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Research Article

New molecular evidence supports the species status of Kaempfer's Woodpecker (Aves, Picidae)

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Abstract

Kaempfer's Woodpecker (*Celeus obrieni*) is the only species of the genus *Celeus* endemic to Brazil. The description of this taxon as a subspecies of the Rufous-headed Woodpecker (*Celeus spectabilis*) was based on a single specimen. While *C. obrieni* and *C. spectabilis* are now considered separate species based on morphological and limited molecular evidence, no study has critically tested the reciprocal monophyly and degree of evolutionary independence between these taxa with several specimens. Herein, fragments of the mitochondrial and nuclear DNA of three recently-collected specimens of *C. obrieni* were analyzed to evaluate the degree of evolutionary differentiation of this taxon with respect to *C. spectabilis*. The results confirm the reciprocal monophyly between the specimens of *C. obrieni* and *C. spectabilis*. The genetic divergence values for the two taxa also support their classification as independent species, given that they are greater than the values recorded among other closely-related but separate species of the same genus. Estimates of the divergence time between *C. obrieni* and *C. spectabilis* indicate that cladogenesis occurred in the mid-Pleistocene, during a period of major climatic fluctuations and landscape change, consistent with the hypothesis of a corridor of open bamboo dominated forests and woodland stretching.

Keywords: Bamboo, biogeography, *Celeus obrieni*, *Celeus spectabilis*, taxonomy.

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Introduction

The woodpeckers of the genus *Celeus* are endemic to the Neotropics. At the present time, 10 species are recognized (Remsen *et al.*, 2012), of which, nine are found in Brazil. The Kaempfer's Woodpecker (*Celeus obrieni* Short 1973) is the only *Celeus* endemic to this country (Winkler and Christie, 2002; CBRO, 2011).

Celeus obrieni (Short 1973) was originally described as a subspecies of the Rufous-headed Woodpecker (*Celeus spectabilis*), based on a specimen collected in 1926 by Emil Kaempfer in the region of Uruçuí, in the Brazilian state of Piauí (Hidasi *et al.*, 2008). More than thirty years later, the South American Classification Committee (SACC - Remsen *et al.*, 2012) re-evaluated the status of *Celeus s. obrieni* based on a comparison of plumage characters with specimens of *C. spectabilis* from Ecuador, Peru, and Bolivia, and concluded that *obrieni* was a distinct species. The total lack of new records over almost a century led some ornithologists to believe that the taxon had become extinct (Tobias *et al.*, 2006). The first record since the original specimen collected by Emil Kaempfer was obtained only in 2006, when the species was rediscovered in Goiatins, in the northeast of the Brazilian state of Tocantins, some 400 km from the type locality (Prado, 2006).

Subsequently, several new records were obtained from the state of Maranhão (Santos and Vasconcelos, 2007; Santos *et al.*, 2010), in addition to the states of Tocantins (Pinheiro and Dornas, 2008), Goiás (Dornas *et al.*, 2009; Pinheiro *et al.*, 2012), and Mato Grosso (Dornas *et al.*, 2011). All of these new records extended the known geographic range of *C. obrieni* quite considerably. Even though, the size of the species population has yet to be defined, since its rediscovery more than 50 individuals have been recorded within an area extending more than one thousand kilometers between extreme localities of distribution. Despite this expansion in the known range of the species, currently estimated at some 280,000 km^2 (Benz and Robbins, 2011; BirdLife International, 2011), a cautious estimate of the total population is 50-250 individuals,

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which is consistent with the IUCN critically threatened (CR) category (IUCN, 2010).

Recently, Benz and Robbins (2011) published a phylogeny for the genus *Celeus* based on molecular and morphological data, including the genetic material from the holotype of *C. obrieni*, collected in 1926. This specimen was identified as the sister taxon of *C. spectabilis*, as predicted by the traditional classification. In their study, four mitochondrial (ND2, ND3, ATP6-8/COIII, and the Control Region) and two nuclear markers (Intron 7 of the β-fibrinogen gene and HMGN2) were analyzed, although only the ND2 and ND3 genes and part of the HMGN2 sequence were amplified successfully for *C. obrieni* (Benz and Robbins, 2011). The results indicated a genetic divergence of approximately 1% between *C. obrieni* and *C. spectabilis* for the mitochondrial marker ND2. The authors suggested that further sampling would be needed to confirm the reciprocal monophyly of these forms and their status as distinct evolutionary lineages (Benz and Robbins, 2011). Nonetheless, Benz and Robbins (2011) treated species treatment for *C. obrieni*.

