RESEARCH ARTICLE

Cryptic diversity and conservation units in the Bahama parrot

M. A. Russello · C. Stahala · D. Lalonde · K. L. Schmidt · G. Amato

Abstract Wide application of genetic approaches has enhanced the detection of cryptic diversity, even in historically well-studied organisms. In addition to improving our knowledge of biodiversity, detection of cryptic diversity can have important management implications within imperiled groups, such as the Cuban parrot complex (*Amazona leucocephala*). Bahama parrots (*A. l. bahamensis*) were once widespread throughout the archipelago, but are now restricted to the two largest islands (Abaco and Inagua). Mitochondrial DNA-based population genetic and phylogenetic analyses revealed the distinctiveness of the Abaco, Inagua and now extirpated Acklins populations, detecting diagnostic character support and reciprocal monophyly indicative of three phylogenetic species. Congruent results were obtained for the Abaco and Inagua populations based on Bayesian clustering analyses of microsatellite genotypic data. Genetic signatures of demographic contraction were identified on Abaco, but not Inagua. These findings were consistent with lower genetics-based estimates of effective population size on Abaco, as well as the disproportionate human impacts reported on the island relative to Inagua. Overall, our results suggest that the taxonomy of the Cuban parrot complex requires revision and that the conservation status of the Abaco phylogenetic species should be immediately elevated to reflect its historical isolation, recent population decline and continued threats to its persistence.

Keywords *Amazona leucocephala bahamensis* · Cuban Amazon parrot · Population genetic · Phylogenetic species · Historical DNA · Museum specimen · Subspecies dilemma

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Introduction

Cryptic diversity continues to be identified as genetics-based approaches, including DNA-barcoding initiatives, are broadly applied to a wide breadth of taxa. Perhaps surprisingly, unrecognized genetic divisions have been detected in megafauna (Beheregaray and Caccone 2007) which historically constitute some of the most thoroughly studied organisms on the planet. Likewise, these groups are among the most imperiled, so that detection of cryptic

diversity contributes immediately and directly to conservation efforts (Russello et al. 2005a; Brown et al. 2007; Poulakakis et al. 2008; Gentile et al. 2009).

Amazon parrots (Genus *Amazona*) are among the most recognizable and coveted of all birds. Their colorful plumage and general ability to mimic speech has endeared them to humans as pets for centuries and, as an unfortunate consequence, contributed to the threatened status of the majority of *Amazona* species in the wild. Cuban parrots (*Amazona leucocephala*) are no exception. Once widespread across all major islands in the Bahamas, Cuba and the Cayman Islands, *A. leucocephala* is currently restricted to six islands in the Greater Antilles (Fig. 1). Declines across the range have been attributed to habitat destruction, hunting, capture for the pet trade, introduction of feral mammals and natural disasters (Snyder et al. 2000; Wiley et al. 2004). Consequently, *A. leucocephala* is listed in Appendix I of CITES and classified as near-threatened by IUCN and BirdLife International, though individual subspecies and populations have been deemed endangered or critically endangered (Snyder et al. 2000; Wiley et al. 2004). Amazon parrots in general, and Cuban parrots, specifically, have been subject to a broad range of ecological and evolutionary studies (recent examples include Eberhard and Bermingham 2004; Ottens-Wainright et al. 2004; Russello and Amato 2004; Brightsmith 2005a, b; Enkerlin-Hoeflich et al. 2006; Ribas et al. 2007; Leite et al. 2008; Matuzak et al. 2008; Wright et al. 2008), yet taxonomic uncertainty remains within *A. leucocephala*, with

unknown consequences for the conservation status of contemporary island populations.

Historically, Cuban parrot taxonomy has been largely based on qualitative assessments of geographical variation in plumage coloration resulting in a number of descriptions and revisions over the past 150 years. There were early suggestions that the Cayman Island populations were distinct from the Cuban populations, warranting separate species status (Cory 1891; Clark 1905). Later, Peters (1928) recognized four subspecies, concluding that the Isla de la Juventud (formerly Isla de Pinos) and Cuban populations were indistinguishable. Currently, there are five formally described subspecies of *A. leucocephala* distributed on Cuba (*A. l. leucocephala*), Isla de la Juventud (*A. l. palmarum*), Great Abaco and Great Inagua (*A. l. bahamensis*), Cayman Brac (*A. l. hesterna*), and Grand Cayman (*A. l. caymanensis*) (Fig. 1).

Recent studies have investigated the distinctiveness of the extant named Cuban parrot taxa. Ottens-Wainright et al. (2004) concluded that four subspecies of *A. leucocephala* (*A. l. hesterna* was not sampled) constitute distinct lineages reflecting current geographic distributions based on results of a phylogenetic analysis of partial mitochondrial DNA (mtDNA) cytochrome *b* (cyt *b*) sequences for 23 individuals of *A. leucocephala* and exemplars from 15 additional *Amazona* species. Improving upon the population sampling of earlier studies, Reynolds and Hayes (2009) examined 18 morphological and plumage characters from all six extant populations of *A. leucocephala* subsp. as

Fig. 1 Distribution map of the *Amazona leucocephala* complex. Subspecies names are indicated in *italics* next to islands upon which they are currently distributed. Inset maps depict Abaco and Inagua islands at a finer scale. Localities of Bahama parrot nests sampled for the current study are also indicated (*filled star*)
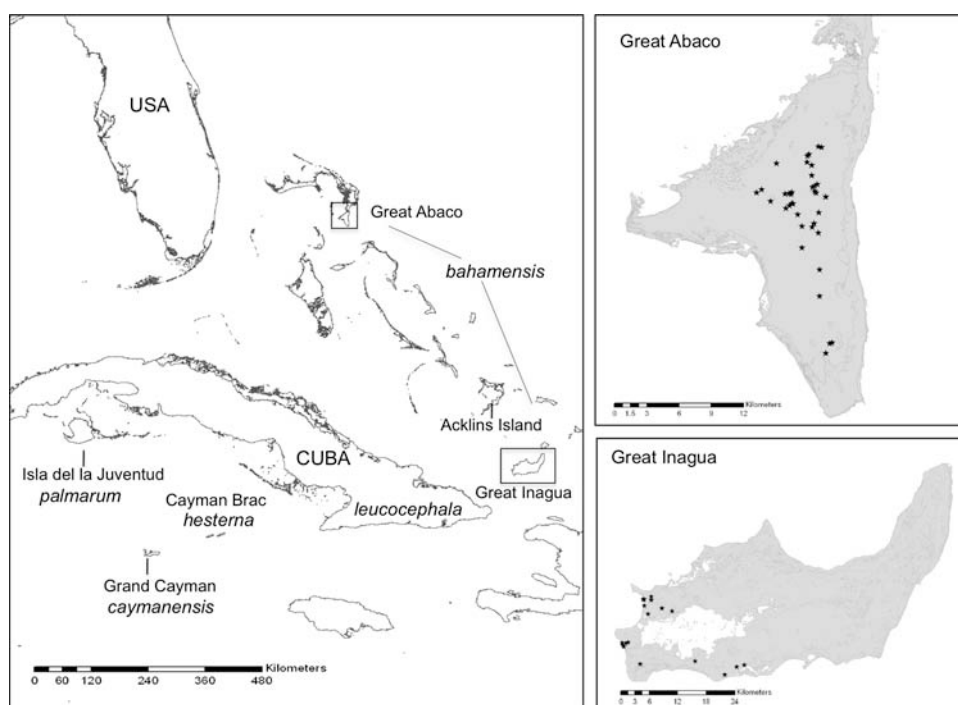
well as one extinct population of the Bahama parrot on Acklins Island to assess taxonomic status. Although no population possessed a single diagnostic character, Reynolds and Hayes (2009) concluded that all seven islands warrant subspecies status based on results of discriminate function analyses. In addition to the four currently recognized subspecies in Cuba and the Cayman Islands, Reynolds and Hayes (2009) proposed splitting the Bahama parrot into three subspecies: *A. l. bahamensis* (extinct subspecies presumably confined to Acklins, Crooked, and Long Cay island group), *A. l. abacoensis* (extant subspecies of Great Abaco), and *A. l. inaguaensis* (extant subspecies of Great Inagua and possibly Little Inagua).

