ORNITOLOGIA NEOTROPICAL 21: 271–281, 2010


# GENETIC VARIABILITY AND STRUCTURE OF OILBIRD (*STEATORNIS CARIPENSIS*) COLONIES IN ANTIOQUIA, COLOMBIA

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**Resumen. – Variabilidad y estructura genética de colonias de guácharos (*Steatornis caripensis*) en Antioquia, Colombia. –** El guácharo (*Steatornis caripensis*) es la única ave frugívora nocturna del mundo, y también es peculiar en que los individuos viven en cavernas y utilizan ecolocalización. Esta especie ocurre en el norte de Sur América y aunque no ha sido catalogada como amenazada, sus características de historia natural únicas pueden hacer que las poblaciones sean más vulnerables a los impactos antrópicos sobre los bosques donde forrajean. Nosotros evaluamos la diversidad genética de cuatro colonias de guácharos en el Magdalena Medio en el departamento de Antioquia secuenciando el gen mitocondrial (mt) NADH deshidrogenasa subunidad 2 (ND2), y evaluando la variabilidad en marcadores RAPD, usando muestras de 59 individuos. Aproximadamente el 17% de los marcadores RAPD mostraron polimorfismo, con evidencia de estructura entre colonias que estaban separadas por 70 Km. En los especimenes analizados encontramos seis haplotipos de ND2, siendo polimórficas seis de las 1041 bases secuenciadas. Se encontró una riqueza haplotípica alta en cada una de las cuatro colonias sin evidencia de estructura entre los sitios. Los resultados sugieren que a pesar de que viven en colonias discretas, estos grupos de guácharos no han sido aislados reproductivamente, con niveles históricos de flujo génico significativos inclusive entre las colonias más distantes. Sin embargo, los bajos niveles totales de variabilidad genética, especialmente en el locus de mtADN, sugieren que la fragmentación de los bosques durante el pasado siglo han aislado las colonias, reduciendo sus tamaños efectivos de población y contribuyendo a la deriva. Las persistentes altas tasas de deforestación en esta región pueden tener efectos a largo plazo sobre la genética de estos demes de esta especie única.

**Abstract. –** The Oilbird (*Steatornis caripensis*) is the only frugivorous nocturnal bird in the world and is also peculiar in that individuals roost in caves and use echolocation. The species occurs in northern South America and although it has not been catalogued as threatened, its unique life history characteristics may make its populations more vulnerable to human impacts on the forests where they forage. We assessed the genetic diversity of four oilbird colonies in the middle Magdalena River drainage in the Antioquia Department of Colombia by sequencing the mitochondrial (mt) NADH dehydrogenase gene subunit 2 (ND2), and also assessing variability in RAPD markers, using samples from a total of 59 individuals. Approximately 17% of the RAPD markers exhibited polymorphism, with evidence of structure among populations that were separated by 70 km. Six different ND2 haplotypes were found in the specimens analyzed, with 6 of 1041 bp polymorphic. Haplotype richness was high in each of the four colonies with no evidence of structure among the sites. The results suggest that despite their living in discrete colonies, these oilbird groups have not been reproductively isolated, with significant historical levels of gene flow between even the most distant colonies. However, the overall low levels of genetic variability, especially in the mtDNA locus, suggest forest fragmentation during the past century may have isolated the colonies, reducing their effective population sizes and contributing to drift. Continuing high rates of defor-

estation in this region may have long term effects on the genetics of these demes of this unique species.
*Accepted 18 May 2010.*

**Key words:** Oilbirds, colonies, mtDNA, RAPD, population structure, genetic variability.

## INTRODUCTION

The Oilbird (*Steatornis caripensis)* is a unique species, being the only nocturnally frugivorous cave-dwelling bird in the world. It also is a phylogenetically important species, since it is the only living member of the family Steathornithidae (Thomas 1999), with various molecular analyses concordant in showing its deep phylogenetic uniqueness (Mariaux & Braun 1996, Larsen *et al.* 2007, Braun & Huddleston 2009, Han *et al.* 2010). In addition, the species is unusual for its echolocation abilities, which only occurs in oilbirds and Indoaustralian swiftlets (Family Apodidae) (Thomassen *et al.* 2005).