In the present study, a multi-locus molecular approach was used to confirm the validity of the species status of *C. obrieni*, an essential initial step in the development of a conservation plan for this threatened taxon. Three recently collected specimens of *C. obrieni* were sequenced, together with individuals representing three other species of the genus (*C. spectabilis*, *Celeus undatus* Waved Woodpecked, and *Celeus grammicus* Scale-breasted Woodpecked) to estimate phylogenetic relationships and pairwise genetic distances within this group.

Materials and Methods

Sampling

Three specimens of *C. obrieni* were collected by MPDS during surveys of bird populations at three sites in the Brazilian state of Maranhão (Figure 1, Table 1) - Serra da Raposa, (06º35'S, 43º37'W), in the municipality of São João dos Patos (specimen registered in the Museu Paraense Emílio Goeldi [MPEG] under the accession number 61549), Fazenda Castiça (05°28' S, 43°13'W), in the municipality of Matões (MPEG 69978), and Fazenda Normasa (05°36'S, 43°28'W), in the municipality of Parnarama (MPEG 69979). Specimens were collected under special license 20902-1 issued to MPDS.

Samples of muscle tissue were obtained from each of the three specimens of *C. obrieni*, together with 16 samples of specimens representing three other *Celeus* species - *C. spectabilis* (n = 5), *C. undatus* (n = 2), and *C. grammicus* (n = 9) - all of which were provided by the ornithological collection of the Goeldi Museum (Table 1). Samples of *C. undatus* and *C. grammicus*, two sister lineages historically regarded as separate species by traditional taxonomy and close to *C. obrieni / spectabilis* (Benz and Robbins, 2011),

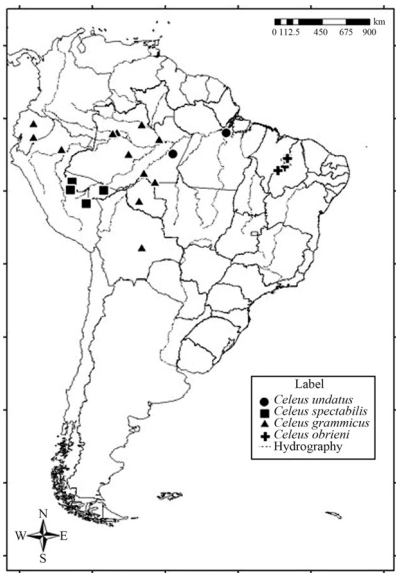

Figure 1 - Map showing the localities where *Celeus* specimens analyzed in the present study were collected.

were included in the analysis so that genetic distances between these species pairs could be contrasted. The samples were divided into aliquots and kept frozen at -20 °C until processing in the UFPA Genetics and Molecular Biology Laboratory.

Extraction, amplification and sequencing of the DNA

Once the samples were processed, the genetic material was extracted using the standard phenol-chloroform protocol, followed by precipitation in sodium acetate and isopropanol (Sambrook *et al.*, 1989). The samples were then electrophoresed in 1% agarose gel, stained with ethidium bromide and viewed under an UV transilluminator to the confirm the successful extraction of genetic material, its integrity and concentration. Using specific primers, the polymerase chain reaction (PCR) technique was used to amplify three regions of the mitochondrial genome (rDNA 16S, Cytochrome *b*, subunit 2 of the NADH dehydrogenase region) and one nuclear marker, intron 7 of the β-fibrinogen gene. The primers described by Sorenson *et al.* (1999) - L-15298 and H-16064 - were used for the cytochrome *b* gene (Cyt *b*), and those presented by Palumbi *et al.* (1991) - L-1987 and H-2609 -for the rDNA 16S (16S) gene. For subunit 2 of the NADH dehydrogenase region (ND2), the primers described by Hackett (1996) were used - H-6313 and L-5215. Part of intron 7 of the β-fibrinogen gene (I7BF) was amplified using the primers (FIB-BI7U and FIB-BI7L) described by Prychitko and Moore (1997).

