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CONSERVATION GENETICS OF THE PLAIN PIGEON (*COLUMBA INORNATA*) IN PUERTO RICO

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ABSTRACT.—Over the last five to eight years, the Puerto Rico Department of Natural Resources, U.S. Fish and Wildlife Service, and University of Puerto Rico have maintained a breeding and recovery program for the Plain Pigeon (*Columba inornata*), which is declining throughout its range. To assist this ongoing effort, variability in nuclear DNA and mitochondrial DNA (in its control region) was quantified for the 20 surviving founders of this program by DNA fingerprinting and polymerase-chain-reaction/dideoxy sequencing, respectively. Compared to unrelated individuals of other birds, the DNA data indicate that a low level of genetic variation exists among the founders, among their captive-bred descendants, and in their source population. This conclusion is evaluated against the recent history of the species in Puerto Rico and then in terms of its importance to the ongoing recovery program. *Received 26 January 1993, accepted 17 November 1993.*

THE PLAIN PIGEON (*Columba inornata*) is an endemic species of the Greater Antilles (Goodwin 1983, Raffaele 1989). Traditionally, three subspecies are recognized: *C. i. exigua* (Jamaica), *C. i. inornata* (Cuba and Hispaniola), and *C. i. wetmorei* (Puerto Rico). This arrangement has been challenged by Banks (1986), who has argued that these subspecies represent a single biological form. On Puerto Rico, the taxonomic status of the species has been further complicated by its recent interactions with humans. Due to habitat destruction and unregulated hunting, the Plain Pigeon may have disappeared from the island in the 1920s, with its last reliable sighting in 1926 (Danforth 1931). Although unconfirmed reports of isolated individuals were made in the interim (Perez-Rivera 1990), it was not until 1963 that the species was rediscovered in east-central Puerto Rico in the municipality of Cidra (Leopold 1963, King 1981). It is unclear whether this extant population is endemic or represents a recent introduction from a neighboring island (Banks 1986, Perez-Rivera 1990).

Regardless of its taxonomic problems, more pressing questions about the Plain Pigeon center on its conservation and recovery (U.S. Fish and Wildlife Service 1982, Lowe et al. 1990). The remaining population on Puerto Rico, which is on the United States list of endangered species, consists of about 250 birds. This species has also declined on its other islands and already may be extinct on Jamaica. In an effort to reverse this decline, a captive-breeding program was initiated in 1984 at the Humacao Campus of the University of Puerto Rico, starting with eight squabs and one juvenile from Cidra. Over the next four years, 13 additional individuals from this population were added to complete the original breeding stock. The program has produced 126 squabs since its inception, with the captive flock now numbering 103 birds (20 remaining founders and 83 surviving descendants). Eight of these descendants were released at Cidra in April 1993 as a further step to re-establish the species throughout the island.

No such conservation programs have been developed or are being planned for the species in Cuba, Hispaniola, and Jamaica. Thus, the ongoing breeding program, maintained by the Puerto Rico Department of Natural Resources, U.S. Fish and Wildlife Service, and University of Puerto Rico, offers the best chance for the preservation and recovery of the Plain Pigeon. If non-Puerto Rican populations are extirpated, descendants of the Cidra population may become the source of individuals for re-establish-

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ing the species throughout the Greater Antilles. However, a major shortcoming of the recovery program has been the lack of information on the genetic variation present in both the captive and wild flocks. Although the precise relationship between fitness and genetic variability remains elusive (Allendorf and Leary 1986), it is the goal of the captive-breeding effort to conserve as much of this variation as possible.

Until recently, individuals in the recovery program were selected for crossing on the basis of reproductive fitness, with the most fecund pairs allowed to produce the majority of offspring. Unfortunately, this strategy is likely to result in significant reductions of genetic variation, since some founders will not have contributed to the gene pool of the descendants (Haig et al. 1990).

