FEATURE ARTICLES

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CONSERVATION PRIORITIES FOR RESPLENDENT QUETZALS BASED ON ANALYSIS OF MITOCHONDRIAL DNA CONTROL-REGION SEQUENCES

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Abstract. The Resplendent Quetzal (*Pharomachrus mocinno*) is a threatened bird species classified into two putative subspecies (*P. m. mocinno* and *P. m. costaricensis*) and distributed in cloud forests of seven countries in Mesoamerica. Because the birds are rare, tissue samples are difficult to obtain, but we analyzed genetic diversity in 25 quetzals from five countries based on 255 bp of domain I of the control region of mitochondrial DNA. Eight haplotypes were detected. Nucleotide diversity for Mexico (*P. m. mocinno*: 0.0021) and Panama (*P. m. costaricensis*: 0.0026) were low, and did not differ from the values estimated for other birds species irrespective of whether they were endangered. A haplotype tree rooted with the Pavonine Quetzal (*P. pavoninus*) recovered two reciprocally monophyletic clades corresponding to each subspecies, so we propose that each subspecies be considered as an evolutionarily significant unit for conservation planning. A minimum spanning network showed the number of genetic differences separating haplotypes within subspecies was small relative to the number of substitutions among them, indicating strong population subdivision (F_{ST} = 0.37). In spite of the limited sampling we propose that in conservation practice Mexico–Guatemala, Nicaragua, El Salvador, and Panama be considered preliminarily as independent conservation management units since they each have unique haplotypes. Additionally, these countries should construct international agreements to protect the natural vegetation corridors among cloud forests of Mesoamerica and to curtail the illegal trade of quetzals.

Key words: evolutionarily significant units, Mesoamerica, mitochondrial DNA, management units, Pharomachrus mocinno.

Prioridades de Conservación para el Quetzal Basadas en el Análisis de la Región Control del ADN Mitocondrial

Resumen. El quetzal (*Pharomachrus mocinno*) es una especie de ave amenazada clasificada en dos subespecies (*P. m. mocinno* y *P. m. costaricensis*) distribuidas en los bosques de niebla de siete países de Mesoamérica. Debido a que ésta es un ave rara, las muestras de tejido son difíciles de obtener, pero pudimos analizar la diversidad genética en 255 pb del dominio I de la región control del ADN mitocondrial en 25 quetzales procedentes de cinco países. Se encontraron ocho haplotipos. La diversidad nucleotídica para México (*P. m. mocinno*: 0.0021) y Panamá (*P. m. costaricensis*: 0.0026) fue baja, pero no difiere de la estimada para otras especies de aves amenazadas o no amenazadas. El árbol de haplotipos enraizado con *P. pavoninus* mostró dos clados recíprocamente monofiléticos, correspon-

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diendo cada uno a cada subespecie, por lo que proponemos que para planes de conservación cada subespecie sea considerada como unidad evolutiva significativa independiente. Una red de distancias mínimas mostró que el número de diferencias genéticas que separa a los haplotipos dentro de las subespecies fue pequeño con respecto al número de sustituciones que existe entre ellas, indicando una fuerte división poblacional (F_{ST} = 0.37). Considerando nuestro muestreo limitado proponemos que para fines de conservación prácticos México–Guatemala, Nicaragua, El Salvador y Panamá sean considerados preliminarmente como unidades de manejo independientes ya que éstos presentan haplotipos únicos no compartidos entre localidades. Además, estos países deberían firmar acuerdos internacionales para proteger los corredores de vegetación naturales entre los bosques de niebla de Mesoamérica y tratar de reducir el comercio ilegal de los quetzales.

INTRODUCTION

The worldwide distribution of the Resplendent Quetzal (*Pharomachrus mocinno*) ranges from southern Mexico across Guatemala, Honduras, El Salvador, Nicaragua, Costa Rica, and Panama. However, the distribution of quetzals is not continuous through these countries, but is determined by the patchiness of cloud forests (Solórzano et al. 2003). These forests, ranging from 1300 to 3000 m elevation, represent the breeding habitats for this species (Stotz et al. 1996).