Subspecies continue to be an ill-defined taxonomic unit with ambiguous links to evolutionary history (Mayr 1963; Zink 2004), however, rigorous conservation unit delimitation in the Bahamas would have direct implications for management strategies in the region. The Bahama parrot was formerly found throughout the Bahamas including Abaco, Great Inagua, New Providence, San Salvador, Long Island, Crooked Island, and Acklins. Describing a visit to what some believe was Acklins or Crooked Island in 1492, Christopher Columbus noted "flocks of parrots which darken the sun…" (Hale 1891; Attrill 1980; Keegan 1992). More recently, populations of Bahama parrots have been estimated at approximately 4,000–10,000 individuals (reviewed in Wiley et al. 2004), restricted to the northernmost and southernmost extremities of the archipelago on Abaco ($n = 1576$–3612; Rivera-Milan et al. 2005) and Great Inagua ($n = 3486$–7490; Rivera-Milan et al. 2005), respectively. In addition to the variation in morphological and plumage characteristics described by Reynolds and Hayes (2009), differences in nesting behavior and flight calls between Abaco and Inagua have also been reported (Gnam and Burchsted 1991; Reynolds 2006).

The present study uses mitochondrial DNA character data to evaluate the distinctiveness of the formally described *A. leucocephala* taxa, including the hypotheses of three distinct taxa of Bahama parrots set forth by Reynolds and Hayes (2009). Microsatellite genotypic data were also used to further investigate patterns of divergence, demographic history and conservation status of the contemporary populations of the Bahama parrot on Abaco and Inagua.

Materials and methods

Sampling

Blood samples were collected over three sampling years. For the Abaco population, a single chick was sampled from each of 38 nests from the southern part of the island in July 2004 (Fig. 1). On Great Inagua, blood samples were collected from a single chick from 20 nests in June 2006 and June 2007 (Fig. 1). Samples were collected following the protocol of the American Ornithologists' Union (Gaunt and Oring 1997). Blood was stored in cryogenic tubes containing a buffer solution (100 mM Tris–HCl, pH 8.0; 100 mM EDTA, pH 8.0; 10 mM NaCl; 0.5% SDS; Longmire et al. 1997) and frozen at −80°C until analysis. All nests were georeferenced (Fig. 1), however due to poaching concerns, specific coordinates are not listed (available from the authors). All samples were collected in accordance with local, national and international regulations.

The 58 field-collected Bahama parrot samples were supplemented with previous collections and museum specimens. The Inagua population sampling was augmented with DNA extracts from two individuals [POW_BAPA(I-CH), POW_BAPA(I-S)] originally collected in Ottens-Wainright et al. (2004) and three museum specimens from the American Museum of Natural History (AMNH174667–174669). In addition, eight museum specimens originally collected from the now extirpated population on Acklins were sampled from the AMNH (AMNH95479), Carnegie Museum of Natural History (CMN30889, CMN30890, CMN30892, CMN30893) and Museum of Comparative Zoology at Harvard (MCZ41021, MCZ47505, MCZ58508).

Exemplars from all other *A. leucocephala* subspecies were obtained as either DNA extracts from Ottens-Wainright et al. (2004) or as toe-pad tissue sampled from the AMNH or MCZ (Table 1). DNA extracts of *A. ventralis* and *A. vittata* originally sampled in Russello and Amato (2004) and accessioned in the Ambrose Monell Collection for Molecular and Microbial Research at AMNH (AMCC110752, AMCC110756) were also used for the current study.

Data collection

DNA was extracted from blood samples using the DNeasy Tissue kit (Qiagen, Inc.) following manufacturer protocols. Museum specimens were handled in a dedicated ancient DNA facility using a modified Qiagen DNeasy Tissue kit protocol (Russello et al. 2007). Other necessary precautions were taken to prevent and detect contamination by contemporary specimens, including use of extraction and PCR negative controls, PCR amplification of short, overlapping fragments (see below), and confirmation of all unique haplotype sequences by way of cloning (Wandeler et al. 2007).

A 697 base pair segment of the mitochondrial genome (mtDNA) including control region 1 (CR1) and two pseudo genes, pND6 and pGlu, was amplified as a single fragment using external primers LThr and CR522Rb (Eberhard et al. 2001) for the DNA extractions from blood samples or, in

