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MINIREVIEW

The multiple applications of DNA barcodes in avian evolutionary studies[1]

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Abstract: DNA barcodes of birds are currently available for 41% of known species and for many different geographic areas; therefore, they are a rich data source to answer evolutionary questions. We review studies that have used DNA barcodes to investigate evolutionary processes in birds using diverse approaches. We also review studies that have investigated species in depth where taxonomy and DNA barcodes present inconsistencies. Species that showed low genetic interspecific divergence and lack of reciprocal monophyly either are the result of recent radiation and (or) hybridize, while species with large genetic splits in their COI sequences were determined to be more than one independent evolutionary unit. In addition, we review studies that employed large DNA barcode datasets to study the molecular evolution of mitochondrial genes and the biogeography of islands, continents, and even at a multi-continental scale. These studies showed that DNA barcodes offer high-quality data well beyond their main purpose of serving as a molecular tool for species identification.

Key words: birds, biogeography, cryptic species, diversification, radiation.

Résumé : Des codes à barres de l'ADN sont présentement disponibles pour 41% des espèces connues et pour plusieurs régions géographiques; ils constituent une riche source de données pour répondre à des questions sur l'évolution. Les auteurs passent en revue les études qui ont été réalisées à l'aide de codes à barres afin d'étudier les processus évolutifs chez les oiseaux au moyen de diverses approches. Les auteurs examinent des études qui ont investigué des espèces en profondeur et où la taxonomie et les codes à barres présentent des discordances. Des espèces qui présentaient une faible divergence génétique intra-spécifique et une absence de monophylie réciproque ont été identifiées comme étant le produit de radiations récentes ou d'hybridation. Par contre, il a été déterminé que les espèces présentant une séparation génétique nette de leurs séquences COI reflétaient plus d'une unité ayant évolué indépendamment. Les auteurs ont également examiné des études qui ont employé de grands jeux de données de codes à barres pour étudier l'évolution moléculaire au sein des gènes mitochondriaux et la biogéographie des îles, des continents et même à l'échelle multi-continentale. Ces études ont montré que les codes à barres de l'ADN procurent des données de grande qualité bien au-delà de leur objectif principal de servir d'outils moléculaires pour l'identification des espèces. [Traduit par la Rédaction]

Mots-clés : oiseaux, biogéographie, espèces cryptiques, diversification, radiation.

Introduction

The DNA barcode project was conceived in 2003 with the intention of building a molecular identification tool for all living species from a standardized genetic fragment (Hebert et al. 2003). After its inception, the library of sequences has grown exponentially, having reached in 2015 the 5 million sequences milestone formally proposed in 2009 (Frézal and Leblois 2008). Of these sequences, more than 47 000 belong to birds from almost 6000 species (according to the Barcode of Life Data Systems (BOLD) available at www.boldsystems.org; access date 25 July 2016), out of a total global avian diversity estimate of 10 473 species (Clements et al. 2015). The avian DNA barcode library includes 34 000 publicly available records with sequences of 4280 species from 37 out of 39 recognized avian orders (Fig. 1). Most avian DNA barcodes were obtained as part of the All Birds Barcoding Initiative (ABBI, Baker et al. 2005; Stoeckle 2005), which is one of the first global campaigns advocated for obtaining the DNA barcodes of a particular taxonomic group.

Birds were one of the first animal groups in which the efficacy of DNA barcoding was tested because of their

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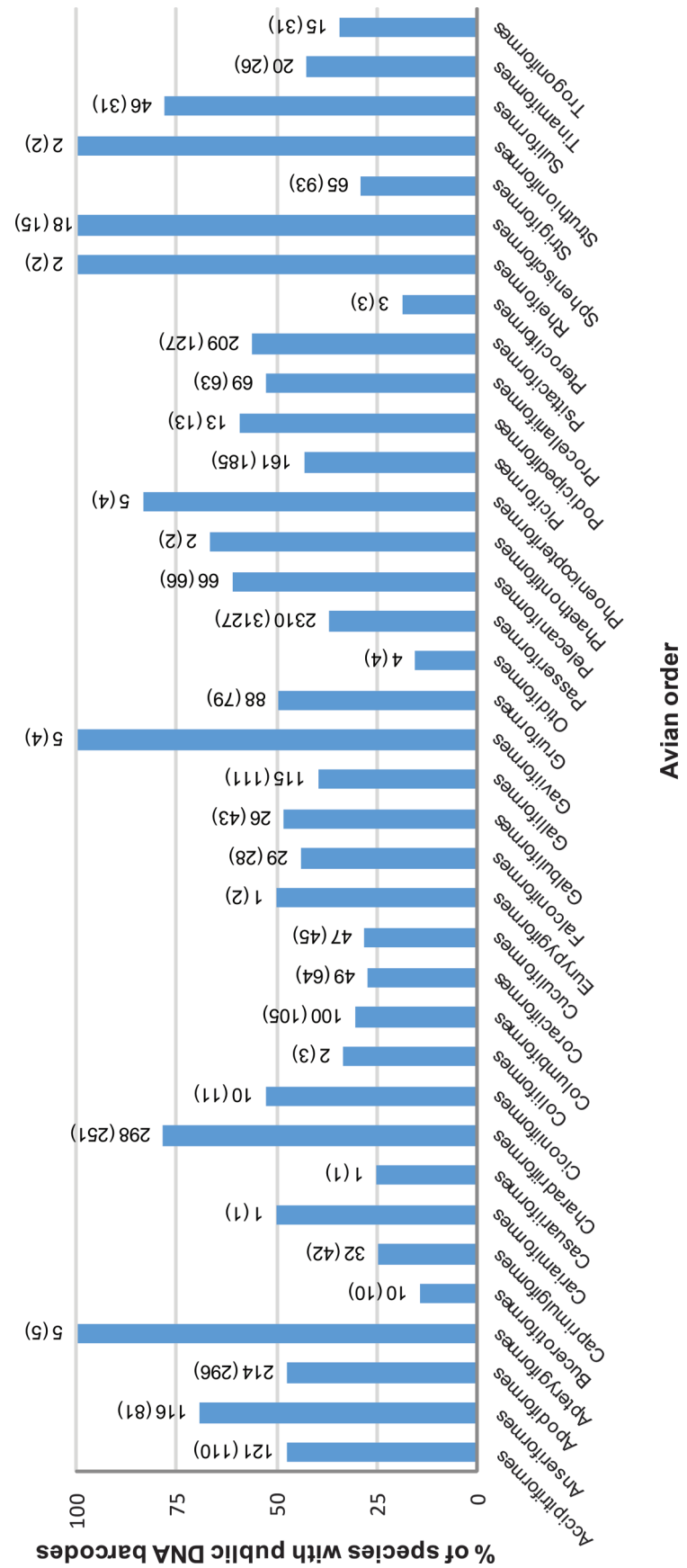


