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Mitochondrial DNA variation and phylogeography of the ferruginous pygmy-owl (*Glaucidium brasilianum*)

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Abstract

Sequences from the mitochondrial cytochrome *b* gene were used to examine patterns of variation within and among populations of the ferruginous pygmy-owl (*Glaucidium brasilianum*) from both North America (including populations from Mexico) and South America. As currently conceived, *G. brasilianum* is paraphyletic, with North American and South American clades representing two distinct groups that should be recognized as the distinct species *G. ridgwayi* and *G. brasilianum*, respectively. Within the *G. ridgwayi* clade, populations from Arizona, Sonora, and Sinaloa are genetically distinct and share no mitochondrial haplotypes with populations occurring in Texas and other regions of Mexico. According to nested clade analysis this separation may be the consequence of past fragmentation that predates the origin of the Sonora desert. In addition, gene flow between the Arizona/Sonora/Sinaloa populations and the Texas/other Mexico populations is practically nonexistent, with estimates being approximately one individual every 10 generations. Collectively, these data suggest that the Arizona/Sonora/Sinaloa clade should be recognized as either a distinct subspecies or phylospecies from the group containing populations in Texas and the remainder of Mexico. These data should be used as guidelines for pygmy-owl recovery and conservation, as they meet the recommendations of task 3 of the pygmy-owl recovery plan that lists genetic data as essential information for pygmy-owl management.

Introduction

The ferruginous pygmy-owl (*Glaucidium brasilianum*, hereafter referred to as pygmy-owl) has an historical range that includes areas in southern Arizona, southern Texas, and regions extending from northern Mexico to Chile (Ridgway 1914, Proudfoot and Johnson 2000). Traditional pygmy-owl habitat in the United States includes mesquite (*Prosopis* spp.) woodlands and cottonwood (*Populus* spp.) forests in the Salt, Verde, and Gila river areas of Arizona, and mesquite brush, ebony (*Pithecellobium* spp.), and riparian areas in the Lower Rio Grande Valley of Texas (Millsap 1987; Gilman 1909). Unfortunately, by the early 1970s land-use practices resulted in a depletion of over 90% of pygmy-owl habitat in Texas (Oberholser 1974), thus severely reducing population sizes. Similar land-use practices and destruction of riparian areas are credited with extirpating the pygmy-owl as a regular nesting species in Arizona (Johnson et al. 1979). In 1999, only 41 adult pygmy-owls were known to exist in Arizona. In 2000 and 2001, population sizes in Arizona were 34 and 36 adults, respectively (U.S. Fish and Wildlife Service 2003).

From a taxonomic standpoint, populations in southwestern Arizona through Colima and Jalisco

in western Mexico as well as populations in southern Texas south to Nuevo Leon and Tamaulipas in eastern Mexico are recognized as a distinct subspecies, *G. b. cactorum* (Friedmann et al. 1950; AOU 1957; U.S. Fish and Wildlife Service 1994, 1997). As a consequence of population declines, the taxonomic uniqueness of pygmy-owls in this region and the separation of populations by political boundaries in the United States and Mexico, the U.S. Fish and Wildlife Service proposed listing pygmy-owl populations in Arizona as endangered and those in Texas as threatened (U.S. Fish and Wildlife Service 1994). The final ruling on this proposal resulted in listing only the Arizona population as endangered (U.S. Fish and Wildlife Service 1997).

Although *G. b. cactorum* is recognized by the American Ornithological Union (1957) and the U.S. Fish and Wildlife Service (1994, 1997), the current taxonomy of the pygmy-owl is complicated, thus making it difficult to objectively characterize overall patterns of geographic variation, a requisite for establishing a comprehensive management plan. For instance, of the approximately 15 recognized subspecies of pygmy-owl (Proudfoot and Johnson 2000), as many as four subspecies have been recognized in North America (Peters 1940; Friedmann et al. 1950; AOU 1957; Phillips 1966; Holt et al. 1999). Peters (1940) recognized populations in southwestern Arizona south to Nayarit in western Mexico as *G. b. cactorum*. Other populations were relegated to *G. b. ridgwayi*, a subspecies occurring in the Lower Rio Grande Valley in Texas southward along the Atlantic slope in eastern Mexico to Panama, as well as from Jalisco, Mexico, south to tropical Central America and the Canal Zone. If patterns of geographic variation are found to support this taxonomic decision, then two separate units of conservation are warranted. Friedmann et al. (1950) suggested that populations of *G. b. ridgwayi* geographically subdivide populations of *G. b. cactorum*, a division Proudfoot and Johnson (2000) considered highly unlikely. Nevertheless, Friedmann et al.'s (1950) recognition of only *G. b. cactorum* occurring in the United States implies one conservation unit. Because only slight differences in size (e.g., wing and tail length), pattern (e.g., streaking and coloration, which grade with local humidity), and vocalization (which are broadly similar over its entire range) are used to characterize subspecies, evaluation of these competing taxonomic treatments is complicated (König et al. 1999; Proudfoot and Johnson 2000).

