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Molecular phylogeny of Threskiornithidae (Aves: Pelecaniformes) based on nuclear and mitochondrial DNA

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ABSTRACT. The family Threskiornithidae includes 13 genera and 32 species, and it is traditionally divided into 2 subfamilies: Plataleinae and Threskiornithinae. We present a phylogenetic reconstruction to test the monophyly of currently accepted subfamilies, including 15 species from both subfamilies and 10 genera of family Threskiornithidae. Phylogenetic trees were inferred on the basis of the mitochondrial 16S rRNA gene and the nuclear intron 7 of β-fibrinogen. Threskiornithidae was recovered as a monophyletic group. Plataleinae formed a monophyletic group, but nested within Threskiornithinae, which was thus paraphyletic. Two major phylogenetic groups were identified: the 'endemic New World clade', including genera endemic to the American continent, and the 'widespread clade', comprising the remaining species. These phylogenetic groups diverged about 39-42 million years ago, i.e., before the separation of South America and Antarctica. Our results agree with an initial vicariance due to Gondwana break-up and subsequent colonization of species from the Old World to the New World.

Key words: Ibis; Plataleinae; Spoonbills; Threskiornithinae

INTRODUCTION

The ibises and spoonbills are classified as a family (Threskiornithidae) that includes 13 genera and 32 species (Matheu and del Hoyo, 1992) of long-legged water birds of medium to large size (50 to 110 cm). The body is elongated, but robust, with a fairly long beak that is the most distinctive morphological element among species (Matheu and del Hoyo, 1992). This family shows its greatest diversity in the tropics, and occurs almost worldwide, except in Antarctica (Matheu and del Hoyo, 1992). The majority of genera occur on only one continent, except *Plegadis* and *Platalea*, which have cosmopolitan distributions (Matheu and del Hoyo, 1992).

Threskiornithidae was traditionally classified in the order Ciconiiformes (Mayr and Amadon, 1951; Wetmore, 1960), which includes waders and long-necked birds. However, recent molecular studies have questioned the monophyly of this order (Sibley and Ahlquist, 1990; Ericson et al., 2006; Hackett et al., 2008), and Threskiornithidae has been reclassified as a member of the order Pelecaniformes (Chesser et al., 2010a). Studies of the systematics within this family are scarce, and little is known about phylogenetic relationships of its genera and species. Threskiornithidae was traditionally divided into two subfamilies that are easily distinguished by morphological differences in their beaks. The subfamily Threskiornithinae (ibises) has been recognized by its long, narrow, and curved beak. The subfamily Plataleinae (spoonbills) was characterized by a long beak with broad and flattened tip (Austin and Singer, 1983). The validity of these two subfamilies has been questioned by some authors (Sibley and Ahlquist, 1990; Fleischer and McIntosh, 2001; Ferreira, 2007; Chesser et al., 2010b). Livezey and Zusi (2007) proposed the subfamily Plataleinae as an independent family. Information based on genetic data should help clarify phylogenetic relationships within this family.

The aim of this study was to reconstruct the phylogenetic relationships between genera of the family Threskiornithidae using mitochondrial and nuclear markers and, thus, to evaluate the monophyly of subfamilies.

MATERIAL AND METHODS

Biological samples

Blood, tissue, and skin samples were obtained from 15 species of 8 genera (Table 1). Since fresh tissue samples were not available, the majority of samples used here were obtained from museum skins. Several species were not found in the museums visited. In addition, DNA is difficult to obtain from old skin samples (older than 50 years). Despite our efforts, it was not possible to amplify all species available in museums. For *Plegadis chihi*, we obtained samples from 2 individuals from 2 distant localities. Sequences from the 16S rRNA gene of 4 species from 3 genera of the family Threskiornithidae were available in GenBank (Table 1) and included in the analyses. A stork, *Mycteria americana* (Ciconiidae: Ciconiiformes), and a heron, *Ardea alba* (Ardeidae: Pelecaniformes), were selected as outgroups (Ericson et al., 2006; Hackett et al., 2008). DNA was extracted from blood samples by a standard phenol-chloroform method. DNA extraction from skin and tissue samples was performed in a DNA clean room using the DNeasy® Blood and Tissue kit (QIAGEN, Hilden, Germany) or NucleoSpin® Tissue (Macherey-Nagel, Düren, Germany), with the addition of 30 µL (0.1 g/mL) DTT for digestion.

