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SHORT COMMUNICATION

Cryptic species in a Neotropical parrot: genetic variation within the *Amazona farinosa* species complex and its conservation implications

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Abstract The application of genetic approaches has enhanced the identification of cryptic species in a wide variety of taxa, often with immediate conservation implications. Here, we employed multilocus DNA analyses to assess genetic variation and its correspondence to taxonomy within the Mealy Amazon (*Amazona farinosa*), a parrot species found in Central and South America. DNA sequence data from four mitochondrial regions and two nuclear introns were used to infer relationships among all five named subspecies in this species complex. Two reciprocally monophyletic groups with strong nodal support were found; one comprised of the two Central American subspecies *guatemalae* and *virenticeps* and one including all three South American subspecies *farinosa, chapmani,* and *inornata*. Molecular characters diagnosed distinct Central American and South American lineages, with an estimated divergence time of 1.75–2.70 million years ago as inferred from cytochrome-*b* (3.5–5.4 % corrected distance). Our data support recognizing Central American and South American Mealy Amazons as separate species worthy of independent conservation management. Furthermore, our results suggest recognition of two separate management units within the South American clade, although further study is required. These findings have important conservation implications as Central American *A. farinosa* are under increased pressure from habitat destruction and collection for the pet trade, yet are listed as of Least Concern due to their current classification as subspecies' subsumed within the species complex.

Keywords Cryptic species · Mealy Amazon · Neotropics · Parrots · Species complex

Introduction

Accurate scientific taxonomy is a foundation for effective conservation efforts, particularly those focusing on individual species as representatives of distinct evolutionary histories (Hazevoet 1996; Mace 2004). However, species status is not always definitively known, especially in morphologically conserved species or within variable species complexes that may conceal genetically distinct species. Application of genetic approaches has enhanced the identification of such cryptic species in a wide variety of taxa, often with immediate conservation implications (Bickford et al. 2007; Russello et al. 2005). These successes include several notable examples from the parrots (Order Psitacciformes; Eberhard and Bermingham 2004; Murphy et al. 2011; Russello et al. 2010), which is one of the most endangered large orders of birds (Birdlife International 2004).

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Parrots of the genus *Amazona* are highly prized in the exotic pet trade due to their colorful plumage and ability to mimic human speech. As a consequence, a majority of *Amazona* species are listed by the IUCN under some category of conservation concern (e.g., near threatened, vulnerable, endangered or critically endangered; IUCN 2011). However, the Mealy Amazon (*Amazona farinosa*) is listed as a species of Least Concern due to an extensive range and large population, even though the species has a declining population trend (IUCN 2011). Central American populations are under particular pressure from habitat loss and collection for the pet trade (Juniper and Parr 1998; J Gilardi pers. comm.)

The *A. farinosa* species complex consists of five recognized subspecies (*farinosa*, *chapmani*, *inornata*, *virenticeps*, and *guatemalae*) ranging from southern Mexico through Central America and Panama, and through the Amazon basin to southern Brazil and along the Atlantic Coastal Forest (Fig. 1). Morphological variation has lead to numerous revisions of this species complex with the Central American forms *A. f. guatemalae* and *A. f. virenticeps* once treated as full species before being subsumed as subspecies of *A. farinosa* (Juniper and Parr 1998; Salvadori 1891). *Amazona kawalli* was once recognized as an aberrant form of *A. farinosa* but is now recognized as a distinct species (Collar and Pittman 1996; Stresemann 1924).

Russello and Amato's (2004) molecular phylogeny of the genus *Amazona* included one representative each of four of the five subspecies of *A. farinosa*. They found that Central American and South American subspecies formed sister clades but the distance between the two groups of subspecies was as pronounced as divisions between other long-recognized *Amazona* species (Russello and Amato 2004). Such a division could have important conservation implications, particularly for Central American populations. It is important to note however that a small sample size, incomplete taxon sampling and lack of vouchered *A. farinosa* specimens limit the conclusions of Russello and Amato (2004). In the present study, we used multilocus DNA sequence analyses derived exclusively from vouchered specimens to explicitly test whether Central and South American *A. farinosa* constitute distinct phylogenetic species (Cracraft 1983) in order to resolve taxonomic uncertainty within this complex and address associated conservation implications.