Oilbirds breed in northern South America from northern Guyana, Trinidad, and Venezuela along the Andes in Colombia, Ecuador, and Peru into Bolivia (Thomas 1999). In Colombia, it can be found throughout the middle Magdalena River drainage in the departments of Antioquia and Santander. In 1998, the forests in the middle Magdalena River drainage had already been reduced by 35% (Etter *et al.* 2006). The deforestation rate there over the past two decades has been 158.4 $km^2$/year (Melo & Ochoa 2002), and if this rate is maintained, all forest in the region will disappear during the next 20 to 30 years.

This represents a threat to the oilbirds there that require large expanses of forest where ripe fruit is available year round, as well as caves or gorges for roosting and nesting sites (Hilty & Brown 1986, Thomas 1999). While oilbirds have a broad diet, they may fly up to 240 km in one night while foraging (Roca 1994). In Venezuela, oilbirds have been reported to perform limited migrations during the non-breeding season as well, presumably in response to deteriorating foraging conditions near the breeding cave, but individuals return to concentrate around the cave each breeding season (Bosque & Ramirez 1988). In contrast, observations by Snow (1961) of oilbird colonies in Trinidad indicated that in this population individuals remained near the same cave year-round and exhibited natal site philopatry.

The observations of fidelity to specific caves (possible natal caves) and the monogamous mating system of oilbirds suggest that each cave colony could be genetically isolated from others. But an inspection for genetic structure among colonies in Venezuela using mtDNA RFLPs (Gutierrez 1994) indicated that there was low overall genetic diversity (possibly due to a recent bottleneck), with high genetic similarity among colonies, suggesting substantial gene flow between them. Thus, observations of behaviour in one location and genetic analysis in another have produced disparate conclusions concerning patterns of oilbird movements and population structure.

The deforestation that has occurred in the middle Magdalena River drainage in Colombia may have influenced oilbird population structure there in one of two ways. If oilbirds avoid dispersing across open areas while foraging, habitat fragmentation may have led to an isolation of demes associated with particular caves located in different forest patches. But if oilbirds do readily traverse deforested areas, habitat alteration might have increased dispersal and the probability of gene flow among caves, as individuals must search more widely for limited food resources in the remaining habitat patches.

It is important to know which of these patterns has occurred in Colombia, because management strategies should consider past

and existing dispersal patterns within and among populations, if the goal is to conserve the genetic diversity within and among these populations of oilbirds (Moritz 1994, Crandall *et al.* 2000). This led us to conduct a study of the levels of genetic variability in, and magnitude of genetic differences among, oilbird colonies occurring in the Antioquia Department, Colombia, using both mtDNA and RAPD markers.

## MATERIALS AND METHODS

*Sample collection and DNA extraction.* Five oilbird colonies are known to occur along a north-south transect along the Cordillera Central in the middle Magdalena River drainage of the Antioquia Department of Colombia. The colonies are separated by distances of 5–40 km, with 60 km separating the most northern and southern caves (Fig. 1). We visited all five colonies and captured oilbirds by placing mist nets in the entrances to the caves during the hours when oilbirds depart to forage (18:00 to 21:00 h). It was logistically impossible to set nets at the second cave (the Nus cave), but we were successful in capturing birds at the Alicante (hereafter referred to as S1), Condor (S3), Danta (S4), and Manantial (S5) sites.

Blood samples were obtained from all captured individuals by venipuncture using sterile 1 $cm^3$ syringes, after which the birds were released. Blood samples were deposited into sterile microcentrifuge tubes containing 1% EDTA and held at 4°C until processed. DNA was extracted using a standard phenol-chloroform method as follows. 30 µl of blood was digested with Proteinase K in digestion buffer (10 mM Tris-HCl, 10 mM EDTA, 50 mM NaCl, and 2% SDS) and incubated at 55°C for 20 hours. The DNA was separated with a mixture of phenol, chloroform and isoamylic alcohol (proportion 25:24:1). The DNA solution was washed with saturated butyl alcohol and precipitated with absolute ethanol. Once the DNA was dry, it was resuspended in 50 µl of TE (50 mM Tris-HCl, pH 8.0; 1 mM EDTA) and stored at -20°C.