Table 1 Sampling of *Amazona leucocephla* subspecies and outgroup taxa

Species	Subspecies	Island	Sample	Nest ID[a]/Museum accession no.[b]
Amazona leucocephala	*bahamensis*	Gteat Abaco	38	BPA401, BPA403, BPA405, BPA406, BPA408, BPA410, BPA412, BPA414, BPA415, BPA418–423, BPA427–432, BPA436, BPA439, BPA441, BPA445, BPA451, BPA455, BPA456, BPA463, BPA467, BPA472, BPA487–489, BPA491, BPA493–495
		Acklins	8	AMNH95479, CMN30889, CMN30890, CMN30892, CMN30893, MCZ41021, MCZ47505, MCZ58508
		Great Inagua	25	BPI601–605, BPI711–714, BPI716–718, BPI720, BPI726, BPI728, BPI731, BPI733, BPI735, BPI736, BPI740, AMNH174667–1764669, POW_BAPA(1-CH), POW_BAPA(1-S)
Amazona leucocephala	*caymanensis*	Great Cayman	8	POW_CAMA(91–95), POW_CAMA(084), POW_CAMA(1), POW_CAMA(RK5-91), POW_CAMA(HILL1), POW_CAMA(HILL4), AMNH174674, AMNH475336
Amazona leucocephala	*hestema*	Cayman Brac	9	MCZ68308, MCZ68310–MCZ68312, MCZ68314–MCZ68317, MCZ81847
Amazona leucocephala	*leucocephala*	Cuba	6	POW_CUAM(L1), POW_CUAM(L2), AMNH399401–99403, AMNH437242
Amazona leucocephala	*palmarum*	Isla de La Juventud	9	POW_CAMA(P1), POW_CAMA(P2), AMNH175001–175004, AMNH175328–175330
Amazona ventralis		Hispaniola	1	AMCC110752
Amazona vittata		Puerto Rico	1	AMCC110756

[a] Acronyms related to Nest ID are Abaco (BPA) and Inagua (BPI)

[b] Acronyms related to Museum accession numbers are Ambrose Monell Collection for Molecular and Microbial Research (AMCC) at the American Museum of National History(AMNH), Carnegie Museum of Natural History (CMN), and Museum of Comparative Zoology (MCZ). Samples provided by Patricia Ottens-Wainright (POW) and used in Ottens-Wainright et al. (2004)

the case of the DNA extractions from museum specimens, using nested PCR and a set of four overlapping fragments not exceeding 180 base pairs in length each [LThr/AleucoCR1_1R (GCTCGAATTACATAGCGCAAA); AleucoCR1_2F (TTATGGCCTAAAAACCGCTCT)/AleucoCR1_2R (GCTTTAAYCCATGAAGCGAAA); AleucoCR1_3F (ATAGATGTATACTAGGACATTAATTGG)/AleucoCR1_3R (GCCTTTCRGGACAAGACATTG); AleucoCR1_4F (ACAAGRACTTATCGGTCACAGC)/CR522Rb]. For the nested PCR, amplification was initially performed with the external primers (LThr/CR522RB; Eberhard et al. 2001). The product of the primary reaction then served as template for another PCR amplification (nested reaction) using the corresponding pair of internal primers. All PCR reactions were carried out on an ABI Veriti thermal cycler in 25 μl reactions containing: ~20–50 ng of DNA, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.5 μM of each primer and 0.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems). Cycling conditions for all primer pairs consisted of 95°C for 10 min, 35 cycles of 95°C for 30 s, 57°C for 30 s, 72°C for 45 s, and a final extension of 72°C for 7 min. Double-stranded PCR products were sequenced using Big Dye 3.1 terminators on an ABI 3130XL DNA sequencer (Applied Biosystems). Sequences were visualized and edited using SEQUENCHER 4.7 (Gene Codes).

Previous work revealed duplication and concerted evolution of the mitochondrial control region in *Amazona* and *Pionus* parrots (Eberhard et al. 2001). The following precautions were taken to ensure amplification of control region 1 (CR1; Eberhard et al. 2001): (1) all PCRs used LThr as the forward primer directly (contemporary) or in a nested PCR (historical), which is immediately upstream of CR1, and was shown to solely amplify CR1 in other species of *Amazona* and *Pionus* (Eberhard et al. 2001); (2) CR2 was amplified and sequenced in a subset of samples (n = 4), allowing comparison of length variation and polymorphism with CR1 from the same samples; (3) haplotype sequences from contemporary and historical samples from the same island (Inagua) were analyzed; (4) all unique haplotypes were cloned and sequenced (as described above) to verify single copy.

Genotypic data were collected at nine microsatellite loci originally characterized in the St. Vincent Amazon parrot (*Amazona guildingii*; AgGT04, AgGT17, AgGT19, AgGT21, AgGT22, AgGT42, AgGT72, AgGT83, AgGT90; Russello et al. 2001; Russello et al. 2005b). All forward primers were 5′-tailed with an M13 sequence [5′-TCCCAGTCACGA-CGT-3′] to facilitate automated genotyping. Specifically, the M13-tailed forward primer was used in combination with an M13 primer of the same sequence 5′-labeled with one of four fluorescent dyes (6-FAM, VIC,

NED, PET), effectively incorporating the fluorescent label into the resulting PCR amplicon (Schuelke 2000). In addition, reverse primers were modified following Brownstein et al. (1996) to improve genotyping. All PCR reactions were carried out on an ABI Veriti thermal cycler in 12.5 μl reactions containing: ~20–50 ng of DNA, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 200 μM dNTPs, 0.08 μM of the M13-tailed forward primer, 0.8 μM of each of the reverse primer and the M13 fluorescent dye-labeled primer, and 0.5 U of AmpliTaq DNA polymerase (PE Biosystems). Reaction conditions for all primers were optimized using a 'touchdown' cycling program which consisted of: 95°C for 10 min; 35 cycles of 95°C for 30 s, annealing for 30 s, and 72°C for 45 s; and a final step of 72°C for 7 min (Russello et al. 2001). The annealing step in the 'touchdown' program decreased 2°C every other cycle from 59°C until it reached 51°C (the ninth cycle) at which point the remaining cycles continued with a 51°C annealing temperature. Fragments were separated on an ABI 3130XL Genetic Analyzer and scored using GENEMAPPER® 4.0 (Applied Biosystems).

Haplotypic variation and population differentiation

Haplotypic (h) and nucleotide (π) diversity (Nei 1987) estimates were calculated based on mtDNA sequences as executed in ARLEQUIN (Schneider et al. 2000). Pairwise genetic distances were calculated in PAUP*4.0b10 (Swofford 2002) assuming the HKY + I + G model of nucleotide substitution as selected according to the Akaike information criterion as implemented in Modeltest (Posada and Crandall 1998). Levels of genetic divergence between samples were calculated with the fixation index (φ_{ST}) (Excoffier et al. 1992) as executed in ARLEQUIN (Schneider et al. 2000). Because the HKY model is not implemented in ARLEQUIN the more inclusive Tamura–Nei (TrN; Tamura and Nei 1993) model with the same parameters for ti/tv rate and α was used. Significance of φ_{ST} for all possible pairwise population comparisons was assessed using 2,000 permutations. Tests for significant geographic structure among populations were conducted using analysis of molecular variance (AMOVA) (Excoffier et al. 1992). MtDNA sequence alignments for all three Bahama parrot populations were further employed to identify diagnostic nucleotide sites by means of population aggregation analysis (Davis and Nixon 1992). The presence of characters fixed within and differing among populations was used as evidence to diagnose distinct units.