Each reaction was conducted in a final volume of 25 μL, containing 4 μL of the deoxynucleotides (1.25 mM), 2.5 μL of 10x *Taq* buffer, 1 μL of $MgCl_2$ (25 mM), 0.5 μL of each primer (200 ng/μL), approximately 80 ng of the total DNA extracted from the samples, 0.25 μL of *Taq* poly-

Table 1 - *Celeus* specimens analyzed in the present study, showing the species name, number of specimens analyzed, identification code, collecting locality, and GenBank accession numbers for the sequences of the different molecular markers analyzed.

Species (number of specimens)	Identification code (Voucher specimen number)	Collecting locality	Cyt-B	16S	ND2	I7BF
C. obrieni (*n* = 4)						
	Cob1109 (MPEG 61549)	Brazil: Maranhão	KC858911	KC858892	KC858943	KC858930
	Cob1399 (MPEG 69978)	Brazil: Maranhão	KC858912	KC858893	KC858944	KC858931
	Cob1414 (MPEG 69979)	Brazil: Maranhão	KC858913	KC858894	KC858945	-
	AMNH242687*	Brazil: Piauí	-	-	JF433290	-
C. spectabilis (*n* = 7)						
	Csp70 (MPEG 58371)	Brazil: Acre	KC858914	KC858895	KC858946	-
	Csp786 (MPEG 61256)	Brazil: Acre	KC858915	KC858896	KC858947	KC858932
	Csp789 (MPEG 61254)	Brazil: Acre	KC858916	KC858897	KC858948	KC858933
	Csp791 (MPEG 61255)	Brazil: Acre	KC858917	KC858898	KC858949	KC858934
	Csp846 (MPEG 61257)	Brazil: Acre	KC858918	KC858899	KC858950	KC858935
	LSUMNS 45460*	Peru: Madre de Díos	-	-	JF433281	-
	LSUMNS 10664*	Peru: Ucayalí	-	-	JF433280	JF433138
C. undatus (*n* = 4)						
	Cun228 (MPEG 61715)	Brazil: Pará	KC858919	KC858900	KC858951	-
	Cun229 (MPEG 61716)	Brazil: Pará	KC858920	KC858901	KC858952	KC858936
	KUNHM 5765*	Guyana	-	-	JF433267	JF433143
	KUNHM 5829*	Guyana	-	-	JF433266	JF433142
C. grammicus (*n* = 14)						
	Cgr74 (MPEG 57567)	Brazil: Amazonas	KC858921	KC858902	KC858953	-
	Cgr89 (MPEG 59384)	Brazil: Amazonas	KC858922	KC858903	KC858954	KC858937
	Cgr102 (MPEG 57021)	Brazil: Amazonas	KC858923	KC858904	KC858955	KC858938
	Cgr103 (MPEG 57020)	Brazil: Amazonas	KC858924	KC858905	KC858956	KC858939
	Cgr168 (MPEG 62597)	Brazil: Amazonas	KC858925	KC858906	KC858957	KC858940
	Cgr180 (MPEG 62598)	Brazil: Amazonas	KC858926	KC858907	KC858958	KC858941
	Cgr376 (MPEG 59385)	Brazil: Amazonas	KC858927	KC858908	KC858959	KC858942
	Cgr380 (MPEG 62599)	Brazil: Amazonas	KC858928	KC858909	KC858960	-
	Cgr613 (MPEG 58677)	Brazil: Amazonas	KC858929	KC858910	KC858961	-
	ANSP 2477*	Ecuador: Morona-Santiago	-	-	JF433272	-
	LSUMNS 6892*	Peru: Loreto	-	-	JF433271	JF433140
	FMNH 389782*	Brazil: Rondônia	-	-	JF433270	JF433141
	LSUMNS 105252*	Bolivia: Santa Cruz	-	-	JF433269	-
	ANSP 3253*	Ecuador: Napo	-	-	JF433268	-
C. elegans (*n* = 3)						
	KUNHM 5764*	Guyana	-	-	JF433261	-
	LSU 4364**	Peru: Loredo	AY940795	-	-	-
	ANSP 4364*	Peru: Loredo	-	-	-	JF433131