*MtDNA sequencing.* The NADH-dehydrogenase subunit 2 (ND2) gene was chosen for sequencing because it is the third most variable mitochondrial gene, it is relatively long, and it amplifies readily (Sorenson 2003). The entire coding region of the ND2 gene was amplified in two overlapping fragments, which were sequenced and assembled to get the complete ND2 gene sequence (1041 bp). These fragments were amplified with primer pairs L5216-H5766 and L5758-H6313 (Sorenson *et al.* 1999) using the polymerase chain reaction (PCR) in 50 µl reactions with 1X PCR buffer (160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl [pH 8.8], 0.1% Tween 20), 2.5 mM $MgCl_2$, 0.25 mM each dNTP, 0.5 µM each primer, 1.25 units *Taq* DNA polymerase (Bioline Inc., Randolph, MA), and 100 ng of genomic DNA. Fragments were amplified as follows: initial denaturation at 95°C for 5 min followed by 35 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and a final extension of 72°C for 10 min. A negative control was included in each run to test for contamination.

PCR products were resolved on 1% agarose gels containing 1 $\mu g/cm^3$ ethidium bromide and purified using the QIAquick gel extraction kit (QIAGEN Inc., Valencia, CA). Fragments were then sequenced on an ABI 3730xl DNA automated sequencer. Sequences and electropherograms were read and edited manually using Chromas Lite 2.01 (Technelysium Pty Ltd) and aligned with BIOEDIT 7.0.9.0 (Hall 1999).

*Random amplified polymorphic DNA (RAPD).* Genomic DNA was amplified in 25 µl reactions containing 1X PCR buffer (160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl [pH 8.8], 0.1%