Genotypic variation and population differentiation

The dataset was screened for null alleles using MICRO-CHECKER (Van Oosterhout et al. 2004). None of the loci exhibited evidence for null alleles. Consequently, subsequent analyses were run based on data at all nine loci. Allelic diversity, observed (H_O) and expected heterozygosity (H_E) were calculated at each locus for the population samplings on Abaco and Inagua using ARLEQUIN (Schneider et al. 2000). Deviation from Hardy–Weinberg (H–W) equilibrium was assessed using exact tests based on the Markov chain method of Guo and Thompson (1992) as implemented in GENEPOP 3.3 (1,000 dememorization, 1,000 batches and 10,000 iterations; Raymond and Rousset 1995). Linkage disequilibrium was investigated for all pairs of loci using GENEPOP 3.3 (Raymond and Rousset 1995). Type I error rates for tests of linkage disequilibrium and departure from H–W expectations were corrected for multiple comparisons using the sequential Bonferroni procedure (Rice 1989).

Levels of nuclear DNA differentiation among populations were estimated by pairwise population comparisons of θ, an analogue of F_{st} (Weir and Cockerham 1984) calculated in GENETIX (Belkhir et al. 2001). Correspondence of geographically separated populations as discrete genetic units was further tested using the Bayesian method of Pritchard et al. (2000) as implemented in STRUCTURE. Run length was set to 1,000,000 MCMC replicates after a burn-in period of 500,000 using correlated allele frequencies under a straight admixture model. The most likely number of clusters in our sample was determined using the ΔK approach (Evanno et al. 2005) by varying the number of clusters K from 1 to 10 with 20 iterations per value of K.

Demographic history

Demographic history was investigated for the Abaco and Inagua populations using both mtDNA haplotypic and microsatellite genotypic-based approaches. Mismatch distribution analyses were implemented in DnaSP v5 (Librado and Rozas 2009) based on the mtDNA control region sequence data. Populations that have experienced a rapid expansion in the recent past show unimodal distributions, while the ones at demographic equilibrium present multimodal distributions (Rogers and Harpending 1992). Demographic parameters τ (Li 1977) and θ_0 were estimated from the data by considering θ_1 as infinite (Rogers 1995). The sum of square deviations (SSD) between observed and expected distributions, and the raggedness index r of the observed distribution of the mismatch classes (Harpending 1994) were computed as test statistics under the null hypothesis of population growth using the parametric bootstrap (1,000 replicates) approach of Schneider and Excoffier (1999) implemented in ARLEQUIN (Schneider et al. 2000).

Genetic signatures of demographic contraction based on microsatellite genotypic data were assessed using three

different approaches: (1) the heterozygote excess test and (2) the mode-shift test, both implemented in the software package BOTTLENECK 1.2.02 (Piry et al. 1999) and (3) the *M*-ratio test using M_P_VAL.exe and critical_M.exe (Garza and Williamson 2001). For the heterozygote excess test, significance was assessed using 10,000 iterations with the Wilcoxon sign-rank test and two different allele mutation models: stepwise mutation model (SMM) and two phase model (TPM) consisting of 10% multi-state change and a variance among multiple steps of 12 as recommended by Piry et al. (1999). For the *M*-ratio test, we used the SMM and TPM mutation models as outlined above assuming a marker mutation rate μ of 5×10^{-4} and a pre-bottleneck N_e ranging from 500 to 5,000 resulting in a value of θ ($4 N_e \mu$) ranging from 1 to 10 and 3.5 base steps for multi-step mutations. For the SMM the amount of single step mutations was $P_s = 1$, while $P_s = 0.9$ was used for the TPM as recommended by Garza and Williamson (2001).

Furthermore, we estimated the effective population size (N_e) of the Abaco and Inagua populations using the algorithm employed in LDN$_e$ (Waples and Do 2008), which implements a recently developed bias correction (Waples 2006). This approach was used because it requires a single microsatellite dataset as opposed to temporal methods that require at least two datasets from the same population. For this analysis, we assumed monogamous mating, and excluded all alleles with frequencies lower than 0.02 from the analyses.

Phylogenetic analyses

Sequences were unambiguously aligned using MUSCLE as implemented in GENEIOUS 4.6 (Biomatters, Ltd.) employing default settings. A Bayesian haplotype tree was reconstructed using MrBayes 3.1 (Ronquist and Huelsenbeck 2003) assuming the HKY + I + G model of nucleotide substitution as selected by Modeltest (Posada and Crandall 1998) as described above. The Hispaniolan (*A. ventralis*) and Puerto Rican (*A. vittata*) parrots were used as outgroups to root the tree, as a previous phylogenetic study revealed their sister group status to *A. leucocephala* (Russello and Amato 2004). The Bayesian phylogenetic analysis ran four simultaneous chains for 2.0×10^6 total generations, each using a random tree as a starting point, the default heating scheme, and saving a tree every 100 generations for a total 20,000 trees. The first 2,000 trees were discarded as burn-in samples and the remaining 18,000 trees were used to construct a majority-rule consensus tree and derive posterior probability values. Violation of a criterion of monophyly was used to indicate incorrect taxonomic assignment.

Results

Data quality

We obtained single-banded PCR products of 697 base pairs (bp) for all amplifications of CR1 in contemporary specimens using external primers LThr and CR522RB (Eberhard et al. 2001). This is the expected size fragment that also includes partial tRNA-Thr (18 bp), pND6 (52 bp) and pGlu (65 bp), all of which were identified upstream of CR1 (562 bp). Similarly, single-banded PCR products were produced for all four overlapping fragments constituting partial tRNA-Thr, pND6, pGlu and partial CR1 in the museum specimens as generated using nested PCR. Other evidence to suggest that all recovered sequences were homologous to the target region included: (1) all unique haplotypes from museum and contemporary specimens were cloned and confirmed to be single-copy; (2) amplification of CR2 using LGlu and CR522RB (Eberhard et al. 2001) in two individuals from Abaco and two individuals from Inagua produced PCR products of the expected smaller size (548 bp) than the targeted CR1; and (3) the three museum specimens from Inagua (amplified over four fragments) shared a single haplotype that was identical to one sampled in contemporary specimens from the same island (amplified in a single fragment).

