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Founded: Genetic Reconstruction of Lineage Diversity and Kinship Informs *Ex situ* Conservation of Cuban Amazon Parrots (*Amazona leucocephala*)

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Abstract

Captive breeding is a widespread conservation strategy, yet such programs rarely include empirical genetic data for assessing management assumptions and meeting conservation goals. Cuban Amazon parrots (*Amazona leucocephala*) are considered vulnerable, and multiple on-island captive populations have been established from wild-caught and confiscated individuals of unknown ancestry. Here, we used mitochondrial haplotypic and nuclear genotypic data at 9 microsatellite loci to quantify the extent and distribution of genetic variation within and among captive populations in Zapata Swamp and Managua, Cuba, and to estimate kinship among breeders (n = 88). Using Bayesian clustering analysis, we detected 2 distinct clusters within the Zapata population, one of which was shared with Managua. Individuals from the cluster unique to Zapata possessed mitochondrial haplotypes with affinities to Cuban subspecies (*A. l. leucocephala*, *A. l. palmarum*); the shared cluster was similar, but also included haplotypes closely related to the subspecies restricted to Cayman Brac (*A. l. hesterna*). Overall mean kinship was low within each captive population (–0.026 to –0.012), with 19 and 11 recommended breeding pairs in Zapata and Managua, respectively, ranked according to mean kinship and informed by molecular sexing. Our results highlight the importance of understanding population history within *ex situ* management programs, while providing genetic information to directly inform Cuban parrot conservation.

Resumen

La cría en cautiverio constituye una estrategia de conservación extendida, sin embargo tales programas rara vez incluyen datos genéticos para evaluar las asunciones de manejo y alcanzar los objetivos de

conservación. La cotorra cubana (*Amazona leucocephala*) es considerada vulnerable, y en Cuba se han establecido múltiples poblaciones en cautiverio a partir de ejemplares de ascendencia desconocida, los cuales han sido capturados en el medio natural o confiscados. En el presente trabajo, empleamos información proveniente de haplotipos mitocondriales y 9 loci microsatélites nucleares para cuantificar la magnitud y distribución de la variación genética dentro y entre las poblaciones cautivas de la Ciénaga de Zapata y Managua, Cuba, así como estimar índices de parentesco entre los reproductores (n=88). Empleando métodos de agrupamiento con base en la inferencia Bayesiana, detectamos 2 grupos dentro de la población de Zapata, uno de los cuales resultó ser compartido con Managua. Los individuos del grupo único de Zapata presentaron haplotipos mitocondriales relacionados con los referidos para las subespecies cubanas (*A. l. leucocephala, A. l. palmarum*). En el grupo compartido ocurrió de modo similar, pero este incluyó además haplotipos cercanamente relacionados con la subespecie restringida a Caimán Brac (*A. l. hesterna*). La media de parentesco global resultó ser baja dentro de cada población (–0.026 a –0.012), con 19 y 11 parejas de reproductores recomendadas para Zapata y Managua respectivamente, clasificadas de acuerdo a las medias de parentesco y el sexado molecular informado. Nuestros resultados resaltan la importancia de comprender la historia poblacional para los programas de manejo *ex situ*, mientras proveen información genética para orientar directamente sobre la conservación de la cotorra cubana.

Subject areas: Conservation genetics and biodiversity; Molecular systematics and phylogenetics
Key words: *Amazona leucocephala*, captive breeding, Cuba, mean kinship, microsatellite

Introduction

In the midst of the current biodiversity crisis, stemming the loss of species requires multifaceted, integrated management strategies. Captive breeding is playing an increasingly important role across taxa to boost wild population sizes through supplementation and to create insurance colonies *ex situ* as a hedge against extirpation from the wild (Conde et al. 2011). Yet, there is increasing recognition in the *ex situ* management community that ongoing programs are not likely to achieve their conservation goals under current strategies (Lacy 2013). Traditionally, pedigrees have been used to manage captive populations under a strategy of minimizing mean kinship (Ballou and Lacy 1995). Although theoretical and empirical studies have shown that this approach may effectively limit the risks of genetic drift and inbreeding in captive populations (Montgomery et al. 1997; Lacy 2000), many such programs are characterized by incomplete or absence of pedigree information. In such cases, simplifying assumptions are required that can have genetic costs. One example is the founder assumption, where captive population founders are assumed to be equally unrelated and noninbred. When this assumption is violated, kinship within the population may be severely underestimated, biasing estimates of mean kinship and inbreeding coefficients on which management decisions are based (Russello and Amato 2004). The use of molecular markers can help overcome some of the challenges associated with the founder assumption by providing tools for empirically estimating kinship and identifying genetically important individuals to inform breeding strategies (Doyle et al. 2001; Russello and Amato 2004).