Presently, the Resplendent Quetzal is classified under the IUCN Red List in the category of ''Lower Risk Near Threatened'' (IUCN 2002), but this classification should be revaluated considering that the species is limited to remnant patches of evergreen cloud forests, most populations are geographically isolated and small, and breeding habitats are being lost at high annual rates (Hanson 1982, Solórzano et al. 2003, P. Bubb, unpubl. data). These habitats are themselves conservation priorities because they harbor high biological and ecological diversity (Hamilton et al. 1995, Nadkarni and Wheelwright 2000, Bubb 2001).

Based on morphological characters, two subspecies of Resplendent Quetzals have been recognized: *P. m. mocinno*, distributed from southern Mexico to northern Nicaragua, and *P. m. costaricensis*, found in Costa Rica and western Panama (Sibley and Monroe 1990). These subspecies apparently are isolated geographically by Lake Nicaragua (8324 km^2) and the absence of the breeding habits in regions adjacent to this lake (Fig. 1). The age of this barrier is not well established but probably arose when the Panamanian Isthmus formed 3 million years ago (Keigwin 1982). Although preliminary partial genetic analysis based on a subunit of ND6, tRNA^{Glu} and control region of mtDNA of 10 individuals suggested genetic differentiation of subspecies (Solórzano et al., unpubl. data), the reproductive behavior (e.g., courtship, chick rearing), breeding season (dry season), diet, and vocalizations appear not to differ between subspecies (SS, pers. obs.).

Although the subspecies concept is artificial, it has practical advantages in conservation because it allows us to identify conservation priorities within species (Ryder 1986). The concepts of ''evolutionarily significant units'' and ''management units'' provide useful tools in genetic conservation (Ryder 1986, Moritz 1994a, 1994b, Frankham et al. 2002). The intent of evolutionarily significant units is to preserve evolutionary heritage across taxa assuming a historical origin for the genetic differences between them. This concept emphasizes the phylogeographic pattern of genetic variation rather than degree of genetic divergence or the quantity of genetic diversity pooled in a certain population. Management units represent populations with significant divergence in allele frequencies, but which have not achieved reciprocal monophyly as required for evolutionarily significant units (Moritz 1994a). The main difference between evolutionarily significant units and management units is that evolutionarily significant units are concerned with historical population structure and serve long-term conservation planning, whereas management units focus on the current population structure and are useful to establish short-term monitoring programs based on genetic discontinuity among populations or unique haplotypes harbored in individual populations (Moritz 1994a, 1994b). The criteria to define these concepts have changed from the initial proposals (cf. Ryder 1986, Moritz 1994a, 1994b), and are still debated (Fraser and Bernatchez 2001).

In the present study, our goal is to identify conservation units for quetzals in Mesoamerica based on mtDNA haplotypes. We discuss our results in the context of genetic conservation rath-