Fig. 1. Percentage of avian species with public DNA barcodes by order. Numbers above columns indicate the number of species with public DNA barcodes and within brackets the number of Barcode Index Numbers (BINs) retrieved among them. The data were obtained from http://boldsystems.org/index.php/TaxBrowser_Taxonpage?taxid=51 (access date 25 July 2016).

Table 1. Number of species studied with DNA barcoding, reported efficacy percentage (as percentage of species that could be identified through their DNA barcodes), number of species indistinguishable through DNA barcodes (and the criteria employed by each study to define this group), and number of species with deep splits in their DNA barcodes as reported by several studies conducted on birds from different geographic areas.

Geographic area studied	No. of species studied	Reported efficacy (%)	No. of species indistinguishable through DNA barcodes	No. of species with deep splits	Reference
North America	642	94	42*,†	15	Kerr et al. 2007
Argentina	500	98	9*,†,‡	21	Kerr et al. 2009*a*
Eastern Palearctic and North America	559	96	18*,†,‡	44	Kerr et al. 2009*b*
Scandinavia	296	94	19*,†,‡	4	Johnsen et al. 2010
Low latitudes of Neotropics	561	93	21*,†	20	Tavares et al. 2011
Korea	154	99	2*,†,‡	2	Park et al. 2011
Netherlands	141	95	6*	1	Aliabadian et al. 2013
Japan	234	97	10*,†,‡	11	Saitoh et al. 2015

*Species show low interspecific divergence in COI.
†Species lack reciprocal monophyly in the gene tree (includes cases of shared DNA barcodes).
‡Species cannot be separated with the diagnostic characters criteria.

very well-developed taxonomy that allowed a sound estimation of the agreement between genetic variation and Linnaean species delimitation (Hebert et al. 2004). All unknown samples can be identified from its cytochrome *c* oxidase subunit I gene sequence (COI, established as the DNA barcoding target region for animals) at least to the genus level, while most can be identified to the species level (93%–99%, depending on the study; Table 1). This is the result of the presence, in most cases, of a lower maximum intraspecific distance in this gene compared to the minimum interspecific distance (i.e., the barcode gap, Meyer and Paulay 2005). There are several analyses, besides the barcode gap concept, that were additionally employed by various studies to evaluate DNA barcode efficacy (see references in Table 1), including the evaluation of the percentage of species that are retrieved as monophyletic clusters in a neighbour-joining tree generated with COI data (or even the use of other, more complex, phylogenetic reconstruction methods such as Bayesian analysis); the analysis of the Barcode Index Numbers (BINs), which are genetic clusters generated by BOLD that correspond in most cases to species (Ratnasingham and Hebert 2013; Fig. 1); and the search for diagnostic characters (e.g., DeSalle et al. 2005), which allow species with low sequence divergence to be differentiated by comparing specific divergent positions in the sequence.

The excellent performance of DNA barcodes to discriminate bird species has turned them into a very powerful tool to highlight cases in need of deeper scrutiny. Inconsistencies between DNA barcodes and taxonomy usually represent interesting evolutionary phenomena (i.e., genetic introgression, hybridization, recent radiations, and cryptic divergence; Stoeckle and Thaler 2014). Therefore, these species constitute good models to perform phylogeographic, phylogenetic, and biogeographic studies to obtain further insights on the speciation process as they can illustrate instances of early and cryptic divergence. In addition, the growing library of standardized COI sequences is also promoting large-scale analyses of avian evolutionary patterns.

Here, we review studies that used DNA barcodes to investigate evolutionary processes in birds covering a diverse set of approaches. Low or null genetic divergence in COI with noticeable phenotypic differentiation between different species has been linked to events of recent radiations (Liebers et al. 2004; Campagna et al. 2010, 2012*a*, 2015). In turn, some cases of deep genetic divergence in DNA barcodes within species were further investigated with other molecular and phenotypic markers and were found to be cases of evolutionarily isolated lineages, some of which even deserved species status (Sanin et al. 2009; Efe et al. 2009; Kerr and Dove 2013; Lavinia et al. 2015*a*; García et al. 2016). Moreover, the large geographic coverage of bird DNA barcoding projects allowed researchers to compare the patterns of genetic divergence across different biogeographic regions to study the diversifying factors acting in each of them (Lohman et al. 2010; Lijtmaer et al. 2011; Tavares et al. 2011; Milá et al. 2012; Campagna et al. 2012*b*; Nishiumi and Kim 2015). DNA barcodes were also employed to study mechanisms and patterns of mitochondrial gene evolution (Kerr 2010, 2011; Stoeckle and Kerr 2012; Stoeckle and Thaler 2014; Lavinia et al. 2016), among many other applications. This mini-review highlights a rich literature employing DNA barcodes for evolutionary study and outlines prospects for further discovery.

