

Application of DNA fingerprinting to the recovery program of the endangered Puerto Rican parrot

M. KELLY BROCK*[†] AND BRADLEY N. WHITE^{‡§}

*Department of Biology, Queen's University, Kingston, ON, Canada K7L 3N6; and [‡]Department of Biology, McMaster University, Hamilton, ON, Canada L8S 4K1

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ABSTRACT The Puerto Rican parrot was reduced to ≈ 13 animals in 1975 and as a conservation measure, a captive population was established from a few founders taken from the wild between 1973 and 1983. The number of successful breeding pairs in captivity has been low, and the captive breeding program has not been as productive as that of the closely related Hispaniolan parrot. Therefore, a genetic study was initiated to examine the relative levels of relatedness of the captive founders using levels of bandsharing in DNA fingerprints. Unrelated captive founder Puerto Rican parrots had the same average level of bandsharing (0.41) as second-degree relatives of the Hispaniolan parrot (0.38, $P > 0.05$), with an inbreeding coefficient of 0.04. High levels of bandsharing ($>40\%$) between pairs of males and females correlated with reproductive failure, suggesting that inbreeding depression is partly responsible for the low number of breeding pairs. Consequently, DNA profiling can be used to guide the captive breeding program for the Puerto Rican parrot, and other endangered species, by identifying pairs of males and females with low levels of bandsharing.

The Puerto Rican parrot *Amazona vittata* is one of the most endangered birds in the world. Its habitat was reduced as a result of colonization of the West Indies by Europeans during the 18th and 19th centuries, and its numbers declined from millions to ≈ 2000 in 1937, followed by further drastic declines to a minimum of ≈ 13 in 1975 (1). Currently, <30 Puerto Rican parrots exist in the wild, and ≈ 65 exist in captivity. The captive population was established in the 1970s to prevent extinction of the species and to bolster the wild population through releases of captive-produced individuals (2). In addition, a captive breeding program for the less threatened and taxonomically related Hispaniolan parrot *Amazona ventralis* was established to provide cross-fostering parents for Puerto Rican parrot eggs and nestlings and as models for testing avicultural practices before they were used on Puerto Rican parrots (3).

The majority of all fledgling young in both flocks descended from four (Puerto Rican parrot) or three (Hispaniolan parrot) founders. The two species have differed in fecundity despite similar founder populations and equivalent environmental conditions and management practices. For example, between 1980 and 1990, 92% (11/12) of Hispaniolan parrot pairs produced fledgling young compared to 46% (6/13) of Puerto Rican parrot pairs (see Table 3). Although there has been no intentional inbreeding among the captive Puerto Rican parrots, annual trends in reproductive performance by Puerto Rican parrots remain poor compared to Hispaniolan parrots. Furthermore, past anecdotal evidence of inbreeding in the wild population of Puerto Rican parrots (1) suggested that inbreeding depression may be a limiting factor in Puerto Rican parrot productivity.

We used DNA fingerprinting to assess the degree of relatedness among captive founder Puerto Rican parrots. Hypervariable multilocus genetic markers like DNA fingerprints are inherited in Mendelian fashion (4) and, therefore, can be used to assess familial genetic relationships by similarity indices or the proportion of bands shared between pairs of individuals (5–7). When frequency distributions of similarity coefficients for known genetic relationships are developed, the degree of relatedness may be estimated for individuals with unknown genetic relationships. Our objective was to determine frequency distributions of DNA fingerprint bandsharing coefficients (BSCs) of captive Hispaniolan parrots with known pedigrees and compare them to captive Puerto Rican parrots.

MATERIALS AND METHODS

DNA was isolated from a total of 70 captive Hispaniolan parrots, including 9 founder group members and their descendants spanning five generations, and 65 Puerto Rican parrots, including 15 surviving captive founder parrots, 30 of their descendants spanning three generations, and 20 parrots descended from all known wild breeding pairs between 1983 and 1989. All captive parrots were maintained at the Luquillo aviary, Palmer, Puerto Rico, by the U.S. Fish and Wildlife Service (8). Sampling from nests in the wild was conducted in 1988 and 1989 when adult parrots were absent from nesting areas in the Caribbean National Forest, Palmer, Puerto Rico. No attempts were made to capture the breeding pairs for sampling, nor other wild adult parrots, because there were no proven safe means of doing so. Nine of the 15 wild nestlings from the 1988/89 nests fledged in the wild, and 6 were retained in captivity. The remaining 8 wild samples were collected from nestlings transferred from nests in the wild to captivity between 1983 and 1987.

