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Cryptic diversity revealed by DNA barcoding in Colombian illegally traded bird species

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Abstract

Colombia is the country with the largest number of bird species worldwide, yet its avifauna is seriously threatened by habitat degradation and poaching. We built a DNA barcode library of nearly half of the bird species listed in the CITES appendices for Colombia, thereby constructing a species identification reference that will help in global efforts for controlling illegal species trade. We obtained the COI barcode sequence of 151 species based on 281 samples, representing 46% of CITES bird species registered for Colombia. The species analysed belong to nine families, where Trochilidae and Psittacidae are the most abundant ones. We sequenced for the first time the DNA barcode of 47 species, mainly hummingbirds endemic of the Northern Andes region. We found a correct match between morphological and genetic identification for 86–92% of the species analysed, depending on the cluster analysis performed (BIN, ABGD and TaxonDNA). Additionally, we identified eleven cases of high intraspecific divergence based on K2P genetic distances (up to 14.61%) that could reflect cryptic diversity. In these cases, the specimens were collected in geographically distant sites such as different mountain systems, opposite flanks of the mountain or different elevations. Likewise, we found two cases of possible hybridization and incomplete lineage sorting. This survey constitutes the first attempt to build the DNA barcode library of endangered bird species in Colombia establishing as a reference for management programs of illegal species trade, and providing major insights of phylogeographic structure that can guide future taxonomic research.

Keywords: Cytochrome coxidase subunit 1, DNA barcoding, CITES, Colombia, Trochilidae, Psittacidae

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Introduction

The Neotropics are widely recognized for their huge diversity in groups such as plants (Gentry 1982), amphibians (Duellman 1999) and birds (Fjeldså 1994). Deforestation and poaching are the major threats for bird species in this region (BirdLife International 2000; Renjifo *et al*. 2002, 2014); where geographically restricted species with high juvenile mortality and low reproductive outputs are particularly vulnerable to human activities and over-exploitation (Nash 1993; Purvis *et al*. 2000). To prevent global extinctions of wild populations, the international conservation movement encouraged governments worldwide to support the 1973 Convention on International Trade in Endangered Species of Wild Fauna and Flora, giving birth to the CITES Secretariat that classifies species depending upon their vulnerability to human exploitation and regulates and promotes legal mechanisms of wildlife trade (CITES Secretariat 2000; Reeve 2002).

To monitor CITES species trade, it is pivotal to accurately identify the species traded. To do so, species diagnostic has been traditionally based on morphological traits. Unfortunately, these diagnostic characters are insufficient when the specimen belongs to a highly diverse taxonomic group or is a juvenile, or when the sample is fragmented or highly processed. A proved strategy to facilitate the identification of specimens or

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biological fragments with no or little diagnostic traits is the use of standard DNA regions, also known as DNA barcodes (Hebert *et al.* 2003, 2004a; Gonzalez *et al.* 2009; Hubert *et al.* 2010; Tavares *et al.* 2011; Gonçalves *et al.* 2015). DNA barcoding ensures a rapid and accurate identification of a broad range of biological specimens using a standard and small gene region of the DNA sequence. Hebert *et al.* (2003) proposed to use a 648 bp fragment of the mitochondrial gene Cytochrome c oxidase subunit 1 (COI) to identify all animal species based on a unique molecular identification system. Although the mitochondrial DNA (mtDNA) by itself has some shortcomings in delimiting or describing species (Ebach & Holdrege 2005; Brower 2006), it has been readily adopted as a key component of an integral taxonomic framework (Sites & Marshall 2003; Padial & De La Riva 2007). Since its appearance, DNA barcoding has been used for rapidly appraising cryptic diversity, which has speeded up the discovery of new species particularly in the Neotropics (Crawford *et al.* 2013). In addition, DNA barcoding allows the identification of illegally traded species by nonspecialized personnel by simply following the standard procedures of molecular processing (Hsieh *et al.* 2001; Caballero *et al.* 2012), therefore enhancing controls on wildlife trade.

The DNA Barcode initiative promotes the development of a public reference library (www.boldsystems.org) of DNA barcode markers of the world's biodiversity (Hebert *et al.* 2004b). There have been large initiatives to barcode North American birds (Kerr *et al.* 2007), as well as birds from Argentina and Brazil (Kerr *et al.* 2009; Tavares *et al.* 2011). Results from these studies have shown unambiguous identifications, except for few cases of hybrid and cryptic species. Interestingly, studies from Argentina (Kerr *et al.* 2009) and the Brazilian Amazon (Tavares *et al.* 2011) have shown that some South American birds exhibit high intraspecific divergences and geographic structure, a pattern that has been reported for samples from large geographic areas with breaks between ecoregions where multiple subspecies have been described (Tavares *et al.* 2011). Therefore, DNA barcoding may be a valuable tool to screen which taxa would benefit from more extensive taxonomic revisions combined with a multilocus approach.

Colombia is a megadiverse country with the largest number of bird species worldwide (*c.a* 1889 species; Bello *et al.* 2014), most of them geographically restricted to the Andean region (Hilty & Brown 1986; Orme *et al.* 2006; Graham *et al.* 2010); however, no previous efforts have been made to build a reliable barcode library. This study represents the first step towards building a DNA barcode library of bird species from Colombia. We focus on the species listed in the CITES appendices looking to contribute with global efforts for controlling illegal species trade, and to provide a basis for subsequent taxonomic studies which will in turn shed light on this biodiversity hotspot.