Methods

We sampled all five recognized subspecies of *A. farinosa* as well as *A. kawalli*; *Amazona auropalliata* and *Amazona amazonica* were included as outgroups. We only used vouchered samples from museum collections and excluded the four specimens from Russello and Amato (2004) due to their undocumented origins. Most samples came from frozen tissues, but five originated from toe pads of study skins and one from a bone fragment of a study skeleton (Online Resource 1).

For frozen tissue samples, total genomic DNA was isolated using a DNeasy extraction kit following manufacturer's protocol (Qiagen, Valencia, CA). Four mitochondrial DNA fragments [12 s rDNA (12S); 16 s rDNA (16S); cytochrome oxidase subunit I (COI); cytochrome-*b* (CYTB)] and two non-coding nuclear introns [tropomyosin alpha-subunit intron 5 (TROP); transforming growth factor β-2 intron 1 (TGFB2)] were PCR amplified (primers in Online Resource 2). Bone and toe pad extractions were conducted in a UV hood in a lab where no vertebrate PCR products have ever been present. Negative extraction and PCR controls were conducted alongside to monitor for

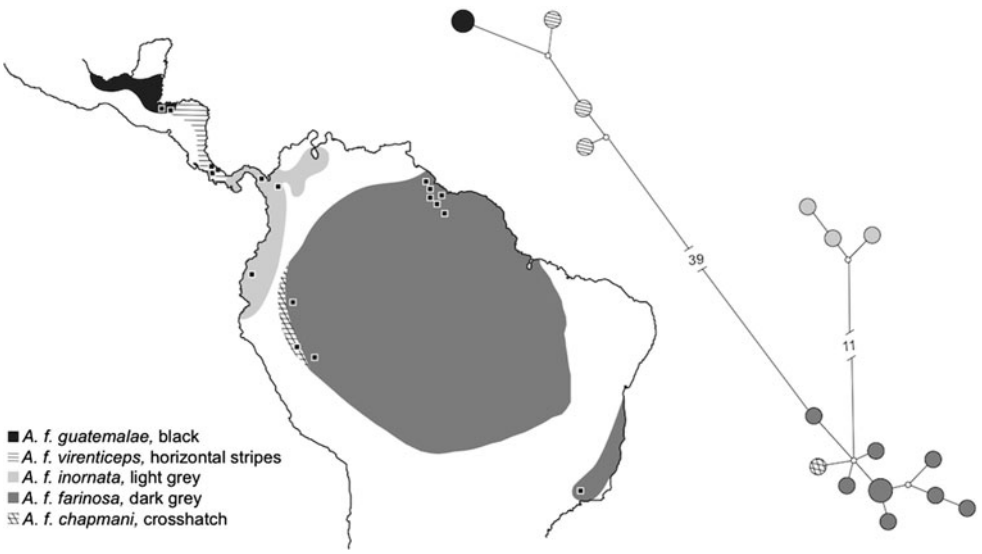

Fig. 1 Sampling localities and ranges of currently recognized subspecies with median-joining network based on 1,157 bp of COI and CYTB summarizing the relationships among subspecies. Size of *circle* is proportional to the number of individuals sharing that haplotype. Branches represent number of mutational steps between haplotypes and are proportional in length to the number of changes from one to six mutations, or otherwise are denoted by numbers on branches

contamination. DNA isolation was conducted with modifications to the DNeasy protocol following Boessenkool et al. (2009) for both bone and toe pad samples. Only COI and CYTB fragments were amplified from these samples using primers that amplified overlapping fragments.

All PCRs were cleaned using QiaQuick 96 PCR purification kit (Qiagen, Valencia, CA) and manufacturer's protocols. Products were sequenced at the University of Chicago CRC DNA sequencing facility, with the same primers used for PCR. Resulting bi-directional sequences were checked and edited using Sequencher 4.10.1 (Gene Codes Corporation, Ann Arbor, MI) and consensus sequences were aligned in Clustal X 2.1 (Larkin et al. 2007) employing default settings for gap opening and extension.