Haplotypic variation and population differentiation in the Bahama parrot

A total of 12 mtDNA CR haplotypes were recovered among the 71 individuals sampled from Abaco, Acklins and Inagua (GenBank Accession No. GU380307–GU380318; see Table S1 for full alignment). The number of haplotypes identified ranged from three (Abaco, Acklins) to six (Inagua), with all haplotypes unique to the island from which they were sampled. Levels of haplotypic and nucleotide diversity were highest for Inagua and comparatively lower in Abaco and Acklins (Table 2). Overall, mean sequence divergence between Bahama parrot haplotypes recovered from the three island populations ranged from 2.8% (Abaco/Inagua) to 3.5% (Acklins/Inagua). Pairwise distances between island populations of Bahama parrots equaled or exceeded values recovered between currently described subspecies of Cuban parrot [1.2% (*leucocephala/palmarum*)–1.7% (*leucocephala/hesterna*); highest = 4.4% (*caymanensis/hesterna*)]. Average pairwise distances between *A. leucocephala subsp.* and outgroup taxa ranged from 5.7% (*A. vittata*) to 7.8% (*A. ventralis*).

Haplotypic variation across the samples was highly structured with significant levels of genetic variation distributed among, rather than within, the three island populations of Bahama parrots ($P < 0.0001$; Table 3a). A

Table 2 Genetic variation within Bahama parrot populations

Population	n	Mitochondrial DNA			Microsatellite			
		No. Haplotypes[a]	Haplotypic diversity, h	Nucleotide diversity, π	Mean no. alleles per locus	% Private alleles	H_O	H_E
Abaco	38	3	0.52 (0.076)[b]	0.0024 (0.016)	3.2	0.34	0.50	0.51
Inagua	25[c]	6	0.69 (0.062)	0.0026 (0.0017)	5.3	0.63	0.69	0.71
Acklins	8	3	0.46 (0.20)	0.0010 (0.0010)	–	–	–	–

[a] All haplotypes are unique to the population in which they were sampled

[b] Values in parenthesis are the standard errors for h and π

[c] Metrices calculated for microsatellites based on data from 22 individuals, excluding museum specimens

Table 3 Genetic divergence among Bahama parrot populations

Population	Source of variation[a]	d.f.	% Of variation	P value
a. Analysis of molecular variance				
Abaco	Among	2	91.49	<0.0001**
Acklins	Within	68	8.51	
Inagua	Total	70		

Population	Abaco	Acklins	Inagua
b. Diagnostic characters and fixation indices[b]			
Abaco	–	0.922	0.906
Acklins	15	–	0.930
Inagua	12	15	–

** Indicates statistical significance ($P < 0.001$)

[a] Among populations, within populations or total based on mtDNA control region sequence data

[b] Number of diagnostic characters (below diagonal) and φ_{ST} (above diagonal) based on mtDNA control region sequence data

similar pattern was revealed by the fixation indices, with all pairwise comparisons highly significant (Table 3b). Likewise, all three island populations were diagnosably distinct from each other, with the number of diagnostic characters detected ranging from 12 (Abaco/Inagua) to 15 (Abaco/Acklins and Acklins/Inagua) across the 697 bp of mtDNA (Table 3b).

Genotypic variation and population differentiation in the Bahama parrot

Genotypes were generated at nine microsatellite loci for all contemporary samples collected on Abaco ($n = 38$) and Inagua ($n = 22$). None of the museum specimens originally collected on Acklins ($n = 8$) and Inagua ($n = 3$) yielded consistent and reproducible genotypic data. For this truncated dataset, less than 2.0% of data were missing. Only locus AgGT83 in the Inagua population significantly deviated from H–W equilibrium following sequential Bonferroni correction. Furthermore, there was no evidence of non-random association of genotypes ($P > 0.05$) in any

of the pairwise tests for linkage disequilibrium performed for all possible pairwise comparisons of the sampled loci.

Similar to the results based on mtDNA, the Inagua population exhibited greater levels of genetic variation than Abaco as reflected in both mean number of alleles per locus (5.3–3.2) and expected heterozygosity (0.71–0.51) (Table 2). Both populations exhibited a high proportion of private alleles, ranging from 34 to 62% for Abaco and Inagua, respectively (Table 2).

Genotypic differentiation between the Abaco and Inagua populations was high ($\theta = 0.22$; $P < 0.001$). Likewise, STRUCTURE analyses revealed that individuals sampled on Abaco and Inagua represent distinct clusters (Fig. 2c), with the distribution of the ad hoc statistic ΔK (Evanno et al. 2005) modal at $K = 2$ (Fig. 2a, b).

Demographic history

Detection of genetic signatures of demographic contraction varied by population and method used. Both Abaco and Inagua exhibited multimodal mismatch distributions (Fig. 3) estimated from the mtDNA sequence data with raggedness values ($r = 0.2872$–0.2882) larger than those expected under population expansion, however neither were significantly different (Abaco: $P = 0.18$; Inagua: $P = 0.06$). Similar results were found based on SSD values (Abaco: SSD $= 0.11$, $P = 0.12$; Inagua: SSD $= 0.08$, $P = 0.12$). A significant excess of heterozygosity was revealed for the Abaco population (eight of nine microsatellite loci; $P = 0.024$ SMM, $P = 0.024$ TPM, Wilcoxon sign-rank test), but not for Inagua ($P = 0.10$ TPM, $P = 0.18$ SMM, Wilcoxon sign-rank test), although seven of nine microsatellite loci exhibited heterozygote excess. The M-ratio was always small for both Abaco and Inagua, falling below the critical value (M_c) simulated for a stable population with the same number of individuals and microsatellite loci used (Garza and Williamson 2001), suggesting a reduction in effective population size [$\theta = 10$; Abaco: $M = 0.67$, $M_c = 0.69$ (TPM): $P < 0.01$, $M_c = 0.80$ (SMM): $P < 0.001$; Inagua: $M = 0.62$, $M_c = 0.69$ (TPM): $P < 0.01$, $M_c = 0.80$ (SMM): $P < 0.001$].
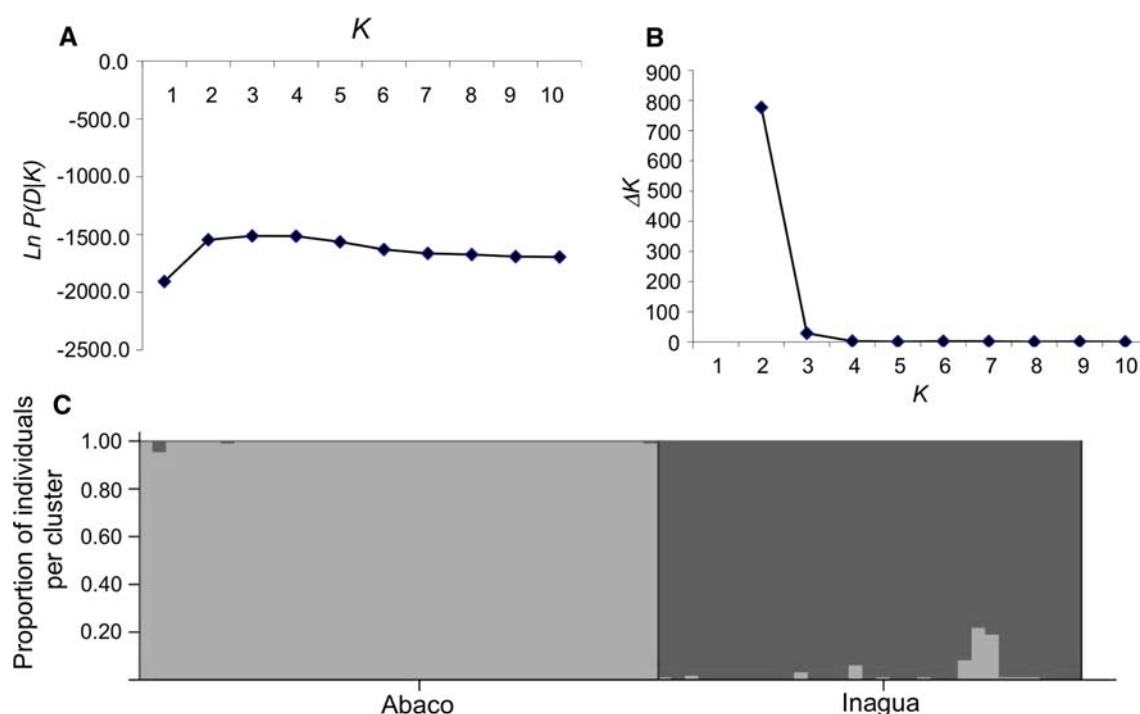
Fig. 2 Results of the Bayesian clustering analysis conducted with STRUCTURE. **a** Log-likelihood profile for the whole dataset for a given number of putative populations (*K*) ranging from 1 to 10, averaged over 20 independent runs. **b** Profile for the ad hoc statistic Δ*K* (Evanno et al. 2005) plotted against various values of *K*, suggesting that 2 is the most likely number of clusters. **c** Results of the population assignment tests performed using STRUCTURE. The relative contributions of each of the two genetic partitions recovered from the data are indicated by color for each individual (column) in each sampled population. The Abaco cluster is represented in *light grey* while Inagua is in *dark grey*