Materials and methods

Sampling collection

We selected tissue samples from the biological and tissue collection of the Institute Alexander von Humboldt (hereafter IAvH-A and IAvH-CT; Arbeláez-Cortés *et al.* 2015) for species included in 2014 list of Colombian CITES bird species (www.checklist.cites.org). DNA was obtained from different tissues: liver and muscle (222), blood (1), feathers (7), and toe pads from museum specimens (52). Samples were collected throughout the whole Colombian territory with emphasis on the Andean region (Fig. 1). Six samples were obtained from specimens confiscated from illegal traders and eight more samples came from individuals in captivity. Museum specimens were captured mainly between 1968 and 1986. Overall, a total of 281 samples belonging to 151 species were processed, and up to six individuals per species were analysed. The taxonomy of the samples was confirmed by checking its associated information and the corresponding specimens from the museums. We used this information as an *a priori* method to validate the resolution of the DNA barcode. Details of the specimens used are provided in the BOLD data set CITES birds from Colombia (DS-IHCB16).

Laboratory procedures

DNA was extracted following the Ivanova *et al.* (2006) spin columns protocol. For feather samples 20 μl of DTT 1 M was added during the lysis phase. For the toe pads from museum specimens, 30 μl of proteinase K and 10 μl of DTT 1 M were added during the tissue digestion phase; the final elusion was split into three consecutive sets of ddH_2O preheated to 56°C. A ~700 bp fragment of COI was amplified using Falco and Vertebrate primers (Kerr *et al.* 2007). Falco and BirdR2 primers (Hebert *et al.* 2004b) were used instead when the Falco-Vertebrate primers failed to amplify. The PCR solution mix included 2.5 μl 10× Taq Buffer containing $(NH_4)_2SO_4$, 1.25 μl $MgCl_4$ 25 mM, 0.25 μl dNTPs (10 μM), 0.25 μl each primer (10 μM), 1U of Taq polymerase (Thermo Scientific) and 1–5 μl of total DNA (5–2500 ng/μl) for a final volume of 25 μl. PCR thermal conditions were 1 min at 94°C followed by 5 cycles of 1 min at 94°C, 1.5 min at 45°C, and 1.5 min at 72°C followed by 30 cycles of 1 min at 94°C, 1.5 min at 51°C, 1.5 min at 72°C, and 5 min at 72°C.

For toe pad samples, three internal primer pairs were used resulting in three overlapping fragments with an average length of 310 bp each (Patel *et al.* 2010). PCR conditions were: final volume of 20 μl, 2.5 μl buffer 10×,

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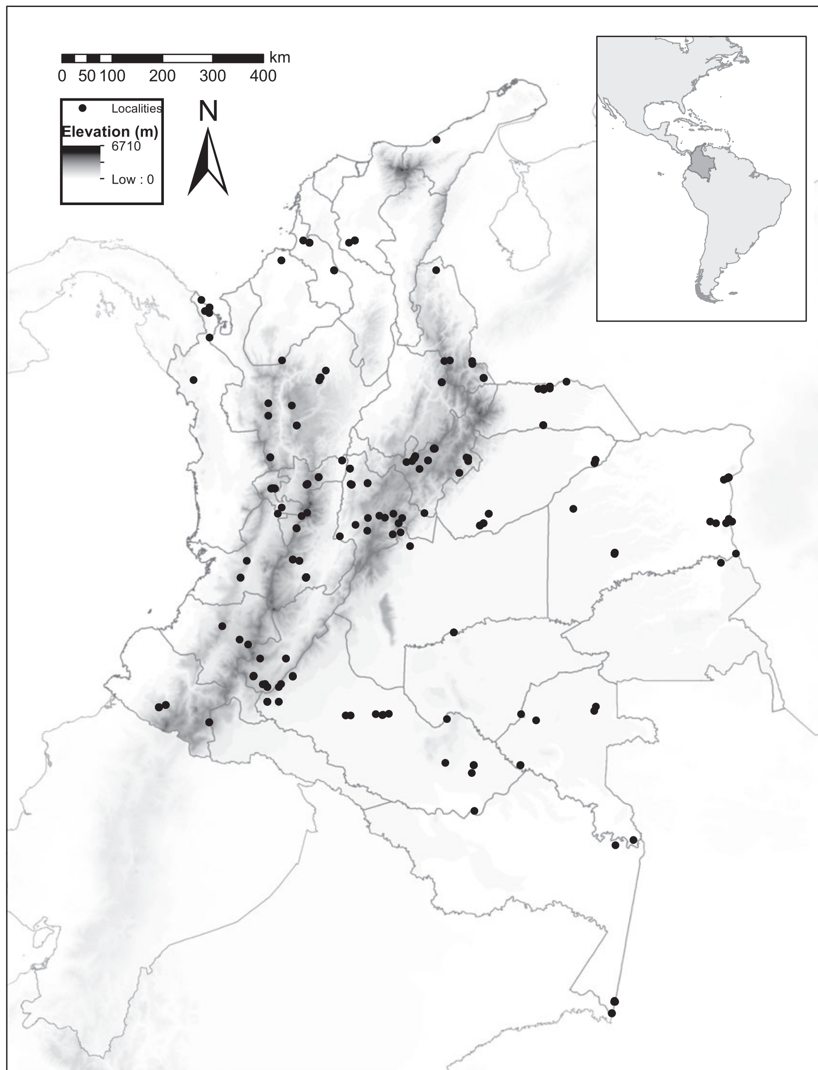

Fig. 1 Localities of collection of the bird samples used.

