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Congruence of phenotypic and genetic variation at the subspecific level in a Neotropical passerine

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The increasing availability of molecular phylogenies has highlighted the issue that genotypic and phenotypic patterns of variation are not always congruent, particularly below the species level. This has led to an ongoing discussion on the validity of the subspecies category and on the use of molecular data to help revise traditional classifications based on phenotypic data. We compared patterns of spatial diversification in genotype and phenotype in the Blue-black Grosbeak *Cyanocompsa cyanoides*, a Neotropical songbird with four recognized subspecies. Variation in phenotype and genotype are partially congruent among the four subspecies. The more genetically divergent subspecies C. *c. rothschildii* is strongly differentiated from the other subspecies in morphological characters, plumage coloration and song. We suggest that this taxon be accorded full species status as *Cyanocompsa rothschildii*. Regarding the remaining diversity within C. *cyanoides*, both phenotypic and genetic markers suggest that it could be divided into two subgroups, but the boundaries of genetic lineages do not coincide with those of subspecies defined using phenotypic data. Lack of complete congruence between phenotypic and molecular markers may be expected, as they are subject to different evolutionary processes. The discordance could also be due to a methodological problem, as subspecies' geographical boundaries were defined on the basis of phenotypic descriptions that were not systematically evaluated. We consider the subspecies to be an informative taxonomic entity, but note that many current subspecific designations for diverse species need extensive reassessment.

Keywords: body size, mitochondrial DNA markers, plumage coloration, song, subspecies.

The use of genetic markers to assess biological diversity has revealed the existence of some discordance between traditional approaches to taxonomic delineation of taxa based on phenotype and that derived from molecular data, particularly at the subspecific level (Winker 2009). For several reasons (James 2010) the subspecies concept continues to receive criticism, mainly from authors who advocate the phylogenetic species concept (Haig & Winker 2010). In the case of avian diversity, a meta-analysis (Zink 2004) of the congruence between taxonomically defined subspecies from North America and molecular phylogenetic results that include these taxa concluded that only 3% of 'traditional' subspecies (i.e. those based entirely on phenotype) were phylogenetically distinct (but see Phillimore & Owens 2006). However, that result is based solely on the analysis of genetic markers and there is no consensus on what degree of genetic differentiation subspecies should show (Patten 2010). Subspecies are defined as populations of a species that differ in certain characters but are not reproductively isolated (Mayr 1982). As genes that are assumed to evolve in a nearly neutral fashion need a long period of little or no gene flow to drift and sort (Hudson & Coyne 2002), reciprocal monophyly of subspecies should not be necessary for their delimitation (Patten 2010). Furthermore, as populations may diverge in the face of gene flow under selection (Nosil 2008), lack of complete congruence between phenotype and molecular markers is expected when reproductive isolation is incomplete (Winker 2009, Patten 2010).

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In addition, there is an important geographical bias in the study of avian diversification, as some biogeographical realms, such as the Neotropics, are generally under-represented in studies of genetic diversification (Phillimore & Owens 2006, Beheregaray 2008, Reddy 2014). This could lead to biased ideas on patterns of avian diversification, as Neotropical species have been shown to have generally higher levels of phylogeographical structure than Nearctic or Palaearctic species (Lovette 2005, Lijtmaer *et al.* 2011). There are several cases of Neotropical bird species in which the number of subspecies and the number of genetic clades are similar, although their geographical boundaries do not necessarily match. Examples include the Lesser Woodcreeper *Xiphorhynchus fuscus* (Cabanne *et al.* 2008), the Ochre-bellied Flycatcher *Mionectes oleagineus* (Miller *et al.* 2008) and the Rufous-tailed Hummingbird *Amazilia tzacatl* (Miller *et al.* 2011). These incongruities can arise through biological processes, but they could be the result of methodological artefacts as well. Most subspecies were described before the simplest statistical analyses, such as the *t*-test, were developed (Remsen 2005), and therefore a modern re-analysis would probably demonstrate that a significant proportion of the subspecies delineated are in need of revision. All of this suggests that the subspecies category is not useless, but that current classification needs reassessment through the incorporation of new molecular information and also a re-examination of phenotypic variation.

We use a Neotropical passerine, the Blue-black Grosbeak *Cyanocompsa cyanoides*, as a model to study the congruence of spatial variation between phenotype and genotype. The Blue-black Grosbeak is distributed from southern Mexico to Brazil, with a continent-wide distribution that is interrupted by the Andes (Orenstein & Brewer 2011, Fig. 1a). The Blue-black Grosbeak is currently classified into four subspecies based mainly on size and plumage colour variation (Todd 1923), but differences among these subspecies are subtle (Orenstein & Brewer 2011), at least to the human eye. Genetic evidence suggests the existence of three reciprocally monophyletic lineages within the species (Bryson *et al.* 2013). The only taxon that occurs east of the Andes, the subspecies *C. c. rothschildii*, has been evolving independently for more than 3 Myr. It has been suggested that it should be accorded full species status (Bryson *et al.* 2013), but no detailed phenotypic analysis has been undertaken to support such a proposal. We complement previous findings with new genetic and phenotypic analyses to assess whether the patterns of phenotypic variation among subspecies are congruent with those of genetic differentiation. We analyse two mitochondrial markers as well as morphological characters (body weight, wing length, beak size) and plumage coloration. We also analyse song, a behavioural character that is learned in Oscines and therefore could be considered to be more plastic than body size or coloration. Lastly, we evaluate whether phenotypic variation supports the proposition of Bryson *et al.* (2013) that *C. c. rothschildii* should be treated as a distinct species.