Numerous studies of threatened and endangered populations and species have employed genetic approaches for both the assessment of population vulnerability and the identification of units of conservation (Avise 1994), and several of these approaches have been applied to avian species (Barrowclough 1992; Haig and Avise 1995; Zink et al. 2000a, b; Eggert et al. 2004; Martinez-Cruz et al. 2004). From a phylogeographic standpoint, historical barriers to dispersal and more recent impediments to gene flow contribute to the fragmentation of a species' range, and these geographically defined units do not always coincide with current subspecific boundaries (Avise 2000).

Therefore, the identification of genetically and phylogenetically defined units provides an objective means of identifying both evolutionary significant units (e.g., phylogenetic species) and management units (Moritz 1994).

As indicated by several authors (Heidrich and Wink 1994; Heidrich et al. 1995; König and Wink 1995; Johns and Avise 1998), mtDNA provides an effective marker for examining phylogeographic structure within species and has proven useful at several geographic scales (Zink 1997, 2002; Zink et al. 1998, 2001). In this paper, mitochondrial DNA (mtDNA) markers are used to investigate patterns of geographic variation and levels of genetic divergence in pygmy-owl populations from Arizona, Texas, and regions in Mexico. Sequences from North American pygmy-owls are further compared to taxa from Eurasia, Africa, and South America. Given the controversies surrounding the uniqueness of pygmy-owl populations, especially those in Arizona, this study is essential to the design of a viable management plan for pygmy-owls (U.S. Fish and Wildlife Service 2003).

Methods

Sampling

Whole blood and tissue biopsies from feather pulp were taken from individual pygmy-owls collected in Arizona and Texas, U.S., and in Nayarit, Sinaloa, and Sonora, Mexico (Figure 1 and Table 1). One-hundred-three specimens were examined from Arizona (n = 14), Mexico (n = 71), and Texas

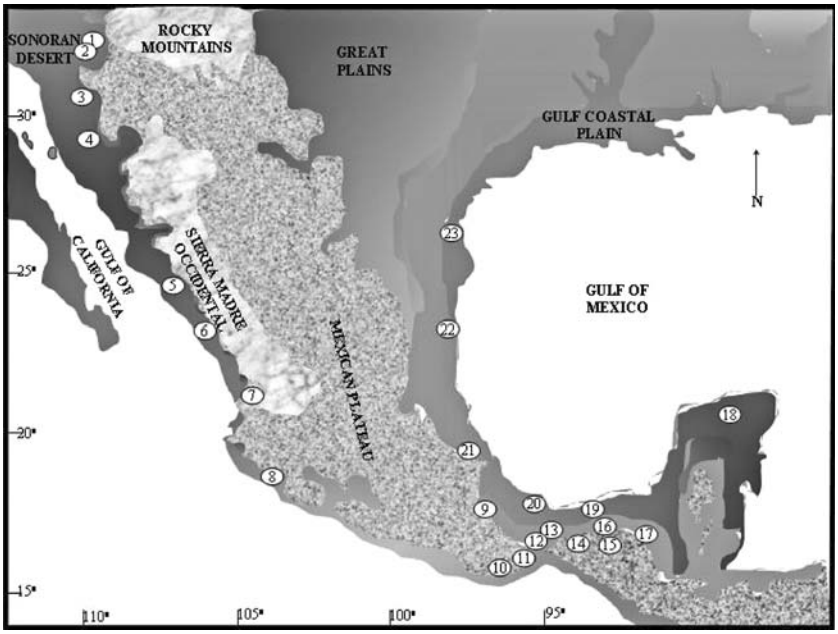

Figure 1. Collection locations for North American *G. brasilianum*. Cross-reference for geographic location with corresponding numbers in Table 1 are: Arizona (1 & 2), Sonora (3 & 4), Sinaloa (5 & 6), Nayarit (7), Michoacan (8), Oaxaca (9–13), Chiapas (14–17), Yucatan (18), Tabasco (19), Veracruz (20 & 21), Tamaulipas (22), and Texas (23).

Table 1. Approximate geographic location of sample areas for *G. brasilianum* from North America. Information from field notes and Encarta® 2000 (Microsoft®, Redmond, WA) were used to obtain latitude and longitude for tissue samples provided by museums. # corresponds to localities in Figure 1 and haplotype letters are the same as in Figures 2 and 3