Study cases where DNA barcodes do not match taxonomy

Low interspecific divergence and lack of reciprocal monophyly among species

As mentioned above, DNA barcodes are capable in most cases of separating bird species. However, there are exceptions, mainly when pairs or groups of species do not show reciprocal monophyly because of their low genetic distance and (or) the presence of hybridization (Table 1). The study of these species that show consider-

able morphological and (or) behavioural differences but lack genetic differentiation in their DNA barcodes allows a better understanding of the evolutionary processes and the ultimate causes of diversification and speciation. In particular, the study of these species provides insights into the role of phenotypic and behavioural traits in the evolution of reproductive isolation between recently diverged lineages.

One of the most striking cases of low interspecific divergence and lack of reciprocal monophyly in DNA barcodes among Neotropical birds is that of the southern capuchino seedeaters, a clade of eight species of *Sporophila* that are mostly sympatric (Kerr et al. 2009*a*; Campagna et al. 2010). This group shows remarkably high plumage colour divergence among males, which also produce distinctive vocalizations, suggesting that species-level designations to them are correct (Campagna et al. 2012*a*). There is also evidence that males from these species react more strongly to male conspecific vocalizations than to hetero-specific ones, indicating that the interspecific differences in vocalizations are in fact detected by the birds, supporting the notion that these are different species (Benites et al. 2015). The first DNA barcode study to include six of these species (among others from Argentina) showed that this was one of the very few cases with low or null interspecific differentiation in COI. Sequences in fact clustered together in the neighbour-joining tree and showed no diagnostic characters that could separate species (Kerr et al. 2009*a*). This was consistent with a previous study of the group with shorter mitochondrial sequences (not including COI) that had shown for the first time the complexity of this group (Lijtmaer et al. 2004). Later on, Campagna et al. (2010) increased the sampling effort to include the DNA barcodes of all species from the clade and two sister species and used more samples per species. The analysis of this enlarged DNA barcode dataset with various phylogenetic reconstruction methods still could not distinguish among species of southern capuchinos (Campagna et al. 2010).

A more extensive analysis that included more mitochondrial markers, microsatellites, and nuclear gene sequences showed that this group of species constitutes a recent radiation with incomplete lineage sorting and hybridization still occurring among these species despite the large morphological and behavioural differentiation (Campagna et al. 2012*a*, 2013). More recently, Campagna et al. (2015) used a reduced-representation genomic approach to examine a much larger number of loci and still could not find genetic markers significantly differentiated among species of southern capuchinos. All these deeper studies, with complete species sampling and large genetic databases, confirm that this group of species represents an exceptional case of continental recent and rapid radiation. The differences in plumage colour and song among these species suggest that sexual selection is probably driving their sympatric divergence and that selection-mediated differences in these characters can evolve much faster than differences in neutral genetic markers.

Another well-known example of a species group in which DNA barcodes are shared interspecifically is the herring gull complex (*Larus* spp., Hebert et al. 2004; Kerr et al. 2009*b*; Johnsen et al. 2010; Kwon et al. 2012; Aliabadian et al. 2013). This group of species, composed of 21 taxa distributed throughout the Holarctic region, are posed as a classic example of a ring species (a chain of contiguous and interbreeding populations whose distribution bends and overlaps at its terminal ends, where local populations do not interbreed, Newton 2003). The herring gull complex populations extend throughout a circumpolar breeding range, showing differences in body size and plumage colouration (Liebers et al. 2004). However, according to the findings of Liebers et al. (2004), who studied genetic variation in the hypervariable part of the control region (HVR-1) and cytochrome *b* (cyt *b*), this species group does not fit completely the ring species model because the distributions of the interbreeding lineages (as determined genetically) do not come in contact at their endpoints. Currently sympatric non-interbreeding lineages would have evolved in allopatry and not as the result of isolation by distance as the ring species model proposes (Liebers et al. 2004). The herring gull complex shows signs of past hybridization and introgression that resulted in shared mitochondrial haplotypes (Liebers et al. 2004), including the DNA barcode region.

There are other cases in which DNA barcodes were not able to separate species that are known to be part of recent radiations, but with fewer species involved. One such case is that of the crossbills (*Loxia curvirostra* and *L. pytyopsittacus*, Johnsen et al. 2010), which experienced a recent ecological speciation process with assortative mating among members within clades in relation to their bill size and calls, but with no differentiation in mitochondrial markers and microsatellites and occasional hybridization (Piertney et al. 2001; Parchman et al. 2006; Summers et al. 2007; Edelaar 2008).

DNA barcoding is expected to fail at distinguishing species that hybridize or that were recently diverged since maternally inherited genes, such as COI, will be shared between them (Hebert et al. 2004). These cases are infrequent among birds, and therefore DNA barcodes are in general a powerful tool to identify avian specimens to species. Interestingly, the studies reviewed here show that even when much more molecular information is analyzed (including fast-evolving and nuclear markers), these species continue to be hard to distinguish genetically despite the fact that they do show considerable phenotypic and behavioural differences. The deeper examination of these species sheds light on the processes of speciation as the reviewed studies suggest that sexual

selection and ecological specialization can lead to rapid phenotypic and behavioural differentiation and assortative mating, without significant genetic divergence and lack of reciprocal monophyly. Also, the deeper scrutiny of these species contribute to locating hybrid zones, identifying mechanisms of reproductive isolation and the bases of phenotypic differentiation, and understanding the evolution of genetic divergence in relation to geographic distribution.