The 20 surviving founders (9 males and 11 females) of the breeding program comprise a representative sample of the remaining wild population at Cidra, as well as of the original stock of the recovery effort. The objective of our study is to estimate genetic variation among these 20 birds and, in the process, relate this variability to their captive-bred descendants and the Cidra population. This variation is quantified for nuclear DNA by DNA fingerprinting (Jeffreys et al. 1985, Geyer et al. 1993) and for mitochondrial DNA (mtDNA) by sequencing of its control region (Desjardins and Morais 1990, Quinn and Wilson 1993). The genetic information is compared to that for other birds, integrated with the recent history of the species in Puerto Rico, and then evaluated for its utility to the ongoing breeding program.

Materials and Methods

About 1.0 to 1.5 ml of whole blood were drawn from the brachial vein (vena ulnaris) of each founder into a 2-cc vacutainer tube with 3.0 mg of EDTA. The tubes were then gently inverted to mix their contents, placed on dry ice for transport to the laboratory, and stored at −70°C until needed. After thawing, total genomic DNA was isolated and purified from 25 to 30 μl of each blood sample by the procedure of Arctander (1988). In this way, 50 to 100 μg of pure, high-molecular-weight DNA was obtained per breeder.

In the DNA-fingerprinting comparisons, 3 μg of total genomic DNA were digested per founder with 40 units of the restriction enzyme *Hae* III. The products of these digestions were electrophoresed on a 0.8% agarose, TAE gel (Sambrook et al. 1989) until all fragments of less than 1.5 kilobases (kb) in length had run off its end. The separated DNAs were transferred by Southern (1975) blotting to a nylon membrane, as recommended by its manufacturer (Schleicher and Schuell). Hybridization of the membrane with probe 33.6 (Jeffreys et al. 1985) and subsequent visualization of the hybridized fragments were accomplished with the DNA-fingerprinting kit from Molecular Biosystems, Inc. The probe 33.6 provided in this kit has been conjugated with the enzyme alkaline phosphatase and, therefore, visualization of the DNA fingerprints relied on specific staining rather than autoradiography.

To increase accuracy, bands were scored as a single fragment only if they shared the same mobility and were consistently resolved as determined from side-by-side comparisons. Furthermore, only the larger fragments were counted (those greater than 6.5 kb) since they were well separated on the gels (i.e. not stacked close to each other like the smaller bands; Jeffreys and Morton 1987). As a final check, DNA fingerprints were also obtained with a second restriction enzyme, *Hinf* I, using the same genomic DNA samples and procedures as for *Hae* III (except that only fragments greater than 7.5 kb were scored). However, because the fingerprint data for the two enzymes could not be regarded as completely independent, these two sets of results were kept separate during all subsequent analyses.

Fingerprint differences among the 20 founders were quantified for the *Hae* III and *Hinf* I results by the same summary statistics used by Gilbert et al. (1990: fig. 2; see also Lynch 1990). To quantify interindividual variation, the difference value (D) was calculated for all possible pairs of founders as the number of different fragments between two birds, divided by the total number represented by them. Average percent difference (APD) was then defined as mean D for all possible pairs of a particular analysis, multiplied by 100. In this manner, APDs were estimated for all 20 founders, as well as for their subgroups as defined by mtDNA haplotype (see below). For each APD, an unbiased estimate of the standard error of the mean (SE, corrected for the nonindependence of pairwise Ds) was calculated according to the equation

$$SE = 100(2\bar{D}[1 - \bar{D}][1 + \bar{D}]/\bar{n}[3 + \bar{D}])^{0.5}, \quad (1)$$

where $\bar{D}$ equals mean D for all possible pairs of the analysis and $\bar{n}$ refers to the average number of scored fragments per individual (Lynch [1990:equation 10], with $\bar{S}$ replaced in his formulae by $[1 - \bar{D}]$).

In the analysis of mtDNA variation, sequence data from the 5′-end of its control region were first obtained for a 21st representative of the Plain Pigeon from Puerto Rico. This individual, which was a first-generation descendant of the breeding program, was selected initially because of its availability of frozen tissue samples (brain and liver). By starting with frozen tissue, it was possible to isolate pure mtDNA in microgram quantities using the standard protocol of Brown et al. (1979). Sequence data from the 5′-end of

TABLE 1. Summary statistics of DNA-fingerprinting data for 20 founders (*n* refers to sample size; i.e. number of individuals [I] or pairwise *D*s [II]).