1.25 μl $MgCl_2$ 25 mM, 0.25 μl dNTPs 10 μM, 0.25 μl each primer 10 μM, 2 μl BSA 10× y 1U Taq polymerase. PCR thermal conditions for all primer combinations consisted in 2 min at 94°C followed by 10 cycles of 20 s at 94°C, 20 s at 55°C and 20 s at 72°C followed by 40 cycles of 20 s at 94°C, 20 s at 50°C, 20 s at 72°C, and 4 min at 72°C. The sequences of all the primers are shown in Table S1 (Supporting information).

In both cases, amplification success was verified in 1% agarose gel stained with Sybr® Safe dye. PCR products were purified by ExoI/FastAP (Fermentas) and sequences were obtained using the chemical reaction BigDye Terminator v. 3.1 in an automated DNA sequencer (ABI 3730XL and 3550 series, Applied Biosystems Inc).

Data analyses

Consensus sequences were assembled from forward and reverse reads and edited manually using the Geneious 7.0.5 (Kearse *et al.* 2012) software. Nucleotide-sequences, traces files and images were uploaded to the BOLD System (www.barcodinglife.org) and are available in the data set DS-IHCB16.

Sequence divergence was estimated with Kimura's 2-parameter (K2P) nucleotide evolution model implemented by BOLD. To graphically represent the species divergence, we generated a Neighbour-Joining (NJ) tree based on K2P sequence divergence. Node support was computed with 1000 bootstrap replicates.

Since species clustering can vary with the algorithm used (Paz & Crawford 2012), we assessed the correspondence between species identification and DNA barcodes following three different methods: (i) the automatically assignment of Automated Barcode Gap Discovery (ABGD) (Puillandre *et al.* 2012) using the web interface (http://wwwabi.snv.jussieu.fr/public/abgd/) and the K2P measure with a Pmax of 0.1 and a relative gap width of 1.0 (ii) the automatically Barcode Index Number (BIN)

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clustering (Ratnasingham & Hebert 2013), and (iii) the "Best Close Match" criterion implemented in the Species Identifier tool of TaxonDNA 1.8 software (Meier *et al.* 2006). The "Best Close Match" considers the barcode assignment to be correct when a query matches all conspecific sequences within the 95th percentile of all intraspecific distances (Meier *et al.* 2006).

In our data set, when we found incongruences between DNA barcode and the specimen identification, we resequenced the samples and revised the specimens with experts in order to verify the identity. In four cases both the expert revision and barcode information concurred, thus we corrected and included these sequences in our data set. For four further samples, we did not have enough guarantee to resolve possible troubleshooting therefore they were excluded from our data set.

Results

We obtained 281 COI sequences belonging to 151 bird species. This corresponds to 46% of the bird species registered in the Colombian CITES checklist. For 261 samples corresponding to 146 species, we obtained sequences longer than 500 bp that were used in subsequent analyses. These species represent seven orders and nine families, being Trochilidae and Psittacidae the most abundant ones (Table 1). Sequence length varied from 218 bp (IAvH-CT 14590-*Amazona ochrocephala*) to 780 bp (IAvH-CT 14733-*Pionites melanocephalus*). For 16 of the toe pads sampled from museum specimens, only one of the three fragments was successfully amplified, obtaining sequences ranging from 218 to 337 bp. Also, for 18 toe pad samples we amplified two of the three fragments obtaining sequences ranging from 308 to 614 bp. For hummingbirds (order Apodiformes), which comprise most of the species listed in CITES for Colombia, we sequenced 95 out of the 152 species represented on the list (Table 1). To the best of our knowledge, we obtained for the first time the barcode records for 38 hummingbirds, four parrots, three hawks and two owl species, mainly endemic of Northern Andes (Table S2, Supporting information).

Based on 160 assessments, the mean intraspecific pairwise genetic distance calculated was 1.37% (0.00–14.61%; Fig. 2a, Table 1). For eight species, intraspecific distances were above 2.5%, which match with the lower 5% of congeneric distances assessed (Table 2, Fig. 2b); and three further species (*Boissonneaua flavescens*, *Glaucidium jardinii* and *Eriocnemis vestita*) appeared to be polyphyletic based on the NJ tree (Fig. 3). Based on 650 comparisons, the mean K2P genetic distance among congeneric species was 8.96%, ranging from 0.15% to 16.53% (Fig. 2, Table 1). Interspecific divergences lower than 2.5% were obtained for 15 species (Table 3).

Barcode clustering

The ABGD analysis reported 143 groups out of the 146 nominal species studied, 86.3% of which were correctly designated to single groups (Fig. 3). This algorithm clumped 15 nominal species in six groups and six further nominal species were split into multiple groups (Table 2, Table 3).

For the TaxonDNA analysis, we excluded seven museum samples with ambiguous bases in their sequences. The 254 sequences with >500 bp left corresponded to 141 nominal species. Using the estimated divergence threshold computed by the Best Close Match method (2.0%), 239 sequences showed a successful match. Seven species were split (Table 2), whereas six species were clumped (Table 3). Overall 92.2% of the nominal species were correctly identified.