PCR amplification and sequencing

A fragment of approximately 600 bp of the 16S rRNA gene (16S) was amplified using primers Tr16SL (5'-CGAGCYRGGTGATAGCTGGTT-3') and Tr16SH (5'-TTACGCTACCT TCGCACGGT-3'). These primers were designed based on the universal primers 16L2a and 16H10 (Hedges, 1994) and on mitochondrial genomes available in GenBank for species of Threskiornithidae and related families.

Approximately 1 kb of the intron 7 of β-fibrinogen gene (FIB-7) was amplified using primers FIB-BI7U (5'-GGAGAAAACAGGACAATGACAATTCAC-3') and FIB-BI7L (5'-TCCCCAGTAGTATCTGCCATTAGGGTT-3') (Prychitko and Moore 1997). Internal FIB-7 primers, designed on the basis of sequences obtained in the present study, were used to amplify museum skin samples: F206 (5'-TTACCAGCCAAATGTCCA-3'), F380 (5'-ATGGTGGCAGTGCTGAGG-3'), F613 (5'-GGGATKSTTTAGACTGG-3'), F819 (5'-GGCGATGGTCAATCTTT-3'), R260 (5'-AGTTTTACCTSCCCTTG-3'), R476 (5'-GCTACCTGTCTCTTTCCTC-3'), R681 (5'-GCAATATCAAYGCAATTT-3'), and R900 (5'-CCTGCCCCTGTACTGAA-3'). Annealing temperatures of the primer pairs and sizes of the amplified fragments obtained for each primer pair were FIBU/R260 = 48ºC (260 bp), F206/R476 = 50°C (271 bp), F380/R681 = 48°C (302 bp), F613/R900 = 50°C (288 bp), and F819/FIBL = 45°C (255 bp).

PCR was carried out in a final volume of 12.5 µL, using 25 mM dNTPs, 10 mM Tris-HCl, 50 mM KCl, 0.4 µM each primer, 2 mM $MgCl_2$, and 1 U *Taq* polymerase (Fermentas, Vilnius, Lithuania). Reactions were performed in an Eppendorf Mastercycler Gradient® thermal cycler (Eppendorf AG, Hamburg, Germany) with the following parameters: 35 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 1 min. PCR products were sequenced using Big Dye Terminator Cycle Sequencing kit (Perkin Elmer) following manufacturer instructions. Sequences were obtained using ABI 3130 (Applied Biosystems, Foster City, CA, USA) at Centro de Estudos do Genoma Humano (USP, São Paulo, SP) and using ABI Prism 3730 (Applied Biosystems) at CREBIO, UNESP, Jaboticabal, SP.

Data analyses

Sequences were edited with BioEdit (Hall, 1999) and deposited in GenBank (accession Nos. for 16S rRNA: JQ910183-JQ9101200 and for FIB-7: JQ910169-JQ910182). Global alignment was carried out with Clustal X2 (Larkin et al., 2007). The alignment of the 16S rRNA gene was complex; conserved or similar segments (3 or more nucleotides) were used as landmarks, and whenever the alignment involved the possibility of assuming a transition or a transversion, transitions were preferred. Pairwise p-distances were obtained in MEGA v4.02 (Tamura et al., 2011).

The best-fit nucleotide substitution model was chosen based on Akaike criterion using jModeltest (Posada, 2008). Base frequencies, proportion of invariant sites and shape parameter of the gamma distribution were also estimated in jModeltest. Gaps in genes (such as FIB-7) can add significant information in topology reconstruction (Pásko et al., 2011), and for this reason, they were considered a fifth character. Phylogenetic analyses were performed with the mitochondrial and nuclear data sets separately. Neighbor-joining trees were obtained using PAUP* 4.0b10 (Swofford, 2003). A heuristic procedure with tree-bisection-reconnection (TBR) branch swapping was used. Maximum parsimony analyses were performed in

PAUP*4.0b10, where initial heuristic searches were conducted with random stepwise addition and TBR branch swapping. Maximum likelihood analyses were conducted in PAUP* 4.0b10 using a heuristic search, where the initial tree was obtained by stepwise addition and TBR. Branch supports for the neighbor-joining, maximum parsimony, and maximum likelihood trees were evaluated using bootstrap with 1000 replicates. Bayesian inference was performed by MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001); 4 chains were run simultaneously for 10 million generations, sampled every 1000 generations and with burn-in of 9000. A species tree based on mitochondrial and nuclear data, including 11 species, was estimated by *BEAST (Heled and Drummond, 2010), using 10 million generations, sampled every 1000 and with a burn-in of 9000. The reciprocal monophyly of subfamilies was tested using the Kishino and Hasegawa (1989) and Shimodaira and Hasegawa (1999) tests. *M. americana* was chosen to root the trees (Ericson et al., 2006; Hackett et al., 2008).