Standard techniques used to extract DNA from whole blood and to obtain DNA fingerprints were described in detail elsewhere (9). Two minisatellite probes were used: human 33.6 probe (4) and the mouse periodicity gene *Per* (10). The human 33.15 minisatellite probe (4) was tested, but it identified few loci and complex alleles of linked fragments in the Hispaniolan parrot (9), which made it unsuitable as a marker system.

The number of loci identified by each probe and the nature of their alleles were analyzed by conducting segregation analyses of bands in the 2- to 23-kilobase size range in the DNA fingerprints of parents and 13 offspring in a Hispaniolan parrot family and parents and 9 offspring in a Puerto Rican parrot family (9, 11, 12). For unrelated parrots, BSCs were calculated for 33.6 and *Per* DNA fingerprints separately, each by $2s/N_i + N_j$, where s is the number of bands shared by a

pair of birds i and j , and N_i and N_j are the total number of bands scored in the DNA fingerprint of each bird (13). Combined probe BSCs were calculated using the same formula, except that s , N_i , and N_j were equal to the total number of bands shared in the 33.6 and *Per* DNA fingerprints of two individuals, and the sum total of bands scored in both DNA fingerprints from both individuals, respectively. BSCs were calculated for breeding pairs and first and second degree relatives in the same manner using *Per* DNA fingerprints only. Bands were considered to be the same if their relative intensities were similar and if the position of the bands were the same using internal size markers as guides (14, 15). Combined BSCs of unrelated captive Hispaniolan parrots and captive Puerto Rican parrots were grouped in 10% intervals and plotted against the proportion of individuals whose BSCs fell within the range of each interval.

All pairwise comparisons in the nonrelative groups of captive Hispaniolan parrots ($N = 36$ among seven bloodlines) and captive and wild Puerto Rican parrots ($N = 67$ among seven bloodlines, and $N = 20$ among six bloodlines, respectively) were used to calculate average BSCs. The standard errors were corrected according to Lynch (7), which accounted for redundancy in the data set that resulted from comparing each individual to every other individual in the group. BSCs of Puerto Rican parrots with known relationships were excluded from the nonrelative data set; however, because there are relatives in the founder group, the number of independent pairwise observations group was corrected. For example, the degree of bandsharing between individuals A and B would be an independent observation. However, the level of bandsharing between individuals A and C (individual B's sibling) would be related to the first observation. Only half as much more information would be gained by comparing individuals A and C, thus this pairwise comparison would be considered a half observation. We used this approach for all relatives in the Puerto Rican parrot founder group and obtained a corrected number of 36 pairwise comparisons for computing the corrected standard error. Average BSCs for first- and second-degree relatives were calculated by taking the overall mean from the means of all bloodlines. For example, the overall average BSC for parents and offspring was calculated by averaging the BSCs of each father with each of his offspring and then taking the overall mean of all families. Likewise, average of BSCs were calculated for each sibship, followed by the overall average for all sibships. The standard errors were not corrected for first- and second-degree relatives, nor for breeding pairs.

One-sample t tests were used to test hypotheses that the average observed Hispaniolan parrot BSCs, from each category of relationship, were equal to values expected for outbreeding populations (6, 16–18). This analysis was used to validate the appropriateness of the captive Hispaniolan parrots as a reference group to which the Puerto Rican parrots may be compared. Two-sample t tests (18) were used to test hypotheses that average observed BSCs (33.6, *Per*, and combined) for unrelated captive founder Puerto Rican parrots was greater than that observed for unrelated captive founder Hispaniolan parrots. Once that was determined, two-sample t tests were used to test whether unrelated captive founder Puerto Rican parrots were as genetically similar as first-degree Hispaniolan parrot relatives or second-degree Hispaniolan parrot relatives. Mated pairs of Puerto Rican parrots were categorized as successful (produced fledglings) or unsuccessful (did not produce fledglings) and the average *Per* BSCs of each category were compared using a Mann–Whitney two-sample t test. One pair of captive successfully breeding Puerto Rican parrots was omitted from the analysis because the female died before tissue samples were collected. Postmortem tissue samples were not available; therefore, a BSC coefficient for that pair could not be

determined. Two types of degrees of freedom were used at the 0.05 level of significance: minimally, the number of blood lines used to average the BSCs in each category of relationship (N_b), and maximally, the total number of pairwise comparisons in each group (N_p). In all cases, null hypotheses were rejected or accepted using either value of degrees of freedom.