Only the mode-shift test did not reveal signatures of demographic contraction, as indicated by normal L-shaped distributions for both populations.

A mean effective population size (N_e) of 70 (95% interval: 32–312) and 270 (95% interval: 65-infinity) individuals were inferred for Abaco and Inagua, respectively, as calculated according to the method of Waples and Do (2008). The overall effective population size to census size ratios (N_e/N) using the mean estimate of census (Abaco: $n = 2386$; Inagua: $n = 4450$; Rivera-Milan et al. 2005) were 0.03 (Abaco), and 0.06 (Inagua). These estimates were lower than the median estimate of 0.11 obtained for 102 species of wild unmanaged populations of various taxonomic groups (Frankham 1995) and 0.14 from a similar review by Palstra and Ruzzante (2008) based on indirect genetic methods.

Phylogenetic analysis

Thirty-two unique *A. leucocephala* subsp. mtDNA haplotypes were identified among the 121 individuals sampled from across the contemporary and former distributions of the Cuban parrot in the Greater Antilles (Fig. 1, Table 1). Two additional haplotypes were sampled from outgroup taxa (*A. ventralis* and *A. vittata*; Fig. 1, Table 1). Of the

699 aligned base pairs of CR1 and upstream elements (partial tRNA-Thr, pND6, pGlu), 91 (13%) characters were variable (including two indels), of which 67 were parsimony informative.

A Bayesian haplotype tree reconstructed three major clades, generally corresponding to current subspecific taxonomy. All haplotypes sampled from the Bahamas formed a well-supported monophyletic group (posterior probability (PP) = 98; Fig. 4). Within the *bahamensis* clade, haplotypes sampled on Abaco, Acklins and Inagua each formed well-supported (PP ≥ 98; Fig. 4) monophyletic groups, but relationships among islands form a polytomy and remain unresolved. In addition, all sampled *caymanensis* haplotypes from Grand Cayman were monophyletic supported by a high posterior probability (PP = 99; Fig. 4). A third major clade (PP = 93; Fig. 4) includes all haplotypes sampled from Cuba including the formally described *leucocephala* (Cuba east of Las Villas province) and *palmarum* (Isla of Juventud) subspecies, as well as all *hesterna* haplotypes sampled from its current distribution in the Cayman islands. The reconstructed topology indicates that the Cuban *palmarum* and *leucocephala* are paraphyletic and possess no diagnostic molecular characters (data not shown), providing little evidence for their evolutionary distinctiveness. In contrast,
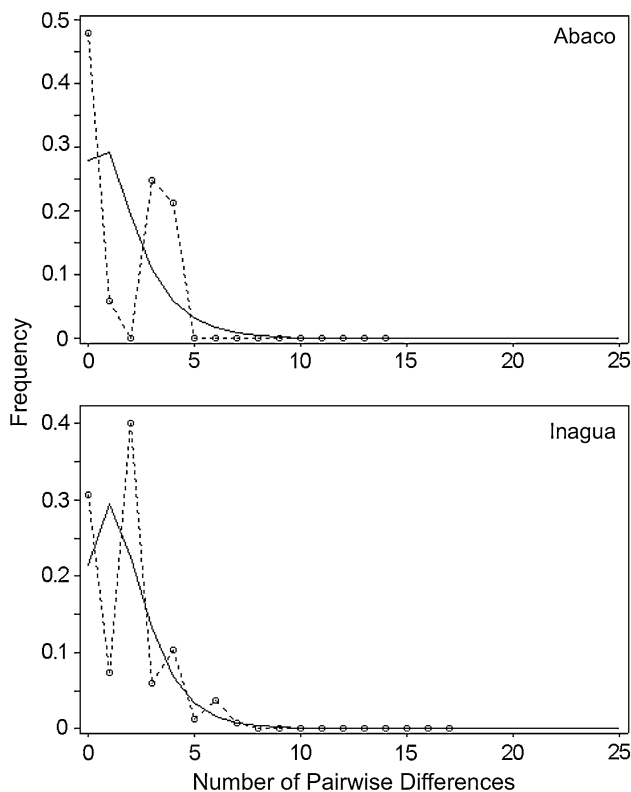
Fig. 3 Mismatch distributions of mtDNA haplotypes in Bahama parrots from Abaco and Inagua. Observed distributions indicated by *dotted line*, with *solid lines* depicting expected distributions under a model of population expansion

hesterna haplotypes, restricted to Cayman Brac, were diagnosably distinct (two characters separated from *palmarum* and *leucocephala*; data not shown) and formed a well-supported monophyletic group (PP = 93; Fig. 4) nested within this otherwise Cuban distributed clade.