Amazon parrots (genus *Amazona*), a group of charismatic and largely imperilled species, continue to face dramatic population declines due to poaching for the pet trade and habitat destruction (Collar 2000). The Cuban parrot (*Amazona leucocephala*) is one such example. Formerly distributed throughout much of the Bahamas, Cuba, and Cayman Islands, *A. leucocephala* is now restricted to 6 islands and is listed as Vulnerable on the 2012 Red List of Cuban vertebrates (González Alonso et al. 2012). Within Cuba, there are 2 traditionally recognized subspecies: *A. l. leucocephala*, found in the eastern mainland; and *A. l. palmarum*, found in the western mainland and on Isla de la Juventud off the southwestern coast (Ottens-Wainright et al. 2004). Genetic studies based on mitochondrial DNA (mtDNA) have determined that these subspecies are not evolutionarily distinct entities (Russello et al. 2010).

Given its conservation status, captive breeding populations of Cuban parrots have been established on the Cuban mainland by the Empresa Nacional para la Protección de la Flora y la Fauna (ENPFF) at Zapata Swamp, Managua, and the National Zoo breeding facilities. The Zapata Swamp breeding facility was founded with 10 individuals in 1982, presumably from the *A. l. leucocephala* subspecies, which were collected from the wild in Zapata Swamp. By 1991, 95 wild individuals had been captured in Zapata Swamp and incorporated into the program. To date, the Zapata Swamp captive population has increased to 117 individuals, including 35 breeding pairs, and is currently managed as a closed population. The breeding facility in Managua was founded in 2002, largely using individuals confiscated from illegal trade. Now numbering 20 breeding pairs, these individuals are thought to have been originally collected in the wild from Isla de la Juventud and belong to the *A. l. palmarum* taxon. Lastly, captive Cuban parrots housed at the National Zoo are exemplars of mixed origin and unknown ancestry and are largely maintained for exhibition. The overall goal of the ENPFF program is to provide a hedge against extinction of the wild population, yet at present, all captive populations are managed in the absence of pedigree and genetic information.

Limited knowledge regarding the origin of and relatedness among the founders as well as the lack of accurate studbooks are major challenges to the effective management of the on-island, Cuban parrot captive breeding programs. Here, we used mtDNA haplotypic, nuclear microsatellite genotypic, and molecular sexing data to: 1) quantify the extent and distribution of genetic variation within and among captive populations in Zapata Swamp and Managua; 2) estimate kinship among individuals of unknown ancestry; and 3) propose management recommendations based on all available demographic and genetic information.

Materials and Methods

Sampling

We collected samples from Cuban parrot captive populations in Zapata Swamp (22°27′10.7″N,–81°08′22.6″W) and Managua

(22°57′04″N,–82°17′28″W), the 2 ENPFF populations established with the expressed purpose of maintaining lineage integrity. Feather samples were collected from 64 adults at Zapata Swamp in August 2011 and from 24 adults at Managua in February 2012 and preserved in 95% ethanol. All samples were collected and transported under CITES import permit # 12CA00292/CWHQ-1 and CITES export permit # C0001456. Total genomic DNA was extracted using the Nucleospin kit (Macherey-Nagel, Düren, Germany) following manufacturer's protocols.

Molecular Sexing

Amazon parrots, including *A. leucocephala*, are phenotypically sexually monomorphic. To fill this knowledge gap, all individuals were molecularly sexed targeting the sex-linked chromo-helicase-DNA-binding gene using the polymerase chain reaction (PCR)-based test and methods described in Russello and Amato (2001).