Table 1 Summary of total CITES bird species in Colombia by order according to the CITES checklist webpage (http://checklist.cites.org/) and total species sequenced in this study. K2P genetic distances for bird species analysed in total and by order. All K2P genetic distances were calculated for sequences with length >500 bp

Categories	Total CITES species	Analysed species	No. samples	Intraspecific distances			Conspecific distances		
				Min K2P	Mean K2P	Max K2P	Min K2P	Mean K2P	Max K2P
Anseriformes	4	1	1	–	–	–	–	–	–
Apodiformes	152	95	184	0	1.50	14.61	0.15	9.35	16.53
Falconiformes	73	12	14	0.15	0.16	0.16	6.00	11.72	15.01
Passeriformes	5	1	1	–	–	–	–	–	–
Piciformes	5	1	1	–	–	–	–	–	–
Psittaciformes	54	29	48	0	0.68	2.00	0.75	6.26	12.28
Strigiformes	25	7	12	0	2.37	7.86	5.77	9.30	11.50
Total	329*	146	261	0	1.37	14.61	0.15	8.96	16.53

*The value of total CITES species included 11 species of orders Galliformes (6), Ciconiiformes (3), Charadriiformes (1) and Sphenisciformes(1) not processed in this paper.

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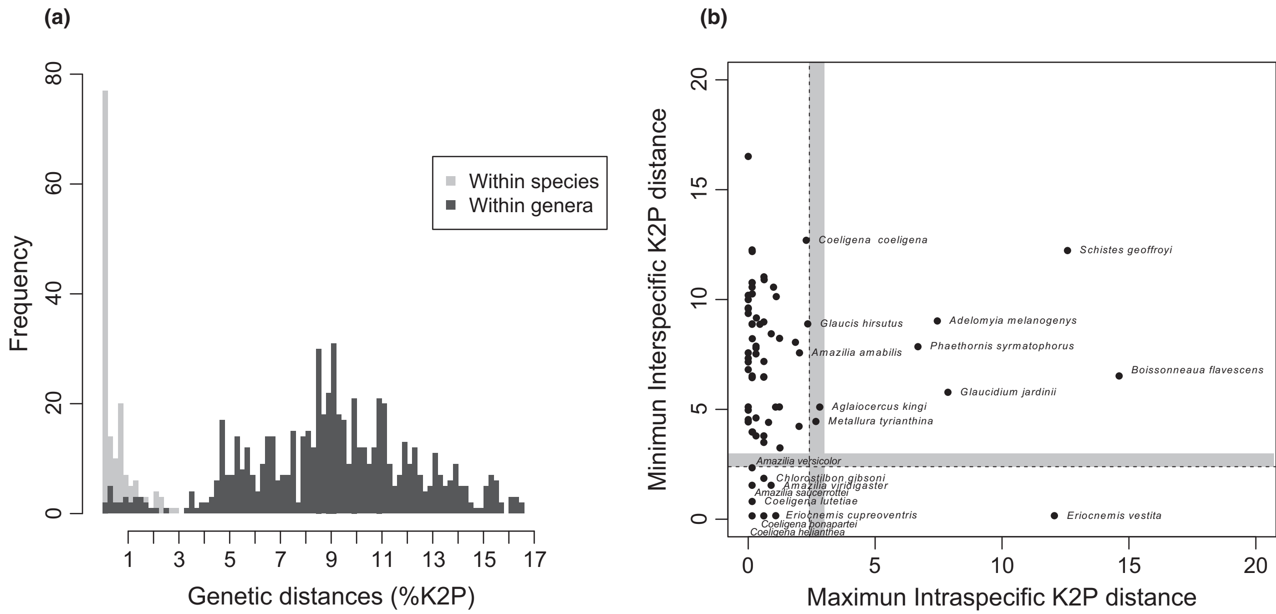

Fig. 2 a) Frequency distribution of K2P distances within (light-grey) and among (dark-grey) species of Colombian CITES birds. b) Comparison of intraspecific versus interspecific distances (K2P) of the COI sequences from individual species, showing the threshold of barcode-gap and troubleshooting species. The grey bars show the visible gap between the 2.5% (corresponding to the lower 5% congeneric distances) and the value immediately above (3.2%).

Table 2 Bird species displaying high K2P intraspecific genetic distances

Nominal species	Clustering method splitting	No. of. sequences	Min intraspecific K2P	Mean intraspecific K2P	Max intraspecific K2P
Adelomyia melanogenys	□○Δ	3	1.07	**5.21**	**7.45**
Aglaiocercus kingi	○Δ	2	**2.81**	**2.81**	**2.81**
Amazilia amabilis	○	4	0.16	1.00	2.02
Boissonneaua flavescens	□○	4	0.93	**9.73**	**14.61**
Coeligena coeligena	○Δ	2	2.28	2.28	2.28
Eriocnemis vestita	□○	4	0	**5.21**	**12.06**
Glaucis hirsutus	○	3	0.92	1.76	2.34
Metallura tyrianthina	○Δ	3	0	1.71	**2.66**
Phaethornis syrmatophorus	□○Δ	2	**6.68**	**6.68**	**6.68**
Schistes geoffroyi	□○Δ	2	**12.58**	**12.58**	**12.58**
Glaucidium jardinii	□○Δ	3	0	**5.22**	**7.86**

Symbols indicate splitting by any of the automatically clustering methods: ABGD (squares), BIN (circles) and TaxonDNA (triangles). All K2P genetic distances were calculated for sequences with length >500 bp. Bold values indicate K2P genetic distances above 2.5% which coincides with the lower 5% of overall congeneric distances.

BIN records were generated for 248 sequences of 137 nominal species, yielding 144 BIN clusters (Fig. 3). The samples without BIN records corresponded to sequences that did not fulfil with barcode compliance standards. We obtained multiple BIN records for eleven species (Table 2) and six species were clumped into single BINs (Table 3). Excluding those cases, 89.7% of the analysed species were identified in congruence with their morphological characteristics.

In order to evaluate the effect of sequences without barcode standard compliance (museum incomplete sequences and sequences with ambiguous results) on the performance of the clustering methods, we executed the ABGD and TaxonDNA analyses for the 248 sequences analysed by BIN, and then compared these with the initial results. No differences were found between the clustered/splitted species in relation to the analyses for all 261 sequences.