Unique haplotypes were identified using DnaSP 5.10 (Librado and Rozas 2009) for a combined data set of CYTB and COI. We constructed a median joining haplotype network using Network 4.6.0.0 (Fluxus Technology Ltd., Clare, England) to estimate relationships among haplotypes. Nuclear and mtDNA sequence alignments for all subspecies were used to identify diagnostic nucleotide sites by means of population aggregation analysis (Davis and Nixon 1992), where character states (nucleotide sites) were summarized for all individuals to estimate population profiles for those states. Individuals with identical population profiles were combined; this was continued iteratively until the only remaining sample aggregates were those separated by fixed character state differences. Separate sample aggregates were used to diagnose distinct phylogenetic species (Cracraft 1983).

Phylogenies were reconstructed using maximum-likelihood (ML) algorithms in Meta PIGA 2 (Helaers and Milinkovitch 2010) for each gene region independently and for a combined data set of all concatenated gene regions. Substitution model parameters were found using the Akaike Information Criteria (AIC) as implemented in Meta PIGA 2 (Helaers and Milinkovitch 2010). Starting trees were constructed using loose neighbor joining and consensus pruning. Nodal support was assessed by bootstrap replicates of a minimum of 100.

Bayesian inference of phylogeny was implemented using Mr. Bayes v3.1.2 (Huelsenbeck and Ronquist 2001) on the combined dataset of all gene regions following the same models that were selected using the AIC for ML analyses. The analysis was run using partitioned likelihood, so that parameters could vary independently for the gene regions in the combined analysis. Four simultaneous chains were run for 1.0×10^7 generations and sampled every 1,000 generations. Stationarity was reached before the first 10,000 generations and the first 10,000 generations were discarded before using the remaining trees to obtain a majority-rule consensus.

Net between group mean genetic distances were calculated using MEGA 5.05 (Tamura et al. 2011) and the Kimura 2-Parameter model (Kimura 1980) with gamma distribution of rates among sites, to account for intra population variation before calculating inter population distances. Standard deviation estimates were calculated using 1,000 bootstrap replicates.

Results

A total of 3,463 bp was obtained for the full dataset including both nuclear and mtDNA: 393 bp of 12S, 526 bp of 16S, 528 bp or 422 bp of COI (some toepads failed to amplify a section), 868 bp or 776 bp of CYTB (some toepads failed to amplify a section), 523 bp of TROP, and 625 bp of TGFB2 (sequences deposited in GenBank Online Resource 1). Evidence we sequenced from mitochondria include: (1) long amplifications or highly specific internal primers with significant overlap; (2) lack of indels or stop codons observed in coding regions; (3) alignment with other recognized parrot mtDNA; and (4) lack of contaminants or secondary bands detected in PCR products.

Within the mtDNA data set, 110 sites were variable, 96 of which were parsimony informative. Transitions outnumbered transversions at a ratio of 11:1. Of these sites, 55 were diagnostic between the Central American and South American subspecies. Within South America, 16 diagnostic characters were detected between *A. f. inornata* and the two other South American subspecies. The TROP and TGFB2 introns contained five variable sites fixed in the homozygous state within individuals, one of which was diagnostic between Central American and South American subspecies and two of which were diagnostic between *A. f. inornata* and the remaining South American subspecies.

A total of 1,157 bp of COI and CYTB data were used to identify 16 unique mtDNA haplotypes (Fig. 1). Three distinct clusters of haplotypes were observed, characterized by no shared haplotypes and a minimum of 11 mutational steps between clusters. Recovered patterns conformed largely to geographic distribution, corresponding to: (1) one Central American cluster (*A. f. virenticeps* and *A. f. guatemalae*); and two South American clusters including (2) *A. f. inornata* and (3) *A. f. farinosa* and *A. f. chapmani*.

ML and Bayesian trees had identical topologies at all major nodes, revealing two reciprocally monophyletic groups of *A. farinosa*, one comprised of Central American subspecies (CA Clade) and one of South American subspecies (Fig. 2). The topology of this tree was likely driven primarily by the mtDNA data, which had a disproportionate number of parsimony informative sites relative to the nuclear data ($n = 96$ versus $n = 5$). Nonetheless, an ML tree recovered from only the nuclear data (Online