Genetic Data Collection

Genotypic data were collected at a panel of 9 microsatellite loci (AgGT04, AgGT17, AgGT19, AgGT21, AgGT22, AgGT42, AgGT72, AgGT83, and AgGT90), originally developed for the St. Vincent parrot (*Amazona guildingii*; Russello et al. 2001; Russello et al. 2005), that have been successfully applied to the Bahama parrot (subspecies of Cuban parrot; Russello et al. 2010). Loci were PCR amplified using an M13-fluorescent labeling technique (Schuelke 2000) on an ABI Veriti thermal cycler in 12.5 µL reactions. Reaction and cycling conditions followed Russello et al. (2010). Loci were coloaded and run on an Applied Biosystems 3130XL DNA automated sequencer. Fragment lengths were determined using the software GENEMAPPER 4.0 (Applied Biosystems, Foster City, CA).

Based on the results of the genotypic data analyses (see below), targeted individuals from Zapata Swamp (n = 8) and Managua (n = 4) were PCR amplified at a 655 base-pair segment of mtDNA including control region 1 using the primers LThr and CR522Rb (Eberhard et al. 2001). PCRs were carried out on an ABI Veriti thermal cycler in 25 µL volumes, with reaction and cycling conditions following Russello et al. (2010). PCR products were purified using ExoSAP-IT (USB® Products, Santa Clara, CA) and sequenced using Big Dye 3.1 terminator chemistry on an ABI 3130XL DNA sequencer (Applied Biosystems). Sequences were visualized and edited using SEQUENCHER 4.7 (Gene Codes, Ann Arbor, MI).

Data Archiving

Primary data underlying these analyses have been deposited in Genbank (KP453974, KP453975, KP453976, KP453977, KP453978, and KP453979) and the Dryad Digital Repository (http://datadryad.org/resource/doi:10.5061/dryad.802bs).

Genotypic Variation and Population Differentiation

Loci were tested for the presence of null alleles using MICROCHECKER (Van Oosterhout et al. 2004). Tests for linkage disequilibrium between pairs of loci and exact tests for deviation from Hardy–Weinberg equilibrium (HWE) were implemented in GENEPOP 3.3 (Raymond and Rousset 1995). Significance levels were adjusted for multiple comparisons using the sequential Bonferroni (Rice 1989). Allelic diversity as well as observed and expected heterozygosities were calculated at each locus, and a list of private alleles were tabulated using GENALEX 6.5 (Peakall and Smouse 2012). Mean population relatedness was estimated using the Queller and Goodnight (1989) method implemented in GENALEX 6.5 (Peakall and Smouse 2012). Inbreeding coefficients (F_{IS}) were calculated using GENETIX (Belkhir et al. 2004).

To determine whether substructure exists within the sample, the Bayesian method of Pritchard et al. (2000) was implemented in STRUCTURE 2.3.4. Run lengths were set to 500 000 Markov Chain Monte Carlo replicates after a burn-in period of 500 000 using correlated allele frequencies under a straight admixture model. We varied the number of clusters (K) from 1 to 8 with 20 iterations per value of K. The most likely number of clusters was determined by plotting the log probability of the data ($\ln \Pr(X|K)$) across the range of K values tested and selecting the K where the value of $\ln \Pr(X|K)$ plateaued. We also calculated ΔK (Evanno et al. 2005) as implemented in STRUCTURE HARVESTER (Earl and vonHoldt 2011). The 20 iterations were averaged using CLUMPP (Jakobsson and Rosenberg 2007) to produce a single result. Levels and significance of population differentiation were determined using the Weir and Cockerham (1984) estimator, θ, as implemented in FSTAT (Goudet 2001).

Kinship Analyses

Pairwise kinship between individuals was calculated in SPAGeDi (Hardy and Vekemans 2002). A mean kinship value was then calculated for each individual and potential breeding pairs were suggested following the "Ranked MK Selection" method described in Ivy and Lacy (2012). Population mean kinships were also calculated as the arithmetic mean of all individual mean kinships.