	Restriction enzyme	
	Hae III	*Hinf* I
I. Fragments per founder[a]		
	11.6 ± 0.4; 9–16; 20	6.6 ± 0.4; 3–10; 20
II. Average percent difference[b]		
A. Among 20 founders	41.3 ± 13.2%; 8.3–71.4%; 190	36.3 ± 16.9%; 7.7–80.0%; 190
B. Among founders with *A*-haplotype	38.8 ± 12.7%; 17.2–61.9%; 66	42.7 ± 17.4%; 9.1–80.0%; 66
C. Among founders with *G*-haplotype	39.9 ± 13.4%; 8.3–68.4%; 28	22.0 ± 14.3%; 7.7–33.3%; 28
D. Among founders with *A*- vs. *G*-haplotypes	43.4 ± 13.3%; 10.0–71.4%; 96	36.0 ± 16.8%; 7.7–75.0%; 96

[a] $\bar{x}$ ± SE; range; *n*.
[b] APD ± SE; range; *n*.

the control region were then collected by using the polymerase chain reaction (PCR) and direct dideoxy sequencing of its asymmetrical amplification products (Allard et al. 1991). The primers used in these amplifications and sequencing were those of S. V. Edwards (HD4 [5′ CCCGACCAGCTGCATCTGTG 3′] and ND6F [5′ ATTAAACGCTACCGCTAAAC 3′] for the heavy and light strands of mtDNA, respectively; unpubl. results), as developed for the same orthologous region of passerine birds. As in all of our PCR experiments (i.e. including those for sequencing the control region of the 20 founders; see below), standard precautions were taken to avoid contamination and to ensure the fidelity of our results (Erlich et al. 1991). The new orthologue was further checked for its accuracy by sequencing it in both directions.

The identity of the newly obtained sequence was established as originating from the 5′-end of the control region by aligning it to that of the published mtDNA genome for the chicken (*Gallus gallus*; Desjardins and Morais 1990) by the LFASTA comparison algorithm (Pearson 1990). This alignment was checked for the presence of conserved elements, characteristic of the control regions for mammals and other birds (Quinn and Wilson 1993, Ramirez et al. 1993).

With this sequence information, new primers were developed, specific for the 5′-end of the control region of the Plain Pigeon (LD1, DL+, DL−, and HD3; Fig. 1). With these new primers, comparable sequence data for the 20 founders were obtained using their genomic DNA samples and either the same PCR and sequencing protocols as before (for 5 individuals, with the asymmetrical PCR preceded by a gel purification step) or the ds-DNA Cycle Sequencing System of Bethesda Research Laboratories (for the other 15). In the latter, the procedures of PCR amplification and dideoxy sequencing were performed together, as recommended by the supplier except that the double-stranded templates were purified with Centricon-30 microcentrifuge tubes (Amicon, Inc.). DNA sequencing by the two approaches revealed a single polymorphism, which allowed for the reanalysis of the DNA-fingerprinting data according to mtDNA haplotype (Lehman et al. 1992). Thus, APDs and their standard errors were calculated for individuals of each haplotype, as well as for those between them.

RESULTS AND DISCUSSION

A similar level of nuclear-DNA variation, as measured by DNA fingerprinting, was suggested by both the *Hae* III and *Hinf* I results for the 20 founders (Table 1). For the two enzymes, APDs for the founders were 41.3% (95% confidence interval of 15.4–67.2%) and 36.3% (3.2–69.4%), respectively. For the *Hae* III fingerprinting data, the APD for founders with different mtDNA types (*A* and *G*; see below) was not significantly greater than those for individuals with the same haplotype (43.4%, 95% confidence interval of 17.3–69.5% vs. 38.8%, 13.9–63.7% and 39.9%, 13.6–66.2% for the *A*- and *G*-classes, respectively; Table 1). The *Hinf* I results also were insignificant (36.0%, 3.1–68.9% for the *A*- vs. *G*-classes compared to 42.7%, 8.6–76.8% and 22.0%, 0.0–50.0% for the other two), even though the APD for the *G*-haplotype was nearly one-half less than those for the others. However, besides being insignificant, this difference was not corroborated by the *Hae* III results, which were based on almost twice as many scored fragments per individual (6.6 vs. 11.6, respectively; Table 1). The DNA-fingerprinting results and mtDNA polymorphism were considered uncoupled (cf. Lehman et al. 1992), as expected for a population with random mating.