#	Location	Latitude & longitude	Haplotypes (no. of individuals)
1	NW Tucson, AZ	032_36′ N, 111_11′ W	C (10)
2	Altar Valley, AZ	031_29′ N, 111_33′ W	C (1), D (2), L (1)
3	Sonora 1	030_40′ N, 111_00′ W	C (1), L (1), M (1)
4	Sonora 2	029_12′ N, 110_47′ W	K (3)
5	Sinaloa 1	024_47′ N, 107_24′ W	D (3), K (1), N (1)
6	Sinaloa 2	023_12′ N, 106_25′ W	D (1)
7	Nayarit	021_20′ N, 104_35′ W	A (3), J (2)
8	Michoacan	018_45′ N, 103_40′ W	A (1), F (1), J (1), O (1), W (1), X (1), CC (1), DD (1)
9	Oaxaca 1	017_03′ N, 096_43′ W	G (1)
10	Oaxaca 2	015_44′ N, 096_27′ W	V (1)
11	Oaxaca 3	016_22′ N, 095_39′ W	A (2), T (1), Z (1)
12	Oaxaca 4	016_34′ N, 095_06′ W	A (1), Q (1), R (1)
13	Oaxaca 5	016_53′ N, 095_01′ W	A (1), G (1)
14	Chiapas 1	016_14′ N, 093_53′ W	A (1)
15	Chiapas 2	016_44′ N, 093_06′ W	H (1), BB (1)
16	Chiapas 3*	017_33′ N, 092_56′ W	A (4), F (1), U (1)
17	Chiapas 4	016_55′ N, 091_21′ W	A (1), I (1)
18	Yucatan	020_40′ N, 088_35′ W	H (1)
19	Tabasco*	018_00′ N, 093_22′ W	A (2), E (3), S (1)
20	Veracruz 1*	018_26′ N, 095_04′ W	A (4), G (1), I (2), Y (1), AA (1)
21	Veracruz 2	020_00′ N, 096_57′ W	A (1), I (1), P (1)
22	Tamaulipas	022_55′ N, 097_56′ W	B (3), G (1)
23	Texas	026_83′ N, 097_70′ W	B (18)

(n = 18). In Arizona and Texas, samples were obtained as part of banding studies conducted from 1994 to 2004. In an effort to minimize bias from the inclusion of siblings, when samples were taken from nestlings, only one individual per nest site was used in the phylogeographic comparisons. In Mexico, adult pygmy-owls were sampled from the following locations: (1) samples from Nayarit (n = 4) in proximity (ca. 10–15 km) to Laguna at Santa Maria del Oro; (2) Sinaloa (n = 5) along the Río Tamazula near (ca. 15–25 km) Culiacán; and (3) Sonora (n = 6), in the proximity of Magnalena de Kino, Hermosillo, and the south-eastern reaches of the Río Yaqui. All sample sites were within 35 km of latitudes and longitudes provided in Table 1. It was assumed that spacing between and within sample areas reduced the probability of examining related individuals derived from the same female lineage. Alcohol preserved tissues of 56 additional pygmy-owls collected in Mexico included: Chiapas (n = 7), Michoacan (n = 8), Nayarit (n = 1), Oaxaca (n = 13), Sinaloa (n = 1), Tabasco (n = 10), Tamaulipas (n = 4), Veracruz (n = 11), and Yucatan (n = 1), Mexico. Accession numbers for samples provided by the University of California Museum of Vertebrate Zoology at Berkeley are MVZFC 22474–22575 and MVZFC 20006.

Sequences from two Old World species (*G. passerinum*, n = 3, *G. tephronotum*, n = 1) were used as outgroups, and in addition to *G. brasilianum*, seven New World species of *Glaucidium* were added to the phylogenetic analysis. These species included the mountain pygmy-owl (*G. gnoma*, n = 1), Andean pygmy-owl (*G. jardinii*, n = 1), yungas pygmy-owl (*G. bolivianum*, n = 1), Amazonian pygmy-owl (*G. hardyi*, n = 2), Austral pygmy-owl (*G. nanum*, n = 1), Peruvian pygmy-owl (*G. peruanum*, n = 1), ferruginous pygmy-owl (*G. brasilianum*, n = 7) from South America, and the Chaco pygmy-owl (*G. tucumanum*, n = 4). Accession numbers for sequences downloaded from GenBank include: AJ003975–AJ003977, AJ003979, AJ003981, AJ003982, AJ003984, AJ003987–AJ003991, AJ003994, AJ003996–AJ003999, and AJ004002–AJ004006 (Wink and Heidrich 1999).

Data collection

Total DNA was extracted using a Qiagen DNeasy kit (Qiagen, Valencia, California). For all individuals, an approximately 1100 bp fragment of the mitochondrial cytochrome *b* gene (cyt *b*) was amplified using the polymerase chain reaction (PCR) (Saiki et al. 1988). Sequences in GenBank for elf Owl (*Micrathene whitneyi*, #MWU89170), northern saw-whet owl (*Aegolius acadicus*, #AAU89172) and long-eared owl (*Asio flammeus*, #AFU89171) were used to design PCR oligonucleotide primers. External PCR primer sets included F14899 (5′-CCCAACATCCGAAART CTCAC −3′) and R15940 (5′-GGATGCTAGT TGGCCGATRAT-3′). In addition to the external primers, six internal primers were designed for nucleotide sequencing (contact GAP for primer sequences). Accession numbers (GenBank) for North American haplotype sequences are AY859373–AY859402.

