

Cloudy with a chance of speciation: integrative taxonomy reveals extraordinary divergence within a Mesoamerican cloud forest bird

MADHVI X. VENKATRAMAN^{1,2}, DEVON A. DERAAD^{1,2}, WHITNEY L. E. TSAI¹, EUGENIA ZARZA¹, AMANDA J. ZELLMER², JAMES M. MALEY¹ and JOHN E. MCCORMACK^{1,2,*}

¹Moore Laboratory of Zoology, Occidental College, Los Angeles, CA 90041, USA

²Biology Department, Occidental College, Los Angeles, CA 90041, USA

Received 23 May 2018; revised 18 September 2018; accepted for publication 19 September 2018

The highlands of Mesoamerica harbour some of the highest biodiversity in the world, especially in cloud forests, but the landscape drivers of this diversification are not well known. Taxonomy in this region has been challenging owing to a lack of consensus about how to test species limits. We apply integrative taxonomy to an emblematic species of the Mesoamerican cloud forests, the unicolor jay (*Aphelocoma unicolor*). We assess divergence along three complementary axes (genetics, phenotype and ecological niche), finding evidence for differentiation among five lineages, currently described as subspecies, in isolated forest patches. DNA suggests that these lineages have long histories of divergence. Multivariate analysis of phenotype, along with an objective method for detecting phenotypic clusters, suggest that at least four of the five lineages are diagnosable. There was also a pattern of increasing ecological divergence through time. The divergence observed among lineages is comparable to other species-level divergences in the genus, arguing for elevation of at least two, and as many as four, lineages within *A. unicolor* to species rank. According to our time tree, cloud forest patches became isolated starting in the Pliocene and continuing into the Pleistocene, suggesting glacial cycles as the main drivers of speciation.

ADDITIONAL KEYWORDS: allopatry – allospecies – environmental niche models – phylogeography – ultraconserved elements.

INTRODUCTION

The Mesoamerican Highlands are a global biodiversity hotspot (Myers *et al.*, 2000). Montane cloud forests within this region are especially diverse, hosting the second-highest biological diversity of all of Mexico's ecoregions despite covering only 1% of the land area. Recent studies estimate that anywhere from 50 to 99% of this cloud forest could disappear by the mid-21st century as a result of forest clearing and climate change (Ponce-Reyes *et al.*, 2012; Rojas-Soto *et al.*, 2012). It is critically important that we properly recognize the diversity of Mexico's cloud forests, including how and when their diversity arose, in assessing their conservation value in the face of existential threats.

The distribution of Mexican cloud forest is naturally patchy, which has led isolated populations to diverge through time (Ornelas *et al.*, 2013; Caviedes-Solis & Leaché, 2018). This patchwork of cloud forest began to take its modern form in the late Miocene and Pliocene when mountain building finished in the region and the low-elevation Isthmus of Tehuantepec formed and was subject to marine inundation (Barrier *et al.*, 1998; and references provided by Ornelas *et al.*, 2013; Mastretta-Yanes *et al.*, 2015). The region was then influenced by Pleistocene glacial cycles, which led to elevational shifting and reorganization of habitats (Mastretta-Yanes *et al.*, 2015). Divergence times of lineages spanning these barriers vary from the middle Miocene to the Pleistocene depending on the taxonomic group, with plants and reptiles generally showing the oldest splits and birds the youngest (Castoe *et al.*, 2009;

*Corresponding author. E-mail: mccormack@oxy.edu

Barber & Klicka, 2010; Daza *et al.*, 2010; Ornelas *et al.*, 2013). From these studies, it seems clear that birds have generally been more influenced by Pleistocene glacial cycles and have maintained higher levels of gene flow over time, whereas many reptile and plant divergences line up temporally with tectonic activity followed by little to no gene flow.

Another issue of Mesoamerican cloud forests has been the taxonomic status of allopatric populations. Mayr (1963) first proposed the idea of superspecies to describe a monophyletic group of allopatric populations that were too differentiated to be considered mere subspecies, with the component populations later being termed allospecies by Amadon (1966). Although allospecies well describes many populations in Mesoamerican cloud forest patches and conveniently fills a gap in the Linnean hierarchy, the term has not been carried forward strongly in systematics. As a result, the taxonomy of differentiated Mesoamerican cloud forest populations has been, in a sense, caught between two popular species concepts: the biological species concept, where the default position might be to consider allopatric populations a single species until a measure of reproductive isolation can be demonstrated among them, and the phylogenetic species concept, where populations might be elevated immediately to species rank on the basis of genetic monophyly (see e.g. Winker, 2016).