Phylogenetic Analyses

Sequences collected in this study (n = 12) were combined with those from Russello et al. (2010, n = 34), culminating in a data set that consisted of representatives of all 5 subspecies of *A. leucocephala* and 2 outgroups: *A. ventralis* and *A. vittata*. Sequences were unambiguously aligned using MUSCLE as implemented in GENEIOUS 6.1 (Biomatters Ltd, San Francisco, CA) using default settings. A Bayesian tree was reconstructed using MrBayes 3.1 (Ronquist and Huelsenbeck 2003) and the HKY+I+G model of nucleotide substitution, the latter selected according to the Akaike information criterion as implemented in Modeltest v.3.06 (Posada and Crandall 1998). The analysis was run using 4 heated chains for 1.1×10^6 generations after a burn-in period of 1.0×10^4, saving a tree every 200 generations thereafter. The first 25% of trees were discarded, and a consensus tree with a posterior probability support threshold of 50% was produced.

Results

Genotypic Variation and Population Differentiation

Two of the 9 loci (AgGT42 and AgGT83) tested positive for null alleles in Zapata Swamp, but not in Managua. In Zapata Swamp, 4 loci were found to deviate from HWE, whereas no loci displayed significant deviation from HWE in Managua. Evidence for linkage disequilibrium was found in 28 of 36 pairwise comparisons of loci in Zapata Swamp, but in only one comparison (AgGT19/AgGT72) in Managua. When tests for linkage disequilibrium were rerun accounting for the substructure revealed in the Zapata Swamp captive population (see below), only 1 of 36 and 7 of 36 pairwise comparisons of loci were significant in Zapata α and Zapata β, respectively. Given these findings and the fact that linkage disequilibrium has not been previously identified at these same loci in wild and captive populations of multiple species of Amazon parrots (Russello and Amato 2004; Wright et al. 2005; Leite et al. 2008), including the Bahama parrot (subspecies of Cuban parrot; Russello et al. 2010), it is likely that these significant associations are an artifact of how the Zapata Swamp captive population was founded through the opportunistic collection of individuals, especially in light of the

finding that breeders assign to multiple divergent lineages (see below). Consequently, all downstream analyses were based on genotypic data at all 9 microsatellite loci. The final data set contained <1% missing data.

The most likely number of clusters in the Bayesian clustering analysis was $K = 2$ ($\Delta K = 133.2$; Figure 1). The cutoff for complete membership to a cluster was set to 0.8, where individuals with membership to a cluster below this threshold considered to be admixed ($n = 6$; Figure 1). We excluded admixed individuals from further analyses in order to evaluate the diversity within lineages at each facility, resulting in a refined data set of 82 individuals. In all further analyses, the samples were treated as 3 populations: Zapata α ($n = 18$); Zapata β ($n = 40$); and Managua β ($n = 24$) based on their STRUCTURE assignment. There was statistically significant differentiation ($P < 0.05$) between all pairwise θ comparisons among the 3 groups; the β lineage in Zapata and Managua exhibited the lowest value (0.043), with higher levels detected for Zapata α and Managua β (0.181), and Zapata α and Zapata β (0.166). Yet, a large number of alleles were private to each of the β populations (Table 1). The Zapata α group had lower levels of heterozygosity (0.64) and number of effective alleles (2.1) than the 2 β groups, which had similar levels of diversity across the indices (H_o = 0.75–0.77, A_e = 4.3–4.4; Table 1). Mean relatedness was low in each group, ranging from –0.059 to –0.026 (Table 1). Managua β had the highest average level of inbreeding, although it was still quite low and not significantly different from zero (0.03; Table 1). Both of the Zapata groups had negative inbreeding coefficients (Table 1).