PCR was performed in 50 μl reaction volumes and included 2.0 μl of 10 mM solution of each primer, 5.0 μl of 10× reaction buffer with 20 mM MgCl, 4.0 μl dNTP mix (0.2 mM each), 0.2 Takara® *Ex Taq* polymerase (Fisher Scientific, Houston, TX), and 1–2 μl of DNA template. The amplification profile included: (1) an initial denaturation at 95 °C for 5 min followed by denaturation at 94 °C for 1 min; (2) a touchdown PCR scheme (Don et al. 1991), whereby the initial annealing temperature was 60 °C for the first cycle followed by 2 °C decrease per cycle for the next two cycles, and 35 cycles with a constant annealing temperature of 56 °C; (3) extension at 72 °C for 1 min; and (4) a final extension time at the last cycle for 72 °C, 4 min. All PCR experiments included negative controls.

Sequencing reactions were done with an ABI PRISM™ dye-terminator cycle-sequencing kit (Applied Biosystems, Foster City, CA) and run on an ABI 377 automated DNA sequencer. All PCR fragments were sequenced for both strands, and multiple sequences for each individual were obtained for DNA isolated from both blood and tissue.

Analytical procedures

Presence of potential nuclear pseudogenes (*sensu* Sorenson and Fleischer 1996; Sorenson and Quinn 1998) was investigated in several ways. First, sequences for each individual were obtained from both blood and other tissue sources. Second, sequences were compared against the GenBank

database by BLAST (blastn and blastx) searches, and all sequences corresponded to previously reported cyt *b* sequences of pygmy-owls and related species. Third, nucleotide composition for each codon position was compared to those reported for other avian taxa (Moore and DeFilippis 1997).

Several population statistics, including haplotype diversity (Nei 1987), nucleotide diversity (π; Nei 1987), theta ($\theta = 2\,\mathrm{Ne}\mu$; Watterson 1975), and number of segregating sites, were estimated using DnaSP version 4.0.6 (Rozas et al. 2003). Standard error of these measurements was determined from a null distribution generated from 10,000 random permutations of the data keeping sample size constant. An analysis of molecular variance (AMOVA, Excoffier et al. 1992) in ARLEQUIN version 2.0 software package (Schneider et al. 2000) was used to test for genetic structure within and between major regions identified through phylogenetic and nested clade analysis. The scaled migration rate (*M*) and scaled divergence time (*T*) values calculated using the Markov chain Monte Carlo method (MCMCM) in MDIV (Nielson and Wakeley 2001) were used to estimate the number of migrates per generation and divergence time between distinct populations in North America. Time to the most recent common ancestor (*TMRCA*) was also calculated using MDIV (Nielson and Wakeley 2001). The length of the Markov chain simulation was 5×10^6 cycles, with 5×10^5 cycles set as the burn-in time. The maximum value for *M*, *T*, and theta was 5.0, 5.0, and 20.0, respectively. Because the existence of a universal mtDNA clock for birds is questionable (Garcia-Moreno 2004), three mutation rates (1, 2, and 3% per million years, MY) were used to estimate divergence time between populations and time since common ancestry. To account for presence of transition-transversion bias, the HKY model (Hasegawa et al. 1985) was employed.

Patterns of mtDNA haplotype variation were investigated in two ways. First, relationships among unique haplotypes were determined through both distance based analyses, using neighbor-joining (NJ, Tamura and Nei 1993), and maximum parsimony (MP) that employed the heuristic search option with equal weighting of characters (TBR, random additions, and 10 replications; *PAUP** 4.0, Swofford 1999). Support for the MP trees was determined with bootstrap replications (1000 replications, Felsenstein 1985). Modeltest v3.06 (Posada and Crandall 1998) and the original MP tree were used to determine the appropriate model for the Bayesian analysis. A Bayesian analysis in MrBayes version 3 was performed and posterior probabilities were obtained using Markov chain Monte Carlo (MCMC) techniques (Nst = 6, Rates = gamma, Ngen = 5,000,000, frequency = 100, chains = 4, Ronquist and Huelsenbeck 2003).

Second, a parsimony network for all haplotypes was constructed following the procedure of Templeton et al. (1992) as implemented in TCS version 1.1.3 (Clement et al. 2000). This network provides a framework for a nested clade analysis (NCA) that employs two distance measures to quantify spatial distribution of haplotypes (Templeton et al. 1995; Templeton 1998, 2004). Association between nested clades and geographic distribution was evaluated in GeoDis version 2 (Posada et al. 2000).

Because the distribution of pygmy-owls in North America is limited to areas below 1400 m in the United States and Mexico and 1900 m in Central America (Proudfoot and Johnson 2000), mountain ranges may constitute geographic barriers for pygmy-owl dispersal. Therefore, options provided in Encarta® 2000 (Microsoft®, Redmond, Washington) were used to determine the shortest distances between collection locations within the known distribution of pygmy-owls (Proudfoot and Johnson 2000), and to create a distance matrix for NCA. Phylogeographic patterns were evaluated following procedures outlined by Crandall and Templeton (1993), Templeton (1998) and Templeton's inference key (2004, http://inbio.byu.edu/Faculty/kac/crandall_lab/geodis.htm).