Where taxonomy is challenging, as in cases along the speciation continuum where species concepts are expected to disagree (De Queiroz, 2007), integrative taxonomy can provide a more holistic portrait of the factors likely either to maintain cohesion as a single species or to promote further isolation towards speciation (Padial *et al.*, 2010; Schlick-Steiner *et al.*, 2010). Integrative taxonomy, which queries many complementary axes of divergence, such as phenotype, genetics and ecological niche, has been bolstered by new technologies and data sources, especially as they intersect with research specimens in natural history collections (Aguilar *et al.*, 2016; Pokrant *et al.*, 2016; Webster, 2017; Younger *et al.*, 2018). When these methods are deployed in combination, integrative taxonomy can provide a roadmap through even some of the most challenging taxonomic scenarios involving recent divergence and cryptic species (Andújar *et al.*, 2017; Fišer *et al.*, 2018; Nogueras *et al.*, 2018).

We apply integrative taxonomy to assess divergence among populations of the unicolored jay (*Aphelocoma unicolor* Du Bus, 1847), an emblematic species of Mesoamerican cloud forests. There are five subspecies recognized in *A. unicolor*, each living in a different cloud forest region in Mexico and Central America (Fig. 1). Prior molecular work using allozymes (Peterson, 1992) and mitochondrial DNA (mtDNA) (McCormack *et al.*, 2011) revealed that these five subspecies are

genetically differentiated by perhaps several million years. Pitelka (1951) had previously found evidence for differences in both morphology and plumage among these subspecies, but a leapfrog pattern of phenotypic variation convinced him against assigning any of the subspecies to species rank. We assess new nuclear and mtDNA variation in unicolored jays, add new information on divergence in their environmental niches and update Pitelka's phenotypic analysis with new specimens, tools and statistical methods. Our goal is to understand the evolutionary history of *A. unicolor*, including divergence times among the lineages, and to determine whether any of them warrant elevation to species status. Given the paucity of studies of Mesoamerican cloud forests, our results will contribute to knowledge of general evolutionary patterns in this endangered ecoregion and provide a model for assessing species limits across its patchy distribution through integrative taxonomy.

MATERIAL AND METHODS

PHENOTYPIC DIFFERENTIATION

We measured 182 *A. unicolor* specimens, including individuals from all five subspecies: 11 *A. u. concolor*, 28 *A. u. oaxacae*, 25 *A. u. guerrerensis*, 82 *A. u. unicolor* and 36 *A. u. griscomi*. We measured the tail and unflattened wing cord to the nearest 0.5 mm using a wing ruler, and tarsus length, upper mandible length (also simply called bill length; posterior of the nares opening to the tip of the bill), lower mandible length, bill depth and bill width to the nearest 0.1 mm using digital calipers. All measurements were made once by M.X.V.

On a subset of the specimens, we quantified plumage reflectance using an Ocean Optics JAZ PX UV spectrophotometer. We took measurements from the nape because this region was indicative of overall plumage colour and showed few differences in wear and moult among specimens. We recorded light reflectance at 10 nm intervals from 300 to 700 nm, resulting in 40 readings per individual. We reduced these 40 readings to 20 variables through binning and then to three major axes of variation with principal components analysis (PCA) in Stata version 11.2. Principal components (PCs) of spectral measurements typically correspond to brightness, hue and chroma (Andersson & Prager, 2006).

To test for differences among subspecies, we first assessed differences between sexes. Although there are modest differences between sexes in most morphological traits, conducting separate analysis by sex did not alter the overall patterns; therefore, we present results with both sexes included. We conducted tests on individual traits with Student's unpaired *t*-tests.