METHODS

Model species

The Blue-black Grosbeak is about 17–18.5 cm in length (Orenstein & Brewer 2011) and forms part of the 'blue clade' of the Cardinalidae (Klicka *et al.* 2007). Females are brown, whereas the male plumage is mostly dark blue, with some patches that appear light-blue to the human eye. The Blue-black Grosbeak has four subspecies: *C. cyanoides concreta* and *C. c. toddi* are exclusive to Mexico and Central America; *C. c. cyanoides* is found from eastern Panama to northern South America west of the Andes; and *C. c. rothschildii* is confined to South America east of the Andes (Orenstein & Brewer 2011, Fig. 1a). The male of *C. c. cyanoides* is described as rich deep blue above with rump paler blue; also the forehead is slightly brighter blue than the crown and with a slightly brighter blue shoulder patch (Orenstein & Brewer 2011). The female is uniformly dull chocolate-brown above and below. The male of *C. c. toddi* is darker blue and larger than the nominate subspecies, and *C. c. concreta* is similar to *C. c. toddi*, but larger and even darker, more bluish-black. Finally, *C. c. rothschildii* males are much brighter than the others, with bright sky-blue on the forehead and shoulder and females are paler brown (Orenstein & Brewer 2011).

Molecular DNA analyses

Genomic DNA was obtained from muscle and blood samples using a glass fibre-based extraction protocol developed by Ivanova *et al.* (2006).

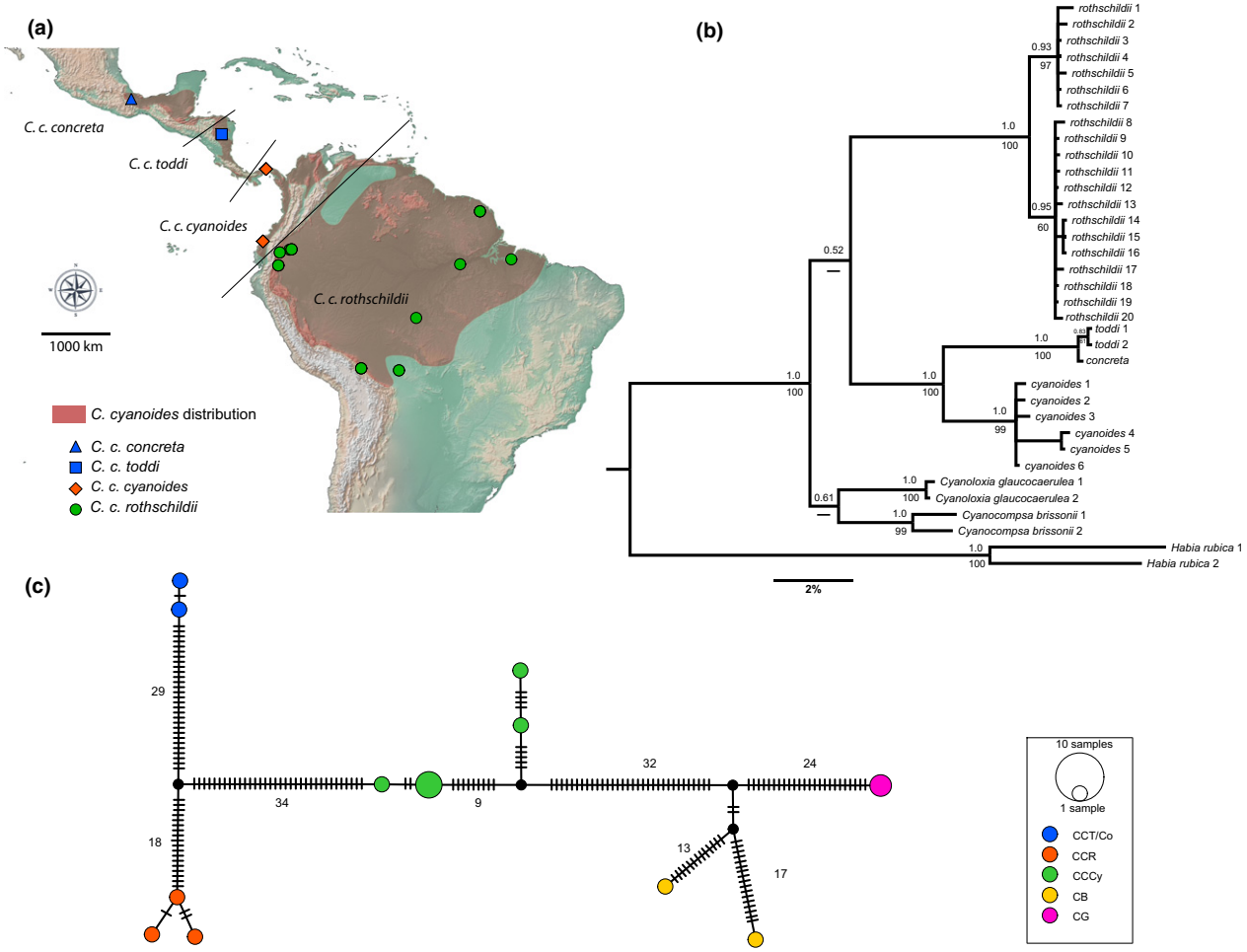

Figure 1. (a) Map showing the distribution of *Cyanocompsa cyanoides* and its currently recognized subspecies, as well as the geographical provenance of tissue samples analysed. The range distribution was based on BirdLife International and NatureServe (2014). (b) Strict consensus tree obtained from the analysis of 814 bp of the COI dataset, showing the three major clades recovered within *C. cyanoides* and the relationships between this species and the other closely related cardinalids sampled in this study. For *C. cyanoides*, samples were coded according to the subspecies they were assigned to. Numbers above and below branches correspond to Bayesian posterior probability and maximum parsimony bootstrap node support values, respectively. (c) Unrooted statistical parsimony network showing the relationships among cyt-*b* haplotypes. CCT/Co = *Cyanocompsa cyanoides toddi/C. c. concreta*. CCCY = *C. c. cyanoides*. CCR = *C. c. rothschildii*. CB = *C. brissonii*. CG = *Cyanoloxia glaucocaerulea*.