Simulation studies (Irwin 2002; Knowles and Maddison 2002) have criticized the validity of NCA's inference key relative to the evaluation of alternative hypotheses (e.g., restricted gene flow, isolation by distance, and fragmentation). Nevertheless, the method does provide a statistical framework for defining genetically distinct populations *a posteriori* through the examination of their geographic distribution and frequency of haplotypes, and according to Templeton (2004) such an approach is complementary to *a priori* procedures discussed by Knowles and Maddison (2002).

Results

Approximately 1100 bp of the cyt *b* gene were obtained for analysis. However, due to a high frequency of ambiguous characters on the 3′-end of GenBank sequences, we reduced the dataset to 899 bp. MP analysis produced 17,280 equally parsimonious trees (length 469 steps, consistency index [CI] = 0.66, retention index [RI] = 0.84, rescale index [RC] = 0.57). Topologies obtained from MP, NJ, and Bayesian analysis revealed similar patterns of relationships among various haplotypes, and these relationships were not congruent with previous designations of species (Figure 2). For instance, *G. brasilianum* clustered into two separate groups. One group contained South American haplotypes representing populations of *G. brasilianum* as well as *G. tucumanum*,

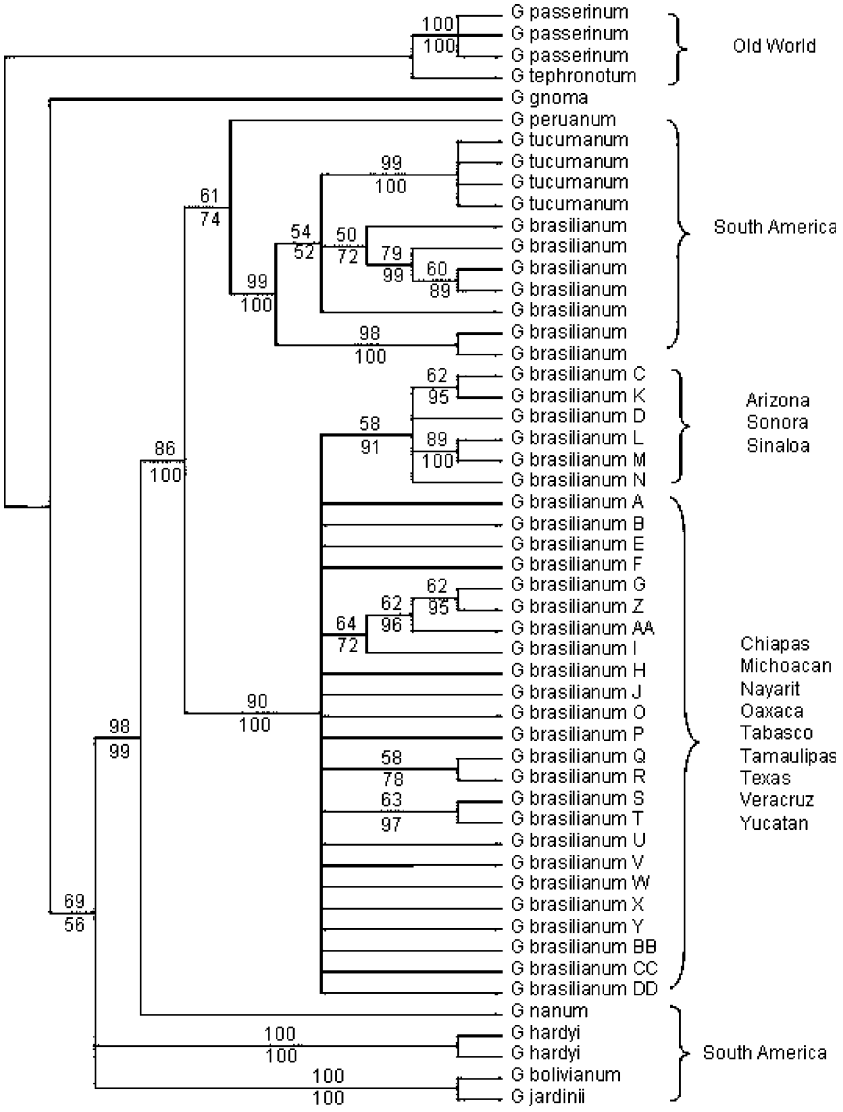

Figure 2. Relationship between members of Glaucidium from Old and New World populations (based on 899 nucleotides of the cytochrome *b* gene). Strict consensus of 17,280 most parsimonious trees generated using MP method with heuristic search (TBR branch swapping, stepwise additions; tree length 469 steps, 209 parsimoniously informative characters); bootstrap support (1000 replications, MP heuristic search with TBR branch swapping) is displayed above branch lines; posterior probability from Bayesian analysis is displayed below corresponding bootstrap values. Uppercase letters following "*G. brasilianum*" from North America correspond to haplotypes in Table 1 and Figure 2.

and this group was sister to *G. peruanum*. Remaining haplotypes of *G. brasilianum* formed a group containing North American populations. Within this predominantly North American group, haplotypes from Arizona, Sonora, and Sinaloa formed a distinct subgroup from the remaining populations of *G. brasilianum* in Texas and Mexico (Table 1 and Figure 2).