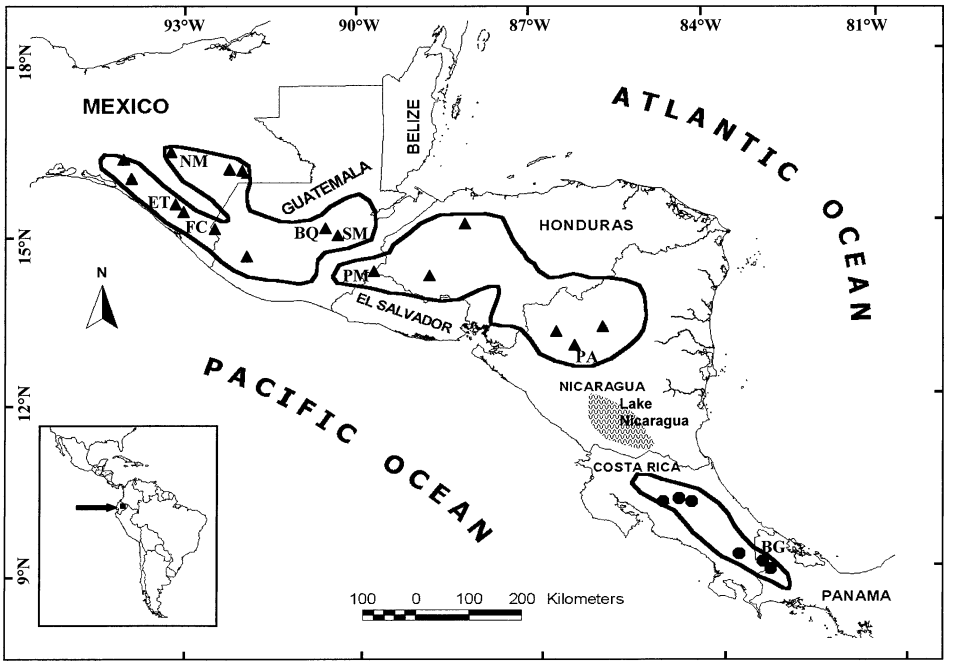

FIGURE 1. Current distribution (symbols) of Resplendent Quetzals in geographically isolated remnant cloud forests (constructed from Hanson 1981, Sibley and Monroe 1990, Solórzano et al. 2003). The past distribution is indicated by bold lines (modified from Johnsgard 2000). Triangles correspond to *P. mocinno mocinno* and circles to *P. m. costaricensis*. Sampled localities: Mexico: NM: Northern Mountains, ET: El Triunfo biosphere reserve, FC: Finca Santa Cruz; Guatemala: BQ: Biotopo Quetzal, SM: Sierra de las Minas biosphere reserve; El Salvador: PM: Parque Montecristo; Nicaragua: PA: Parque Arenal; Panama: BG: Bajo Grande. One sample was not located because its origin is uncertain (ZOOMAT Zoo donation). The outgroup sample PP (*P. pavoninus*) is from Ecuador (inset).

er than a structural analysis of the control region variation and composition, which will be published elsewhere (Solórzano et al., unpubl. data).

METHODS

We conducted fieldwork in eight localities (Fig. 1), collecting blood samples during the breeding season to guarantee the origin of the samples. Additionally, the ZOOMAT Zoo in Chiapas, Mexico, made two blood samples of *P. m. mocinno* available. Although the origin of one of these birds is uncertain because the adult male was confiscated, the trader reported it came from Guatemala. The other one is from Finca Santa Cruz, Mexico (Fig. 1). All 25 samples were stored at ambient temperature in buffer solution (Hillis et al. 1996) before being transported to the laboratory and stored at −70°C.

The two tissue samples for the outgroup, the Pavonine Quetzal (*P. pavoninus*), originated in Ecuador (Fig. 1) and were donated by the Academy of Natural Sciences of Philadelphia, Pennsylvania.

DNA ISOLATION, PCR AMPLIFICATION, AND SEQUENCING

Total genomic DNA was isolated from blood and tissue samples using standard proteinase-K and SDS treatment, followed by extraction with phenol-chloroform and a final ethanol precipitation (Sambrook et al. 1989). We amplified 255 bp from the hypervariable domain I of the mtDNA control region using primers ND62LQTF and H438 (Solórzano et al., unpubl. data). PCR amplification reactions contained 0.1 μM of each primer, 100 μM of each dNTP, 2.0 mM $MgCl_2$, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 0.5 units TaqPol (Boehringer, Ingelheim, Germany) and 1 μL of total genomic DNA. The samples were sequenced with ^{33}P-ATP using a Thermosequenase kit (Amersham Biosciences, Chalfont St. Giles, UK) according to the manufacturer's in-

TABLE 1. Haplotypes identified from the hypervariable domain of the control region of mtDNA in two subspecies and 25 individuals of the Resplendent Quetzal sampled across its range in Mesoamerica. Refer to Figure 1 for sampling locations. ''Zoo'' refers to a quetzal at ZOOMAT confiscated from an unknown location. Position is read vertically; dots indicate similarity with haplotype G.

	Sampling location									Position										
Haplotype	Northern Mtns.	El Triunfo	Finca Santa Cruz	Biotopo Quetzal	Sierra de las Minas	Parque Montecristo	Parque Arenal	Bajo Grande	Zoo	1 5	2 3	3 0	3 2	4 4	6 1	1 2 4	1 2 5	1 3 0	1 4 7	1 7 2
G[b]								1		C	T	C	A	C	A	G	A	T	C	A
A[a]	1	3	1		1				1	.	.	.	.	T	G	A	G	C	A	T
B[a]		5		1	2					.	.	.	G	T	G	A	G	C	A	T
C[a]							1			.	C	T	.	T	G	A	G	C	A	T
D[a]						1				.	.	T	.	T	.	A	G	C	A	T
E[b]								6		.	.	.	.	.	.	.	.	.	A	.
F[b]								1		T	.	.	.	.	.	.	.	.	A	.
H[b]								1		.	C	.	.	.	.	.	.	.	A	.