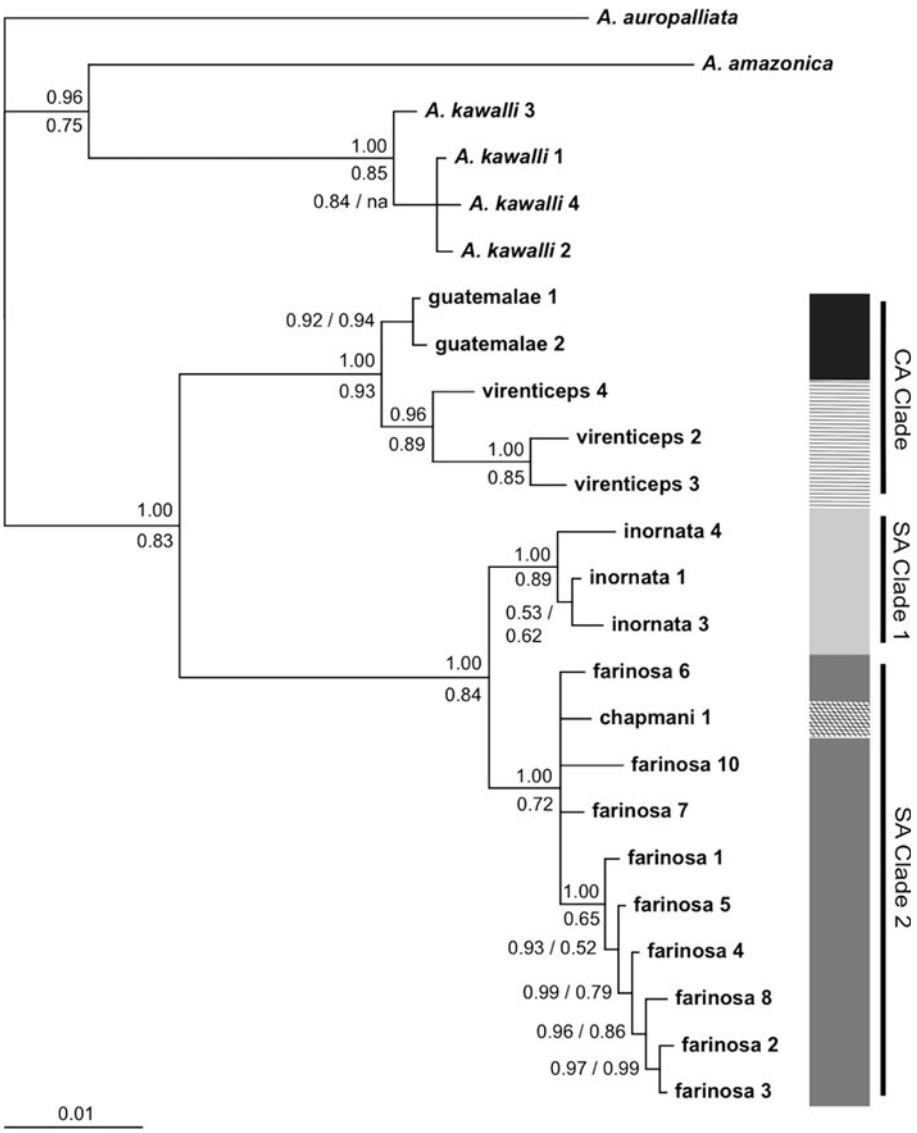

Fig. 2 Bayesian phylogenetic tree based on combined analyses of both nuclear and mtDNA. Numbers on nodes are Bayesian posterior probabilities (above node or before slash) and ML bootstrap values (below node or after slash). Three major clades are indicated: (*1*) CA Clade comprised of the subspecies *A. f. guatemalae* and *A. f. virenticeps*, (*2*) SA Clade 1 comprised of *A. f. inornata*, and (*3*) SA Clade 2 comprised of *A. f. farinosa* and *A. f. chapmani*

Resource 3) showed little topological conflict with the trees recovered from the combined data. Within the Central American clade, there appears to be distinct and well-supported structure between the currently recognized subspecies *A. f. guatemalae* and *A. f. virenticeps*. The South American clade also exhibits strong structure with respect to the subspecies *A. f. inornata* (SA Clade 1), which consistently forms a monophyletic group. Conversely, *A. f. chapmani* and *A. f. farinosa* (SA Clade 2) form a paraphyletic assemblage, sister to *A. f. inornata.* On a broader level, *A. kawalli* forms a well-supported clade with the South American *A. amazonica*.