Estimates of time to the most recent common ancestor (tMRCA) were obtained by BEAST 1.6.1 (Drummond and Rambaut, 2007) for two separate data sets and for concatenated sequences. The analyses were performed assuming a relaxed molecular clock, with a log-normal distribution, using 10 million MCMC runs and sampled every 1000 trees. Evolutionary rates were estimated for each gene using two calibration points obtained at the TimeTree site (Hedges et al., 2006). The first calibration refers to the separation between the families Ardeidae and Ciconiidae, which has 3 date estimates: 75 million years ago (myr) based on 5 nuclear genes (Brown et al., 2007), 88 myr based on 13 mitochondrial genes (Brown et al., 2008), and 80.7 myr based on 4 mitochondrial and 3 nuclear genes (Fain et al., 2007). Thus, to include all these estimated dates, we assumed that this divergence occurred 82 myr, with standard deviation of 3.5 myr. The second calibration point refers to the separation between the families Ardeidae and Threskiornithidae estimated to have occurred 69 myr (Brown et al., 2007) or 77 myr (Brown et al., 2008). Thus, we assumed that this event occurred 74 myr with a standard deviation of 2.5 myr.

RESULTS

Fifteen partial sequences of the mitochondrial 16S rRNA gene and 11 sequences of the FIB-7 gene were obtained for species of the family Threskiornithidae (Table 1). The 16S sequences ranged from 550 to 560 bp. Alignment resulted in 589 characters, of which 223 were variable (162 parsimony informative) and 13 showed indels. The majority of the FIB-7 sequences ranged from 893 to 920 bp, except for *Plegadis falcinellus* and *P. chihi* (USA sample), which had 949 bp, due to a duplication of a 29-bp sequence. Alignment resulted in 977 sites, of which 192 were variable (93 parsimony informative) and 15 showed indels. The best-fit nucleotide substitution model for 16S was TPM2uf + G (gamma = 0.198), and for FIB-7 the best model was TVM + G (gamma = 1.657).

Species tree showed similar topology in comparison to trees based on separate data sets (16S and FIB-7 genes), that where obtained by different phylogenetic methods (Figures 1-3). The monophyly of the family Threskiornithidae was strongly supported by all analyses (Figures 1-3). The results obtained did not reject the hypothesis of monophyly of the subfamily Plataleinae, but indicated that the subfamily Threskiornithinae is paraphyletic. The monophyly of the Threskiornithinae was also rejected by the Kishino and Hasegawa (1989) and Shimodaira and Hasegawa (1999) tests for both genes ($P < 0.0001$).

Table 1. Species analyzed, collection localities and dates, registration numbers of the samples, and GenBank accession numbers.

Taxon	Locality	Date	Sample	Accession No. (16S)	Accession No. (FIB)
Bostrychia hagedash	Kenya	1959	NMK B223[2]	JQ910190	-
Eudocimus albus	Captivity	2006	AMNH PAC1657[1]	JQ910184	JQ910170
Eudocimus ruber	Amapá/Brazil	2007	LGA M226[3]	JQ910183	JQ910169
Geronticus eremita	-	-		EU144041*	
Nipponia nippon	Niigata/Japan	-		NC_008132*	
Phimosus infuscatus	Araguaiana/Brazil	2009	MZUSP 83734[2]	JQ910185	JQ910171
Platalea alba	-	-	NMK B236[2]	JQ910193	JQ910176
Platalea ajaja	Amapá/Brazil	2007	LGA M335[3]	JQ910194	JQ910175
Platalea leucorodia	Taiwan	-		NC_012772*	
Platalea minor	-	-		NC_010962*	
Plegadis chihi	Rio Grande do Sul/Brazil	2008	LGA CH57[3]	JQ910195	JQ910178
Plegadis chihi	Utah/USA	1994	AMNH PRS1911[1]	JQ910196	JQ910180
Plegadis falcinellus	New York/USA	2003	AMNH PAC1127[1]	JQ910197	JQ910179
Plegadis ridgwayi	Bolivia	1958	AMNH S803360[2]	JQ910198	-
Pseudibis papillosa	Dangs/India	1954	AMNH S778593[2]	JQ910189	-
Theristicus caudatus	São Paulo/Brazil	2008	LGA TC[1]	JQ910187	JQ910173
Theristicus caerulescens	Mato Grosso/Brazil	2007	MZUSP 79231[2]	JQ910186	JQ910172
Theristicus melanopis	Rio Negro/Argentina	2005	AMNH PRS2838[1]	JQ910188	JQ910174
Threskiornis aethiopicus	-	-	AMNH PRS2383[1]	JQ910192	-
Threskiornis molucca	Rennell/Solomon Islands	1995	AMNH MKL12[1]	JQ910191	JQ910177
Mycteria americana	Amapá Brazil	2007	LGA L218[3]	JQ910199	JQ910182
Ardea alba	Mato Grosso/Brazil	2008	LGA T183d[3]	JQ910200	JQ910181