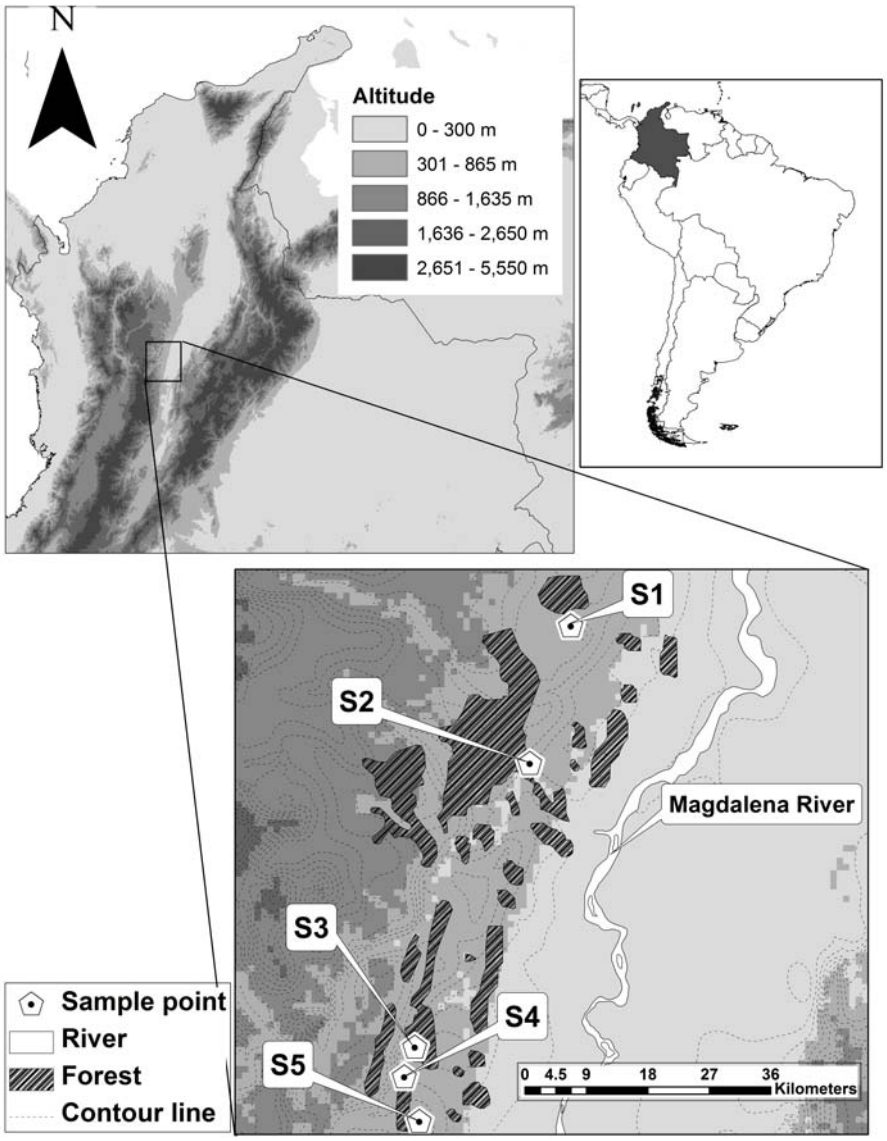


FIG. 1. Map showing the locations of the oilbird colonies examined in this study. Exact locations were: Alicante (S1; 06°28.92'N, 74°38.01'W), Nus (S2; 06°18.03'N, 74°41.24W), El Condor (S3; 05°55.55'N, 74°50.33'W), Danta (S4; 05°49.67'N, 74°49.98'W), and Manantial (S5; 05°53.20'N, 74°51.17'W).

Tween 20), 3 mM $MgCl_2$, 0.2 mM each dNTP, 1 μM 10-mer primer, and 1 unit *Taq* DNA polymerase (Bioline Inc., Randolph, MA). The cycling program began with an initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 1 min, 36°C for 1 min, 72°C for 2 min, and a final extension step at 72°C for 10 min. A negative control was

added in each run to test for contamination. All PCR reactions were performed on a PTC 200 thermalcycler (MJResearch Inc. USA, Waltham, MA) and special care was taken, performing three independent amplifications for each of the primers used and using only reproducible bands for the analyses. The set of 20 primers analyzed are shown in Table 1. Amplification products were resolved on 1% agarose gels containing 1 $\mu g/cm^3$ ethidium bromide. Gels were run at 80 volts for 1 to 2 hours, depending on the sizes of the fragments. The bands were then visualized under UV light and photographed. Only bands that were reproducible were considered in this study.

*Data analysis.* The following calculations and analyses were performed for both markers using the software Arlequin 3.1 (Excoffier *et al.* 2005). Population differences were assessed calculating the overall and pairwise $F_{ST}$ indices. Slatkin's linearized genetic distances also were calculated, and the significance of the genetic distances and $F_{ST}$ values were tested by 10,000 permutations with a significance level of 0.05. The effective number of migrants also was obtained and an analysis of molecular variance (AMOVA) was conducted using the pairwise distance method. The statistical significance of different sources of variation was estimated using 10,000 random permutations.

For mtDNA data, haplotypes were determined and aligned using DAMBE (Xia & Xie 2001). The software DnaSP (Rozas *et al.* 2003) was used to calculate the following parameters for each population and for all individuals examined: (i) Haplotype (gene) diversity (H) and (ii) Nucleotide diversity (ð).

For RAPD data we assigned a number to each RAPD band and treated them as binary unit characters, 1 (present) or 0 (absent). Polymorphism was calculated as the percentage of polymorphic bands for each population, and for the complete sample. Average gene diversity was calculated over all loci for each population with Arlequin 3.1 (Excoffier *et al.* 2005). The software AFLPsurv (Vekemans *et al.* 2002) was used to calculate 1000 bootstrap matrices of Nei's genetic distances between populations; these matrices were used to draw 1000 trees and to calculate a consensus tree with the software Phylip 3.67 (Felsenstein 1989) using the neighbor-joining method.

## RESULTS

A total of 59 adult oilbirds were sampled (S1, n = 21; S3, n = 11; S4, n = 10; S5, n = 17) and no evidence of contamination of the DNA samples used for either the mtDNA or RAPD analyses was found.