Discussion

Conservation management is directly tied to taxonomic status, as evident by the prominent role taxonomy plays in major protective legislation globally including, but not limited to, the World Conservation Union (IUCN) Red List of Threatened Species, Convention on International Trade in Endangered Species of Wild Flora and Fauna (CITES), Brazil's Lista Nacional das Espécies da Fauna Brasileira Ameaçadas de Extinção, Canada's Species at Risk Act, Australia's Environmental Protection and Biodiversity Act, South Africa's Biodiversity Act, The United States Endangered Species Act (ESA)and others (reviewed in Haig et al. 2006). In all legislation listed above, subspecies are formally recognized as units for protection and, in some cases, represent a large proportion of listed taxa (ex. 25% of ESA-listed taxa are at the subspecific rank; Haig et al. 2006).

Yet, the biological relevance of subspecies has been widely debated since the 1950s (Wilson and Brown 1953). Ernst Mayr, who wrote the most influential book on speciation analysis (Mayr 1942), also grappled with the concept of subspecies. Although in early writings he clearly assigned evolutionary status to subspecies (Mayr 1942), later work directly acknowledged the subjectivity associated with this level of taxonomic classification, explicitly stating that subspecies are not units of evolution (Mayr 1963). Avian subspecies are a prime example, with recent surveys finding that 97% of Nearctic and Palearctic continentally-distributed subspecies (Zink 2004), and 62% of continental and island-dwelling subspecies from seven biogeographic realms (Afrotropics, Australasia, Indo-Malaysia, Nearctic, Neotropics, Oceania, Palearctic; Phillimore and Owens 2006) lack the population genetic structure indicative of historically independent units. Within the context of conservation, the "subspecies dilemma" has long been recognized and broadly debated (Ryder 1986; Barrowclough and Flesness 1996; Phillimore and Owens 2006).

We share the view of many that an accurate taxonomy should reflect evolutionary history. The phylogenetic species concept (PSC) offers such an approach, directly linking patterns of evolution with species status (Cracraft 1983). Under the PSC *sensu* Cracraft (1983), a species is the smallest diagnosable cluster of individual organisms within which there is a parental pattern of ancestry and descent, essentially rendering the subspecies rank moot. Although this has caused consternation in some (see Isaac et al. 2004; Haig et al. 2006), the ambiguity associated with subspecies, especially in avian taxonomy, may hinder evolutionary studies and prove misleading to conservation (Zink 2004; Johnson et al. 2005).

For the purposes of conservation, diagnosis has been explicitly proposed (Vogler and Desalle 1994) and broadly applied for designating conservation units in a wide range of taxa. Consequently, under this criterion, conservation unit delimitation is directly tied to taxonomic assignment (e.g. phylogenetic species). Although there are some limitations to this approach (discussed in Goldstein et al. 2000), the objectivity, reproducibility, operationalism, and direct link to the products of evolutionary history and subsequent taxonomic assignment make this an effective method for unambiguously designating units for conservation management.

Taxonomy of the Cuban parrot complex

The current study represents the most comprehensive treatment of Cuban parrot systematics based on molecular character data. The reconstructed phylogeny revealed well-supported monophyletic groups corresponding to

Fig. 4 Bayesian haplotype tree depicting relationships among sampled *Amazona leucocephala* haplotypes and two outgroups relative to their geographic and taxonomic distributions. Bayesian posterior probabilities (>50%) are indicated above the branches. Sample names are as in Table 1
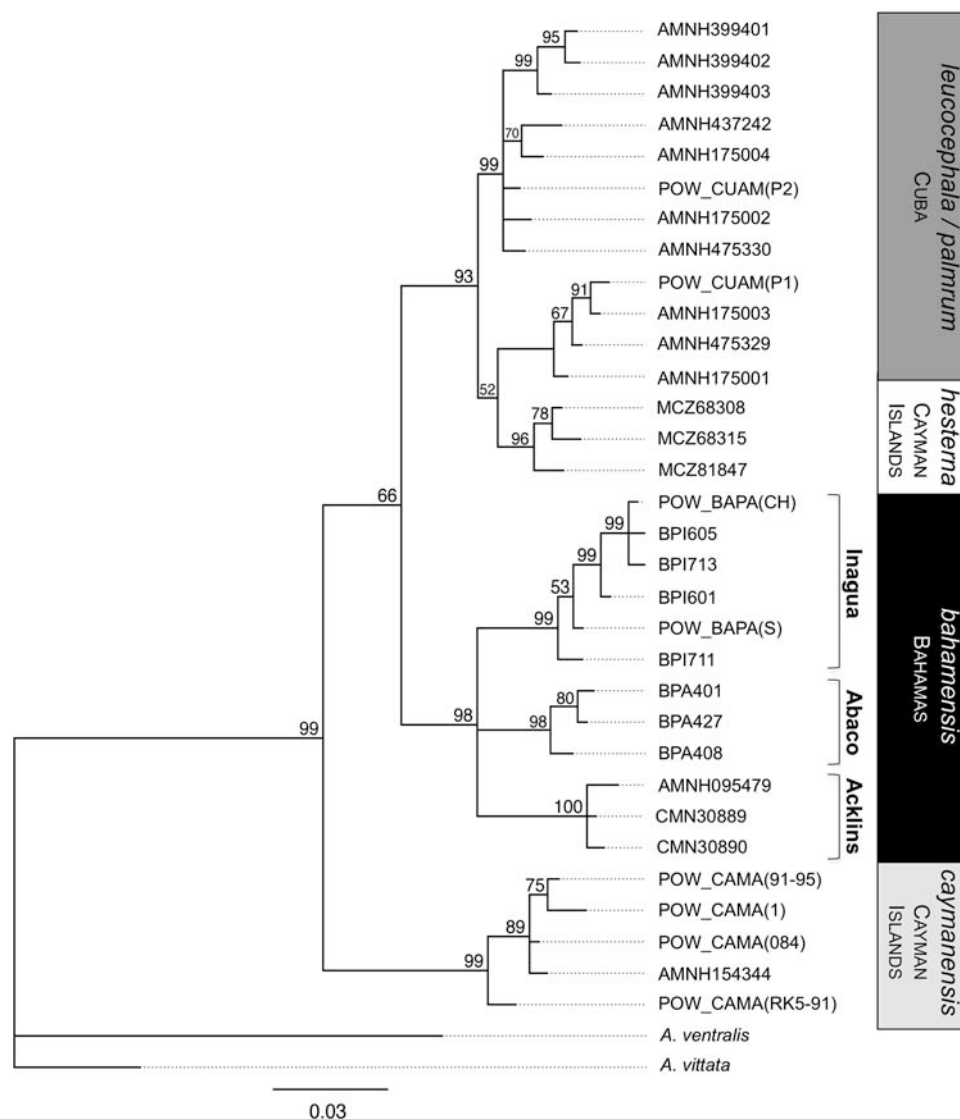
three formally described subspecies (*A. l. bahamensis*, *A. l. caymanensis*, and *A. l. hesterna*). Individuals from the remaining two subspecies (*A. l. leucocephala*, *A. l. palmarum*) lacked diagnostic character support, both molecular (this study) and morphological (Reynolds and Hayes 2009), and violated a criterion of monophyly. Overall, the molecular phylogeny was consistent with the taxonomy of Peters (1928), who suggested synonymizing the Isla de Juventud (*A. l. palmarum*) and Cuban (*A. l. leucocephala*) subspecies. These results are further consistent with the reconstructed phylogeny of Ottens-Wainright et al. (2004), but counter to their conclusions. Despite the paraphyly of *A. l. palmarum* and *A. l. leucocephala* in the cyt *b* tree presented in Fig. 4 of Ottens-Wainright et al. (2004), the authors concluded that they were distinct lineages and used the observed "weak differentiat(ion)" to suggest that these were "the last populations to become genetically independent." Our alternative interpretation of the cyt *b* results in tandem with the