*sequences obtained from Benz and Robbins (2011). ** sequences obtained from Webb and Moore (2005).

merase (5 U/μL, *Taq* DNA Polymerase, Recombinant - Invitrogen) and sterile distilled water to complete the final reaction volume. The PCR for each of the genetic markers was run in a thermocycler (GeneAmp, PCR System 9700 - Applied Biosystems). For the mitochondrial markers (rDNA 16S, Cyt *b*, and ND2), the protocol began with 3 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, one minunte at 55 °C, two min at 72 °C, with a final extension of 7 min at 72 °C. For the nuclear fragment (I7BF), the protocol was 3 min at 94 °C, followed by 35 cycles of 20 s at

94 °C, 15 s at 50 °C, and 1 min at 72 °C, with a final extension of 7 min at 72 °C.

The amplified products were purified using an ExoSAP-IT kit (Amersham Pharmacia Biotech. Inc., UK). The purified samples were used for the sequencing reaction, using the dideoxy-terminal method (Sanger *et al.*, 1977) with reagents of the Big Dye kit (ABI Prism™ Dye Terminator Cycle Sequencing Ready Reaction - Applied Biosystems, USA), following the manufacturer's instructions. Reagents not incorporated during the reaction were eliminated through ethanol washes. The gene fragments resulting from this sequencing reaction had their nucleotide sequences determined by an ABI 3500 automatic sequencer (Applied Biosystems). Sequences obtained from GenBank for the Cyt *b* (Webb and Moore, 2005), ND2, and I7BF segments (Benz and Robbins, 2011) were also used in the present analysis (see Table 1 for access numbers).

Sequence alignment

The sequences obtained by electrophoresis were aligned automatically using the CLUSTAL-W application (Thompson *et al.*, 1994), with the parameters suggested by Schneider (2007). The file generated by this procedure was converted into the FASTA format and transferred to the BioEdit sequence editor (Hall, 1999) for visual inspection of the alignment and possible correction of the codification of observed insertions or suppressions. The nucleotide composition, transition/transversion rates, polymorphic sites, and divergence rates (P distances) within and between species were calculated using the MEGA software, version 4.0. (Tamura *et al.*, 2007). The DnaSP program, version 5.10 (Librado and Rozas, 2009) was used to determine the haplotypes obtained, and the Network program, version 4.6 (Bandelt *et al.*, 1999) was used to produce a haplotype network.

Statistical analyses

The best fitting evolutionary model for each separate gene region and the concatenated data was determined by the Akaike Information Criterion (AIC), run in jModeltest 0.1.1 (Posada, 2008). The phylogenetic arrangements of the species were obtained using the PAUP* program, v. 4.0b 10 (Swofford, 2002), for the production of maximum parsimony (MP) and maximum likelihood (ML) trees, with the bootstrap support for nodes being based on 1000 pseudo-replicates (Felsenstein, 1985), which provides an estimate of the confidence limits for the arrangement of each tree. The Bayesian Inference analysis was run in MrBayes v3.0b4 (Huelsenbeck and Ronquist, 2001) with three heated chains and one "cold" chain, each with five million generations, sampled every 100 generations, with the application of the stopping rule command. The sequences of the species *Celeus elegans* - Chestnut Woodpecker were obtained from GenBank (Table 1) for use as the outgroup to root the phylogenetic arrangements.

The BEAST 1.7.2 program (Drummond and Rambaut, 2007) was used to visualize the phylogenetic relationships among the different taxa by generating species trees using the *BEAST procedure (Heled and Drummond, 2010), with 60 million generations. This approach is based on the prior selection of the most appropriate evolutionary model for each gene, which is adapted to the database for the generation of the species tree. This approach contrasts with the simple concatenation of the different models, with only a single model being selected for the concatenated data. The results of this analysis were visualized in Tracer v. 1.5 (Drummond and Rambaut, 2007) to determine the quality of the Markov chain search process. The trees were visualized and edited in FigTree v. 1.3.1 (Drummond and Rambaut, 2007). The divergence time among the *Celeus* species was estimated based on a relaxed molecular-clock analysis of the species tree, assuming a 2.1% nucleotide substitution rate per million years for Cyt *b* (Weir and Schluter, 2008), using the *BEAST methodology implemented in BEAST 1.7.2 software.