Kinship Analyses

Population mean kinships were low in each of the 3 groups (–0.026 to –0.012), although the range of values within each group was large (Table 1). Potential mating pairs were set based on data from molecular sexing (Supplementary Table 1 online) and the Ranked MK method (Ivy and Lacy 2012; Supplementary Table 2 online). High

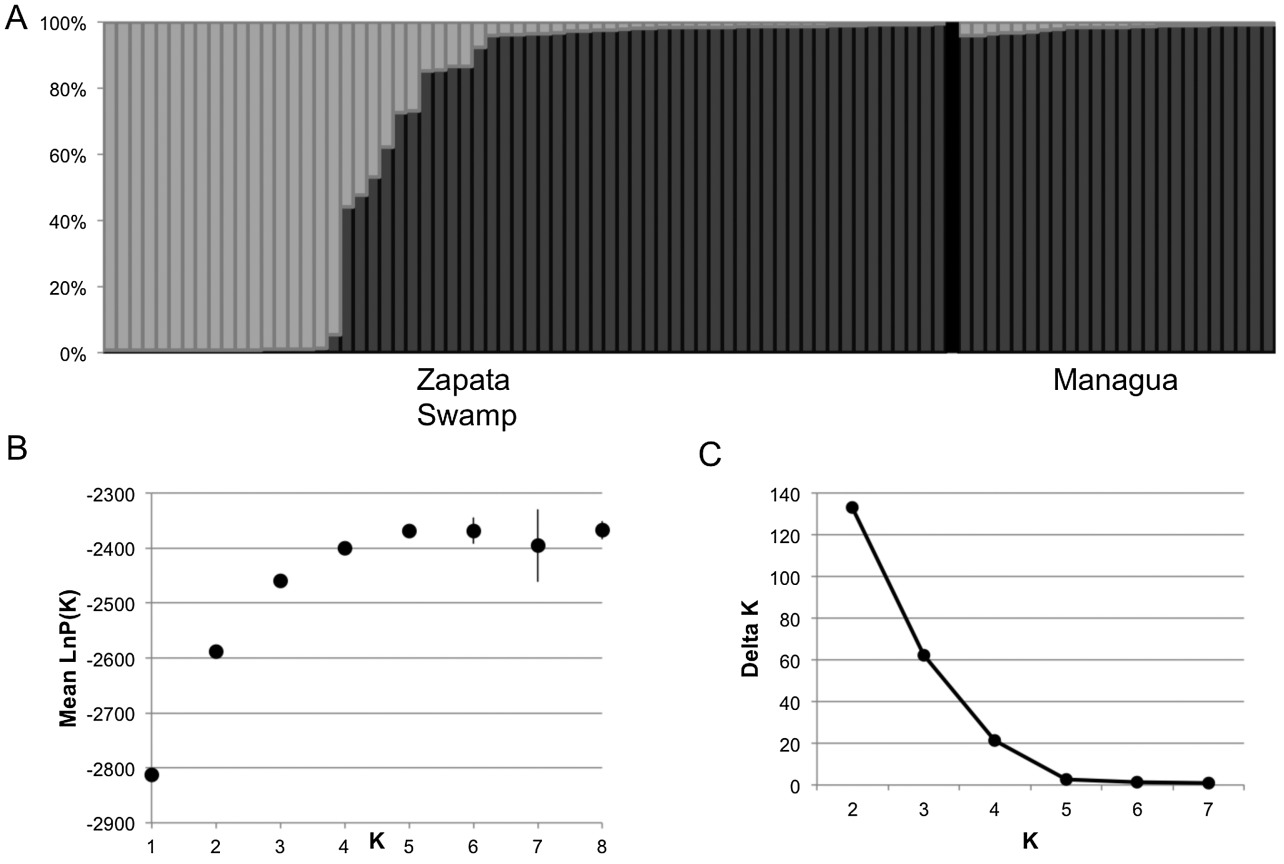

Figure 1. Bayesian clustering according to the approach of Pritchard et al. (2000) as implemented in STRUCTURE. (**A**) Bar plot showing the results for the inferred $K = 2$. Each color represents an inferred genetic cluster (light gray referred to in the text as α, dark gray as β); each bar on the *x* axis represents an individual, with the *y* axis displaying the percentage of membership to each genetic cluster. (**B**) The corresponding plot of ln P(*K*) for every value of *K*. (**C**) The plot of Δ*K* (Evanno et al. 2005) for detecting the number of *K* groups that best fit the data.