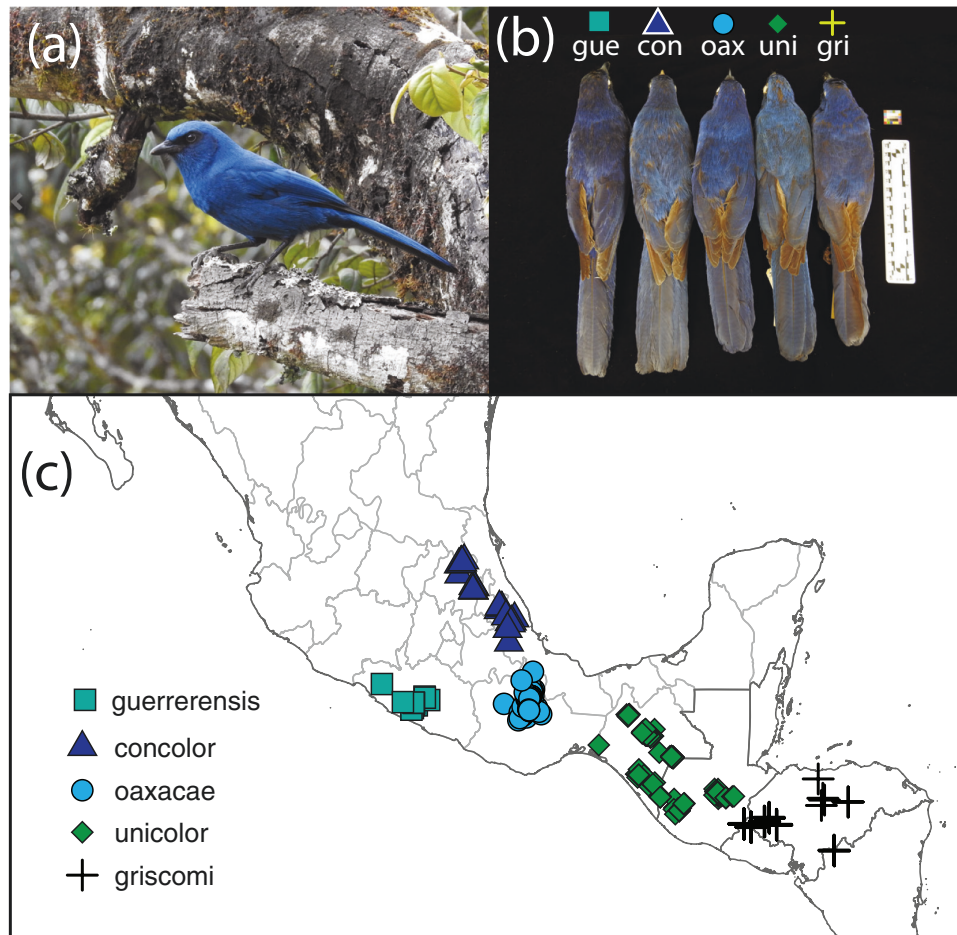


Figure 1. A, a unicolored jay (*Aphelocoma unicolor unicolor*) from Reserva de Biósfera Sierra de las Minas, Guatemala (Macauley Library ML85163771, photograph by Daniel Aldana). B, specimens representing *A. u. guerrerensis* (MLZ 45972), *A. u. concolor* (NMNH A9096), *A. u. oaxacae* (MLZ 33558), *A. u. unicolor* (MLZ 45360) and *A. u. griscomi* (AMNH 327521). C, distribution map of *A. unicolor* subspecies drawn from eBird observations.

We also conducted a discriminant function analysis in Stata v.11.2 using the seven morphological traits and the three plumage PC axes. We also used a normal mixture modelling approach to phenotypic clustering to identify phenotypic clusters objectively within multivariate space (McLachlan & Peel, 2000). Normal mixture models predict the number of unique phenotypic groups without a priori assumptions about species limits. We adapted an R script from Cadena *et al.* (2017) that uses the R package mclust v.5.0 (Scrucca *et al.*, 2016) for model selection, giving probabilities for the presence of any number of objective phenotypic groups present in multivariate space. We ran model selection based on only the two most explanatory PC axes, to avoid overfitting of outliers owing to limited sample size within subspecies. After finding that lineages on different sides of the Isthmus were reciprocally monophyletic in DNA (see Results below), we conducted normal mixture models on each side of the

Isthmus separately to restrict the delimitation analysis to the most closely related lineages. Overlap in phenotypic space by more distantly related lineages was assumed to be caused by convergence.