The analysis of the wild Puerto Rican parrots was somewhat limited by the fact that only nestlings could be sampled. Therefore, to approach the question of how genetically similar unrelated wild Puerto Rican parrots might be to each other, the average level of bandsharing between the combined probe DNA fingerprints from unrelated nestlings were compared to those from unrelated captive founder Puerto Rican parrots, using two-sample t tests. An analysis of variance was used to test whether or not the average levels of bandsharing between wild nestlings (full siblings), siblings in the captive founder group, and siblings produced in captivity were the same. This analysis was used to gain some information about the genetic status of the pairs of Puerto Rican parrots breeding in the wild.

Average band frequency was calculated for the founder group of captive Puerto Rican parrots by estimating the frequency of 43 bands (identified by the *Per* probe) among seven captive Puerto Rican parrots founders representing each family bloodline (one individual was picked at random in cases where there were siblings). Based on the average frequencies of bands and alleles, an inbreeding coefficient for the captive founder group of Puerto Rican parrots was estimated according to Kuhnlein *et al.* (19). A similar analysis was conducted using 83 bands identified among the nine captive Hispaniolan parrot founders.

RESULTS

The general attributes of the Hispaniolan and Puerto Rican parrot DNA fingerprints were comparable to those reported for other species (Fig. 1 and Table 1). Only one instance of a new length variant was observed in *Per* DNA fingerprints of the Hispaniolan parrot family, suggesting a mutation rate in these loci (4×10^{-4}) similar to other species (11, 15, 21). The instances of band linkage and allelism were low and similar to the level found in humans (<10% of all bands scored) (11, 15). The lower number of paternal loci observed in the 33.6-probed DNA fingerprints of the Hispaniolan parrots does not appear significant. In a human pedigree, where an average of 20 bands were scored per individual, only 13 maternal loci were detected by the 33.6 probe whereas 20 paternal loci were detected (11). In the Hispaniolan parrot, only 12 paternal bands were scored, 4 of which were shared with the mother and 2 of which were linked. The range of bands scored per individual parrot (nonrelatives) for either probe was 10–23: a slightly narrower range than that reported for humans (15).

The average BSC for unrelated Hispaniolan parrots was comparable to that reported for other species (16, 22). Based on an average allele frequency of 0.10, we found that the observed *Per* BSC for each category of relationship was not different from expected values based on Hardy–Weinberg equilibrium (Table 2; $P > 0.05$) (6, 16). The distribution of BSCs from unrelated captive founder Puerto Rican parrots was somewhat closer to 1, whereas the distribution of BSCs of unrelated Hispaniolan parrots was closer to 0 (Fig. 2). This was the first indication that the Puerto Rican parrots were inbred. In fact, the average combined BSC of unrelated Puerto Rican parrots, 0.44 ± 0.09 ($N_b = 7$ and $N_p = 36$), was significantly greater than the average BSC of unrelated Hispaniolan parrots, 0.17 ± 0.02 ($N_b = 7$ and $N_p = 36$; $P < 0.05$). Furthermore, the average *Per* BSC for unrelated Puerto Rican parrots, 0.41 ± 0.12 , was less than that of