Samples were either collected by the Ornithology Division of the Museo Argentino de Ciencias Naturales 'Bernardino Rivadavia' (MACN), or loaned by several institutions (Supporting Information Table S1). We sequenced two mitochondrial genes: cytochrome-*c* oxidase subunit I (COI, 694 bp) and cytochrome-*b* (cyt-*b*, 1031 bp). For thermocycling conditions and primers we followed Arrieta *et al.* (2013) and Lijtmaer *et al.* (2012) for cyt-*b* and COI, respectively. Sequences were edited and aligned using CodonCode Aligner 4.0.4 (CondonCode Corporation, Dedham, MA, USA). As these are protein-coding genes, we visually examined the sequences to ensure the absence of indels and stop codons within the reading frame. Additionally, we mined from GenBank all publicly available COI sequences of C. *cyanoides* previously generated by Tavares *et al.* (2011) and Milá *et al.* (2012). GenBank accession numbers for all sequences used here (both generated in this study and previously available) are provided in Table S1.

Phylogenetic analyses were based on the COI dataset because of its greater sample size (a total of 29 individuals representing the four subspecies of C. *cyanoides*; Table S1). We included samples of Ultramarine Grosbeak *Cyanocompsa brissonii*,

Glaucous-blue Grosbeak *Cyanoloxia glaucocaerulea* and Red-crowned Ant Tanager *Habia rubica* for use as outgroups (Table S1). Gene trees were inferred through Bayesian and maximum parsimony methodologies using MrBayes 3.2.2 (Ronquist *et al.* 2012) and TNT 1.1 (Goloboff *et al.* 2003), respectively. In the case of the Bayesian analysis, we performed two independent runs of 10 million generations, sampling trees every 100 generations under default priors for all parameters. The standard deviation of split frequencies between runs was always < 0.01, indicative of convergence. We ensured that both runs reached stationarity using TRACER 1.5 (Rambaut & Drummond 2007). For all parameters, the effective sample sizes (ESS) were > 200 and the potential scale reduction factor (PSRF, Gelman & Rubin 1992) was equal to 1, suggesting that we had an adequate sample of the posterior probability distribution. We discarded the first 25% of the sampled trees as burn-in and the remaining 75 000 trees of each run were combined to generate a 50% majority rule consensus tree. We selected the best-fit model of nucleotide substitution for COI prior to the analysis using the Bayesian information criterion (BIC) implemented in jModelTest 2.1.1 (Darriba *et al.* 2012). The model implemented was the HKY (Hasegawa *et al.* 1985) with gamma-distributed rate variation across sites (+G) and a proportion of invariable sites (+I). As for the maximum parsimony analysis, we ran a heuristic search based on 1000 random-addition sequence replicates (RAS) coupled with the tree-bisection-reconnection (TBR) branch-swapping algorithm, saving 10 trees per replication. A strict consensus tree was estimated from all the most parsimonious trees. To estimate node support we conducted a bootstrap analysis that consisted of 1000 pseudoreplicates of 100 RAS + TBR, saving 10 trees per replicate. Additionally, we used cyt-*b* data to generate an unrooted statistical parsimony network with POPART 1.0 (Leigh & Bryant 2015).

Finally, we used both mitochondrial loci to estimate mean uncorrected genetic distances among and within subspecies using MEGA 5.0 (Tamura *et al.* 2011), and to perform analyses of molecular variance (AMOVAs) in ARLEQUIN 3.5 (Excoffier & Lischer 2010) with individuals grouped by subspecies. Pairwise Φ_{ST}-values were computed using uncorrected genetic distances and significance was tested through 2000 random permutations.

Analyses of plumage coloration

To objectively describe plumage colour variation among subspecies, we used reflectance spectra measurements taken on museum skins (see Supporting Information Table S2 for collections consulted). We selected museum skins from both sexes in good condition and with complete data regarding sex and collection locality. We assigned each specimen to a subspecies based on its collection locality and the geographical ranges of each subspecies as described by Clements *et al.* (2014). We measured plumage reflectance spectra on eight plumage patches: chest, belly, forehead, crown, cheeks, back, rump and wing coverts. We measured a total of 10 specimens of *C. c. concreta* (five males and five females), 11 specimens of *C. c. toddi* (five males and six females), 10 specimens of *C. c. cyanoides* (five males and five females) and 12 specimens of *C. c. rotschildii* (seven males and five females). All reflectance measurements were taken by N.C.G. using an Ocean Optics USB 2000 spectrophotometer with a PX-2 xenon light source (effective range of emission 220–750 nm) calibrated against a WS-1 diffuse reflectance white standard (Ocean Optics, Inc., Dunedin, FL, USA). Plumage was illuminated and the reflected light was collected with a bifurcated probe located perpendicularly and 4 mm away from the museum skin surface. The probe was placed on a rubber holder that kept it at a fixed distance from the surface and isolated from ambient light. Measurements were taken three times per patch. The spectrophotometer resolution was 0.35 nm, and each spectrum was the average of five readings with an integration time of 100 ms and a boxcar smoothing function of five. We recalibrated the equipment before measuring plumage reflectance on each individual.