High intraspecific divergence

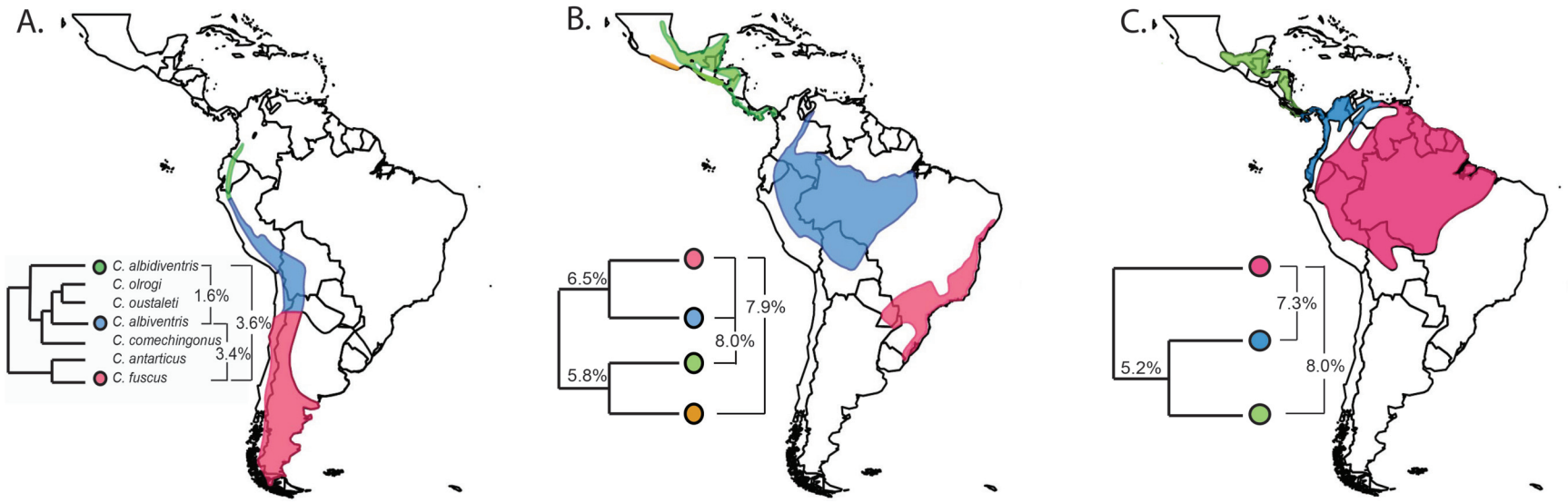
Despite the well-known taxonomy of birds, all DNA barcode studies conducted to date have found species that showed deeply diverging lineages within them (Table 1). These taxa are thus flagged as candidate cryptic species (i.e., more than one evolutionarily independent lineage with no evident phenotypic differentiation among them). Species can be defined as metapopulation lineages that evolve independently (unified species concept), in which characteristics such as reciprocal monophyly, morphological diagnosability, reproductive isolation, etc., are contingent properties that species may or not acquire during their existence (de Queiroz 2005). These properties, although not indispensable to assign species status, can be employed as evidence supporting taxonomic re-evaluations (de Queiroz 2005). In that sense, determining if highly divergent lineages are in fact cryptic species and the assessment of their geographic boundaries requires a comprehensive sampling across the whole species distribution. To gather further evidence supporting taxonomic splits, it is desirable to evaluate phylogenetic relationships between the observed clades with additional molecular markers, as well as to assess the occurrence of variation in phenotypic traits such as song, morphology, and plumage colouration (which can be indicators of potential reproductive isolation between genetic lineages as these characters are often involved in avian sexual interactions).

Studies of DNA barcodes of Neotropical birds have found several species with large intraspecific genetic splits, showing complex geographic patterns (Table 1). There are many different areas of occurrence of these splits and varying levels of intraspecific genetic divergence and geographic patterns among species occupying similar distributional ranges (Kerr et al. 2009*a*; Tavares et al. 2011; Milá et al. 2012). Many of the highly divergent species are polytypic, with several taxonomically recognized subspecies, but the number and geographic distribution of the genetic lineages discovered in COI are not always congruent with those of the subspecies, suggesting that observable phenotypic differentiation and genetic divergence in neutral markers might occur at different rates.

Next, we review three case studies of cryptic divergence within Neotropical passerines of wide distribution that were studied in depth after DNA barcodes showed large differentiation. These data sources only partially agree with subspecies delimitations based on traditional taxonomic classifications, but genetic lineages within these species are often accompanied by consistent differentiation in phenotypic characters. These studies help in taxonomic revisions and also illustrate different geographic patterns of genetic diversification within Neotropical avian species.