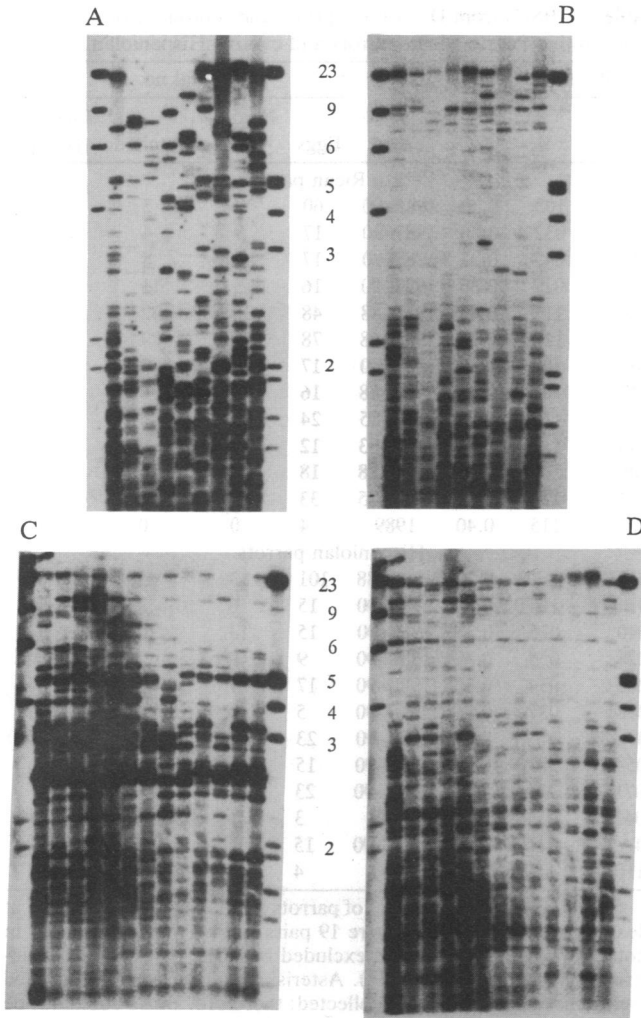


FIG. 1. DNA fingerprints of captive founder Hispaniolan parrots (A and B) and captive founder Puerto Rican parrots (C and D) identified by minisatellite probes *Per* (A and C) and 33.6 (B and D). The genomic DNA lanes are flanked on either side by molecular size markers as indicated in kilobase pairs.

first-degree Hispaniolan parrot relatives, 0.61 ± 0.02 ($N_b = 19$ and $N_p = 200$; $P \approx 0.05$), but not different from second-degree Hispaniolan parrot relatives, 0.38 ± 0.03 ($N_b = 11$ and $N_p = 69$; $P > 0.05$; Fig. 3). Also interesting is the fact that while we found an overall similarity in the level of bandsharing between unrelated captive Puerto Rican parrots and second-degree Hispaniolan parrot relatives, we detected no differences in the level of bandsharing between first-degree Puerto Rican parrot relatives, 0.65 ± 0.02 ($N_b = 11$ and N_p

Table 2. BSCs from *Per* DNA fingerprints of captive Hispaniolan parrots

Relationship	<i>E</i>	BSC
Nonrelatives	0.19	0.19 ± 0.02
First degree		
Parent/offspring	0.61	0.59 ± 0.02
Full siblings	0.62	0.63 ± 0.03
Second degree		
Half-siblings	0.44	0.43 ± 0.03
Grandparents	0.46	0.32 ± 0.06
Aunts/uncles	0.43	0.39 ± 0.04

Data are mean \pm SEM. Expected levels of bandsharing for different orders of relationship (*E*) were based on Hardy-Weinberg equilibrium as reported by Honma and Ishiyama (6).

= 117) and first-degree Hispaniolan parrot relatives ($P > 0.05$; Fig. 3). This was attributed to the fact that pairs of Puerto Rican parrots and Hispaniolan parrots that produced viable offspring had similarly low levels of bandsharing, 0.34 ± 0.04 ($N_p = 5$) and 0.29 ± 0.02 ($N_p = 9$), respectively ($P > 0.05$). However, the average BSC of Puerto Rican parrot pairs unsuccessful at producing offspring, 0.47 ± 0.03 ($N_p = 7$), was significantly higher ($P < 0.05$). Only one breeding pair of Puerto Rican parrots had a high BSC of 0.48, whereas the remaining four breeding pairs had BSCs < 0.40 . All mated Hispaniolan parrot pairs had BSCs < 0.40 (Table 3). It seems, therefore, that the difference in the number of pairs with low levels of bandsharing correlates with the difference in fecundity observed between the two species.