NMK = National Museums of Kenya; AMNH = American Museum of Natural History; LGA = Laboratório de Genética de Aves (UFSCar); MZUSP = Museu de Zoologia da Universidade de São Paulo. Classification follows Matheu and del Hoyo (1992). [1]Tissue. [2]Skin. [3]Blood. *Sequences downloaded from GenBank; (-) = data not available.

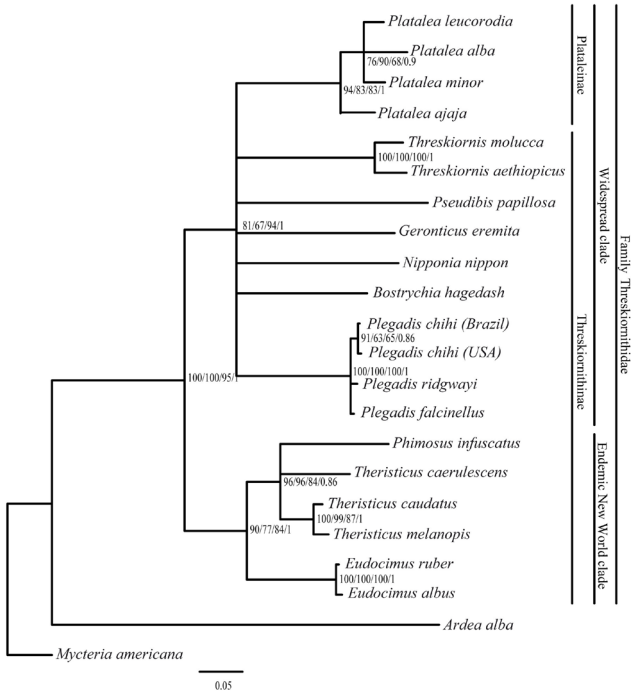

Figure 1. Bayesian tree based on 589 bp of the 16S rRNA gene. Numbers on branches are bootstrap values for neighbor-joining, maximum parsimony, and maximum likelihood, and posterior probability for Bayesian inference, respectively. The scale bar indicates 0.05 nucleotide substitutions per site.

The genera *Eudocimus*, *Phimosus*, and *Theristicus* of the subfamily Threskiornithinae clustered at a separate clade that we named the 'endemic New World clade'. The remaining species was grouped in a second clade named the 'widespread clade', which included the subfamily Plataleinae and representatives of the Threskiornithinae subfamily (*Bostrychia hagedash*, *Geronticus eremita*, *Nipponia nippon*, *Pseudibis papillosa*, *Threskiornis aethiopicus*, *Threskiornis molucca*, *Plegadis chihi*, *P. falcinellus*, and *P. ridgwayi*). The relationships within the 'widespread clade' could not be established (Figures 2 and 3).