For the mtDNA sequence data, 451 base positions (bp) from the 5′-end of its control region were initially obtained for the individual represented by frozen tissue (Fig. 1). For each of the 20 founders, a comparable region of this orthologue was then collected between primers

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          .         .         .         .         .         .         .
ACgtacCGCTAAACGATCCACTATTAATGCATgtacCTAGACATTAACCCCAACGGGCAAAATCCCCTCA    70
   --------------------------- (LD1)

          .         .         .         .         .         .         .
AACACAGCCATCCTTCCAGAgtacCTGAAATGCAATGATACCTAAGACATTCCACACTATAACCTCgtac   140
                                                              (DL-) ----
                                                            ----------

          .         .    *    .         .         .         .         .
TAAACCCATAAACAGTTAATATgtacATACCTCCAAAACAACACGGAAGTGCCTTAATACACACTATGAT   210
--------------------
---------- (DL+)

          .         .         .         .         .         .         .
TGgtacCGCCCATAACTGAGATATCTCCTGAAgtacACAAAGCAGgtacCAGGTTATCTATTAATCTTAC   280


          .         .         .         .         .         .         .
ACCTCACGTGAAACCAGCAACTCGACGCGAGAAGTATCCATCACGACTAGCTTCAGGCCCATTCTTTCCC   350


          .         .         .         .         .         .         .
CCTACACCCTAGCACGACTTGCTCTTTTGCGCCTCTGGTTCCTATGTCAGGGCCATAACTTGCCGATTCC   420
                                                    (HD3) ----------


          .         .         .
CATGAACTTGCTCTTCACAGATGCAGCTGGT                                          490
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Fig. 1. Mitochondrial DNA sequence of 5′-end of control region for Plain Pigeon represented by frozen tissue. The single variable position (site 165 with *A*–*G* polymorphism) is highlighted with asterisk. This polymorphism is associated with one of *Rsa* I sites in sequence (which are denoted in lowercase lettering). Dashes correspond to new primers used for PCR amplification and sequencing, with their sequences being: (LD1) 5′ TACCGCTAAACGATCCACTATTAATGCA 3′ (L); (DL+) 5′ CTATAACCTCGTACTAAACCCAT 3′ (L); (DL−) 5′ TTAACTGTTTATGGGTTTAGTAC 3′ (H); and (HD3) 5′ AAGAGCAAGTTCATGGGA-ATCGGCAAGT 3′ (H) (L and H refer to light and heavy strands of mtDNA, respectively). Identity of this sequence was confirmed by presence of following conserved elements, characteristic of control regions for mammals and other birds (Quinn and Wilson 1993, Ramirez et al. 1993): F box (sites 277–305); D box (382–406); and C box (428–451, which remains incomplete for Plain Pigeon). This mtDNA sequence has been deposited in GenBank data base (accession number M98393).

LD1 and HD3, with 250 or more base pairs apiece obtained for 15 individuals (with two of these completed for the entire segment). Thus, more than 6.2 kb of orthologous sequence information were resolved for the 20 founders and the additional bird. Nevertheless, only one sequence polymorphism was detected (an *A*–*G* difference at position 165), with the *A*- and *G*-haplotypes occurring at frequencies of 60.0% (12 founders) and 40.0% (8), respectively, for a haplotypic diversity of 0.49 (Nei 1987:260). The two mtDNA haplotypes varied by percent sequence divergences of 0.27 to 0.61%, with the range due to the unequal amounts of sequence information available for each bird. Within-group variation (i.e. average interindividual divergence; Nei 1987:256) for the 20 founders was calculated as 0.22%. The individual represented by frozen tissue (the only captive-bred descendant of the breeding program sequenced in this study) carried the *A*-haplotype (Fig. 1), in agreement with that of its mother.