The reflectance spectra obtained were analysed with the 'pavo' package (Maia *et al.* 2013) in R 3.1.0 (R Development Core Team 2014). Males and females were analysed separately. We averaged the three readings obtained for each plumage patch and individual thereby obtaining a single reflectance spectrum with one reflectance value per nanometre between 300 and 700 nm. We first used the Vorobyev and Osorio (1998) visual model implemented in 'pavo' to estimate the perceptual distance (ΔS) in the chromatic component of plumage colour for each plumage patch

between all individuals for each sex separately. This model is based on the sensitivity of the photoreceptors in an avian retina and their relative abundance, and how the measured reflectance spectra stimulate those while taking into consideration ambient factors such as illumination and background. ΔS is expressed in terms of just noticeable differences (*jnd*); according to avian visual modelling, the discrimination threshold lies between one and two *jnd* depending on the intensity of ambient illumination (Eaton 2005). Thus, values > 2 should be differentiable by birds under all light conditions. For this calculation, we made use of an average UV spectral sensitivity (Endler & Mielke 2005) to determine the absorbance at each wavelength for each cone type. There are no data about the specific sensitivity of each cone type in C. *cyanoides*, but it is likely that it belongs to the UV-sensitive group (Ödeen *et al.* 2011). We selected an ideal white illuminant for our calculations and used the Eurasian Blue Tit *Cyanistes caeruleus* relative cone abundance (1 : 2 : 2 : 4) and a Weber fraction of 0.05. We calculated the mean ΔS within subspecies (between individuals of the same subspecies) and between subspecies (between individuals of the subspecies being compared). As mean ΔS between subspecies exceeded mean ΔS within subspecies only in four plumage patches of males (forehead, crown, cheeks and coverts), we restricted our subsequent analyses to these patches.

We then described colour in the aforementioned patches using spectral parameters typically used to characterize plumage coloration (Montgomerie 2006). We estimated hue as the wavelength where the maximum reflectance occurs ($\lambda_{R_{max}}$). We calculated plumage mean brightness following the formula $\sum R_{(300–700)}/401$, where 401 is the total number of data points in each spectrum. UV chroma is usually described in studies of avian coloration because it may serve as a private channel of communication (Håstad *et al.* 2005, but see Renoult *et al.* 2013), and we estimated it as the ratio between the light reflected between 300 and 400 nm and the light reflected over all wavelengths ($\sum R_{(300–400)}/(\sum R_{(300–700)}$). Finally, as an indicator of plumage colour saturation we estimated overall chroma as $(R_{max}–R_{min})/(\sum R_{(300–700)}/401)$. We used these four spectral variables extracted from the reflectance spectra of the four plumage patches of males as the dataset for a principal components analysis (PCA) and extracted all principal components (PCs) with eigenvalues > 1. PCs were unrotated. We then tested for differences among subspecies in their scores for the corresponding PCs with a one-way ANOVA followed by Tukey's contrast tests. All analyses were performed in SPSS 15.0 for Windows (SPSS, Chicago, IL, USA).

Morphology

We measured beak size and wing length on a subset of the same study skins from which colorimetric measurements were taken. We measured a total of 10 specimens of C. *c. concreta* (five males and five females), 10 specimens of C. *c. toddi* (five males and five females), eight specimens of C. *c. cyanoides* (four males and four females) and 11 specimens of C. *c. rothschildii* (six males and five females). We measured three linear variables that represent beak size: beak length (measured from the point at which the feathers of the forehead in their natural position cease to hide the culmen to the tip of the beak) and beak width and depth (vertically) at the level of the nostrils (Baldwin *et al.* 1931). We used these variables to perform a PCA and obtained one variable (PCbeak) that represents beak size. All three linear variables correlated significantly and positively with PCbeak, which explained 100% of the observed variance. PCbeak showed no differences between males and females ($t = -1.36$, $P = 0.19$), and thus we pooled data when investigating differences among subspecies.

Wing length was taken in a straight line from the farthest anterior point on the anterior edge of the wrist joint to the tip of the longest primary (see Baldwin *et al.* 1931). Both left and right wings were measured to obtain the mean wing length per individual. Wing length was significantly shorter in females ($t = -5.79$, $P < 0.001$), and therefore differences in wing length among subspecies were evaluated for each sex separately.

Few of the aforementioned study skins reported body mass. Thus, additional data were obtained from Verea *et al.* (1999) and from several institutions (Supporting Information Table S3) through VertNet (www.vertnet.org) or directly provided by institution staff. We estimated mean body mass for each subspecies based on data from 14 females and 12 males of C. *cyanoides concreta*, 11 females and 28 males of C. *c. toddi*, 14 females and 31 males of C. *c. cyanoides*, and 44 females and 57

males of C. *c. rothschildii*. There were no significant differences between males and females ($t = -0.67$, $P = 0.504$), so we pooled data when investigating differences among subspecies.

Differences in body mass and wing length among subspecies were tested with a one-way ANOVA followed by Tukey's contrast tests. Differences in PCbeak among subspecies were tested with a Welch's *F*-test, as the assumption of homogeneity of variances was not met. We then performed pairwise comparisons to determine which subspecies differed using *post hoc* tests with Dunnett's T3 correction. All analyses were performed in SPSS 15.0 for Windows.

Song analysis

Recordings used in this study were obtained from several sound libraries (Supporting Information Table S4). We analysed recordings of 12 individuals of C. *c. concreta*, 13 of C. *c. toddi*, 19 of C. *c. cyanoides* and 29 of C. *c. rothschildii*. We avoided using recordings that were referenced as being from a female or a juvenile. For each recording, a spectrogram was generated and analysed in RAVEN 1.4 (Bioacoustics Research Program, Cornell Lab of Ornithology, Ithaca, NY, USA; see http://www.birds.cornell.edu/raven), using a 512 fast Fourier transform length with a 50% overlap and a Hann window of 512 samples. We selected one song per individual and measured the following variables on the spectrogram: minimum, maximum and emphasized frequencies, song frequency bandwidth, song duration, number of notes, mean note duration, note rate and mean inter-note interval length, number of inflections and inflection rate. An inflection is a point in a note at which frequency changes from ascending to descending, or vice versa. We defined a note as any continuous trace in the temporal axis of a spectrogram, or groups of continuous traces separated by < 0.015 s. The note rate was calculated as the number of notes divided by the song duration. The emphasized frequency was obtained using the 'Max Frequency' option in RAVEN 1.4.