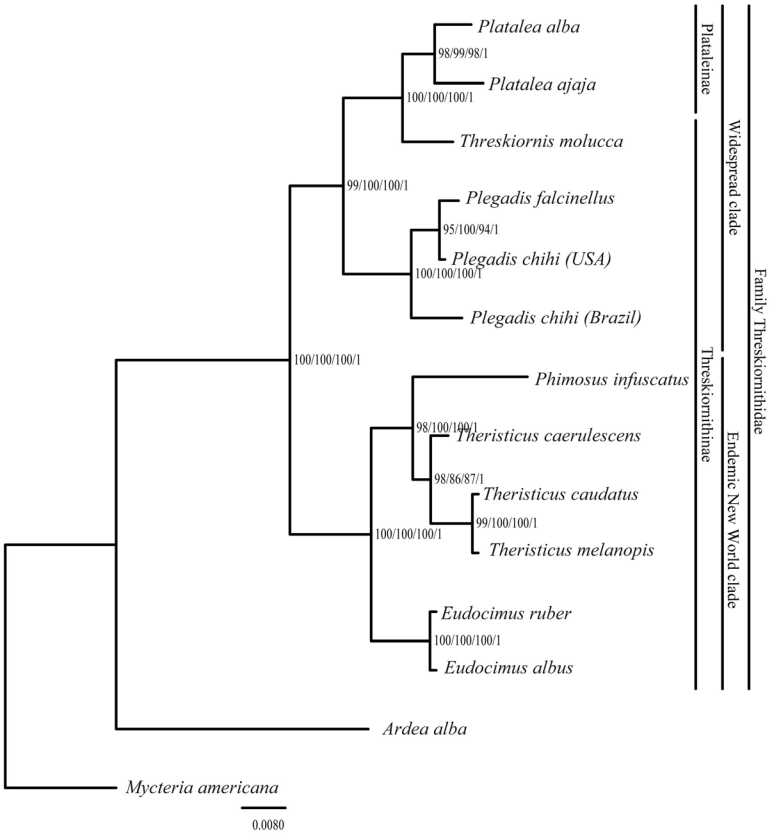

Figure 2. Bayesian tree based on 977 bp of the intron 7 of β-fibrinogen gene. Numbers on branches are bootstrap values for neighbor-joining, maximum parsimony, and maximum likelihood, and posterior probability for Bayesian inference, respectively. The scale bar indicates 0.008 nucleotide substitutions per site.

The average tMRCA based on the 16S data for the family Threskiornithidae was 38.8 myr, with a confidence interval that ranged from 25.6 to 54 myr. The average tMRCA based on FIB-7 was 41.8 myr, with a confidence interval between 30.9 and 53.4 myr. The average tMRCA based on both markers was 39.6 myr, with a confidence interval between 30.1 and 50.6 myr. A chronogram based on FIB-7 data is shown in Figure 4.

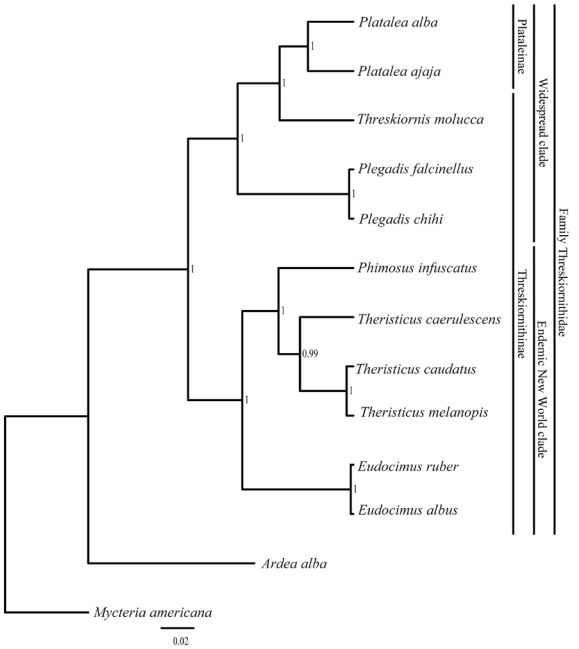

Figure 3. Species tree based on 1566 bp of concatenated 16S rRNA and intron 7 of β-fibrinogen genes. Values on branches are posterior probabilities. The scale bar indicates 0.02 nucleotide substitutions per site.