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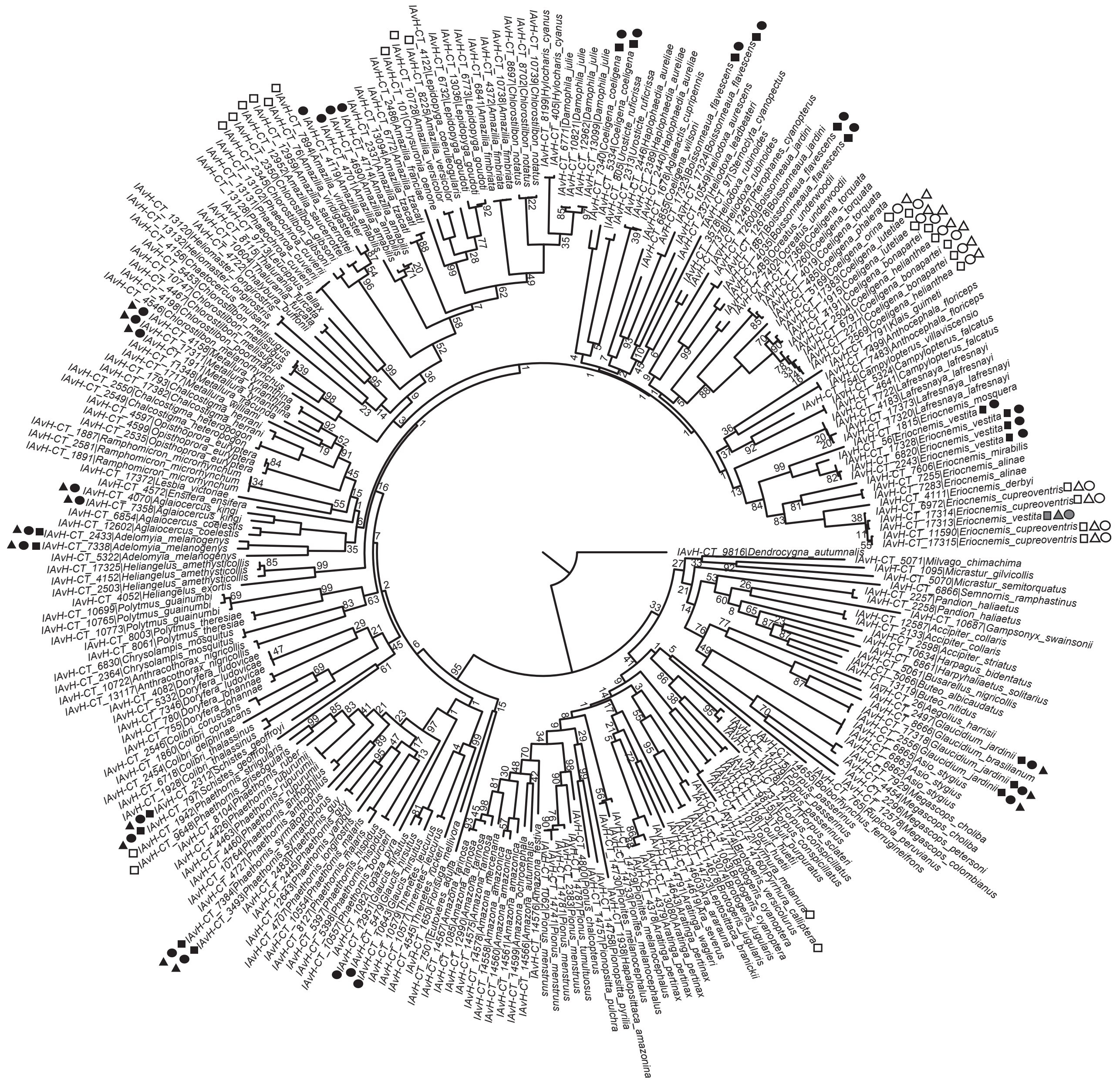

Fig. 3 Neighbour-Joining (NJ) dendrogram obtained from mitochondrial COI sequences of Colombian CITES birds showing the molecular relationships of split (filled) and clumped (blank) species according to ABGD (squares), BIN (circles) and TaxonDNA (triangles) clustering methods. Numbers in each node represent posterior probability values < 0.9 after bootstrap with 1000 replicates.

Discussion

Clustering based on COI sequences was 86–92% congruent with morphological species delimitation of the Colombian bird species included in the CITES checklist, depending upon the algorithm used. Although these results indicate that most species were clearly distinguishable from related species, the percentage of congruency is slightly lower than that found for North American, Brazilian and Argentinean bird species (94%, 93% and 98% respectively) (Kerr *et al*. 2007, 2009; Tavares *et al*. 2011). In this study, conflicting cases corresponded to samples from either splitting or clumping of nominal species (Tables 2 and 3).