Average absolute nucleotide difference among recognized species was 8.9% ± 4.0%, and average difference with Jukes and Cantor correction was 9.6% ± 4.6%. Absolute nucleotide difference among *G. brasilianum* in North and South America was 2.7%. Within population variance (AMOVA) of North and South America populations of *G. brasilianum* was 19.0% ($\theta_{st} = 0.81$, $P < 0.001$). AMOVA among groups in North and South America was 77.7% ($\theta_{ct} = 0.78$, $P = 0.007$), and variance among populations within groups was 3.2% ($\theta_{sc} = 0.15$, $P < 0.001$).

Haplotype diversity of North American pygmy-owls

Of the 103 individuals examined from Mexico and the United States, 30 unique haplotypes (approximately one in every three individuals) were revealed. Absolute sequence difference among haplotypes ranged from 1 to 11 substitutions or 0.1–1.0%. Number of polymorphic sites was 30, with 16 being parsimony informative. Average variation per sequence (Theta-W) was 7.64, and variation per site was 0.0085. Nucleotide diversity (π) with and without Jukes and Cantor correction was 0.0043. In comparison to Mexico, with 27 haplotypes, both Arizona and Texas had extremely low levels of average haplotype diversity, with three and one haplotype, respectively. Arizona shared one haplotype with Sonora and one haplotype with Sinaloa. No haplotypes from Arizona, Sonora, or Sinaloa were shared with the remainder of either Mexico or Texas. Texas shared one haplotype with Tamaulipas, Mexico.

Phylogeographic patterns of haplotype diversity in North America

AMOVA (1023 permutations) of the two groups (Arizona-Sonora-Sinaloa and Texas-rest of Mexico) of *G. brasilianum* from North America showed considerably more variance (55.2%) among groups ($\theta_{ct} = 0.55$, $P = 0.007$) than variance among populations within groups, 14.1% ($\theta_{sc} = 0.32$, $P < 0.001$). Variance within populations was 30.7% ($\theta_{st} = 0.69$, $P < 0.001$), marginally less than half the value shown between *G. brasilianum* from North and South America. Based on the results from the MDIV analysis, the posterior distribution for M revealed a maximum value of 0.10, suggesting on average of one individual female exchanged between the two populations every 10 generations. The posterior distribution for T revealed a sharp peak at 0.58. With mutation rates of 1, 2 and 3%, estimated divergence time for the two populations was 3.14 MY, 1.57 MY and 1.04 MY, respectively, and estimates for time to the most recent common ancestor (TMRCA) were 4.43 MY, 2.21 MY, and 1.48 MY, respectively.

A single haplotype network (Figure 3) was obtained with TCS analysis. Testing for associations between haplotype distribution and geographical location, NCA (Chi-square) identified seven clades that violated the null hypothesis of panmixia, showing significant D_c, D_n, or I–T (interior–tip nodes) values (Table 2). Inference for six of the seven clades implied restricted gene flow (RGF) with isolation by distance (IBD). For clade 1–1, significant differences in D_c and D_n values in haplotype B (geographically Tamaulipas and Texas), coupled with the known geographic distribution of pygmy-owls, indicated RGF with IBD occurred between Texas-Tamaulipas and populations in Chiapas, Michoacan, Nayarit, Oaxaca, Tabasco, and Veracruz. Significant differences in clade 1–7 occurred between tip (haplotype C, geographically Arizona and Sonora) and interior nodes (haplotype K, geographically Sinaloa and Sonora). Using the known distribution of pygmy-owls and inferences from NCA, information provided in clade 1–7 suggested RGF with IBD occurred between Arizona-Sonora and Sinaloa. Inferences for the total cladogram (4–1) yielded past fragmentation between clade 3–1 (geographically Chiapas, Michoacan, Nayarit, Oaxaca, Tabasco, Tamaulipas, Texas, Veracruz, and Yucatan) and clade 3–2 (geographically Arizona, Sinaloa, and Sonora). Thus, NCA supports two distinct populations in North America and suggests past fragmentation as the demographic event separating the two groups (detailed results of NCA are available from the author). In the initial TCS network, populations of *G. brasilianum* from South America were included. However, because