1. Bar-winged cinclodes (*Cinclodes fuscus*, Figure 2A): In the project to barcode the birds of Argentina, two allopatric genetic clusters were identified in the COI sequences of this widespread Andean–Patagonian species, with an average genetic distance between them of 4.35% (range from 4.21% to 4.68%, Kerr et al. 2009*a*). Sanin et al. (2009) employed additional mitochondrial markers (COII and ND3) and studied the species throughout its geographic distribution, including several samples from the five most widespread subspecies of this polytypic species. The study led to the discovery of three lineages within *Cinclodes fuscus* (two of which included more than one subspecies) that are actually more closely related to other species of *Cinclodes* than to conspecifics. As a result of the phylogeographic analyses based on these mitochondrial markers, *C. fuscus* as previously considered was found to be paraphyletic (Sanín et al. 2009), which was in agreement with previous observations made on the geographic variation in morphology and behaviour in this species (Jaramillo 2003), and it was therefore split. Now three different species are considered: the buff-winged cinclodes (*C. fuscus*) in southern South America, the cream-winged cinclodes (*C. albiventris*) in the central Andes, and the chestnut-winged cinclodes (*C. albidiventris*) in the northern Andes (Sanin et al. 2009, Remsen et al. 2016).
2. Red-crowned ant tanager (*Habia rubica*, Figure 2B): When the Argentine dataset was combined with that of Bolivia, resulting in a wider geographic coverage in the Southern Cone of South America, a large set of widely distributed Neotropical species showing large splits in their DNA barcode sequences was found (Lavinia et al. 2015*b*). One of the most striking cases was that of the red-crowned ant tanager that showed around 7% of average genetic divergence between the samples from the Atlantic forest in Argentina and the Yungas of Bolivia (Lavinia et al. 2015*b*). A parallel DNA barcode analysis of the birds of Mexico and Guatemala also found deep genetic divergence within this species in its northern area of distribution, which led to a large collaborative project between researchers from different countries (Lavinia et al. 2015*b*). Lavinia et al. (2015*a*) performed a large-scale phylogeographic analysis of red-crowned ant tanagers using both mitochondrial and nuclear genetic markers together with phenotypic characters (song and plumage colouration). Apart from shedding light on the evolutionary history of this taxon, the results showed that it

Fig. 2. Distribution maps for Neotropical passerine species (based on BirdLife International and NatureServe 2015) with large genetic splits in their DNA barcodes, and the geographic distribution of each genetic lineage detected among these illustrated in different colours. Also, a schematic representation of the phylogenetic relationship and the average percentage of mitochondrial gene divergence between the observed genetic lineages are shown. (A) *Cinclodes* spp. (that were previously considered as *Cinclodes fuscus*); genetic distances correspond to concatenated COII and ND3 sequences reported by Sanin et al. 2009. (B) *Habia rubica*; percentages of genetic divergence between lineages correspond to the concatenated sequences of COI and cyt *b* genes reported by Lavinia et al. 2015*a*. (C) *Cyanocompsa cyanoides*; genetic divergence between lineages correspond to COI sequences reported by García et al. 2016. The maps were constructed employing the "maptools" package (Bivand and Lewin-Koh 2016) for R 3.3.0 (R Core Team 2016).



includes four divergent phylogroups, three of which also show phenotypic divergence that is concordant with this division, suggesting that the red-crowned ant tanager is currently comprised of at least three different species (Lavinia et al. 2015*a*).

3. Blue-black grosbeak (*Cyanocompsa cyanoides*, Figure 2C): This species belongs to the family Cardinalidae, and it is distributed from southern Mexico south to northwestern Brazil. García et al. (2016) observed deep DNA barcode divergence (up to 7.98%) clustered in three subgroups within this species. Large genetic divergence within this species was also present in other mitochondrial genes (cyt *b* and ND2; García et al. 2016 and Bryson et al. 2014, respectively). Furthermore, the objective and quantitative analysis of variation in body size, plumage colour, and vocalizations across the species distribution showed that even though there are differences in these characters among the four recognized subspecies of blue-black grosbeak, these differences are not completely congruent with genetic patterns (García et al. 2016). Only the subspecies *C. c. rothschildii*, distributed east of the Andes Cordillera in South America, showed congruent and large differences in all of the studied characters with respect to the other subspecies, and the genetic and morphological patterns observed suggest that this subspecies could be elevated to species status (Bryson et al. 2014, García et al. 2016).

DNA barcode studies also detected species with large intraspecific divergence in the Northern Hemisphere (Table 1). For instance, Saitoh et al. (2015) DNA barcoded 234 species within the Japanese Archipelago and found 11 candidate cryptic species with two or more supported genetic clusters. Further on, Saitoh et al. (2015) compared COI sequences from Japan with sequences from Eurasia, and 19 species were found to have large genetic splits. Johnsen et al. (2010) studied COI variation in 296 Scandinavian bird species and found large genetic splits in four species within Scandinavia and 19 species of trans-Atlantic distribution (see further details below).

One case of large intraspecific divergence in COI that was further studied among Northern Hemisphere species is that of the northern fulmar (*Fulmarius glacialis*), which was found to possess a large genetic distance in its DNA barcodes between its allopatric populations from the Atlantic and Pacific Oceans (maximum divergence of 3.2%; Kerr et al. 2007; Johnsen et al. 2010). However, these studies only included one specimen from the Pacific population, and this species possesses highly variable plumage colouration, with dark, light, and intermediate morphs present in both populations with varying frequencies and distributions, which confused their taxonomic treatment (Kerr and Dove 2013). To determine if the genetic divergence between these allopatric populations extends to other loci, Kerr and Dove (2013) studied nuclear and mitochondrial markers, including the melanocortin-1 receptor gene (*MCR1*) that has been associated with melanin colour variation in the plumage (Mundy 2005), with an extensive sampling of both populations. They found deep divergence in all mitochondrial markers but little differentiation in nuclear markers and shared nuclear sequences between the Atlantic and Pacific populations, suggesting an early-stage divergence. The *MCR1* gene showed many variable sites, but its genetic diversity seemed to be more associated with geographic distance than with plumage morph (Kerr and Dove 2013). Based on these results, the authors recommended that the Atlantic and Pacific populations of northern fulmar be considered separate species (Kerr and Dove 2013).