Results

A total of 3113 base pairs were sequenced, of which 2234 belonged to the mitochondrial markers (ND2, Cyt *b*, and 16S), while the other 879 represent the nuclear segment, I7BF. The analyses were complemented by sequences obtained from GenBank, belonging to the four species analyzed here, together with *C. elegans* (Webb and Moore, 2005; Benz and Robbins, 2011), which was used as the outgroup for the phylogenetic analyses. No evidence was found that the mitochondrial segments analyzed here may have been nuclear copies (NUMTs) of the mitochondrial genome, based on standard analytical criteria (Lacerda *et al.*, 2007; Rêgo *et al.*, 2010).

The nucleotide composition of the mitochondrial sequences was broadly similar among the four species, with an AT content of 51% for the mitochondrial sequences and 64% for the nuclear ones. Most changes in the mitochondrial sequences were transitions, at rates four to ten times higher than those recorded for transversions, indicating a lack of saturation in the sequences. This ratio was more balanced (ts/tv = 1.7) for the nuclear I7BF segment. A total of 28 amino-acid substitutions were recorded for the two coding regions (Cyt *b* and ND2). Of these, 10 were exclusive to *C. obrieni* and *C. spectabilis.*

A total of 66 variable sites were identified for Cyt *b*, 97 for ND2, 16 for 16S, and nine for I7BF in the 19 *Celeus* specimens examined. In all, 170 of these 188 sites were informative for phylogenetic analysis. There were 36 variable sites between *C. obrieni* and *C. spectabilis*, of which 26 were informative for parsimony analysis, with ND2 and Cyt*b*, once again, being the most variable.

The genetic divergence values (p distance) recorded within and between species for the four gene segments are presented in Tables 2 and 3. Within taxa, the greatest diver-

Table 2 - Mean pairwise distances (p distances) and standard deviations for *Cyt b* (752 bps; below the diagonal) and ND2 (1041 bps; above the diagonal) for the four *Celeus* species analyzed in the present study. Intraspecific distances in parentheses.

	C. obrieni (0.002 ± 0.001)	*C. spectabilis* (0.002 ± 0.001)	*C. undatus* (0.007 ± 0.002)	*C. grammicus* (0.002 ± 0.001)
C. obrieni (0.000 ± 0.000)	-	0.012 ± 0.003	0.073 ± 0.007	0.075 ± 0.007
C. spectabilis (0.003 ± 0.001)	0.013 ± 0.004	-	0.072 ± 0.007	0.075 ± 0.008
C. undatus (0.000 ± 0.000)	0.076 ± 0.009	0.068 ± 0.009	-	0.007 ± 0.002
C. grammicus (0.005 ± 0.002)	0.077 ± 0.009	0.069 ± 0.009	0.005 ± 0.002	-

Table 3 - Mean pairwise distances (p distances) and standard deviations for 16S (441 bps; below the diagonal) and I7BF (879 bps; above the diagonal) for the four *Celeus* species analyzed in the present study. Intraspecific distances in parentheses.

	C. obrieni (0.001 ± 0.001)	*C. spectabilis* (0.000 ± 0.000)	*C. undatus* (0.000 ± 0.000)	*C. grammicus* (0.001 ± 0.000)
C. obrieni (0.000 ± 0.000)	-	0.002 ± 0.001	0.004 ± 0.002	0.004 ± 0.002
C. spectabilis (0.000 ± 0.000)	0.005 ± 0.003	-	0.002 ± 0.002	0.002 ± 0.002
C. undatus (0.000 ± 0.000)	0.034 ± 0.008	0.029 ± 0.007	-	0.000 ± 0.000
C. grammicus (0.000 ± 0.000)	0.036 ± 0.008	0.032 ± 0.008	0.002 ± .002	-

gence was observed in Cyt *b* and ND2, with lower levels being observed in 16S and I7BF. Divergence between *C. obrieni* and *C. spectabilis* was much higher for Cyt *b* (1.3%) and ND2 (1.2%) than that observed between *C. undatus* and *C. grammicus* - Cyt b (0.5%) and ND2 (0.7%). The haplotype network for Cyt *b* and ND2 indicated a clear differentiation between *C. obrieni* and *C. spectabilis*, with no shared haplotypes (Figure 2). As expected for a relatively well-conserved marker, the 16S fragment presented only a single haplotype for each species, separated by two mutations.