The average band frequency (v_i) in the DNA fingerprints of seven Puerto Rican parrots selected at random from sibling groups representing an original captive founder family line was 0.3614 and was 0.1966 for the DNA fingerprints of all nine Hispaniolan parrots. The average allele frequencies (q) were 0.205 and 0.095, respectively, for each group of parrots. By using the following equation, $v_i = q^2 + Fq(1 - q) + 2q(1 - q) - 2Fq(1 - q)$, an inbreeding coefficient (F) was estimated as 0.04 for the founder group of Puerto Rican parrots and essentially 0 for the founder group of Hispaniolan parrots.

The similarity of combined probe BSCs of unrelated captive founder Puerto Rican parrots (0.44) and unrelated nestlings from recent wild nests, 0.51 ± 0.03 ($N_p = 20$), indicated that wild parrots were also inbred ($P > 0.05$). Furthermore, BSCs between some captive founders (three parrots) and wild parrots were high, with a combined probe bandsharing average of 0.49 ± 0.10 . To gain some insight about the genetic status of the six pairs of Puerto Rican parrots that bred in the wild at different times during the 1980s, we compared the average levels of bandsharing of full siblings from recent wild nests (0.68 ± 0.02 , $N_b = 6$), captive founder siblings (0.54 ± 0.07 , $N_b = 4$), and siblings produced in captivity (0.67 ± 0.02 ,

Table 1. Comparison of analyses of DNA fingerprints from unrelated Puerto Rican parrots, Hispaniolan parrots, barn swallows (*Hirundo rustica*), and humans

Parameter	<i>Per</i>			33.6		
	PRP	HP	BS	PRP	HP	HUM
Mean BSC	0.42	0.19	0.24	0.45	0.16	0.14
SD	0.12	0.07	0.13	0.10	0.08	0.09
Bands per individual, mean no.	16.2	16.8	15.5	18.4	17.6	18.1
Shared bands, mean no.	6.7	3.2	3.8	8.4	2.9	2.6
Loci, mean no.	9.5	13.6	13.5	10.0	14.7	16.3
Maternal loci, no.	7.0	13.0	13.7	10.0	17.0	16.4
Paternal loci, no.	8.0	14.0	14.3	7.0	7.0	16.2

PRP, Puerto Rican parrots; HP, Hispaniolan parrots; BS, barn swallows; HUM, humans; SD, standard deviations of mean BSCs. SDs were used instead of corrected standard errors to make the parrot data comparable to the barn swallow and human data. Data for *Per* in the barn swallows were from ref. 20 and for 33.6 in humans were from ref. 15.

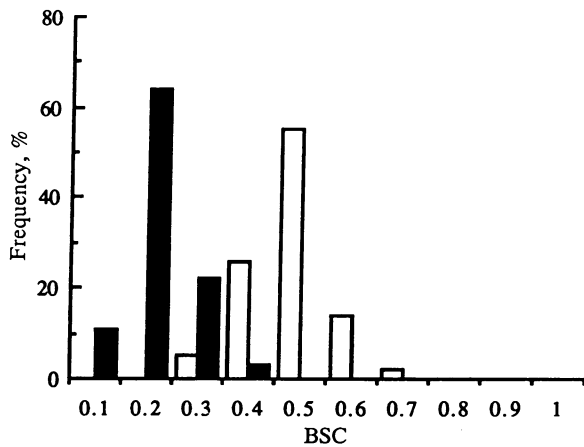


FIG. 2. Frequency distribution of BSCs from combined 33.6 and *Per* DNA fingerprints from unrelated captive founder Hispaniolan parrots (solid bars) and unrelated captive founder Puerto Rican parrots (open bars). The BSCs from each pair of individuals were grouped into 10 intervals from 0 to 0.10, 0.11 to 0.20, 0.21 to 0.30, . . . , 0.91 to 1.0.

$N_b = 4$). There were no differences in the overall level of bandsharing between the sibling groups ($P > 0.05$). We concluded, therefore, that as in captivity, only pairs of Puerto Rican parrots in the wild with low levels of bandsharing were successfully producing offspring.