inferences based on control region data presented here suggest that *A. l. palmarum* and *A. l. leucocephala* are not distinct evolutionary entities.

Of the monophyletic lineages, the two Cayman island subspecies were not sister taxa and in fact, exhibited the highest percent sequence divergence of any pairwise comparison between *A. leucocephala* taxa. These results are consistent with documented behavioral differences. While *A. l. caymanensis* is loud and gregarious, moving in large flocks, *A. l. hesterna* is more secretive for much of the year, flying below the canopy and traveling in small groups (Wiley et al. 2004; Reynolds and Hayes 2009). It has been suggested that this comparably different behavior exhibited by *A. l. hesterna* has an affinity to the Lesser Antillean *Amazona*, where predation risk is lower (as on Cayman Brac) than that observed on Grand Cayman and other Greater Antillean islands (Enkerlin-Hoeflich et al. 2006).

Regarding the Bahama parrot, we found evidence for three distinct lineages corresponding to the two extant island populations on Abaco and Inagua and the extirpated population on Acklins. All three island populations were diagnosably distinct (12–15 nucleotide characters) and formed well-supported monophyletic groups (PP ≥ 98; Fig. 4). Consequently, the Abaco, Inagua and Acklins parrots represent three phylogenetic species and distinct units for conservation. These results based on molecular character data are in concordance with the findings of Reynolds and Hayes (2009), who suggested independent taxonomic and conservation status (albeit at the subspecies level) for these three island populations.

Cryptic diversity in the Bahama parrot

Given the lack of diagnostic morphological characters, our findings of unrecognized genetic subdivisions between Bahama parrot island populations are suggestive of cryptic diversity within this system of already notable conservation status. There is a burgeoning literature surrounding the identification of cryptic species, analyzing trends in discovery and significance for disciplines from macroecology (Isaac et al. 2004) to conservation (Beheregaray and Caccone 2007; Bickford et al. 2007). A recent review proposed that the distribution of cryptic species may be non-random across taxonomic groups and biomes, leading to important implications for evolutionary theory, biogeography and conservation planning (Bickford et al. 2007). One suggestion has been that charismatic megafauna, such as parrots (and birds in general), have been subject to a disproportionate amount of taxonomic scrutiny relative to other groups, with the added attention promoting a higher rate of cryptic species discovery (Isaac et al. 2004; Bickford et al. 2007). However, a subsequent meta-analysis has shown that cryptic species are almost evenly distributed among major metazoan taxa and biogeographical regions when corrected for species richness and study intensity (Pfenninger and Schwenk 2007; but see Trontelj and Fiser 2009).

On a broad scale, ascertainment bias, both taxonomic and geographic, associated with cryptic species detection should continue to be a consideration when dealing with large-scale biodiversity conservation initiatives. Yet, recognition of cryptic diversity remains important within the context of global, regional and single-species biodiversity conservation, even in well-studied groups (Ceballos and Ehrlich 2009).

Conservation implications

The identification of cryptic diversity in Bahama parrots has immediate conservation implications. Currently considered as stable but vulnerable (Wiley et al. 2004), the splitting of Bahama parrots into three distinct phylogenetic species transforms the conservation status of this group. First, the conservation status of the Abaco phylogenetic species should immediately be elevated, given the small census and effective parrot population sizes [mean N_c = 2386 (Rivera-Milan et al. 2005); mean N_e = 70 (current study)], and the increasing human population on the island (30% growth from 10,000 to 13,000 from 1990 to 2000; reviewed in Wiley et al. 2004). The increased development and road construction associated with human population growth on Abaco promises to exacerbate threats that have emerged since the mid-20th century, including: (1) destruction of native broadleaf and pine woodlands that make up primary parrot habitat; (2) illegal hunting; (3) capture for the pet trade; (4) predation by feral cats and raccoons; and (5) hurricanes, including well-documented damage to parrot habitat in 2008 (Wiley et al. 2004; Stahala pers. com.). These threats have likely contributed to a large population decline, even before the recent human population growth, as indicated by the detectable signatures of demographic contraction in the Abaco parrot population. Second, the status of the Great Inagua parrots may indeed be stable. This island boasts robust census and effective parrot population sizes [mean N_c = 4450 (Rivera-Milan et al. 2005); mean N_e = 270 (current study)], relatively high population genetic variation (Table 2), remarkably little human presence (reduced from 5,000 in 1900 to <1,000 in 2000; reviewed in Wiley et al. 2004), and a significant portion of parrot habitat already protected in the Inagua National Park (74,360 ha; Wiley et al. 2004). Lastly, rather than an extirpated population, the Acklins island phylogenetic species is now extinct.

Detailed genetic analyses are revealing important new insights into the evolutionary histories of some of the most iconic and threatened species on the planet (Russello et al. 2005a; Brown et al. 2007; Gentile et al. 2009). The present work further demonstrates the importance of applying an evolutionary perspective to conservation management, combining population genetic, phylogenetic and historical DNA analyses of extant and extirpated taxa to resolve taxonomic uncertainty and designate conservation units within the Cuban parrot complex, taxa long-revered by local inhabitants and European explorers and now considered flagships for regional conservation.

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