The most appropriate evolutionary models selected by the jModeltest program for the Bayesian Inference (BI) criterion of the four markers were HKY (rRNA 16S), TPM3uf+G (Cyt *b*), HKY+I (ND2), and F81 (I7BF). The topology obtained for the species tree indicated a high degree of statistical support for both the *C. obrieni* / *C. spectabilis* and the *C. undatus* / *C. grammicus* groupings (Figure 3). Similarly, the phylogenetic analyses of the concatenated data provided by the MP, ML, and BI approaches confirmed the results of the species tree, particularly the distinction between *C. obrieni* and *C. spectabilis*, which represent two reciprocally monophyletic groups, with branch support values of over 95% in all the analyses (data not shown). The estimated divergence time obtained based on the Cyt *b* marker indicated that the cladogenesis of *C. obrieni* and *C. spectabilis* was probably completed between 400,000 and 800,000 years ago, which coincides with the mid-Pleistocene (Figure 3).

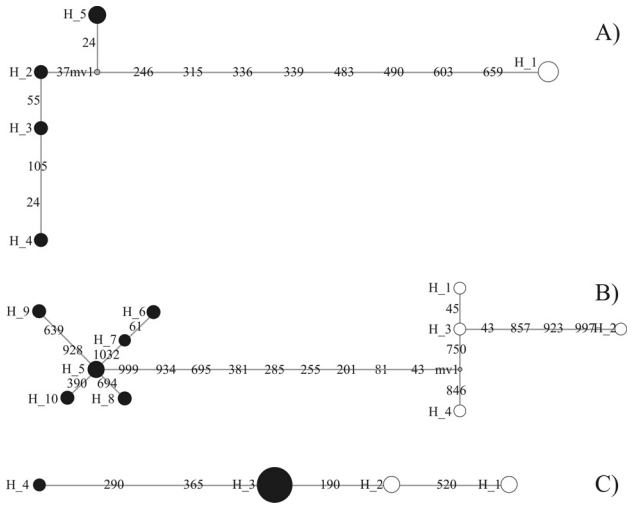

Figure 2 - Haplotype networks for *Celeus obrieni* (black circles) and *Celeus spectabilis* (white circles) for three molecular markers - Cyt *b* (A), ND2 (B), and I7BF (C). Haplotypes are represented by circles, the sizes of which are proportional to their frequencies. Numbers on the lines that join the circles correspond to the positions of the divergent nucleotides in the region studied.

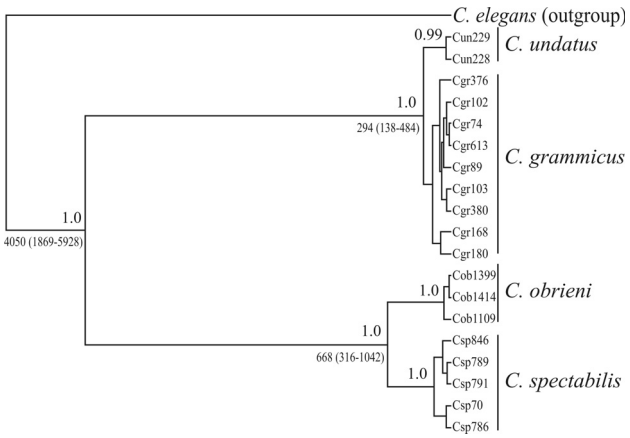

Figure 3 - Bayesian Inference for the species tree based on the mitochondrial and nuclear data run in *BEAST. Posterior probability values are shown above branches, whereas divergence times from the common ancestor are shown below branches.