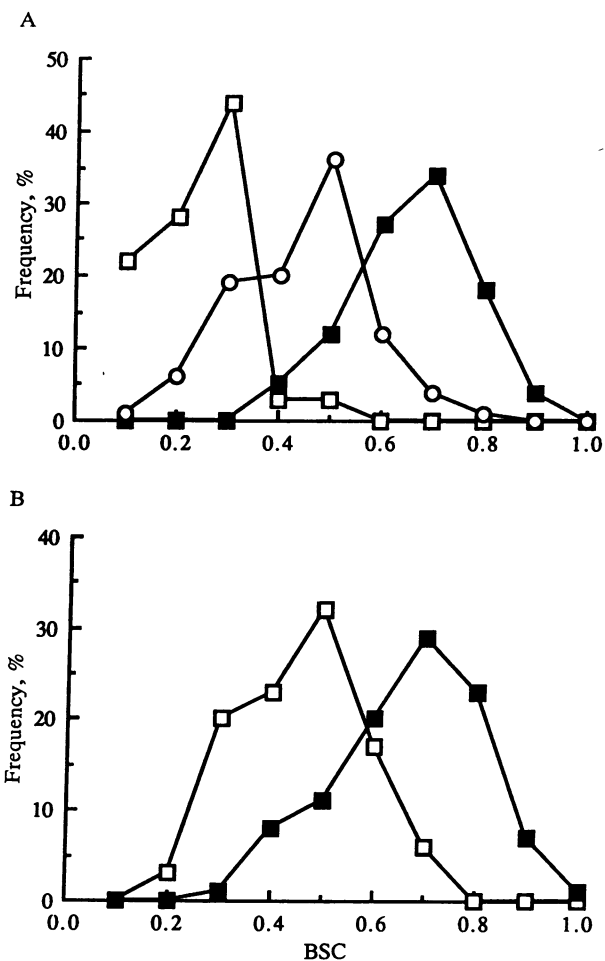


FIG. 3. Frequency distributions of *Per* BSCs. (A) Unrelated Hispaniolan parrots (□), first-degree relatives (■), and second-degree relatives (○). (B) Unrelated Puerto Rican parrots (□) and first-degree relatives (■). BSCs were grouped as in Fig. 2.

Table 3. BSCs from DNA fingerprints and reproductive data from captive Puerto Rican parrots and captive Hispaniolan parrots

Pairs		<i>Per</i> BSC	Years	Total no.			
♂	♀			Eggs	Fertile eggs	Hatched	Fledged young
Puerto Rican parrots							
017	103	0.30	1985-90	60	9	2	1
083	032	0.36	1987-90	17	9	4	2
023	049	0.21	1987-90	17	9	8	6
077	029	0.36	1988-90	16	15	11	9
111	112	0.48	1981-88	48	36	22	19
109	110*	ND	1978-88	78	55	29	21
106	108	0.47	1989-90	17	0	0	0
107	108	0.55	1986-88	16	0	0	0
117	115	0.53	1982-85	24	0	0	0
106	113	0.27	1980-83	12	0	0	0
106	105	0.53	1985-88	18	0	0	0
083	116	0.56	1980-85	33	0	0	0
107	115	0.40	1989	4	0	0	0
Hispaniolan parrots							
4M72	4F73	0.26	1980-88	101	83	45	40
4B379	1F70	0.30	1988-90	15	6	3	3
040	025	0.38	1987-90	15	13	10	9
028	055	0.39	1988-90	9	9	7	5
206	034	0.20	1988-90	17	7	6	6
4981	037	0.26	1988-90	5	4	4	4
4A180	5F74	0.31	1987-90	23	21	18	15
054	309	0.22	1988-90	15	7	6	5
071	221	0.26	1989-90	23	1	1	1
042	051	0.39	1989	3	0	0	0
4A579*	1F70	ND	1988-90	15	6	3	3
HP1*	1F70	ND	1982	4	4	3	2

Successfully breeding pairs of parrots were those that produced at least one fledgling. There were 19 pairs of Puerto Rican parrots in total; however, 6 pairs were excluded because males were considered immature (<5 years old). Asterisks indicate parrots that died before tissue samples were collected; therefore, BSCs could not be determined (ND). Some pairs were manipulated to lay more eggs per year than the normal clutch size of four eggs. Some eggs broke and fertility was not always determined. There were 20 pairs of Hispaniolan parrots in total; however, 3 pairs were excluded because males were <5 years of age, and 5 pairs were excluded because they were full-sibling matings.