Recovered net between group mean genetic distances, based on CYTB, represent 3.5–5.1 % sequence divergence between CA Clade and SA Clade 2, 0.7–1.5 % between SA Clade 1 and SA Clade 2, and 3.8–5.4 % between CA Clade and SA Clade 1. Divergence within clades was considerably lower, 0.2–0.7 %.

Discussion

We employed multiple genetic loci, complete taxon sampling, and complementary analytical methods to evaluate the validity of the current taxonomy of the *A. farinosa* species complex. The results showed strong nodal support and congruent topologies supporting distinct Central American and South American clades. Our mitochondrial haplotype network, and to a lesser extent, the combined evidence phylogenies, also support a division within the South American clade between the northern subspecies and the two subspecies found in the Amazon Basin. Our

findings have general significance to understanding patterns of avian speciation in the Neotropics, and specific implications for the conservation status of *A. farinosa.*

We recovered genetic distances of 3.5–5.4 % between Central American and South American subspecies in CYTB, a level of divergence similar to that of a wide array of congeneric bird species pairs for the same locus. For example, approximately half of the species pairs of the 88 genera analyzed by Johns and Avise (1998) exhibited genetic distances equal to or less than that observed between Central American and South American *A. farinosa*. Applying the conventional rate of 2 % sequence divergence per million years for CYTB (Tarr and Fleischer 1993; but see Lovette 2004), the Central American and South American mtDNA lineages possibly diverged 1.75–2.7 mya during the late Pliocene to early Pleistocene after the formation of the Isthmus of Panama ~3.5 mya (Coates 1997). Other Neotropical parrots exhibit this Central America/South America pattern of diversification and magnitude of divergence (Eberhard and Bermingham 2005). This pattern is also well supported by numerous other avian genera where the formation of the Panama land bridge facilitated a large increase in the colonization rate and diversification of South American avian lineages north into Central America (Weir et al. 2009).

A second more recent split within South America was revealed in both the haplotype network and phylogenetic tree between *A. f. inornata* (SA Clade 1) and *A. f. farinosa* and *A. f. chapmani* (SA Clade 2). Applying the 2 % sequence divergence rate for CYTB (0.7–1.5 %), this split appears to have taken place in the mid to late Pleistocene ~500,000 years ago. Whether this split occurred as a result of a vicariance event or a range expansion followed by vicariance is unclear, but it is well supported by our data. Our sole sample from the isolated population in the Atlantic forest of Brazil was not substantially different from other SA Clade 2 samples, but we recommend a closer examination of this disjunct population using a larger sample size.

Correct identification of species is critical for effective conservation, particularly under IUCN procedures that generally assess conservation status at the species level only. The haplotype network showing no haplotype sharing, congruent topologies of ML and Bayesian phylogenetic trees showing reciprocal monophyly, and large genetic distance estimated from CYTB sequences all support recognition of Central American and South American Mealy Amazons as separate conservation units. Likewise, the large numbers of diagnostic characters ($n = 56$) detected suggest that the Central American (*A. f. guatemalae* and *A. f. virenticeps*) and South American (*A. f. farinosa*, *A. f. inornata*, and *A. f. chapmani*) Mealy Amazons are distinct phylogenetic species. This split is also supported by well-described phenotypic differentiation (Forshaw 2010; Juniper and Parr 1998). A case for recognition of *A. f. inornata* as a separate taxon from *A. f. farinosa* and *A. f. chapmani* could also be made based on the lack of haplotype sharing, well-supported reciprocal monophyly, number of diagnostic characters ($n = 18$), and geographic separation of the two clades, however, additional sampling across their distributions are required to minimize the potential for over-diagnosis.

The splitting of Central American and South American *A. farinosa* into separate species, as supported by our data, would allow for the individual assessment and appropriate categorization of the threat level facing these different populations. The Central American Mealy Amazon Parrots are under increased pressure from habitat loss and collection for the pet trade, making their population especially vulnerable and in need of immediate evaluation and possible reclassification. Revision of species limits in this complex could also have immediate consequences for planned releases of confiscated birds from the illegal pet trade (J Gilardi pers. comm.). Our data suggest such releases should be confined to individuals from the same taxonomic unit as the population in the area of release to avoid potential genetic incompatibilities (Burbidge et al. 2003).

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