*MtDNA sequence variation and genetic diversity.* Sequences are available on GeneBank, accession numbers EU683820–EU683878. The mitochondrial origin of the sequenced DNA fragment was supported by the absence of stop-codons in the coding gene region (ND2) which is inconsistent with nuclear copies (Zhang & Hewitt 1996, Sorenson & Quinn 1998).

The average base composition in the sequence from the 59 individuals was 32% A, 22% T, 36% C and 10% G. These values are in accordance with the base composition reported for the ND2 gene of *Gallus gallus* by Desjardins & Morais (1990) (GenBank accession number NC001323). Approximately 0.58% of the surveyed region was found to be variable (six out of 1041 base pairs). All of these changes were transitions, with no transversions in the analyzed sequence (Table 2).

A total of six haplotypes were identified in the 59 individuals analyzed. None of the haplotypes were private, but varied from being present in two to 18 individuals and in two to four populations. Populations differed signifi-

cantly in haplotype frequencies (Table 3).

TABLE 1. Sequences of RAPD primers and total number of bands analyzed. References: [1]Paterson & Snyder 1999; [2]Nusser *et al.* 1996; [3] Baublys *et al.* 2002; [4] Gauer & Cavalli-Molina 2000.

| Primer | Sequence (5´→3´) | Total no. of scorable bands |
|---|---|---|
| UBC 376[1] | CAGGACATCG | 8 |
| UBC 389[1] | CGCCCGCAGT | 12 |
| UBC 370[1] | TCAGCCAGCG | 9 |
| UBC 489[2] | CGCACGCACA | 11 |
| UBC 412[2] | TGCGCCGGTG | 13 |
| UBC 283[2] | CGGCCACCGT | 14 |
| UBC 237[2] | CGACCAGAGC | 11 |
| OL3[3] | CTCACCCGTC | 7 |
| OL4[3] | CAATCGCCGT | 12 |
| OL5[3] | CAAACGTCGG | 7 |
| OL6[3] | GTCCACACGG | 8 |
| OL7[3] | ACGCCGTACG | 10 |
| OL9[3] | TCCGCTCTGG | 9 |
| OL12[3] | GATGACCGCC | 9 |
| OPA01[4] | CAGGCCCTTC | 9 |
| OPF01[4] | ACGCATCCTG | 8 |
| OPH03[4] | AGACGTCCAC | 7 |
| OPH13[4] | GACGCCACAC | 9 |
| OPH18[4] | GAATCGGCCA | 7 |
| OPH19[4] | CTGACCAGCC | 9 |
| Total | | 189 |

Haplotype richness (number of haplotypes found/number of individuals sampled) was lowest in S1 (0.2381) and highest for S3 (0.4545). Haplotype and nucleotide diversity did not differ markedly among populations, with haplotype diversity ranging from 0.733 to 0.787, while nucleotide diversity values ranged from 0.00175 to 0.00241, with S4 exhibiting the lowest values for these two parameters.

*RAPD polymorphism.* A total of 189 bands were amplified from the RAPD-PCR reactions, 33 of which were polymorphic. The sizes of the bands were between 230 and 2500 base pairs. Each individual possessed a unique RAPD phenotype over the 33 polymorphic bands. Percent polymorphic loci in each population ranged from approximately 15% in S1 (28 polymorphic bands) to 16,5% in S5 (31 polymorphic bands). Only one marker was found exclusively in one population (S5), but it occurred in low frequency (only two individuals); all other polymorphic markers were shared by at least two populations. Mean genetic diversity in each population ranged from 0.0508 to 0.0589, averaging 0.0560 for the overall population (Table 4).

*Population structure and gene flow.* The two molecular markers yielded contrasting results. The mtDNA data showed no significant overall or specific pairwise $F_{ST}$ values, and the AMOVA analysis revealed that all variation was due to variation within populations. In contrast, RAPD markers yielded a significant $F_{ST}$ of 0.0586, with non-significant pairwise comparisons among S3, S4, and S5, but significant $F_{ST}$ values for the comparisons of S1 with these latter three sites. The AMOVA analysis considering S1 and S5 as separate groups showed that approximately 13% of the variation was due to variation among these two populations and 87% to within population variation.