Efe et al. (2009) studied the genetic divergence among subspecies of Sandwich terns (*Thalasseus sandvicensis*) that breed in the Mediterranean and Atlantic coasts of Europe as well as the Atlantic coasts of southern North America south to the Argentine Patagonia. Through the analysis of DNA barcodes, together with two other mitochondrial genes and two nuclear markers, the authors determined that the New World and Old World populations were highly divergent and paraphyletic, as the American clade was recovered as sister to *T. elegans* (Efe et al. 2009). The authors proposed that these two Sandwich tern lineages should be granted species status, but this proposal was not accepted by the taxonomic authorities due to limited sampling (Remsen et al. 2016).

There are still many species where DNA barcodes highlighted profound genetic splits that are in need of deeper scrutiny. This is particularly true for the Neotropics, where these splits occur more often and with more complex geographic patterns compared to the Nearctic and Palearctic (Lijtmaer et al. 2011; Tavares et al. 2011; Milá et al. 2012). The results obtained in those cases that have been analyzed in depth indicate that it is very likely that COI divergence is accompanied by differentiation in other genetic loci and in behavioural and phenotypic traits, making these species a promising research subject for ornithology and evolutionary biology.

General patterns of genetic variation investigated through DNA barcodes

Various studies have taken advantage of the many DNA barcode datasets already obtained for birds across large geographic areas, either separately or combined, to investigate general patterns of genetic diversification and their underlying causes. Next, we summarize their diverse research topics and their main findings.

Mitochondrial gene evolution

The large COI database allows the comparison of sequences across many taxa to study patterns of genetic diversity related to molecular evolutionary forces. In this section, we review studies that have used COI public sequences to evaluate causes of genetic variation such as

molecular evolutionary rate, selective sweeps, and pseudogene occurrence in DNA barcodes.

The cyt *b* gene has been by far the mitochondrial locus most used for molecular clock calibrations in birds (e.g., Shields and Wilson 1987; Paxinos et al. 2002; Weir and Schluter 2008), and its rate has been used as a universal measure for mitochondrial genes in general. However, there is evidence indicating that the evolutionary rate is not constant for different mitochondrial loci and even varies across taxa (e.g., Lovette 2004; Pereira and Baker 2006; Patané et al. 2009; Eo and DeWoody 2010; Pacheco et al. 2011). In particular, some of the studies showing the difference among loci used DNA barcodes. For instance, Lijtmaer et al. (2011) estimated an evolutionary rate for COI 35% slower than that of cyt *b* based on the comparison of sequences from both genes from 51 pairs of sister species, which is similar to what was obtained previously through intrageneric comparisons (Aliabadian et al. 2009). Kerr (2011), as part of a broader analysis of COI evolution, compared whole mitochondrial genomes from 22 avian species and showed that COI is the least variable mitochondrial gene, implying that its evolutionary rate is the slowest of the protein-coding mitochondrial loci (a result consistent with that of Pacheco et al. 2011). In a more comprehensive study, Lavinia et al. (2016) used DNA barcodes to compare the evolutionary rate of COI in relation to that of cyt *b* across 556 species from 10 different avian orders, which allowed them to estimate the genetic differentiation for both genes between 278 phylogenetically independent pairs of species. Their results showed that COI evolves on average 14% slower than cyt *b*, but indicated that this number is very variable among different orders of birds, ranging between 2% and 49% slower. In addition, cyt *b* evolved faster than COI in very closely related species, but the rates became similar as the divergence between species increased (Lavinia et al. 2016). This could be related to the presence of a lower functional constraint in cyt *b* that allows it to initially accumulate mutations more rapidly than COI, although this hypothesis still needs to be formally evaluated.

Selective sweeps occur when positive selection acting on one site of a gene sequence causes the loss of variation in this gene and in other linked sites and have been proposed as a common mechanism that could affect mitochondrial sequence diversity. Kerr (2011) used the large COI sequence library of birds to explore the occurrence of selective sweeps by analyzing the relationship between its intra- and interspecific variation. Selective sweeps have been discussed as a potential mechanism that could explain the pattern of low sequence variation within species irrespective of species age that was found within North American birds in COI (Hebert et al. 2004; Kerr et al. 2007). The results did not show evidence supporting selective sweeps acting on DNA barcodes divergence; in birds, the COI gene seems to evolve mainly due to purifying selection (negative selection), while variation between closely related species would occur as a result of drift (Kerr 2011). In accordance with these findings, Stoeckle and Thaler (2014) tested the predictions of the neutral theory of molecular evolution by comparing the intraspecific COI variation in relation to population size in 142 species from 15 different avian families. Intraspecific variation in COI was low, independent of population size (Stoeckle and Thaler 2014). The authors proposed that other forces, such as extreme purifying selection (including at synonymous sites) and continuous adaptive evolution, act more intensively on mitochondrial gene evolution than neutral processes (Stoeckle and Thaler 2014). Furthermore, Hill (2016) hypothesized that the COI gene, and other linked mitochondrial genes, play a key role in the speciation process, which in turn, would explain the fact that COI variation is low within species and high between them. According to this hypothesis, COI co-evolves with nuclear genes that act in cellular respiration and thus mutations in mitochondrial genes generate barriers to gene flow by establishing mito-nuclear incompatibilities.