Table 1. Diversity indices and number of breeding pairs for each of the genetic clusters of *Amazona leucocephala* at the 2 breeding facilities

	N	BP	H_o	H_e	P_A	A_e	MK (range)	$R_{Q\&G}$	F_{IS}
Zapata α	18	8 (5)	0.64	0.50	1	2.1	–0.026 (–0.317, 0.555)	–0.059	–0.24
Zapata β	40	19 (14)	0.77	0.75	19	4.3	–0.012 (–0.129, 0.317)	–0.026	0.03
Managua β	24	12 (11)	0.75	0.75	16	4.4	–0.022 (–0.129, 0.222)	–0.043	–0.02

Along with the population mean kinship, the lowest and highest pairwise kinships within the population are reported. *N*, number; BP, number of breeding pairs with number of high priority pairs in parenthesis; H_o, observed heterozygosity; H_e, expected heterozygosity; P_A, private alleles; A_e, number of effective alleles; MK, mean kinship; $R_{Q\&G}$, population mean relatedness; F_{IS}, inbreeding coefficient.

priority breeding pairs with inbreeding coefficients below the population mean kinship were identified within all 3 groups (Table 1, Supplementary Table 1 online).

Haplotypic Variation and Phylogenetic Analysis

To identify lineage of origin, 12 individuals were sequenced (4 each from Zapata α, Zapata β, and Managua β with STRUCTURE membership coefficients >0.98 to the corresponding cluster) at a 655-bp mtDNA fragment that overlapped with data collected from a previous phylogeographic study of the Cuban parrot complex (Russello et al. 2010). Here, we detected 6 haplotypes, none of which were sampled in previous studies of the complex (Russello et al. 2010). Five of the recovered haplotypes belong to the *leucocephala/palmarum* clade identified in Russello et al. (2010), whereas the other haplotype is closely related to published haplotypes recovered from vouchered specimens of the *hesterna* subspecies from the Cayman Islands (Figure 2). All individuals sequenced from Managua β and the Zapata α STRUCTURE clusters fell within the *leucocephala/palmarum* clade, whereas all Zapata β individuals clustered with *hesterna* (Figures 1 and 2).