[a] Identified in *P. m. mocinno*.
[b] Identified in *P. m. costaricensis*.

structions. The sequencing reactions were electrophoresed for 2 hr on a denaturing (6M urea) 6% polyacrylamide gel at a constant 70 W. The gel was vacuum-dried at 80°C for 1 hr and then exposed to X-ray film for 48 hr. Since *P. mocinno* has been reported to be heteroplasmic for the control region (Solórzano et al., unpubl. data) we used only a portion of the sequence with no observable double peaks. When the amplified product of the outgroup (*P. pavoninus*) was sequenced, double peaks appeared in domain I but the flanking NADH6 gene showed only a single sequence. To resolve this problem the PCR products from the outgroup were cloned into pGEM®-T Easy Vector System according to the manufacturers recommendations (Promega, Madison, Wisconsin). The clones were sequenced with BigDye® Terminator V3.1 cycle sequencing kit (Applied Biosystems, Foster City, California) using an ABI Prism 310 Genetic Analyzer. Five cloned products were sequenced, and after verifying that each had the same control-region sequence they were used in subsequent genetic analyses.

GENETIC ANALYSIS

After aligning sequences with Clustal X (Thompson et al. 1997), overall haplotypic (*h*) and nucleotide diversity per site (π) were calculated (Rozas and Rozas 1999) for the 25 samples as a group (regardless of their origin), as well as individually for Mexico (8 samples) and Panama (9 samples). Nucleotide composition and the transition:transversion ratio (*R*) were estimated for the 25 samples (Kumar et al. 2001).

Correspondence between genetic variation and geographic distribution was tested with a Mantel test (Liedloff 1999) by computing correlations between pairwise genetic distances (Kimura 2-parameter) and geographic separation (km; georeferenced to UTM units with ArcView-GIS; ESRI 1996) under assumptions of an isolation by distance model. We constructed a neighbor-joining (NJ) tree rooted with *P. pavoninus* to estimate the phylogenetic relationships among haplotypes of both subspecies. Tree robustness at the nodes was estimated by bootstrap analysis (Kumar et al. 2001).

We estimated the degree of genetic differentiation between subspecies (F_{ST}), and derived a minimum spanning network from the genetic distances estimated in Arlequin 2.0 (Schneider et al. 2000).

RESULTS

GENETIC DIVERSITY AND NUCLEOTIDE COMPOSITION

The sequences of 255 bp of mitochondrial DNA control region from 25 specimens of *P. mocinno* representing two subspecies (*P. m. mocinno* and *P. m. costaricensis*) revealed eight haplotypes (GenBank accession numbers AY607565–AY607572) based on 12 segregating sites, 10 of which were transitions and two transversions (Table 1). The homologous segment obtained for *P. pavoninus* differed from *P. mocinno* at 52

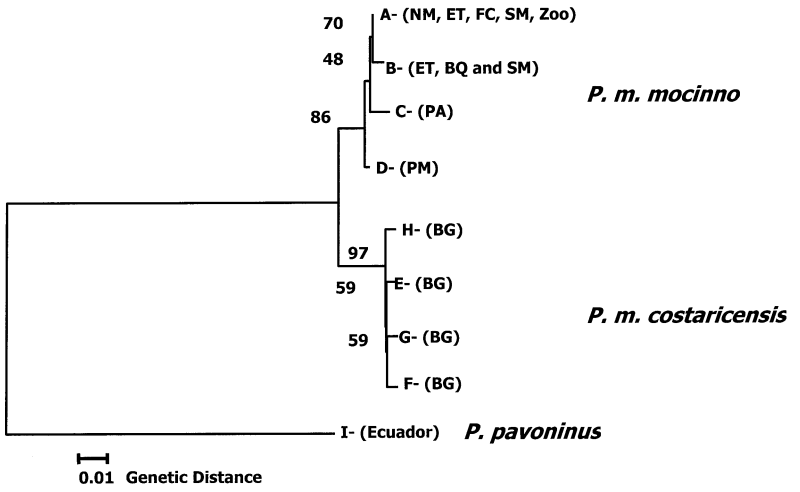

FIGURE 2. Genealogical relationships among control-region haplotypes of Resplendent Quetzals. The neighbor-joining tree was constructed using the Kimura 2-parameter model of substitution, and was rooted with a sequence from Pavonine Quetzal. Bootstrap values shown at the nodes were computed with 1000 replicates of the data. Localities of the haplotypes are in parentheses.

