CytB	COI	MPP
Among *Eudocimus* species ($n = 2$)	0.5-0.9%	0.8-1.1%	0.6%	0.4-0.8%
Among *Plegadis* species ($n = 3$)	0.4-0.7%[1]		0.9%[2]	
Among *Theristicus* species ($n = 3$)	2.2-8.4%[1]			
Among *Platalea* species ($n = 4$)	3.1-6.9%[1]	1.5-8.9%[3]		
Among parapatric birds ($n = 24$)	1.3 ± 1.2%[2]	3.1 ± 2.5%[2]	3.6 ± 3.3%[2]	

[1]Ramirez *et al.* (2013).
[2]Aliabadian *et al.* (2009).
[3]Chesser *et al.* (2010).

Table 4. Likelihood values for species delimitation according to Knowles and Carstens (2007) obtained in SpedeSTEM2. M: mitochondrial data set; N: nuclear data set; N+M: combined data set; lnL: logarithm of likelihood; *k*: number of species on species tree (including outgroup: Buff-necked Ibis); LRT: likelihood ratio test.

Data set	-lnL	*k*	LRT	*P*-value
M	1,177.460	3	293.520	0
	1,324.220	2		
N	3,262.836	3	0.058	0.820
	3,262.865	2		
N+M	723.485	3	0.051	0.820
	723.510	2		

supports our interpretation of results. Gonçalves *et al.* (2010) found no genetic structure among three populations of the Scarlet Ibis. Moreover, there is no evidence of genetic structure among White Ibis populations from North America (Stangel *et al.* 1991). The White Ibis has a very low level of natal and breeding philopatry as well as a nomadic movement pattern (Frederick *et al.* 1996; Heath *et al.* 2009), biological characteristics that can contribute to panmixia.

Our genetic data based on mitochondrial genes indicate that the Scarlet and White ibises are two separate groups, since these birds share no mtDNA haplotypes. The genetic distances were comparable to those found among other species of the family Threskiornithidae (Chesser *et al.* 2010; Ramirez *et al.* 2013) (Table 3). It is important to remember that this family has many species complexes. For example, the three species of the genus *Plegadis* have similar genetic distance values, but these species have been considered as a superspecies (Sibley and Monroe 1990). On the other hand, species of the genus *Platalea* have higher genetic distance values (Table 3); however, the genus has very differentiated species, even with some species placed in monospecific genera. Genetic distance values between the Scarlet and White ibises were lower (but within the range of values) than those found between 24 pairs of parapatric species (Table 3; Aliabadian *et al.* 2009). Threskiornithidae has lower mutation rates of mtDNA in comparison to other groups of birds (Eo and DeWoody 2010), which may explain why we found lower distances than Aliabadian *et al.* (2009). Another explanation would be the recent separation into two lineages, estimated to be two million years ago (Ramirez *et al.* 2013).

One nuclear marker (MPP) showed differentiation between the Scarlet and White ibises, while other nuclear markers revealed polyphyletic patterns, likely due to incomplete lineage sorting and/or the retention of ancestral polymorphism. However, we recovered a strong signal of speciation using nuclear markers based on different coalescent analyses of species delimitation. The coalescent results support the hypothesis of two evolutionary lineages. Moreover, the Bayesian delimitation revealed the highest value for this speciation event, even with nuclear marker data, and the gsi_T statistic supported a significant degree of differentiation, with significant values for both the mitochondrial genes and MPP gene. Species delimitation analysis, as proposed by Knowles and Carstens (2007), based on mitochondrial data supports the hypothesis of two different lineages. However, nuclear data did not reject any hypotheses. This result would be expected if one supposes a recent separation of these lineages.

The results suggest that Scarlet Ibises from the southern end of their range, and White Ibises from North America show strong differentiation that can be interpreted initially as being separate species. However, as the total area of geographic distribution of the Scarlet and White ibises was not sampled, the correct position of the White Ibis from South America, and the overall question of speciation within the contact zone, merits further investigation. We hope to obtain samples from the South American white form, which has been proposed as either a subspecies of White Ibises (Patten 2012) or of Scarlet Ibises (Hancock *et al.* 1992). The first step was done in this preliminary study that standardized the methodological genetic approach, selected candidate genes and started to clarify this taxonomic question.

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