Slatkin's linearized genetic distances also were not significant for the mtDNA dataset and the number of migrants per generation (Nm) between all pairs of populations was infinite, but for the RAPD markers, we found significant genetic distances between populations S1–S3, S1–S4, and S1–S5 (Table 5). Nm was approximately 4, 3, and 2 between the pairs S1 S3, S1 S4, and S1 S5, respectively. The consensus tree using genetic distances also showed that S1 was the most differentiated population, forming a separate branch (100% consensus). Also, 89% of the trees showed that populations S3 and S5 were the most genetically similar.

TABLE 2. Values of polymorphism parameters found in the mitochondrial gene ND2 in each population.

| Population | No. individuals | No. polymorphic bases | % polymorphic bases | No. synonymous changes | No. non-synonymous changes |
|---|---|---|---|---|---|
| S1 | 21 | 5 | 0.48% | 3 | 2 |
| S2 | 11 | 6 | 0.58% | 4 | 2 |
| S3 | 10 | 5 | 0.48% | 3 | 2 |
| S4 | 17 | 5 | 0.48% | 3 | 2 |
| Total | 59 | 6 | 0.58% | 4 | 2 |

## DISCUSSION

Genetic variability in the mtDNA ND2 gene was low for the populations of oilbirds analyzed, since only six haplotypes were found (a haplotype richness of 0.1), with only six polymorphic sites in a total of 1041 bp. Other species of birds have shown higher levels of polymorphism and haplotype diversity for this gene. Threatened populations of Dupont's Lark (*Chersophilus duponti*) from Spain were found to have 16 polymorphic sites within the entire 1041 base pair sequence of the ND2 gene, with 10 haplotypes documented in the population (García *et al.* 2008). Likewise, 18 variable sites were found in introduced populations of the House Finch (*Carpodacus mexicanus*) in North America, with 30 haplotypes present despite having presumably experienced a founder effect upon colonization (Hawley *et al.* 2008). In the Vitelline Warbler (*Dendroica vitellina*) of the Cayman Islands, 11 polymorphic sites were found in the ND2 gene despite having sampled only 28 individuals (Markland & Lovette 2005). Gutiérrez (1994) found 12 haplotypes in four Venezuelan populations of *S. caripensis* and suggested they may have experienced past bottlenecks.

The RAPD markers in this study also showed low levels of variability, as compared to results of studies of other bird populations. RAPD polymorphism levels as high as 85.96% have been reported for the Amazonian Rufous Gnateater (*Conopophaga lineata*) (Dantas *et al.* 2007) and 74% in a population of Common Loon (*Gavia immer*) in the USA (Dhar *et al.* 1997). Other studies report values only slightly higher than the 17% we documented, including 21.6% for Australian brown bandicoot (*Isoodon obesulus*) (Cooper 2000) and 28% for Leach's Storm Petrel (*Oceanodroma leucorhoa*) (Patterson & Snyder 1999).

Although both markers revealed relatively low levels of genetic variability in the individuals sampled, they produced incongruent results with respect to the existence of population structure. While the mtDNA analyses produced no evidence of genetic differences among the sites, implying panmixia, the RAPD data documented significant genetic differences among S1 and the remaining three sites sampled, consistent with an isolation-by-distance model. The lack of evidence for genetic structure in the distribution of ND2 haplotypes could be related to its overall low amount of variation, reducing its power to detect structure even if it occurs. The sample sizes per site were low, so it is possible that we failed to detect private alleles that provide the finest scale information on population structure. The same pattern of lower genetic variability in mtDNA and higher genetic variability in nuclear markers (microsatellites) has been shown for the endangered Spanish Imperial Eagle (*Aquila adalberti*) in a severely fragmented population (Martínez-Cruz *et al.* 2004).