We performed a PCA on song variables and extracted all principal components with eigenvalues > 1. PCs were Varimax-rotated. We then tested for differences among subspecies in their scores for the corresponding PCs with a one-way ANOVA followed by Tukey's contrast test. Differences in PC2 among subspecies were tested with a Welch's *F*-test because the assumption of homogeneity of variances was not met. We then performed pairwise comparisons to determine which subspecies differed using posthoc tests with Dunnett's T3 correction. All analyses were performed using SPSS 15.0 for Windows.

RESULTS

Genetic analyses

Cyanocompsa cyanoides was recovered as monophyletic but with low support (posterior probability (PP) = 0.52) in the Bayesian analysis of COI, whereas monophyly was not recovered in the maximum parsimony (MP) analysis. Similarly, the relationships among C. *cyanoides* and the other members of the 'blue clade' of the Cardinalidae sampled were supported with low posterior probability in the Bayesian tree and remained unsolved in the MP analysis.

Both Bayesian and MP analyses recovered three deeply divergent COI lineages within C. *cyanoides* (Fig. 1b): one clade comprising all individuals of the subspecies *rothschildii*, and two sister clades, one representing the subspecies *cyanoides* and the third a cluster of individuals of the subspecies *toddi* and *concreta*. These three clades were recovered with high node support (PP of 1.0 and MP bootstrap support > 99%; Fig. 1b). As DNA sequence divergence between individuals from subspecies *toddi* and *concreta* was very low (0.16%) at the COI locus, we lumped these two subspecies together into a single group called '*toddi/concreta*' in all further analyses. Lastly, the cyt-*b* statistical parsimony network showed the same clustering and differentiation pattern among groups within C. *cyanoides* (Fig. 1c).

Mean genetic distances in the mitochondrial DNA among all groups within C. *cyanoides* were deep (from 5.2 to 8% for COI and from 5.4 to 6.7% for cyt-*b*; Table 1). The AMOVA indicated that almost all genetic variation within our dataset was attributable to differentiation among groups (91.3% for COI and 88.5% for cyt-*b*). Genetic distances were greater between *rothschildii* and the other groups, '*toddi/concreta*' and *cyanoides*, than between the latter two clades (Table 1). Except for one non-significant comparison, all pairwise Φst values among groups were high and significant (Table 1). Differentiation between *rothschildii* and the other groups within C. *cyanoides* was similar

Table 1. Pairwise comparisons among subspecies of *Cyanocompsa cyanoides* and between *C. cyanoides* and *Cyanocompsa brissonii* and *Cyanoloxia glaucocaerulea* based on the mitochondrial markers (COI, 814 bp; cyt-*b*, 1031 bp). Above the diagonal: pairwise Φst values; significant values ($P < 0.05$) in bold. Diagonal: mean uncorrected genetic distances within species or subspecies. Below the diagonal: mean uncorrected genetic distances between pairs of species or subspecies. Genetic distances are expressed in percentage values.

	COI					Cyt-*b*				
Species and subspecies	*cyanoides* ($n = 6$)	*concreta/toddi* ($n = 3$)	*rothschildii* ($n = 20$)	*C. brissonii* ($n = 2$)	*Cyanoloxia glaucocaerulea* ($n = 2$)	*cyanoides* ($n = 3$)	*concreta/toddi* ($n = 2$)	*rothschildii* ($n = 6$)	*C. brissonii* ($n = 2$)	*Cyanoloxia glaucocaerulea* ($n = 2$)
cyanoides	0.93	**0.86**	**0.91**	**0.83**	**0.88**	0.25	0.97	**0.87**	0.81	0.98
concreta/toddi	5.18	0.11	**0.93**	0.91	0.98	5.42	0.1	**0.87**	0.76	0.99
rothschildii	7.27	7.98	0.67	**0.9**	**0.92**	6.23	6.72	1.1	**0.75**	**0.87**
C. brissonii	6.67	7.45	6.93	1.87	0.77	6.53	6.88	6.1	3.02	0.63
Cyanoloxia glaucocaerulea	6.6	6.58	7.18	4.54	0.14	6.91	7.41	7.11	4.44	0.1

or even greater than the divergence observed between the subspecies of *C. cyanoides* and the other species of the 'blue clade' of the Cardinalidae considered in this study (Table 1, Fig. 1c).

Finally, we also found two haplogroups with high support within the *rothschildii* clade showing a pattern of east–west differentiation (1.24% for COI and 1.90% for cyt-*b*) within the Amazon basin (Fig. 1b,c). The two individuals from Santa Cruz, Bolivia (*rothschildii2* and *rothschildii8*), clustered with different *rothschildii* haplogroups, suggesting this area might represent a contact zone between the two divergent mitochondrial lineages. This sampling locality is not even within the geographical range described for *C. cyanoides*. We are currently investigating this interesting pattern in more depth with more comprehensive genetic and geographical sampling.

Plumage coloration

The mean ΔS between subspecies exceeded mean ΔS within subspecies for only four plumage patches of males (forehead, crown, cheeks and coverts; Table 2), whereas mean ΔS for each colour patch of the females was similar when we compared plumage colour within subspecies and between subspecies (as an example, we also show in Table 2 the averages of the ΔS values for the same patches considered for males). However, all comparisons were > 2, which is the theoretical maximum threshold of visual differentiatiion of the Vorobyev-Osorio model. This means that differences in colour in these patches would be perceptible by the birds under all light conditions. For males, ΔS values for forehead, crown, cheeks and coverts were considerably larger in the comparisons involving *C. c. rothschildii* than in the comparisons among the remaining subspecies or within any of them (Table 2).