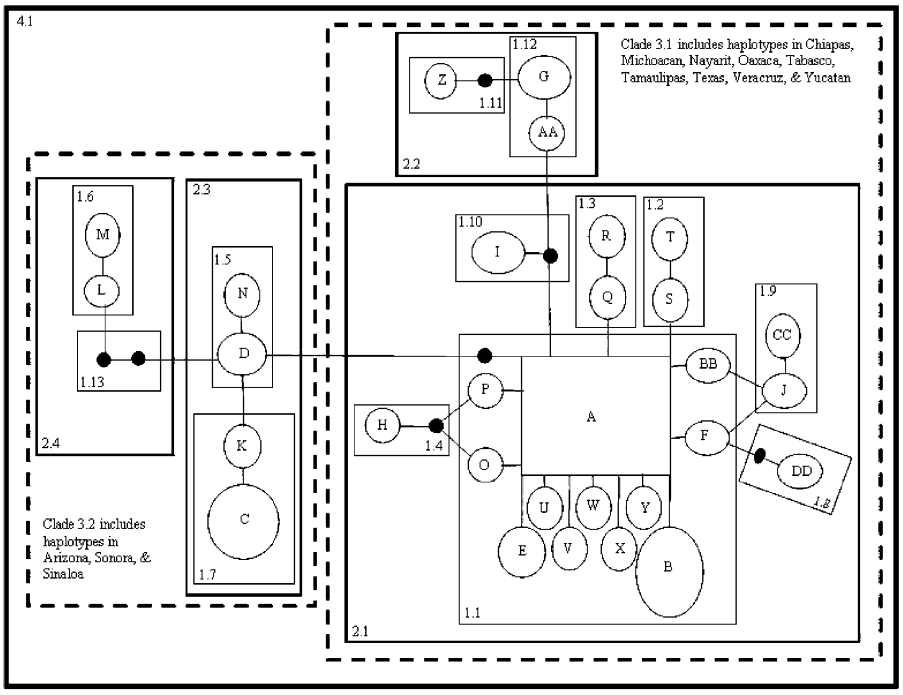

Figure 3. Haplotype network and associated nested design for nested clade analysis of North American pygmy-owls. Each branch represents a single step mutation. Black circles represent hypothetical unsampled haplotypes; lettered circles and ovals represent sampled haplotypes shown in Table 1 and displayed geographically in Figure 1. North American haplotypes C, D, K, L, N, and M form an Arizona, Sonora, and Sinaloa group. The remaining North American haplotypes form a Nayarit, Michoacan, Oaxaca, Chiapas, Yucatan, Tabasco, Veracruz, Tamaulipas, and Texas group. Size of circles and ovals represents haplotype frequency. The lettered square "A" represents the root of the network. The level of the nested clade is given; 1–*x* for 1-step, 2–*x* for 2–27 step, 3–*x* for 3-step, and 4–*x* for the 4-step clade, "*x*" is the number identifying individual clades.

Table 2. Summation of nested clade analysis with permutational chi-square probabilities for geographical structure of clades in Figure 2, from 1,000,000 resampling events. The probability of obtaining a chi-square value greater than or equal to the observed statistic by random chance is given as *P*. Clades with a probability of ≤ 0.05 were excluded from this table. Inferences were obtained following Templeton (1998) and the latest version of the inference key available online at http://bioag.byu.edu/zoology/crandall_lab/geodis.htm. Abbreviations for inferences are: RGF, restricted gene flow; IBD, isolation by distance; PF, past fragmentation

Clades	Permutational			
	χ^2 statistic	*P*	Chain of inference	Inference
Haplotypes nested in 1–1	273.83	0.005	1–2–3–4–NO	RGF with IBD
Haplotypes nested in 1–7	12.00	0.005	1–2–3–4–NO	RGF with IBD
1-step clades nested in 2–1	161.39	0.012	1–2–3–4–NO	RGF with IBD
1-step clades nested in 2–3	15.92	< 0.001	1–2–3–4–NO	RGF with IBD
2-step clades nested in 3–1	27.45	0.083	1–2–3–4–NO	RGF with IBD
2-step clades nested in 3–2	10.81	0.033	1–2–3–4–NO	RGF with IBD
Total cladogram	101.00	< 0.001	1–2–11–17–4–9–NO	PF

G. brasilianum from South America was more than 14 steps from *G. brasilianum* from North America, South America was excluded from Figure 3 and NCA. Fst (Hudson et al. 1992, DnaSP 4.0.6) for the two major clades in North America was 0.32. Phylogenetically, Arizona and Texas populations are unique, with no shared haplotypes (Figures 2 and 3). Populations from Sonora and Sinaloa, Mexico, were distinct from remaining populations in Mexico and grouped closest to haplotypes in

Arizona. Similarly, populations from Texas and Tamaulipas, Mexico (haplotype B), may constitute a distinct group. Pygmy-owls from Arizona differed by as much as 1.0% from pygmy-owls in Oaxaca, Mexico, and by as much as 0.7% from pygmy-owls in Texas.

Discussion

Currently, both North and South American populations are recognized as *G. brasilianum* (AOU 1957, 1998, 2004). Nevertheless, König et al. (1999) proposed the recognition of North American population of *G. brasilianum* as a distinct species, *G. ridgwayi*. Analysis of mtDNA variation in several *Glaucidium* species supports the recommendation that populations of *G. brasilianum* from Mexico, Texas, and Arizona represent a phylogenetically distinct group from populations occurring in South America. Phylogenetic support for the recognition of *G. tucumanum* by König et al. (1999) seems less compelling.