TABLE 3. Variable nucleotide sites within the 1041 bp of the ND2 gene sequenced in this study organized in haplotypes and their frequencies in the populations sampled.

| Haplotype | Position in ND2 coding sequence | | | | | | Frequency in populations | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 240 | 462 | 810 | 876 | 997 | 1033 | S1 | S3 | S4 | S5 | Total |
| 1 | C | A | C | C | A | A | 3 | 1 | 0 | 5 | 9 |
| 2 | C | A | T | T | G | G | 3 | 0 | 2 | 1 | 6 |
| 3 | T | A | T | T | G | G | 5 | 5 | 2 | 6 | 18 |
| 4 | C | A | T | C | A | A | 2 | 2 | 0 | 2 | 6 |
| 5 | C | G | T | C | A | A | 0 | 1 | 1 | 0 | 2 |
| 6 | C | A | T | T | A | A | 8 | 2 | 5 | 3 | 18 |

TABLE 4. Number of RAPD markers found in each colony and the whole population. Standard deviation is shown in parentheses.

| Population | No. markers | No. polymorphic markers | % polymorphism | Expected heterozygosity |
|---|---|---|---|---|
| S1 | 187 | 28 | 14.9733 | 0.0508 (0.0271) |
| S3 | 187 | 29 | 15.5080 | 0.0589 (0.0327) |
| S4 | 187 | 29 | 15.5080 | 0.0558 (0.0315) |
| S5 | 188 | 31 | 16.4894 | 0.0524 (0.0282) |
| Total | 189 | 33 | 17.4603 | 0.0560 (0.0287) |

Another hypothesis to explain the lower genetic variation, and hence lack of evidence for genetic structure, in the mitochondrial ND2 gene is that rare private alleles at this locus have recently been eliminated due to genetic drift. If gene flow among colonies is mediated by individuals encountering new caves, or encountering other individuals and following them to new caves, during foraging excursions, then the deforestation this region has experienced over the past century may have reduced the rate of gene flow among sub-populations and reduced their effective population sizes. Bottlenecks should reduce the number of mitochondrial alleles more rapidly that nuclear loci because mtDNA has a four-fold lower effective population size, thanks to its haploidy and homoplasy (Moore 1995). A number of other species also have shown low levels of mtDNA variation, as compared to their nuclear DNA (Hoelzel & Dover 1991, Martínez-Cruz *et al.* 2004).

Our data do not permit an examination of whether males, females, or both have been responsible for the historical levels of gene flow among these colonies, but we found no evidence of natal site philopatry to specific caves, as Snow (1961) reported for oilbird colonies on Trinidad (based on observations of marked individuals). However, mark-recapture studies might easily miss recording rare cases of exchange of individuals between breeding caves that nevertheless would be sufficient to prevent their genetic divergence (Hanski 1999).

In Colombia, *S. caripensis* is not considered threatened (Renjifo *et al.* 2002), but there is only one protected area (Parque Nacional Natural Cueva de los Guácharos) that includes a colony of oilbirds, and the region where most colonies exist (our study area) lacks protection (Instituto Humboldt 1998).

One factor argued to be important in defining priority conservation areas is the presence of species whose unique biology and evolutionary history renders them more susceptible to human impact (Vane-Wright *et al.* 1991, Crozier 1997, Purvis *et al.* 2000, Mace *et al.* 2003). Oilbirds clearly fall into this category, arguing that this region of Colombia should be given some form of protected status in an effort to insure the continued presence of the species given its evolutionary distinctiveness, peculiar life history, and needs for large tracts of forest around its breeding caves.

## ACKNOWLEDGMENTS

This study was made possible by a grant from the Dirección de Investigaciones of the Universidad Nacional de Colombia, Sede Medellín. We thank Isabel Melo, Jose Manuel Ochoa and Samuel Gaviria for help in fieldwork. We thank Professor Néstor Mancera for administrative collaboration and for reviewing the manuscript, Professor Mauricio Camargo of the Laboratory of Population Genetics of the Universidad de Antioquia, where molecular analyses were conducted; and Professor Gabriel Bedoya of the Laboratory of Molecular Genetics of the Universidad de Antioquia, where computational analyses were performed. We also thank Carlos Ortiz for help with the figure.

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