We obtained three principal components (colour PCs) with eigenvalues > 1 for the spectral variables of the four plumage patches of males; PC1 represented 54% of the variation in the original dataset and was positively and significantly (loadings higher than 0.7) correlated with the brightness and chroma of the four patches and the UV chroma of the crown and coverts. PC2 accounted for 16% of the original variation and related positively to UV chroma of the cheeks and the hue of coverts, whereas colour PC3 explained only 9% of the original variance and was only

Table 2. Mean ± sd colour perceptual distance expressed in terms of ΔS (*jnd*) for comparisons between and within subspecies of the Blue-black Grosbeak. Comparisons were made for the mean reflectance spectra of crown, cheeks, coverts and forehead. Values for comparisons within subspecies are in the table diagonal. Values for comparisons between subspecies shown above the diagonal are for males and those below the diagonal are for females. Comparisons between subspecies that involved the males of the subspecies *Cyanocompsa c. rothschildii* are in bold.

	concreta	*cyanoides*	*toddi*	*rothschildii*
concreta	1.40 ± 4.45 / 3.50 ± 1.84	2.92 ± 0.92	2.39 ± 0.81	**7.66 ± 1.21**
cyanoides	3.53 ± 1.23	1.95 ± 0.45 / 2.75 ± 1.29	2.11 ± 0.67	**5.09 ± 1.34**
toddi	4.26 ± 1.90	3.31 ± 1.57	2.17 ± 0.74 / 3.97 ± 1.73	**5.97 ± 1.30**
rothschildii	4.29 ± 1.41	3.21 ± 1.35	3.35 ± 1.29	2.69 ± 0.64 / 3.38 ± 1.04

related to the hue of the crown. The one-way ANOVA detected significant differences among subspecies only for PC1 ($F = 116.38$, $P < 0.001$), and Tukey's contrast test showed that this variable differed significantly between all pairs of subspecies ($P < 0.010$), with the exception of between C. *c. cyanoides* and C. *c. toddi* ($P = 0.22$). Similar to what we found when comparing the values of ΔS, differences in PC1 were much greater when the contrast involved C. *c. rothschildii* (Fig. 2a). This result indicates that the coloration of the considered plumage patches of C. *c. rothschildii* is brighter and more saturated and has a higher UV component in the crown and coverts. Differences between the other pairs of subspecies are subtler, with C. *c. concreta* having the lowest value for PC1 (Fig. 2a).

Morphology

All comparisons of body mass between subspecies indicated significant differences ($P < 0.01$), except between C. *c. cyanoides* (mean ± sd 30.14 ± 2.91 g) and C. *c. toddi* (29.16 ± 2.04 g; Fig. 2b). *Cyanocompsa c. concreta* is the largest subspecies (32.05 ± 2.53 g) and C. *c. rothschildii* is the smallest (24.81 ± 2.96 g, Fig. 2b). Differences in mean body mass between C. *c. rothschildii* and the other subspecies (*c.* 4.5–7.5 g) were considerably greater than differences between any of the other subspecies (0.9–2.9 g).

PCbeak also showed significant differences between subspecies (Welch's $F = 20.61$, $P < 0.001$). *Cyanocompsa c. concreta* has the highest mean value for PCbeak (i.e. its beak is the largest in the three linear measures considered) and C. *c. rothschildii* has the smallest beak. All comparisons of beak size between subspecies were significant, except between C. *c. toddi* and C. *c. concreta* and between C. *c. toddi* and C. *c. cyanoides* (Fig. 2c).

Wing length shows a similar pattern both for females and for males: C. *c. concreta* has the longest wings, C. *c. rothschildii* the shortest, and C. *c. toddi* and C. *c. cyanoides* have intermediate lengths. However, differences are not significant for males ($F = 0.67$, $P = 0.59$) and only between C. *c. rothschildii* and C. *c. concreta* (Tukey's $P = 0.001$) and between C. *c. rothschildii* and C. *c. cyanoides* (Tukey's $P = 0.034$) for females.

Song

We extracted three principal components (song PCs) with eigenvalues > 1 that accounted for 78% of the variation in the original dataset. PC1 accounted for 29% of the original variation, and was positively and significantly (loadings higher than 0.7) correlated to note rate and negatively to note duration and internote interval duration. PC2 represented 28% of the original variation and was positively related to maximum frequency, bandwidth and song duration. PC3 represented 21% of the original variation and correlated positively to the number of inflections and inflection rate. One-way ANOVA identified significant differences among subspecies for PC1 ($F = 22.86$, $P < 0.001$) and PC3 ($F = 8.80$, $P < 0.001$), and the Welch's *F*-test also detected significant differences for PC2 (Welch's $F = 4.05$, $P = 0.014$). *Cyanocompsa c. rothschildii* had the greatest mean value for PC1 and differed significantly from all other groups (Fig. 2d). Thus, the song of C. *c. rothschildii* has shorter notes produced at a higher rate. *Cyanocompsa c. rothschildii* had the smallest value for PC3, and Tukey's contrast test showed that it also differed significantly from all the other subspecies for this PC (although only marginally from C. *c. toddi*): its song also has fewer inflections, produced at a lower rate. *Cyanocompsa c. cyanoides* had the greatest value for PC2, and differences with C. *c. concreta* and C. *c. toddi* were significant.