Barcode gap

The presence of a gap between the intra- and interspecific genetic distances is the main requirement for successful barcode identification (Hebert *et al*. 2004b; Aliabadian

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Table 3 Bird species displaying low K2P genetic distances among species

Nominal species in each cluster	Clustering method grouping	No. of. sequences	Intraspecific distances			Distants among species		
			Min K2P	Mean K2P	Max K2P	Min K2P	Mean K2P	Max K2P
Amazilia viridigaster	□	2	0.90	0.90	0.90	1.54	2.07	2.66
Amazilia saucerrottei	□	2	0.15	0.15	0.15			
Chlorostilbon gibsoni	□	2	0.61	0.61	0.61			
Chrysuronia oenone	□	1	–	–	–	2.34	2.42	2.49
Amazilia versicolor	□	2	0.15	0.15	0.15			
Coeligena lutetiae	□○∆	2	0.15	0.15	0.15	0.15	0.95	2.16
Coeligena orina	□○∆	1	–	–	–			
Coeligena bonapartei	□○∆	3	0.15	0.40	0.61			
Coeligena helianthea	□○∆	2	0.15	0.15	0.15			
Eriocnemis cupreoventris	□○∆	4	0	0.31	1.08	0.16	9.68	12.94
Eriocnemis vestita	□○∆	5	0	5.21	12.06			
Phaethornis griseogularis	□	1	–	–	–	2.40	2.40	2.40
Phaethornis striigularis	□	1	–	–	–			
Pyrrhura melanura	□	1	–	–	–	0.75	0.75	0.75
Pyrrhura calliptera	□	1	–	–	–			

Symbols indicate clustering by each method: ABGD (squares), BIN (circles) and TaxonDNA (triangles). All K2P genetic distances were calculated for sequences with length >500 bp.

et al. 2009). Previous studies have used a 2.4% K2P threshold for Argentinean species (Kerr *et al.* 2009) and 2.7% K2P threshold for North American birds (Hebert *et al.* 2004b) following the 10× criterion. Also, while Chaves *et al.* (2015) found an overlap between intra- and interspecific genetic distances rather than a barcoding gap in Neotropical birds, Tavares *et al.* (2011) defined a 1.5% K2P threshold for Brazilian species based on the minimum genetic distance found for the lower 5% of congener's comparisons. In this study, we found a visible gap at K2P values among species between 2.4% and 3.2% (Fig. 2b), which coincides with the minimum genetic distance found for the 5% of congeneric comparisons.

Cryptic diversity and geographic patterns

The topographic complexity of the Andes Cordillera, characterized by a number of geographical barriers, resulted in many bird species exhibiting deep intraspecific divergences, reflecting the challenge of DNA barcoding for Neotropical species (Moritz & Cicero 2004). In Colombia, this difficulty is further enhanced as this mountain range is a single mass in the south of the country that splits towards the north into three different mountain systems, known as the Eastern, Central and Western Cordilleras. Such complexity may promote speciation though vicariance by acting as geographic or ecological barriers limiting the dispersal of individuals (Ruggiero & Hawkins 2008; McGuire *et al.* 2014; Smith *et al.* 2014; Mendoza *et al.* 2015).

Some samples from different cordilleras exhibited high intraspecific divergence, and interestingly, several of these high divergences matched with recognized subspecies and/or phylogenetic clades. For instance, in the Coquettes hummingbirds (Fig. 4a–c), samples of *Aglaiocercus kingi* (K2P = 2.18%) coming from different cordilleras corresponded to either the subspecies *A. kingi mocoa* (Central Cordillera) or *A. kingi emmae* (Western Cordillera), which differs in the colour of their throat and tail (Ayerbe-Quiñones 2015; del Hoyo *et al.* 2016). Although nowadays both are considered subspecies, *A. kingi mocoa* has been historically treated as a separate species (e.g., Cory 1918; Peters 1945; Salaman & Mazariegos 1998). Regarding *Metallura tyrianthina*, we found high genetic divergence between samples from the Western and Eastern Cordilleras (K2P = 2.66%, Fig. 4b); this result is in agreement with recent multilocus analyses that found high genetic structure across major topographical barriers in this particular species (Benham *et al.* 2015). Samples of *Adelomya melanogenys* clustered in two different groups with sequences from the Western and Central Cordillera (K2P = 7.45%, Fig. 4c) corresponding to the subspecies *A. melanogenys cervina* (Ayerbe-Quiñones 2015) and the sequence from the western part of the Eastern Cordillera corresponding to a recently proposed new subspecies *A. melanogenys sabinae* (Donegan & Avendaño 2015). In fact, recent analyses based on multilocus data confirmed six phylogroups in *Adelomya* (Chaves & Smith 2011; Chaves *et al.* 2011); our samples correspond to clades D and F described in Chaves & Smith (2011) the former clade being restricted to the northern section of the Eastern Cordillera in Colombia.

Other cases of high genetic divergence between hummingbird subspecies were found. For instance, *Coeligena*