sites. Average nucleotide composition of the 25 Resplendent Quetzal sequences exhibited a high proportion of adenine residues (36%), followed by cytosine (25%), thymine (23%), and guanine (16%). Nucleotide diversity for the *P. mocinno* samples as a group was $\pi = 0.0171 \pm 0.0015$, with a haplotype diversity (h) of 0.8180 ± 0.0470. However, as expected these parameters were lower when Mexico ($n = 8$, $\pi = 0.0021 \pm 0.0005$, $h = 0.5360 \pm 0.1230$), and Panama ($n = 9$, $\pi = 0.0026 \pm 0.0009$, $h = 0.5830 \pm 0.1830$) were treated separately.

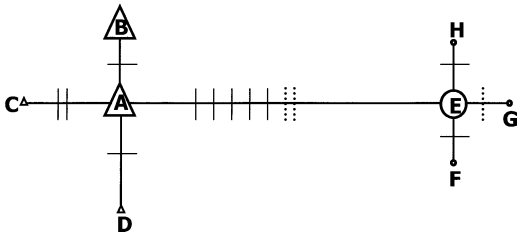

FIGURE 3. Minimum spanning network showing mutational differences among the eight haplotypes found in Resplendent Quetzals representing the subspecies *P. m. mocinno* (triangles) and *P. m. costaricensis* (circles). The size of each symbol is proportional to haplotype frequency, and the lengths of the branches are proportional to the genetic distance between haplotypes. The solid and dotted crossmarks represent the number of transitions and transversions, respectively, between haplotypes.

DISTRIBUTION OF GENETIC DIVERSITY

The correlation between geographical and genetic distances was consistent with a model of isolation by distance ($r^2 = 0.81$, $P = 0.003$). However, the largest genetic distance (0.0406) was between haplotypes F from Panama and C from Nicaragua.

The single population sampled in Panama (Fig. 1) had four private (unique) haplotypes (E, F, G, and H), whereas the three populations sampled in Mexico had two haplotypes (A and B) that were shared with birds sampled from Biotopo Quetzal and Sierra de las Minas, Guatemala (Table 1). Haplotypes C and D, from Nicaragua and El Salvador respectively, were unique to each of these populations. The NJ tree showed a clear split between the putative subspecies *P. m. mocinno* (86% bootstrap support) and *P. m. costaricensis* (97% bootstrap support), and a shallow separation for haplotypes from different populations within the *P. m. mocinno* range (Fig. 2).

The minimum spanning network (Fig. 3) illustrated the small number of genetic differences separating haplotypes within subspecies, and the larger number of substitutions among them. Genetic distances among haplotypes in different subspecies were 3–10 times higher than between haplotypes within each subspecies. Taking haplotype frequencies into account this translates to an F_{ST} value of 0.37 among subspecies.

DISCUSSION

Overall nucleotide diversity (0.0171) in the Resplendent Quetzal in Mesoamerica was similar to that estimated for some endangered species (e.g., cheetahs [*Acinonyx jubatus*], $\pi = 0.0130$, Freeman et al. 2001), but higher than for others (e.g., Florida Grasshopper Sparrow [*Ammodramus savannarum floridanus*], $\pi = 0.0060$–0.0130, Bulgin et al. 2003). However, as the values obtained for Mexico (*P. m. mocinno*) and Panama (*P. m. costaricensis*) were similar to those recorded in other bird species irrespective of whether they were endangered or not (Marshall and Baker 1997, Fry and Zink 1998, Kvist et al. 1999, Griswold and Baker 2002), it is clear that a species endangerment level cannot be derived simply from measures of genetic diversity. The most obvious reason for this is that this estimator is a product of the evolutionary history of lineages studied (Nei 1987) and probably does not reflect recent ecological processes. In Resplendent Quetzals, the value of π, like the minimum spanning network, illustrates the small number of genetic differences separating haplotypes within subspecies, and the larger number of substitutions among them probably reflects genetic diversity related to historically larger population size that declined recently due to loss of habitat. Although data are sparse about the historical and recent changes of quetzal distribution in Mesoamerica, between 1970 and 2000 about 661 km^2 of cloud forests were destroyed in southern Mexico (Solórzano et al. 2003), causing the loss of 80% of the Chiapas nesting habitat. Moreover, the current abundance of quetzals is very low (0.5–1.5 individuals per ha, Solórzano et al. 2003). Considering that fragmentation and destruction of forest is a common problem in Mesoamerica (Bubb 2001) it is likely that local extinctions and a general decline in abundance has occurred. Analyses over generational time scales or comparisons with a historical genetic diversity baseline would be required to distinguish trends in loss of genetic variability and its association with an increased risk of extinction. While this is not yet possible due to lack of data, the present study can act as such a baseline for future periodic monitoring of genetic parameters and assessment of extinction risks for the species.