Discussion

The conservation of species is often hampered by the uncertain taxonomic status of different forms, and the reliable definition of such status is a primary preoccupation in conservation biology (Assis *et al.*, 2006). The use of molecular techniques is fundamental to the analysis of the taxonomic status of most uncertain forms (Silveira and Olmos, 2007; Lacerda *et al.*, 2007), although this approach is limited by the need for an adequate supply of biological material from which DNA can be isolated. *Celeus obrieni* presents a good example of the importance of this approach, given that the existence of a single specimen, deposited at the American Museum of Natural History in New York in 1926, was used for the confirmation of the species status of this form in relation to *C. spectabilis* (Benz and Robbins, 2011). However, the availability of only a single specimen from almost a century ago led these authors to emphasize the need for a larger sample in order to confirm the reciprocal monophyly of these forms, and the reliability of the evolutionary lineages identified. The present study provides additional support for the status of *C. obrieni* as a valid species, distinct from *C. spectabilis*, thus reconfirming the findings of Benz and Robbins (2011). The present findings were based on a larger number of *C. obrieni* specimens, with the results of all the analyses - intra- and interspecific divergence values, haplotype networks, and species trees - confirming reciprocal monophyly between *C. obrieni* and *C. spectabilis*, with a high degree of statistical support.

In addition, the comparative analysis indicated a greater degree of divergence between *C. obrieni* and *C. spectabilis* in comparison with *C. undatus* and *C. grammicus*, and the species tree (Figure 1) was consistent with the complete separation of these four taxa. These analyses, however, did not include the specimens used by Benz and Robbins (2011), given that sequences for the genetic markers used in both studies were not available for all the specimens.

One important detail in the results of Benz and Robbins (2011) is that the mtDNA divergence value within *C. grammicus* was larger than those recorded between the *grammicus* and *undatus* forms, resulting in a paraphyletic arrangement. Nonetheless, a number of substitutions observed in the nuclear loci indicated a different arrangement, in which the taxa were clearly separated, indicating the possibility that the mtDNA data were the result of introgression. Given this, Benz and Robbins (2011) indicated the need for a more definitive evaluation of the taxonomic arrangement of this group (*grammicus-undatus*) based on a larger sample from a wider range of populations and a larger number of independent genetic markers for the evaluation of possible gene flow, especially in the western Amazonian populations of *grammicus*, and in the eastern contact zone mentioned above, to provide a more conclusive assessment of the status of these taxa. The low distance values separating *undatus* and *grammicus* that we found also call into question the specific distinctiveness of these two taxa.

While *C. obrieni* had long been considered a subspecies of *C. spectabilis* (Short, 1982), based on the availability of a single specimen, subsequent morphological and ecological data indicated that *C. obrieni* and *C. spectabilis* are distinct, albeit closely-related species (Santos and Vasconcelos, 2007; Remsen *et al.*, 2012). The geographic distribution of the two species is probably related to the availability of habitats with bamboo (Pinheiro *et al.*, 2012). In the western Amazon basin, *C. spectabilis* is dependent on patches of humid bamboo forest, in which it forages and reproduces (Kratter, 1997, 1998; Whittaker and Oren, 1999; Guilherme and Santos, 2009). Tobias *et al.* (2006) also highlight that the known geographic range of *C. obrieni* (from the type specimen) coincided with open and forested Cerrado habitats, interspersed with narrow and discontinuous belts of gallery forest. New observations (Pinheiro and Dornas, 2008; Pinheiro *et al.*, 2012) indicate that this species is specialized for occupation of patches of bamboo habitat within this landscape, especially those dominated by *Guadua paniculata*, in which approximately 98% of the records of *C. obrieni* foraging behavior were obtained at Cerrado sites. The marked dependence of the species on a bamboo habitat could be related to the dietary preferences of this bird species (Kratter, 1997; Winkler and Christie, 2002). The heterogeneous distribution of *C. obrieni* within the Cerrado may thus be related to this dependence on bamboo habitats, which are relatively widely dispersed within this biome.