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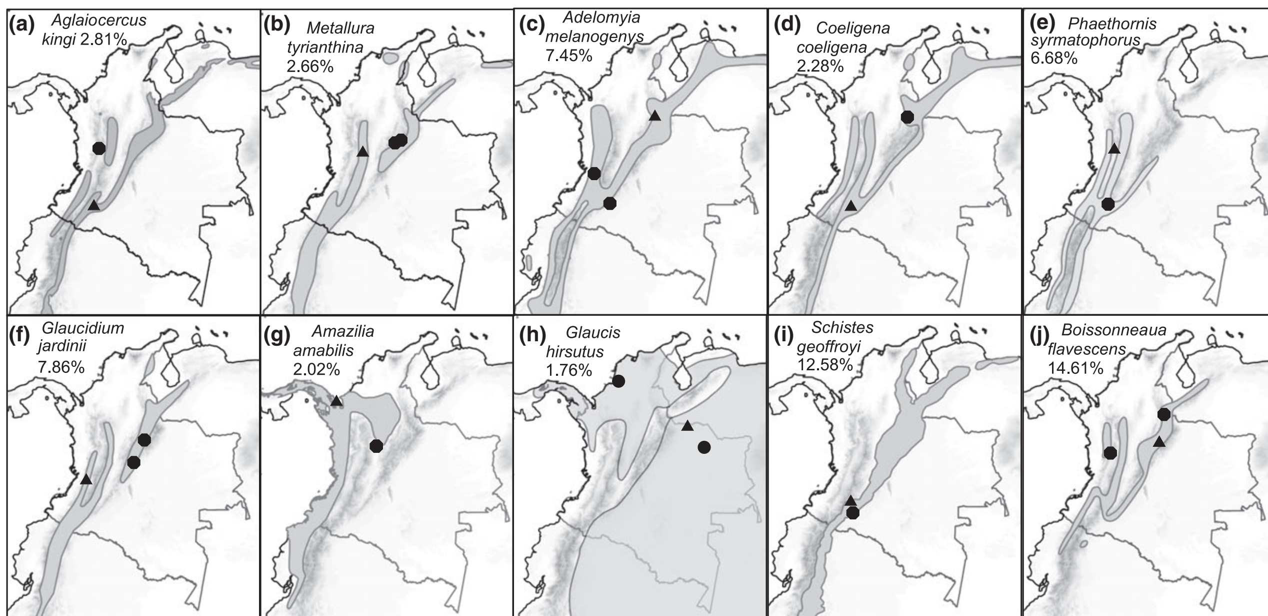

Fig. 4 Sampling localities of CITES birds with high intraspecific divergences based on K2P genetic distances of the COI barcode. a) *Aglaiocercus kingi* b) *Metallura tyrianthina* c) *Adelomyia melanogenys* d) *Coeligena coeligena* e) *Phaethornis syrmatophorus* f) *Glaucidium jardinii* h) *Amazilia amabilis* i) *Glaucis hirsutus* i) *Schistes geoffroyi* j) *Boissonneaua flavescens*. The area of species distribution is displayed in grey. Triangles and circles correspond to different BINs.

coeligena (K2P = 2.28%, Fig. 4d), showed two different DNA clusters corresponding to samples from subspecies *C. coeligena obscura* (southern sample) and *C. coeligena columbiana* (northern sample). These two subspecies differ in size and coloration (Cory 1918; Ayerbe-Quiñones 2015; del Hoyo *et al*. 2016). Samples of *Phaethornis syrmatophorus* from the Central and Eastern cordilleras also showed high intraspecific divergence values (K2P = 6.68%, Fig. 4e), this result is in agreement with previous studies reporting morphological differentiation in this species. Indeed, the eastern clade is recognized as the subspecies *P. syrmatophorus columbianus*, which differs from *P. syrmatophorus syrmatophorus* in having dark brown throat and breast (del Hoyo *et al*. 2016). Hinkelmann & van den Elzen (2002) raised the subspecies *P. syrmatophorus columbianus* to the species level based on morphological differences; the genetic distances found in this study support their hypothesis, although, this category has not been formally accepted.

An interesting case of high genetic divergence was found in *Glaucidium jardinii* between samples from the Eastern and Western cordillera (K2P = 7.86%, Fig. 4f). However, *G. nubicola*, morphologically very similar to *G. jardinii*, also occurs in the same localities from the Western Cordillera where *G. jardinii* was collected (Robbins & Stiles 1999). As both species can only be distinguished vocally, by weight or by subtle coloration patterns (del Hoyo *et al*. 2016), western samples might have been misidentified. Morphological traits and weight of our samples are not conclusive to distinguish between both species (S. Cordoba pers. comm.). Gathering more genetic and population information to better distinguish these two species is critical as *G. nubicola* is vulnerable and highly threatened by forest degradation (del Hoyo *et al*. 2016).

Cases of high intraspecific genetic divergence do not always concur with long distances or geographical barriers between sampled localities. For instance, the samples of the Emerald *Amazilia amabilis* from lowland localities were clustered in two different BINs and their K2P intraspecific distance was of 2.0% (Fig. 4g), while the mean intraspecific distance in hummingbirds was of 1.4%. Considering the overall pattern of high net diversification rates shown by Emeralds in other studies (McGuire *et al.* 2014), we should not expect multiple BINs in a single species. Instead, the overall pattern described by McGuire *et al.* (2014) is in agreement with the low divergence between other *Amazilia* species (*A. viridigaster* and *A. saucerrottei*) showing mean K2P values of 1.56%. Likewise, the sequences of *Glaucis hisrutus* were clustered in two different BINs despite their mean K2P intraspecific distance being 1.76%. In this case, the divergence between BIN clusters is not related to a geographical or ecological barrier (Fig. 4h). However, one of our samples is clustered in a BIN with other sequences from Trinidad and Tobago, French Guiana,

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Guyana and Brazil; whereas the remaining samples are clustered in a BIN with sequences from Peru, Panama and Bolivia. This species represents a very interesting case that needs further investigation.

Another example is that of *Schistes geoffroyi*, which shows extremely high intraspecific divergences between samples collected <100 km apart (K2P = 12.58%), but in opposite slopes of the south part of the Eastern cordillera (Fig. 4i). *Shistes albogularis*, a former subspecies of *S. geoffroyi*, is distributed on the Western and Central cordilleras (del Hoyo *et al*. 2016). Some authors still refer to *S. g. geoffroyi* and *S. g. albogularis* as conspecifics (Ayerbe-Quiñones 2015) whereas others consider them as different species (Cory 1918; Ridgely & Greenfield 2001). It is plausible that the samples analysed correspond to both *S. geoffroyi* and *S. albogularis* in a small area of contact. Finally, four samples of *Boissonneaua flavescens* were clustered in two different groups, one group represented two specimens from the Western and the Eastern cordilleras collected in elevations ranging 2300 and 2600 m.a.s.l that correctly matched other *Boissonneaua* species in our data set, and the other group represented two specimens from the Eastern cordillera collected at 3200 m.a.s.l that were not monophyletic with other *Boissonneaua* (Fig. 4j). In order to verify these results, we sequenced additional genes ND2 and ND4 for the four samples of this species and found that they correctly matched other *Boissonneaua* sequences from Genbank (results not shown). One possible explanation for this result is that the COI region of the samples from high elevation corresponds to pseudogenes, although the quality of the sequences was good and did not have stop codons.