Our results indicate that there is geographical structuring of populations with increasing geographic distance. This suggests that gene flow among populations occurs primarily among adjacent populations and is constrained as distance increases. The clear geographic differentiation between subspecies implies that they are largely isolated genetically, but this needs to be confirmed in future studies by sampling more extensively in their zone of contact. Radio-telemetry studies (Powell and Bjork 1994, 1995) showed that an individual quetzal can traverse 50 km during its seasonal migrations and can visit other breeding areas and lower forests, but all individuals tagged returned to their past breeding locality. However, all of these tagged individuals were caught as reproductive adults, so the extent of natal philopatry is unknown. Regarding the rapid destruction and fragmentation of breeding habitats it is clear that conservation programs for quetzals and their habitats must consider this distance as a minimum acceptable to avoid increased genetic fragmentation of populations. In the present study, distances less than 50 km occurred between El Triunfo and Finca Santa Cruz (32 km) and Sierra de las Minas and Biotopo Quetzal (34 km), and this is consistent with the observation that populations there shared haplotypes. However, populations sampled in Guatemala and Mexico, separated by more than 300 km, also shared haplotypes. Unless long-distance migration occurs, this suggests that recent destruction of quetzal habitats that provided connectivity between these two areas has only recently caused geographical separation and the genetic effects have not yet become apparent. Another possible explanation is that the sequences we utilized do not provide sufficient resolution to track microgeographic structuring of populations, and thus longer sequences of the complete control region may be needed in future studies.

In the present study, we obtained very few blood samples mostly because of the rarity of quetzals and the high rates of both nest poaching and nest predation. These factors limited our analyses, and the identification of genetic priorities consequently suffered. However, since these are the first genetic results for quetzals we make the following preliminary recommendations. Because the mtDNA control-region sequences show that *P. m. mocinno* and *P. m. costaricensis* exhibit reciprocal monophyly, each subspecies should be managed as two independent evolutionarily significant units (Moritz

1994b). Therefore, Mexico, Guatemala, Honduras, El Salvador, and Nicaragua should join efforts in the task of conservation of *P. m. mocinno* regardless of political borders. The same is required of Costa Rica and Panama to preserve *P. m. costaricensis*.

With the small sample sizes available at present it is difficult to define management units, which require genetic distinctiveness of lineages, though not at the level of reciprocal monophyly. The shallow branches within each of the subspecies assemblages of haplotypes suggests that different populations of *P. mocinno* have been fragmented relatively recently, as haplotypes in either assemblage differ by only 1–2 substitutions. More extensive sampling is required to determine if the localities (countries) with unique haplotypes represent discrete genetic units. In spite of the very limited sampling within *P. mocinno*, populations in Mexico–Guatemala, El Salvador, Nicaragua, and Panama might be preliminarily separate management units, as we found haplotypes were not shared among these countries. Therefore, concerted international efforts may be much better for the long-term conservation of a widely distributed species that spans international borders.

The present study confirms the need for significant increases in efforts to increase the chance of long-term survival of quetzals in Costa Rica and Panama (for *P. m. costaricensis*) and Nicaragua, El Salvador, and Guatemala–Mexico (for *P. m. mocinno)*. Furthermore, as this study shows that quetzal populations in each of these countries have low but apparently unique genetic diversity they should also be preserved under national and international agreements. This conservation task should also include the monitoring, protection, and restoration of quetzals and their breeding and migration habitats. Finally, the activities of wild-bird dealers who buy dead and living quetzals for sale to private collections, aviculturists, and zoos, must be curbed significantly or eliminated because of the threat they pose to the quetzal's survival. This illegal trade has already impacted wild populations and has exacerbated the decline in numbers of this rare species (Johnsgard 2000).

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