Nuclear copies of the mitochondrial genes, known as pseudogenes or numts, are problematic for the efficacy of DNA barcodes in identifying species because these are non-coding sequences that accumulate random mutations, and they can be occasionally sequenced instead of the true COI sequence (Kerr 2010; Stoeckle and Kerr 2012). Most pseudogenes possess STOP codon mutations and (or) indels and can therefore be easily identified and excluded from the database. However, some cryptic pseudogenes lack this characteristic and show small genetic divergence with respect to the actual COI sequence (Stoeckle and Kerr 2012). These are particularly problematic to DNA barcoding in bird families where general primers show less affinity for the mitochondrial copy of the gene, such as Tyrannidae, or when amplification is made from blood samples that contain a higher number of nuclear DNA copies relative to mitochondrial ones (Kerr 2010). Because pseudogenes are inserted in the nuclear DNA, they possess a lower evolutionary rate than the mitochondrial COI gene (COI nucleotide substitution rate was found to be 2–14 times greater than that of numts, and mean intraspecific sequence divergence between the true mitochondrial COI and numt sequences was 14.2%, Kerr 2010). However, as they accumulate random mutations it is more common to find second base mutations and non-synonymous changes in pseudogenes (Kerr 2010). Stoeckle and Kerr (2012) employed a frequency matrix analysis to identify rare variants over a database of more than 11 333 DNA barcode sequences from 2706 species of birds, which allowed the identification of cryptic pseudogenes (representing 0.1% of the database) due to presence of multiple very low frequency non-synonymous substitutions. These sequences involved four species (*Nothoprocta ornata*, *Empidonax alnorum*, *Cnemotriccus fuscatus*, and *Branta canadiensis*) and in some

cases, when additional sequences of these species were available, resulted in high intraspecific divergence in DNA barcodes (6%–12%, even among individuals collected in the same locality, Stoeckle and Kerr 2012). Therefore, the sequencing of COI pseudogenes, although infrequent, can mislead to the presumption of cryptic divergence within species and the splitting of conspecifics into more than one BIN. However, some clues can suggest the amplification and sequencing of numts such as the presence of various DNA barcode haplotypes in samples from a single locality, the presence of a genetic cluster grouping two or more species with low divergence among them (usually in addition to other clusters of each of these same species, which are formed with the true, mitochondrial sequences), the observation of multiple peaks in the trace files, and the use of blood samples as DNA source (Kerr 2010; Stoeckle and Kerr 2012). Also, the frequency matrix approach may be useful to flag spurious sequences in large DNA barcode databases (Stoeckle and Kerr 2012).

Taxonomic-wide biogeographic studies

Bird species that inhabit islands are always an attractive subject for researchers studying biogeography and evolution. These species are often reproductively isolated from populations living in the continent, especially in more remote islands, and diversifying factors act more intensively there, varying according to the patterns of colonization and extinction of each species (Newton 2003). Several studies have focused on island species and (or) populations to study their biogeographic patterns through DNA barcodes (Lohman et al. 2010; Campagna et al. 2012*b*; Nishiumi and Kim 2015). In contrast, continental species are more likely to show similar patterns in their genetic structure, reflecting a common geological history (Newton 2003). Many studies focused on continental species of different realms to study general biogeographic patterns of COI variation as a means to determine the diversifying factors that affected them (Johnsen et al. 2010; Lijtmaer et al. 2011; Tavares et al. 2011; Milá et al. 2012; Chaves et al. 2015). We review these studies in the next paragraphs.

Lohman et al. (2010) evaluated the levels of genetic divergence in DNA barcodes of seven widespread species of birds from South East Asia to determine the degree of genetic isolation of their Philippine populations. Their results showed that in all cases populations from the Philippines showed differentiation with respect to their counterparts from the continent, with various phylogenetic patterns reflecting different colonization histories. Six of these seven species could in fact be separate species endemic to the Philippines, which needs confirmation with further molecular, morphological, and behavioural evidence (Lohman et al. 2010). Similarly, Campagna et al. (2012) studied the genetic structure of nine passerine species that regularly breed in the Malvinas – Falkland Islands in the South Atlantic through their DNA barcodes and other additional mitochondrial and nuclear markers. Three of the studied species showed moderate genetic divergence with respect to their continental conspecific populations, and a fourth population (*Troglodytes aedon cobbi*) showed considerable genetic (2.2% average divergence in COI sequence) and behavioural differentiation with respect to populations of *T. aedon* from the continent (Campagna et al. 2012*b*). Consequently, this population was given species status by the South American Classification Committee (*T. cobbi*, Remsen et al. 2016) and constitutes a new endemic species for the Malvinas – Falkland Islands (Campagna et al. 2012*b*).

Usually, islands are thought to be biodiversity sinks, with species inhabiting there having migrated from the continent. Nishiumi and Kim (2015) mined DNA barcode sequences of species from the Holarctic from BOLD and constructed neighbour-joining phylogenetic trees to identify cases of reverse colonization, i.e., from Japanese islands to mainland Asia, by observing the resulting topology in relation to the geographic origin of the samples. This analysis allowed them to identify, within 118 studied species, five species as strong potential candidates to be reverse colonizers, while 39 species breeding in Japanese islands had a genetic structure consistent with a continental origin. The study provided insight into the history of Japan's avifauna and its colonization patterns, and highlighted species in need of further examination (with more genetic markers, a broader sampling, the use of more sound phylogenetic reconstruction methods, such as maximum likelihood and Bayesian analysis, and the estimation of divergence time between populations) to confirm the reverse colonization hypothesis.