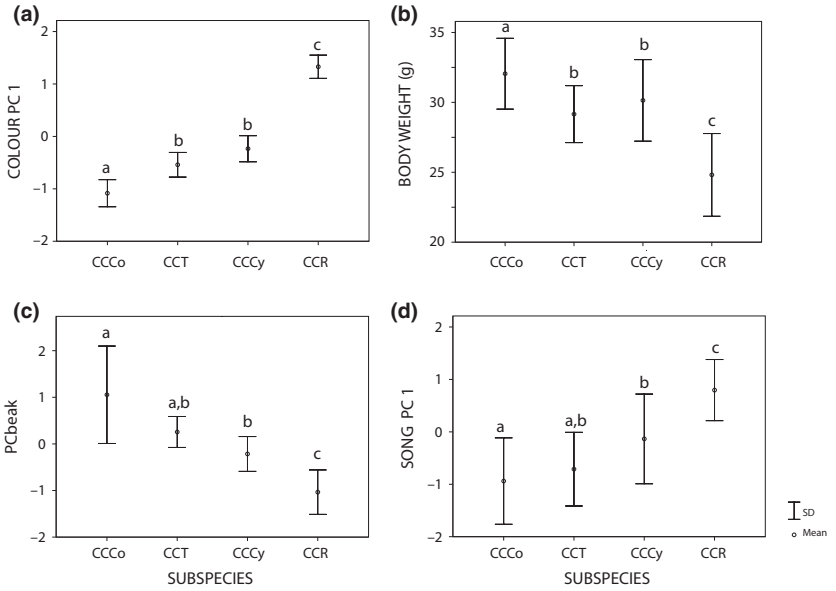

Figure 2. Plumage coloration, morphology and song analysis results. (a,c,d) Scores for Colour PC1, PCbeak and Song PC1 for each subspecies, respectively. (b) Body weight for each subspecies. Mean ± sd are shown in all cases. Taxa that differed significantly after Tukey's contrast tests (or *post hoc* tests with Dunnett's T3 correction for PCbeak) are identified with different letters. CCCo, *Cyanocompsa cyanoides concreta*; CCCy, *C. c. cyanoides*; CCT, *C. c. toddi*; CCR, *C. c. rothschildii*.

Cyanocompsa c. toddi and *C. c. concreta* exhibited no differences for any PC axis.

DISCUSSION

We analysed genotypic and phenotypic variation within the Blue-black Grosbeak to assess whether the patterns of geographical variation at both levels were congruent. We found deep differentiation for two mitochondrial markers and several phenotypic (body mass, beak size and plumage coloration) and behavioural (song) traits. The information provided by both sources of information (phenotype/behaviour and genotype) is partially congruent, indicating that the species as currently recognized could be divided into three subgroups, but the boundaries of these subgroups do not fully coincide. Similar results have been recovered for other species, wherein the number of subspecies and genetic lineages is equal or very similar but boundaries are not always congruent (Cabanne *et al.* 2008, Miller *et al.* 2008, 2011). Additional studies of Neotropical species may demonstrate this to be a common pattern, in contrast to Nearctic species in which there tends to be less congruence between phenotypic and genotypic makers (e.g. Zink 2004).

Genetic vs. phenotypic variation

Milá *et al.* (2012) first showed deep genetic differentiation between the *cis*-Andean and *trans*-Andean South American populations of the Blue-black Grosbeak, but reported low phenotypic variation (in plumage coloration, assessed from a human perspective, see below). Bryson *et al.* (2013) provided evidence that this species actually comprises three evolutionary independent lineages. The range boundaries of two of these lineages are congruent with those of subspecies *rothschildii* and *cyanoides*, whereas the third lineage would comprise individuals that correspond to subspecies *toddi* and *concreta* (Bryson *et al.* 2013). Our results corroborate those of Milá *et al.* (2012) and Bryson *et al.* (2013) with the use of additional molecular markers and individuals, as well as with a complementary methodology of analysis.

The taxon range boundaries of the subgroups that could be defined on the basis of molecular markers and phenotypic variation partially coincide. *Cyanocompsa c. rothschildii* is the most

differentiated subspecies, both in phenotype (including plumage colour, body and beak size, and song) and in genotype. A previous study that compared closely related lineages (subspecies or sister species) separated by the Marañon River valley in Peru found that those lineages that differed most in plumage coloration also showed greater differentiation in molecular markers, indicating they had been isolated for a longer period (Winger & Bates 2015). Therefore, even though differentiation in phenotype and genotype may require different time periods to be achieved, congruence between them is expected after a certain amount of time. It is not surprising then that C. *c. rothschildii* shows the greater phenotypic differentiation, as it has been evolving independently from the other lineages for at least 3 Myr (Bryson *et al.* 2013). However, contrary to Winger and Bates (2015), body mass and beak size showed the same pattern of variation as plumage coloration. Thus, their finding that evolution of morphometric traits shows greater idiosyncrasy and stasis than plumage coloration should perhaps not be extrapolated to other species.

The deep genetic differentiation found between C. *c. rothschildii (cis*-Adean) and the other *trans*-Andean subspecies is congruent with previous evidence that supports the role of the Andes as one of the main drivers of avian diversification in the Neotropics, promoting divergence between lowland populations on each side of the mountain range (Cheviron *et al.* 2005, Rheindt *et al.* 2008, Weir & Price 2011, Fernandes *et al.* 2014, Harvey & Brumfield 2015). Less is known about phenotypic divergence between *trans*-Andean taxa, as most previous phylogenetic and phylogeographical analyses failed to include other sources of evidence in addition to mitochondrial or nuclear markers. In one of the few exceptions, levels of genetic and phenotypic (plumage colour) divergence were studied in 12 Amazonian bird species that are found on both sides of the Andes (Milá *et al.* 2012). Degrees of both genetic and phenotypic differentiation were not consistent when comparing each side of the Andes and east and west Amazonia: some species showed greater differentiation across the Andes, some showed greater differences across the Amazon, and there were some cases where divergence was similar in both comparisons (Milá *et al.* 2012). Altogether, this suggests that even though C. *cyanoides* east of the Andes showed the greatest differences both in phenotype and in genotype, the importance of the Andes relative to other barriers or vicariant events is not equivalent across taxa. Differences in the role of the Andes among taxa could be explained by several factors such as contrasting ecologies (i.e. habitat specialists vs. generalists), differing dispersal abilities and different elevational ranges (Burney & Brumfield 2009, Smith *et al.* 2014). In this context, our results add more evidence to the idea that the effect of the Andes is even greater for species with limited dispersal abilities, e.g. lowland understorey forest specialists such as C. *cyanoides* (Moore *et al.* 2008, Burney & Brumfield 2009, Orenstein & Brewer 2011).