In other species of birds, APDs generally are 70% or greater for unrelated individuals versus 50% or less for first-degree relatives (parents/offspring and full siblings). Examples include House Sparrows (*Passer domesticus*; Burke and Bruford 1987, Wetton et al. 1987), Dunnocks (*Prunella modularis*; Burke et al. 1989), Purple Martins (*Progne subis*; Morton et al. 1990), Indigo Buntings (*Passerina cyanea*; Westneat 1990), Barn Swallows (*Hirundo rustica*; Smith et al. 1991), Blue Tits (*Parus caeruleus*; Kempenaers et al. 1992), Spotted Sandpipers (*Actitus macularia*; Or-

ing et al. 1992), Hispaniolan Parrots (*Amazona ventralis*; Brock and White 1992), and Stripe-backed Wrens (*Camplyorhynchus nuchalis*; Piper and Rabenold 1992). In the Plain Pigeon, the APDs for the 20 founders (for *Hae* III, 41.3%, 95% confidence interval of 15.4–67.2%; and for *Hinf* I, 36.3%, 3.2–69.4%) are about one-half of those for unrelated individuals of other species. Only 2 of the 190 comparisons for the *Hae* III results (71.4%, involving the same founder in both cases) and 5 for the *Hinf* I data (involving the same individual in each instance, but one different from that above) are associated with *D*s of 70% or greater. Instead, the APDs of the founders are most similar to those for first-order relatives of other birds (Table 1 and references above).

For the 5′-end of the control region, the average interindividual divergence for the founders (0.22%) is less than those for all known populations of Grey-crowned Babblers (*Pomatostomus temporalis*; Edwards 1992), Lesser Snow Geese (*Chen caerulescens*; Quinn 1992), Dunlins (*Calidris alpinus*; Wenink et al. 1993), and Ruddy Turnstones (*Arenaria interpres*; Wenink et al. 1994). With few exceptions, the latter exceed the former by severalfold. Thus, both sets of DNA data indicate that the 20 founders of the recovery program are characterized by low levels of genetic variability compared to unrelated individuals of other birds. As these 20 comprise a random sample from the Cidra population, the same conclusion applies to the remaining wild flock in Puerto Rico, which has remained small (<250 individuals) since passing through a severe bottleneck between 1926 and 1958 (Lowe et al. 1990). Small population size, therefore, provides one obvious explanation for the reduced genetic variation of this species on the island (Nei et al. 1975, Denniston 1978). The alternative possibility that the low levels are due to sampling of DNA regions evolving at unusually slow rates is considered unlikely, since this pattern is exhibited by both nuclear and mtDNA genomes.

Furthermore, the possibility of inbreeding in the reduced Cidra population is raised by the close similarities between the APDs of the 20 founders and first-degree relatives of other birds (see above). In Puerto Rican Parrots (*Amazona vittata*), where inbreeding has occurred, reproductive success has been related with genetic differences as measured by DNA fingerprinting (Brock and White 1992). Following the recommendations of the Brock and White study, the best way to minimize the likelihood of inbreeding depression in the recovery program is to focus on founders and their descendants that are genetically distinct. In this regard, matings should favor males and females with the highest *D*-values. Of the 99 potential pairings of male/female founders, 28 are associated with *D*s of greater than 40.0% for both *Hae* III and *Hinf* I (ca. the APDs of all 20 individuals for both enzymes; Table 1). Such matings maximize the genetic differences among individuals and, given the results of Brock and White (1992), presumably their reproductive successes too.

The availability of a known pedigree, spanning three generations of the 83 surviving descendants, provides the means to check that each of the 20 original founders is contributing to the offspring (Lacy 1989, Haig et al. 1990). In this way, problems of inbreeding and drift due to the preferential pairing of founders and descendants can be minimized while still emphasizing those who are genetically distinct. Genetic diversity thereby is maximized while equalizing the genome-equivalent contributions of the original founders. This strategy is most likely to preserve the greatest amount of genetic variation in the breeding program.

Copies of our individual DNA fingerprinting results and mtDNA haplotype identifications have been deposited with the Puerto Rico Department of Natural Resources. The next critical steps of this research are to compile information on the reproductive successes of the 20 founders and to obtain comparable molecular data for their descendants. Although it will take time to complete, such research will greatly enhance the significance of our findings by establishing further the relationship between genetic variation and fitness.

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