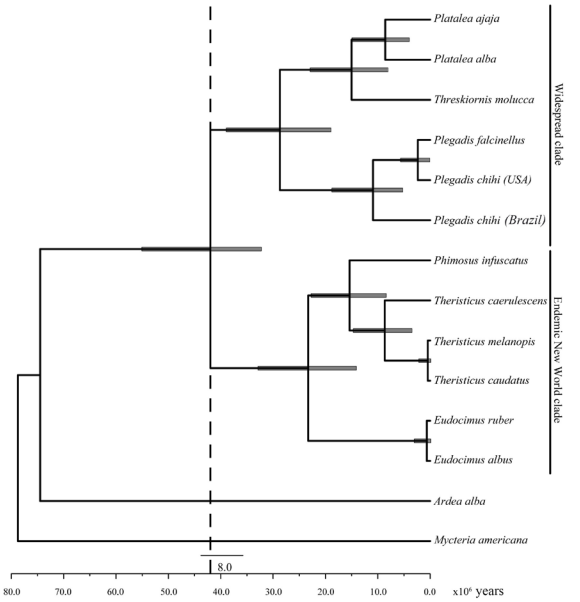

Figure 4. Chronogram based on 977 bp of the intron 7 of β-fibrinogen gene using relaxed molecular clock. Time was expressed in millions of years. Dashed line indicates the initial divergence within Threskiornithidae. Bars indicate the 95% confidence interval of the highest posterior density.

DISCUSSION

This phylogenetic study is the first one with a satisfactory representation of species of Threskiornithidae and that included both mitochondrial and nuclear DNA data. The mitochondrial 16S rRNA gene data set included 59% species and 76% genera from the family, while the analyses of nuclear FIB-7 included 34% species and 54% genera from this family. Although the sampling of Threskiornithidae species was partial, the inferred phylogenies support the monophyly of the family, which has never been seriously questioned (Remsen et al., 2012).

On the other hand, the division of the family Threskiornithidae into two subfamilies was not corroborated by our results. The subfamily Plataleinae was recovered as a monophyletic group, and it was nested within a clade containing species of the subfamily Threskiornithinae. Spoonbills (genus *Platalea*) were initially described as belonging to the family Plataleidae Bonaparte 1838, but they were subsequently included along with subfamily Threskiornithinae Poch 1904 in the family Threskiornithidae Richmond 1917. This last classification is still currently accepted (Remsen et al., 2012). However, according to Brodkorb (1963), the best name for this group should be Plataleidae, since the name Plataleidae has precedence over Threskiornithidae. The subfamily Threskiornithinae was recovered as paraphyletic, in agreement with previous studies based on DNA-DNA hybridization data (Sibley and Ahlquist, 1990), mitochondrial sequences (Fleischer and McIntosh, 2001; Chesser et al., 2010b), and some morphological studies (Ferreira, 2007). However, our findings differ from the traditional taxonomy and the proposition of division of the family Threskiornithidae into two families: Threskiornithidae and Plataleidae (Livezey and Zusi, 2007).

Our data recovered two reciprocally monophyletic groups within the family Threskiornithidae. An ‘endemic New World clade’ was strongly supported and included only genera endemic to the Americas (Figures 1-3). The second ‘widespread clade’ grouped the remaining species, including Old World endemic genera and genera that occur both in the New and Old Worlds. One of the first hypotheses about the relationships between species of the family Threskiornithidae was inferred on the basis of data from DNA-DNA hybridization of samples from 11 species from 8 genera (Sibley and Ahlquist, 1990). This study included representatives of the genera *Cercibis* and *Mesembrinibis*, which were not represented in our study; however, we added data on *Geronticus*, *Nipponia*, *Phimosus*, and *Pseudibis*, which were not previously studied. The phenogram obtained by Sibley and Ahlquist (1990) suggested that *Platalea* and *Threskiornis* were sister taxa and that they were closely related to other taxa from the subfamily Threskiornithinae. Interestingly, Sibley and Ahlquist (1990) found an apparent division into two clades: one with New World taxa and the other grouping the Old World taxa. Fleischer and McIntosh (2001) presented an unpublished parsimony tree built by other authors based on the 12S rRNA gene, which showed Plataleinae species related to Old World species of ibises, but unfortunately, this tree did not have support values. In that study, the genus *Plegadis* was closely related to a clade that included *Eudocimus*, *Apteribis*, and New World ibises. Ferreira (2007), in a study of cranial osteological characters, also recovered Plataleinae as monophyletic and Threskiornithinae as paraphyletic. Two groups were identified within Threskiornithinae: the first one including *Phimosus*, *Mesembrinibis*, *Eudocimus*, *Theristicus caerulescens*, *Geronticus*, and *Pseudibis*, and the second comprising *Bostrychia carunculata*, *Theristicus melanopis*, *Lophotibis*, *Cercibis*, and *Platalea* (Ferreira, 2007). The monophyly of the subfamily Plataleinae was strongly supported by cytochrome b and ND2 mitochondrial

markers by Chesser et al. (2010b), but since only 3 species of Threskiornithinae were included in that study, no conclusions were drawn about this subfamily.