The present-day distribution of *C. obrieni* includes the Brazilian states of Goiás, Maranhão, Piauí, and Tocantins (Pinheiro and Dornas, 2008; Dornas *et al.*, 2011), and more recently, Mato Grosso (Pinheiro *et al.*, 2012). This distribution encompasses wooded environments - gallery and semideciduous forests, both with bamboo habitats - within the Cerrado biome, and reduces the distance (to approximately 1000 km) between the known range of *C. obrieni* and the nearest recorded localities for *C. spectabilis* in the Brazilian state of Acre in the southwestern Amazon basin (Guilherme and Santos, 2009). The available data on the distribution of *C. obrieni* and *C. spectabilis* indicate a pattern different from that of other birds dependent on bamboo habitats, such as *Syndactyla ucayalae, Anabazenops dorsalis, Cercomacra manu, Drymophila devillei*, and *Ramphotrigon megacephalum*, whose stronghold lies in southwestern Amazonia (Kratter, 1997). When they occur outside this area, these species tend to be associated with minor tracts of bamboo habitats, which form a diagonal from southwestern Amazonia, passing through the states of Rondônia and Mato Grosso to southeastern Pará, in the region of Carajás, reaching even the Belém area of endemism in easternmost Amazonia (Parker *et al.*, 1997; Lees *et al*., in press). Unlike those aforementioned species, which are lo-

cally but continuously distributed in this area, the distributions of *C. obrieni* and *C. spectabilis* appear to be truly disjunct, with the latter restricted to western Amazonia and the former to its eastern portion (Kratter, 1997). If this patterns holds, the allopatry of *C. obrieni* and *C. spectabilis* may help explain the comparative divergent phenotypes and genotypes when compared to another phylogenetically close sister species pair of *Celeus,* such as *C. undatus* and *C. grammicus*.

The evidence suggests that in the past, these open bamboo forests were more widely distributed than in the present day, extending eastwards towards the Cerrado, with offshoots that reached as far south as the Atlantic Forest of southeastern Brazil. This major corridor of open forest habitats would provide a historic connection between the geographic ranges of the two species. According to the molecular clock analysis, the cladogenetic event that led to the separation of *C. obrieni* and *C. spectabilis* took place during the climatic fluctuations and associated habitat modifications of the mid Pleistocene. It is worthy of note that the 2.1% nucleotide substitution rate used in the present study is based on average across many unrelated taxa (Weir and Schluter, 2008), therefore, such conclusions must be interpreted with caution.

The climate cycles and related shifts in the distribution of habitats that occurred during the Pleistocene have been identified by several biogeographers (Prance, 1987; Haffer, 2001; Silva and Bates, 2002) as the primary factor driving recent speciation processes in both forested and savanna environments in South America. In particular, the climatic fluctuations of the mid-Pleistocene would have provoked a reduction and fragmentation of the forest cover, leading to the isolation of the ancestral populations of *C. obrieni* and *C. spectabilis*. The geomorphological evidence indicates that a single major refuge would have formed in the center of the present-day distribution of the Cerrado biome during this period, when even gallery forests may have shrunk to the point of interrupting the connection with adjacent areas of forest (Ab'Saber, 1983). The subsequent warming of the climate would have led to an expansion of the forest cover, expanding from the refugia to more peripheral areas, and re-establishing the connectivity of the gallery forests.

Because the latest Brazilian checklist of endangered species was completed in 2003, before Kaempfer's Woodpecker was rediscovered, it is currently not listed as a threatened species in Brazil, but the IUCN lists it globally as critically endangered (BirdLife International, 2011). However, the recent extensions reported in the known geographic range of the species, which now includes Mato Grosso (Dornas *et al.*, 2011), may contribute to a re-evaluation of the species' status in the near future (IUCN, 2010). On the other hand, Pinheiro *et al.* (2012) have concluded that the specialization of *C. obrieni* for foraging in *G. paniculata* bamboo patches within the Cerrado may determine its low population densities and its high degree of intolerance of anthropogenic impacts. This would make the species vulnerable to extinction, especially in the current context of agricultural expansion in the Brazilian Cerrado, where large tracts of natural habitat are being converted into soybean and rice plantations (Silva and Bates, 2002). The destruction of local ecosystems has accelerated considerably in recent years (Klink and Machado, 2005). Over the short term, the development of effective conservation strategies for *C. obrieni* will depend on the collection of more reliable data on population size and viability, for which molecular markers will provide a fundamentally important analytical tool.

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