DNA barcode libraries have also been employed to study continental birds and their biogeographic history. Tavares et al. (2011) combined DNA barcodes obtained for the birds of Brazil with those of Argentina and studied the genetic structure of Neotropical species of birds at a large scale. They found that, although more than 75% of the studied species have low intraspecific divergence in COI (i.e., less than 1% maximum pairwise divergence), the number of species of this realm with large genetic splits is larger than in North American birds. This is likely a consequence of the higher effect of glacial cycles isolating populations in North America and generating speciation (Lijtmaer et al. 2011; more details below). In addition, Neotropical species show complex biogeographic patterns with a large range of intraspecific genetic distances in species of the same areas, which could reflect the occurrence of multiple diversifying events at different geological times in this region, multiple dispersal events after a large isolation process, or considerable variation in the rate of evolution among species (Tavares et al. 2011). In the lower latitudes of the Neotropics, Milá et al. (2012) have studied genetic diversification between eastern and western Amazonian populations of tropical for-

est species and also compared these with trans-Andean populations. They found large levels of intraspecific divergence among these geographic areas in most cases. Genetic divergence between these populations was not proportional to phenotypic variation in plumage colouration, with some species with large genetic divergence having low colour differentiation between populations and vice versa (Milá et al. 2012). These results reinforce the notion that the tropics could possess more species than those recognized by current taxonomy based on phenotypic traits, and also that Neotropical species possess higher genetic structure in comparison to species from temperate zones (Milá et al. 2012). Concordantly, Chaves et al. (2015) studied the phylogenetic relationship within species of the Brazilian Cerrado and the Atlantic Forest through Bayesian tree reconstructions of their DNA barcode sequences and found that 10.4% of them are non-monophyletic. Overall, these large-scale studies suggest that the number of Neotropical species could be largely underestimated and that an increase in the geographic and taxonomic coverage of DNA barcode surveys will facilitate the identification of cryptic species and diversifying factors acting in this geographic region.

The extensive coverage of DNA barcode datasets of birds has also been used to study species divergence across large geographic barriers to gene flow at a multicontinental scale. Johnsen et al. (2010) evaluated the levels of divergence in 78 Holarctic species between their Palearctic and Nearctic populations. Nineteen of these species showed two highly different genetic clusters that corresponded to the populations on either side of the Atlantic Ocean, while three of these 19 species showed paraphyletic patterns (Johnsen et al. 2010). Most of these species have discontinuous geographic distributions and are inland breeders (Johnsen et al. 2010). Lijtmaer et al. (2011) also compared COI sequence variation at a multicontinental scale; by merging DNA barcode datasets from birds of Argentina (a representative avifauna of the Southern Neotropics), the Nearctic, and the Palearctic they estimated species ages for these realms to compare the patterns of avian diversification among regions. Average nearest congeneric neighbour and sister species distances in DNA barcodes were significantly higher for species from Argentina in comparison with those from the Nearctic, indicating that the higher avian diversity of the Neotropics would be a consequence of lower extinction rates rather than higher recent speciation rates (Lijtmaer et al. 2011). Overall, the results suggested that Pleistocene glaciations had a greater effect on shaping the genetic structure of species in the Nearctic than in either of the other two biogeographic areas studied.

Final remarks

Even though DNA barcodes were conceived as a tool for the genetic identification of specimens to species (Hebert et al. 2003), it is certain that the large sequence library generated by the project provides high-quality data to answer many biological questions. Here, we have reviewed a series of research studies that, with very different scopes, made use of DNA barcodes to investigate evolutionary processes acting on avian species.

The COI sequence library deposited in BOLD possesses many key aspects that allow these many applications. (*i*) Although some geographic areas are better represented than others, the avian DNA barcode library has a very large geographic coverage. In the future, as DNA barcoding projects of birds continue to advance, especially in the most biodiverse areas of the planet, the species representation in the library will be more balanced between different avian realms and taxonomically broader. (*ii*) It has an unbiased taxonomic representation; i.e., different taxonomic groups are more or less equally present in the database (Fig. 1), since most sampling is not associated with specific taxonomic studies. Rather, many barcodes are obtained through regional-scale campaigns that aim to be as taxonomically comprehensive as possible. (*iii*) The DNA barcode sequences have very high quality standards such as low number of ambiguities, minimum sequence length, bidirectional sequencing, standardized sequence alignment, etc. And, (*iv*) each sequence in the library is linked to a specimen voucher deposited in a curated collection which, besides having a large amount of specimen data associated (such as voucher type, collection locality, sex, stage, photographs, and up-to-date taxonomy), allows the verification of the taxonomy in case it is needed. In addition, the data are permanently curated (although errors may last in the database for a while until proper action is taken), and therefore even though errors do exist on BOLD their frequency is much lower than in other sequence databases (such as GenBank).

The general patterns of diversification unveiled by the large-scale analyses allow the identification of geographic areas in need of further scrutiny. The deeper study of genetic divergence (with additional molecular markers), accompanied by phenotypic data, in species that show large splits in their COI gene aids the revision of taxonomy. Genomic approaches have grown considerably in the past years (Toews et al. 2016), and their application to study cases where DNA barcodes fail to distinguish species will help understand the underlying evolutionary causes (Campagna et al. 2015). The DNA barcodes of birds have provided, and will undoubtedly continue to provide, the opportunity to address questions about avian evolution well beyond its primary purpose of identifying species. These evolutionary studies in turn provide helpful information to design future avian DNA barcoding projects and improve the efficacy of DNA barcodes to identify species genetically.

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