DISCUSSION

Jeffreys *et al.* (11) showed that different DNA minisatellite probes detect different families of hypervariable loci, but the general attributes of the DNA fingerprints (e.g., number of bands scored, average number of bands shared, and levels of allelism and linkage) were similar. We observed this in the Hispaniolan parrots and Puerto Rican parrots; for example, two probes gave similar bandsharing information from unrelated conspecifics. It has been questioned whether DNA fingerprints give reliable estimates of relatedness (5, 7). Indeed, it would be difficult to assess the relationship between two individuals drawn at random from a population. With reference information however, it is possible to estimate the degree of similarity of a group of individuals. Obviously, the population from which the reference information is drawn is important: a population of conspecifics would be preferred. However, it is unlikely that an extant nonendangered reference population of conspecifics will exist for endangered endemic island species. Nevertheless, we feel that a taxonomically related species can give reliable reference information for this type of analysis. For many endangered species, this may be the only method for deriving such information. There are some caveats of course, including the assumptions that hypervariable loci of DNA fingerprints are selectively neutral, that the level of genetic variation identi-

fied in DNA fingerprints were comparable between two closely related species prior to the decline of the endangered species, and that present day species differences are due to contractions of the endangered population. These assumptions are not unfounded; Flint *et al.* (23) showed that loss of genetic variation (measured from DNA fingerprints) from human Polynesian populations was not due to selection but to population bottlenecks and small population sizes.

Kuhnlein *et al.* (19) demonstrated a linear dependence of band frequency on inbreeding. In their experiment, inbreeding coefficients and average band frequencies were known and used to estimate average allele frequencies. For the Puerto Rican parrot and the Hispaniolan parrot, the inbreeding coefficients of the captive founder groups were unknown, thus they were estimated using the relationship with average band and allele frequencies. The estimated inbreeding coefficient for the captive founder Puerto Rican parrots is higher than the maximum value (2%) desired by animal breeders to avoid adverse effects of inbreeding depression (24). This finding is of particular importance because the inbreeding coefficient is a relative measure, usually calculated by pedigree analysis on the assumption that the reference individuals are unrelated (25). For captive breeding populations, the reference individuals are the founders, usually of unknown genetic origin. For an endangered species, the assumption that the founders are unrelated, or noninbred, may be incorrect. For example, by assuming that the founder Puerto Rican parrots were unrelated (except for those known to be siblings), a pedigree analysis would indicate that the inbreeding coefficient of the captive population is 0. In that case, inbreeding depression may not be recognized as a factor partly responsible for the poor reproductive performance of pairs in Table 3. Consequently, resources may be allocated to measures (such as changes in facility design or management protocols) that probably will not effect an increase in productivity.

The implications of this study are clear. Not only have we obtained valuable information directly applicable to the conservation of the Puerto Rican parrot, but we also feel that this work will serve as a model for other conservation programs. We demonstrated that there is an association between the levels of bandsharing in DNA fingerprints, inbreeding, and reproductive success in a captive breeding program for an endangered species. Our data indicate that the captive Puerto Rican parrot flock was founded by second-degree relatives (Fig. 3). More importantly, we discovered that pairs that produced fledgling young had BSCs at the lowest end of the distribution (Table 3). In some species, BSCs of comparable values (or higher) have been reported for nonrelatives, but those are for species with traits that were artificially selected (19), domesticated (17), or species with small confined populations (26). It appears that the Puerto Rican parrots are inbred as a result of continual population decline and that low reproductive performance is due to inbreeding depression.

We may enhance captive breeding programs (and minimize inbreeding) by using DNA fingerprints to identify genetically desirable pairs of males and females. For the Puerto Rican parrot, those would be pairs with BSCs ($Alu I/Per$) <0.40 . For instance, we looked at alternative pairings of males and females in Table 3 and found that male 107 and female 116 have a BSC of 0.11: we suggest that these two parrots be paired. In cases where BSCs are <0.40 , but no offspring are produced, such as male 106 and female 113, behavioral incompatibility may cause infertility. Therefore, we suggest giving the male a choice of females with whom he shares low levels of bandsharing and with whom he may be behaviorally

compatible. For example, male 106 has BSCs of <0.40 with three other founder females. Alternatively, artificial insemination may be used to overcome other types of problems associated with poor reproductive performance (27), such as behavioral problems or physical handicaps, thus DNA profiling can be used not only to identify optimal semen donors for receptive females but also to verify the paternity of resulting offspring.

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