For the remaining lineages within C. *cyanoides*, the patterns of genotypic and phenotypic variation are mixed. Subspecies *concreta* and *cyanoides* exhibited differentiation in both mitochondrial loci as well as in plumage coloration and body mass. Similar differences in phenotype were found when we compared *concreta* and *toddi*, but they showed no differentiation at mitochondrial loci. The opposite is true for subspecies *toddi* and *cyanoides*: they showed no variation in plumage coloration and body mass, but there are well-supported differences at mitochondrial markers. Finding an explanation for such a complex pattern is challenging because these incongruities between phenotypic and genotypic variation might arise as a consequence of multiple interacting factors whose influence appears, at the same time, difficult to prove with our dataset. A possible explanation could be that despite all subspecies being found in virtually the same type of habitat (Orenstein & Brewer 2011), micro-scale variation within the environment may result in contrasting ecologically based selection pressures that drive phenotypic divergence of *concreta* vs. *cyanoides* and *toddi* (even in the presence of gene flow with *toddi* populations) while promoting convergence between the latter two taxa. C*yanocompsa c. concreta* occupies the northern extreme of the species distribution and is found up to 900 m above sea level, whereas *toddi* and *cyanoides* reach 1200–1400 m.

Variation in beak size and along one of the song PCs showed significant differences between subspecies *concreta* and *cyanoides*, but neither differed significantly from subspecies *toddi*. This could be due to variation in these traits being clinal. Alternatively, individuals that are assigned to subspecies *toddi* could come from two different groups, supporting the previously mentioned idea that

subspecies geographical boundaries based on phenotype should be re-examined.

Taxonomic implications

It was previously suggested that C. *c. rothschildii* should be elevated to species status, but the proposal was based only on molecular evidence (Bryson *et al.* 2013), with differences in phenotype only briefly mentioned. Our study constitutes the first quantification and objective analysis of phenotypic variation within C. *cyanoides*. The levels of genetic, phenotypic and behavioural differentiation found between C. *c. rothschildii* and the other subspecies strongly support the recommendation of Bryson *et al.* (2013) that this subspecies be considered a separate species, *Cyanocompsa rothschildii*.

In the case of the remaining lineages within C. *cyanoides*, subspecies *concreta* could be a valid subspecies even if it does not show genetic differences with *toddi* because subspecies are not expected to be reproductively isolated from each other and their monophyly should not be necessary for their delimitation (Patten 2010). Many authors agree that the problem of the subspecies category is not that they do not represent genetically differentiated lineages but that phenotypic variation should be re-assessed with modern techniques (Patten 2010).

Methodological perspective

It is noteworthy that part of the colour variation we reported should be perceivable from a human perspective; and that such variation is in agreement with previous subjective descriptions of differences among subspecies. For example, C. *c. concreta* is described as the darkest subspecies, whereas C. *c. rothschildii* plumage is the brightest (Orenstein & Brewer 2011), and we found that males of subspecies *concreta* had the lowest brightness values and those of subspecies *rothschildii* the highest.

However, the highly significant differences in plumage coloration that we report here contrast with the lack of colour divergence suggested by Milá *et al.* (2012). In their study, colour divergence between South American individuals of the Blue-black Grosbeak east and west of the Andes (corresponding to C. *c. rothschildii* and C. *c. cyanoides*, respectively) was evaluated with a four-code key based on arbitrary criteria derived from subjective human-eye-based analyses. Methods based on human perception allow the analysis of large numbers of specimens, which is extremely useful to derive general ideas on phenotypic variation across many species. However, these general ideas may not apply when the trait considered is perceived differently from the human and avian perspectives, such as structural coloration with a UV component. An important part of the colour variation we found occurs in the UV range, which may explain the differences in our results with those of Milá *et al.* (2012). It is worth mentioning that even though we do not have direct evidence that C. *cyanoides* perceives UV coloration, we consider that description of the presence and variation of UV coloured patches is highly relevant, as several studies have shown not only the presence of UV-sensitive photoreceptors in bird eyes but also their significant role in foraging and sexual signalling in birds (Cuthill 2006).

CONCLUSIONS

Current subspecific taxonomy needs extensive reassessment, not because species do not show intraspecific genetic structure but because traditional classification of taxa was based on subjective, non-standardized descriptions of phenotypic differences in the absence of the availability of molecular DNA data. What is more, there are many cases in which there is genetic differentiation below the species level, and almost as many phylogroups as subspecies can be found within a species, but their current delimitation does not fully agree, as for the Blue-black Grosbeak. Future studies like ours might show that this pattern is actually common within the Neotropics. Thus, the notion that avian subspecies rarely show genetic differentiation can be explained partially by the predominance of studies focusing on continental subspecies from North America and Eurasia, where the proportion of subspecies that show phylogeographical structure is significantly lower than that of the Neotropics (Lovette 2005, Lijtmaer *et al.* 2011).

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SUPPORTING INFORMATION

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

Table S1. Individuals used in the genetic analyses.

Table S2. Study skins from where colour, beak and wing length data were derived.

Table S3. Body weight data used to calculate mean body mass per subspecies.

Table S4. Recordings used for song analyses.