Clumped species

We found two cases of low interspecific genetic distances that represent a complex case for barcode identification. The first is in the *Coeligena* genus, as *C. bonapartei*, *C. helianthea*, *C. lutetiae* and *C. orina* showed K2P distances values between 0.15% and 2.16% and were clumped as a single species in all the analyses performed (Table 3; Fig. 3). Based on a multilocus study, Parra *et al*. (2009) found low genetic divergence in this genus and supported the monophyly in *C. lutetiae*, but not in *C. bonapartei* and *C. helianthea*. Interestingly, these two species differ strongly in several morphological characters and are not usually sympatric, although they occupy opposite slopes of the same cordillera. Parra *et al*. (2009) recognized both species as an interesting case for further detailed studies on the efficacy of phenotypic differentiation in reproductive isolation. The second case was a single sample that morphologically corresponded to *Eriocnemis vestita* (IAvH-CT 17313), but its COI sequence clustered within the *Eriocnemis cupreoventris* clade (Fig. 3). This sample was collected in a "paramo" region at 3100 m.a.s.l., a location where both species overlap. The collectors reported unusual aspects in the plumage of this specimen (O.A. Acevedo-Charry pers. comm). Further analysis (e.g. examining nuclear loci and morphological traits) is needed to test for hybridization or incomplete lineage sorting.

Taxa representativeness

We included half of the parrot species registered in Colombia using specimens collected either in expeditions to several pristine sites conducted before 1995, or in captivity due to illegal trade activities. Parrots are the most trafficked birds and their capture historically has implied habitat degradation through illegal methods (Beissinger & Snyder 1992; Meyers 1994). Using primers of small overlapping fragments of COI, we were able to obtain sequences from toe pad samples for specimens collected since 1968. Indeed, in the effort to recover DNA from museum specimens, we obtained the first DNA barcodes for restricted and endemic parrot species such as *Bolborhynchus ferrugineifrons*, *Pionopsitta pulchra*, *Pionopsitta pyrilia* and *Pyrrhura calliptera*. Among these, *P. calliptera* and *B. ferrugineifrons* are categorized as vulnerable in the IUCN red list of endangered species, and their records are very scarce (Renjifo *et al*. 2002, 2014).

Unfortunately, our analyses included a low number of samples from raptors. In Colombia, 73 raptor species are included in CITES appendices, yet we only sequenced 12 of them (Bildstein *et al*. 1998). Raptors specimens are rare in biological collections since they are not easy to sample (Wang & Finch 2002). To date, there are records for 62 out of the 73 raptor species registered in CITES for Colombia in the BOLD system, yet these records come from Brazil, Peru, Argentina and Panama.

Implications and further analysis

Gonçalves *et al*. (2015) recently reported the use of DNA barcode for identification of avian eggs being transported illegally in a Brazilian airport. Thanks to DNA identification, they were able to designate these eggs to parrots and owl species. This information could serve to direct conservation strategies for regional, national and international authorities. In fact, it provides a tool to easily identify traded species or their parts, and in several cases it also provides valuable information about the origin of the captured specimen. This information can highlight regions of major poaching that together with an accurate identification of the specimens can be used to update species management conservation plans.

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Although bird species have been well studied taxonomically, we keep deciphering their taxonomic and evolutionary relationships. For instance, between 2000 and 2014, 29 new species of birds were described in Colombia using bioacoustics, morphology and genetic data (Caycedo-Rosales *et al*. 2014). Our results based on a single gene provide a good idea of what has been recently described using multiple genes (e.g. Krabbe *et al*. 2005; Donegan *et al*. 2010; Lara *et al*. 2012), and establish a promising way to reveal potential cryptic diversity. DNA barcoding paves the road to inquire for cryptic diversity, although determining specific status of each mtDNA lineage should be accomplished through an integrative approach to taxonomy (Will *et al*. 2005). Our long-term goal is to complete the DNA barcode library of bird species in Colombia, the country with largest number of bird species worldwide, in order to provide baseline for species delimitation (e.g., Toews & Irwin 2008), information that is critical to environmental authorities (Gonçalves *et al*. 2015).

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M.A.G. contributed with research design, supervised laboratory procedures, data analysis and revisions of the document. S.S. and F.F. participated in the field survey, tissue collecting and species identification. D.L.A. contributed with the first version of research design and provided access to tissues of IAvH collection. M.F.T., N.T. and A.M.M. performed laboratory procedures. A.P. and A.M.M. developed the administration of databases and uploaded the information to Bold System. A.M.M. wrote the paper. All authors contributed with suggestions and revision to previous versions of the paper.

Data accessibility

DNA sequences: BOLD dataset CITES birds from Colombia (DS-IHCB16).

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. PCR primer sequences used for the amplification of complete or small fragment of ~700 pb COI region.

Table S2. List of species and number of specimens used in the study. Asterisks refer to new records in Bold System contributed by this study.

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