The results obtained in the present study suggest the subdivision of Threskiornithidae into two clades, consistent with the results of Sibley and Ahlquist (1990) and different from those found by Ferreira (2007). The genus *Plegadis* fell within the 'widespread clade' (Figures 1-3), but in a different position to that proposed by Fleischer and McIntosh (2001), which placed this genus in the 'endemic New World clade'. The existence of two major clades, one including only New World taxa and the other with both New and Old World taxa (widespread clade), has also been observed in other orders of birds: Gruiformes, Caprimulgiformes, Apodiformes, and Passeriformes (Cracraft, 2001; Ericson et al., 2002). This distribution has been interpreted as resulting from the breakup of Gondwana, which formed the two major clades, followed by subsequent colonization of the New World by Old World taxa (Cracraft, 2001; Ericson et al., 2002). The average tMRCA for the family Threskiornithidae (38 to 41 myr) agrees with the separation of South America and Antarctica, given that some connection between these two continents existed until about 30 to 35 myr (Cox and Moore, 2010).

Among the main taxonomic controversies concerning the family Threskiornithidae is the status of *Platalea ajaja* and *P. flavipes*, which have been classified by some authors in the monotypic genera *Ajaja* and *Platibis*, respectively (Pinto, 1938; Hellmayr and Conover, 1948; American Ornithologists Union, 1998). In the present study, *P. ajaja* appeared basal in relation to other 3 species of the genus *Platalea* included in the analysis based on the 16S rRNA gene (Figure 1). *P. ajaja* was also the most divergent species in the genus: genetic distances between *P. ajaja* and other species of the genus ranged from 0.055 to 0.069, while distances between other pairs of species ranged from 0.031 to 0.049. However, we found that intrageneric distances ranged between 0.020 to 0.084 and intergeneric distances ranged from 0.069 to 0.166. Chesser et al. (2010b) found a clade with *P. leucorodia*, *P. minor*, *P. regia*, and *P. alba*, while *P. flavipes* and *P. ajaja* were placed outside this clade and relationships between these 2 species were not well resolved. Therefore, our results were similar to those described by Chesser et al. (2010b).

Another controversy is presented by *T. caerulescens*, generally included in this genus following Steinbacher (1979), but sometimes classified in the monotypic genus *Harpiprion* (Pinto, 1938; Hellmayr and Conover, 1948; Hancock et al., 1992). All species of *Theristicus* were included in our analyses; 16S data showed a poorly supported monophyly, but FIB-7 data supported this clade with *T. caerulescens* as basal to the other two species (*T. melanopis* and *T. caudatus*), which shared the same FIB-7 haplotype. The two latter species were considered conspecific by Hellmayr and Conover (1948) and Hancock et al. (1992). Genetic distances between *T. caerulescens* and the other two species determined in this study were 0.009 on the basis of FIB-7 data, and ranged from 0.076 to 0.084, according to the 16S rRNA gene. These distances are low in comparison to those observed for species of different genera (from 0.020 to 0.066 for FIB-7 and 0.069 to 0.166 for 16S). Our data did not support the separation of these species into different genera, as suggested by Hancock et al. (1992).

Plegadis falcinellus and *P. chihi* were considered superspecies by Steinbacher (1979), and conspecific by Palmer (1962). These species have been reported to breed in sympatry in Louisiana (American Ornithologists' Union, 1998). The present study included two individuals of *P. chihi* from the opposite ends of its distribution (US and south Brazil). In the analysis based on 16S (Figure 1), these two individuals fell in a poorly supported clade and they were grouped in a polytomy with *P. ridgwayi* and *P. falcinellus*. In the analysis based on FIB-7

(Figure 2), *P. chihi* from the US was more closely related to *P. falcinellus* (sharing a 29-bp duplication) than to *P. chihi* (from south Brazil), showing that *P. chihi* could be paraphyletic. A more detailed analysis of the genus *Plegadis* with a more representative geographical sampling is needed to clarify this issue.

In summary, our results indicate that Threskiornithidae is composed of two major clades: the first one with only New World representatives and the second including taxa from both the New and Old Worlds. This division probably represents a Gondwanan origin of these clades. We suggest that the current classification into two subfamilies, Threskiornithinae and Plataleinae, should be further revised on the basis of more comprehensive sampling and using more genetic markers.

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