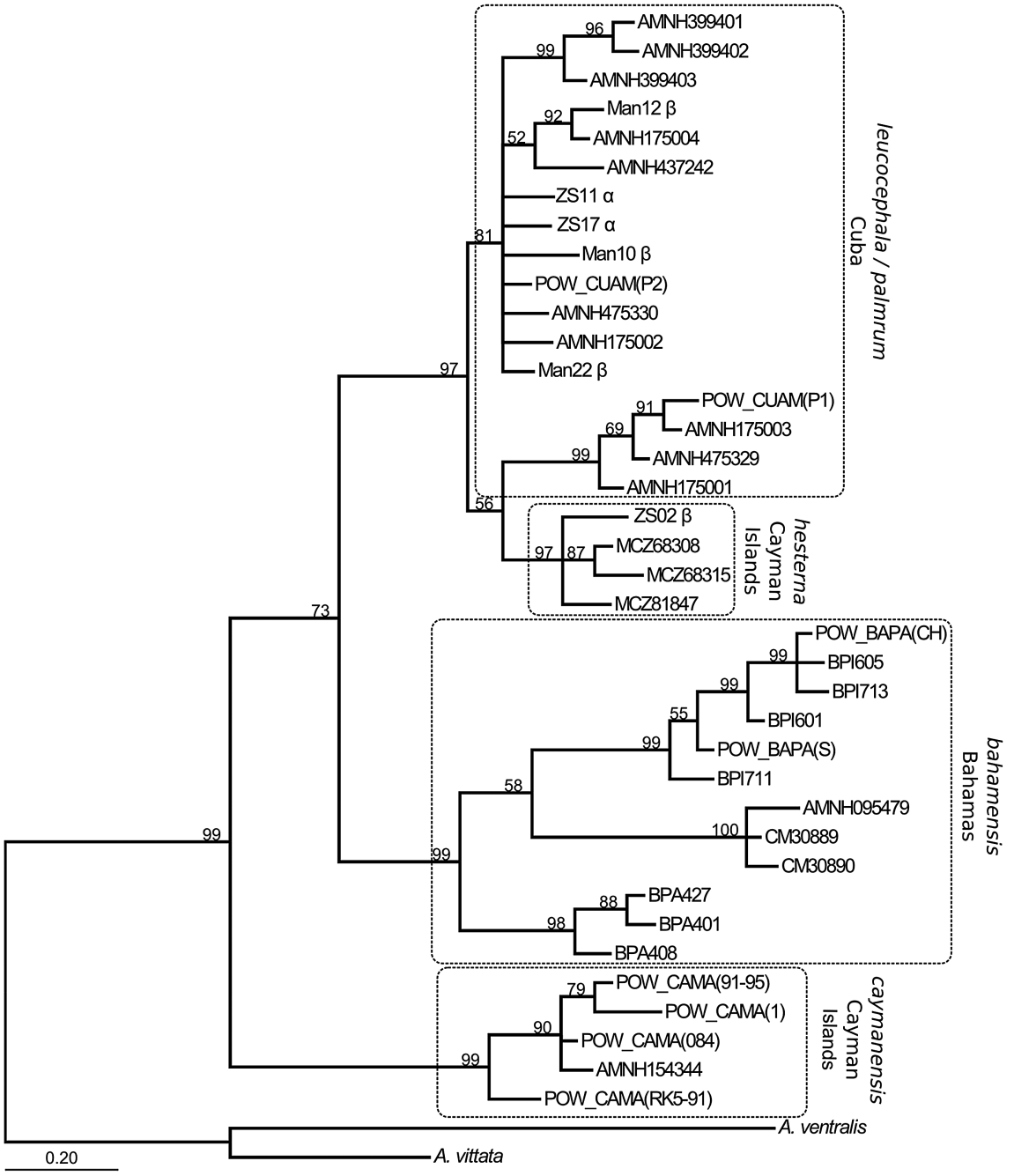

Figure 2. Bayesian tree based on the mitochondrial control region, reconstructed from Russello et al. (2010), depicting relationships among *Amazona leucocephala* haplotypes and the placement of individuals newly sequenced in this study. The symbols next to the haplotypes recovered from the Zapata (ZS) and Managua (Man) populations correspond to their membership to the microsatellite genetic clusters (α or β) identified in the STRUCTURE analysis. Bayesian posterior probabilities (>50%) are indicated above the branches.

Discussion

With the decline of wildlife populations worldwide, scientifically managed captive breeding and reintroduction programs continue to constitute an important component of integrated conservation strategies. Under ideal conditions, founders of captive populations are chosen based on their individual genetic traits so that the *ex situ* population will be representative of the breadth of genetic diversity *in situ* (Witzenberger and Hochkirch 2011). In many cases, however, the founders of captive populations are opportunistically collected and of unknown origin. Such was the case with the founders for the Managua (individuals confiscated from the pet trade) and Zapata Swamp (collected from the wild without prior genetic evaluation) captive populations of Cuban parrots.

To date, program activities have been directed toward managing the historically recognized Cuban parrot subspecies separately at the different sites (*A. l. leucocephala* at Zapata Swamp, *A. l. palmarum* at Managua). Our genetic results, considered in tandem with those from a previous study of Cuban parrot phylogeography (Russello et al. 2010), revealed an entirely different history. The Zapata Swamp captive population can be split into at least 2 distinct lineages based on the genotypic data, with 4 individuals possessing a mtDNA haplotype that forms a well-supported clade with those sampled from vouchered specimens of a third subspecies of Cuban parrot restricted to Cayman Brac (*hesterna*; Figure 2). The remaining individuals sequenced from the Zapata Swamp and Managua captive populations are interspersed within the *leucocephala/palmarum* clade, but there is no genetic evidence that these subspecies are distinct entities (Russello et al. 2010). Moreover, the housing (and breeding) of individuals that originated from otherwise distinct natural populations very likely contributed to our findings of large-scale linkage disequilibrium within the Zapata Swamp captive population, as predicted by population genetic theory for subdivided populations with recent contact (Nei and Li 1973).