Patterns of mtDNA variation also provide strong evidence of two genetically distinct units in North America, one in Arizona, Sonora, and Sinaloa, and the other in Texas, Tamaulipas, and regions of South-Central Mexico. These results are congruent with earlier taxonomic studies that recognized birds from these regions as distinct subspecies (van Rossem 1937; Peters 1940; Phillips 1966; König et al. 1999). Using revised nomenclature, the Arizona, Sonora, and Sinaloa group and the other group in Texas, Tamaulipas, and regions of South-Central Mexico, would be recognized as *G. r. cactorum* and *G. r. ridgwayi*, respectively. The separation is probably the consequence of northern expansion of the pygmy-owl range and barriers to gene flow provided by the Sierra Madre Occidental and the Sierra Madre Oriental, because pygmy-owls rarely occur above 1300 m (Proudfoot and Johnson 2000).

Hewitt (2000) proposed that decline in genetic diversity at the edge of an organism's range may be considered a signature of the magnitude and direction of population expansion. Hence, with only three haplotypes in Arizona, one haplotype in Texas, and 27 haplotypes in Mexico, results from this study indicate northern expansion and recent colonization of Arizona, Sonora, and Sinaloa, and Texas and Tamaulipas, with low levels of divergence reflecting a recent common ancestry (Hewitt 2000). Based on estimated derived from MDIV, dates for divergence time for the Arizona/Sonora/Sinaloa populations and Texas/Tamaulipas/South Central Mexico populations range between 1.04 and 3.14 myr.

Comparing two models (Dispersal-Vicariance Analysis and Brooks Parsimony analysis) and the data from Zink et al. (2000, see also Zink and Blackwell-Rago 2000, Zink et al. 2001), Brooks and McLennan (2001) observed a strong vicariant relationship between avian fauna of Baja California-California and the Sonoran Desert. In addition, moderate support was obtained for a vicariant relationship between avian fauna of Baja California-California and the Sonoran Desert and areas of the Chihuahuan Desert and Sinaloan shrubland of western Mexico. An average genetic distance of 5.1% for 35 species of North American songbird suggests that speciation of this avifauna coincides with expansion of large glacial ice sheets, climatic oscillations, and major changes in the flora of the Northern Hemisphere (Webb and Bartlein 1992; Hewitt 1996; Klicka and Zink 1997, 1999; Cody et al. 2002). Flora of this region continued to change throughout the Pleistocene and the emergence of the Sonoran Desert (Cody et al. 2002). Hence, a combination of geographic barriers and shifting vegetation regimes, caused by environmental changes, may have restricted gene flow between populations of pygmy-owls in Arizona, Sonora, and Sinaloa and the remainder of Mexico and Texas.

Molecular studies of owls (Heidrich and Wink 1994; Heidrich et al. 1995; Barrowclough et al. 1999), and many other birds, have revealed similar geographic subdivisions (Avise 1994; Wink 1995; Wittmann et al. 1995; Wink et al. 1996; Zink et al. 1998, 2001). Because there are distinct differences between the Arizona, Sonora, and Sinaloa populations of Mexico and other localities in Mexico and Texas, mtDNA analysis in this study indicates that pygmy-owl populations in North America represent separate management units that taxonomically can be considered two distinct subspecies.

Based on the haplotypic separation that exists between the pygmy-owl populations of Arizona, Texas, and regions of South-Central Mexico, data from this study do not indicate genetic isolation between the distinct populations in the US and

those immediately across the border in either Sonora or Tamaulipas, Mexico. However, because NCA implies some restricted gene flow between the Arizona-Sonora and Sinaloa population, caution should be demonstrated when developing management plans for endangered pygmy-owls in Arizona. For example, management agencies may consider excluding the Sinaloan group when estimating potential gene flow, immigration through dispersal, and projected recovery of pygmy-owls in Arizona. Because genetic data provide a snap-shot of the past and recognition of genetically distinct units plays only one role in conservation policy (Barrowclough 1992), current demographic data should also be considered in developing management policies for pygmy-owls in Arizona.

There are relatively few examples of deep nuclear divisions without concomitant mtDNA separation (Zink 1997; Palumbi et al. 2001). However, because mtDNA restricts analysis to maternal lineages, other genomic regions (e.g., microsatellites) should be studied to test these conclusions. By examining both maternally and biparentally inherited genetic markers, one may obtain a detailed assessment of the genetic structure of pygmy owl populations. If other genetic markers, such as microsatellites, show low levels genetic variation within the Arizona-Sonora population and similar geographic subdivisions among North American populations, these data should be used as guidelines for pygmy-owl recovery (task 3 of the pygmy-owl recovery plan lists genetic data as essential information for pygmy-owl management, U.S. Fish and Wildlife Service 2003). In addition, research should be conducted to determine the point of separation and to ascertain the cause of RGF with IBD within the Arizona, Sonora, and Sinaloa group. If RGF with IBD resulted from urban and agricultural expansion in Arizona, Sonora, and Sinaloa, the span of isolation was approximately 75 yrs (an extremely short time span in population genetic terms).

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