As there are no formal studbooks and limited records associated with these programs, it is difficult to interpret these patterns. Zapata Swamp facility staff indicated that confiscated birds of unknown origin have been introduced into the population (Alonso A, personal observation). Consequently, it is possible that individuals from the *hesterna* subspecies endemic to Cayman Brac have been unintentionally included in the breeding pool. However, results from the Bayesian clustering analysis based on microsatellite data are not congruent with this pattern. Zapata Swamp individuals with affinity to the *hesterna* clade are members of the β cluster, which is shared with Managua and individuals that are part of the *leucocephala/palmarum* clade on the mtDNA tree. Yet, the lack of genotypic data from reference individuals from *hesterna* limits our ability to accurately infer a genetic contribution from this non-Cuban mainland subspecies. Additional studies of wild populations across the Cuban parrot complex, combining mtDNA and nuclear DNA data with morphological assessments, will be required to disentangle the relationships among these formally recognized taxa and to further assess their relative contributions to the captive populations on mainland Cuba.

Generally, captive breeding programs strive to preserve evolutionarily distinct lineages to maximize adaptive diversity and minimize potential for outbreeding depression. In the case of Cuban parrots, subspecies do not correspond to the lineages detected based on microsatellite data. Instead there is one lineage that is found at both breeding facilities, and one that is unique to Zapata Swamp, with an unclear geographical basis. Until surveys of wild populations can confirm the substructure detected in this study, we recommend following the precautionary principle by only establishing breeding pairs that belong to the same lineage (Edmands 2007) and removing admixed individuals from the breeding programs. This strategy reduces the size of the breeding pool for each lineage, which may have long-term impacts on levels of inbreeding and genetic diversity. There has been much discussion regarding the impacts of lumping and splitting endangered taxa, potentially resulting in unnecessary extinctions (Frankham et al. 2012, 2014; Russello and Amato 2014). However, the β clusters at Zapata and Managua are currently characterized by reasonably high levels of heterozygosity, low levels of relatedness, and inbreeding and do not show indications of population decline (data not shown). Likewise, the α cluster at the Zapata Swamp facility, although possessing low levels of allelic diversity (Table 1), still maintains low mean relatedness and levels of inbreeding.

Given the recommendations for preserving lineage integrity, it will be especially important for managers to make scientifically informed breeding decisions to minimize the genetic consequences of small population size. Here, we paired molecular sexing information with the Ranked MK method (Ivy and Lacy 2012) to design breeding pairs at each facility that maintain the genetic lineages and minimize mean kinship (Supplementary Table 2 online). By following this pairing scheme, inbreeding should be minimized and maximal genetic diversity retained. As mean kinship and the associated ranking of pairings are relative, when new individuals are added to the breeding pool, all of the pairings will need to be reevaluated.

Conclusions

This study highlights the utility of molecular approaches to the applied aspects of managing captive breeding programs in both the short-term and long-term. On a very practical level, correctly identifying the sex of individuals and thus establishing proper breeding pairs will increase the immediate success of the breeding program. In the long-term, designing breeding pairs that minimize mean kinship and preserve distinct lineages will help retain the genetic diversity of the founders, reducing the genetic consequences of maintaining small populations in captivity. As the genetic and demographic management of captive populations requires an iterative approach, ongoing evaluations are required, particularly if new founders are incorporated into the program. At the very least, the knowledge gained regarding the relatedness among current breeders can be used to inform future pedigree-based approaches to minimize mean kinship.

Scientifically managed *ex situ* breeding programs are most effective when directly integrated with conservation strategies and comprehensive study of wild populations. In this case, our lack of knowledge of the extent and distribution of genetic variation *in situ* limited our ability to identify the origin of the multiple lineages detected *ex situ*, reducing the gene pool of optimal breeders in captivity and potentially limiting the recognition of otherwise cryptic diversity in the wild. Consequently, future surveys of the wild populations of Cuban parrots are of critical importance for informing an interactive *in situ/ex situ* population management approach to help maintain the viability of this species of national and international conservation significance.

Supplementary Material

Supplementary material can be found at http://www.jhered.oxfordjournals.org/

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