GENETIC DIFFERENTIATION

We created a phylogeny of unicolored jays using target enrichment of thousands of ultraconserved elements (UCEs) for 12 individuals representing the five subspecies (Table 1) and rooted with a Florida scrub jay (*Aphelocoma coerulescens*) outgroup. Data collection took place early in our effort to establish a pipeline for obtaining UCEs from museum specimens and before a later publication outlining best practices (McCormack *et al.*, 2016). Thus, a major limiting factor for sample inclusion was our ability to obtain high-quality results from older museum specimens. For the three subspecies west of the Isthmus of Tehuantepec for which

Table 1. Specimens used for genetic analysis

Museum	Specimen number	Subspecies	Location	Mitochondrial DNA	Ultraconserved elements
FMNH	394008	<i>A. u. concolor</i>	Tlanchinol, Hidalgo, Mexico	x	x
FMNH	343729	<i>A. u. concolor</i>	Tlanchinol, Hidalgo, Mexico	x	x
FMNH	343590	<i>A. u. guerrerensis</i>	Sierra de Atoyac, Guerrero, Mexico	x	x
FMNH	343593	<i>A. u. guerrerensis</i>	Sierra de Atoyac, Guerrero, Mexico	x	x
FMNH	343592	<i>A. u. guerrerensis</i>	Sierra de Atoyac, Guerrero, Mexico	x	x
FMNH	343591	<i>A. u. guerrerensis</i>	Sierra de Atoyac, Guerrero, Mexico	x	x
FMNH	346831	<i>A. u. oaxacae</i>	Nudo de Zempoaltepetl, Oaxaca, Mexico	x	x
FMNH	343597	<i>A. u. oaxacae</i>	Cerro de Zempoaltepetl, Oaxaca, Mexico	x	x
KU	45892	<i>A. u. oaxacae</i>	Vista Hermosa, Oaxaca, Mexico	x	
KU	45893	<i>A. u. oaxacae</i>	Vista Hermosa, Oaxaca, Mexico	x	
FMNH	393749	<i>A. u. oaxacae</i>	Cerro de Zempoaltepetl, Oaxaca, Mexico	x	x
FMNH	393681	<i>A. u. oaxacae</i>	Cerro de Zempoaltepetl, Oaxaca, Mexico	x	x
MLZ	16791	<i>A. u. griscomi</i>	El Chorro, Ocotepeque, Honduras	x	x
MLZ	16813	<i>A. u. griscomi</i>	El Sillon, Ocotepeque, Honduras	x	
MLZ	56736	<i>A. u. unicolor</i>	5 miles SW Ciudad Las Casas, Chiapas, Mexico	x	x
MLZ	56738	<i>A. u. unicolor</i>	5 miles SW Ciudad Las Casas, Chiapas, Mexico	x	

FMNH, Field Museum of Natural History; KU, University of Kansas Biodiversity Institute and Natural History Museum; MLZ, Moore Laboratory of Zoology.

fresh tissue or modern museum specimens were available (*A. u. concolor*, *A. u. oaxacae* and *A. u. guerrerensis*), we were able to replicate sampling from a previous study (McCormack *et al.*, 2011) and add new samples. However, for the two subspecies east of the Isthmus (*A. u. unicolor* and *A. u. griscomi*), for which we had to rely on DNA from older museum specimens, we obtained high-quality UCE data from only one individual per subspecies.

We obtained mtDNA data from 16 individuals from off-target by-catch of the UCE protocol, including some of the individuals where UCE data were not obtained. We extracted data for the cytochrome *b* (*Cytb*) gene because this gene has the best estimates for its molecular

clock rate across birds (Weir & Schluter, 2008). We aligned mtDNA reads for *Cytb* in Geneious v.8.1.9 and analysed alignments in BEAST using a strict molecular clock and 1.105% (range: 0.765–1.445%) as the estimate of the divergence rate based on biogeographical calibrations for this gene in birds (Weir & Schluter, 2008).

Detailed methods for capturing and sequencing UCEs are found elsewhere (Faircloth *et al.*, 2012; McCormack *et al.*, 2016). In brief, we extracted genomic DNA from fresh tissue or from toe pads of museum specimens using a Qiagen (Valencia, CA, USA) DNeasy Blood and Tissue extraction kit. For fresh specimens, we sheared DNA to a size distribution with its peak between 400 and

600 bp using a Bioruptor ultrasonicator (Diagenode). All historical samples produced DNA fragments smaller than this. We prepared libraries for each sheared sample with a KAPA (Boston, MA, USA) LTP library preparation kit for the Illumina platform, attaching custom indexing tags (Faircloth & Glenn, 2012) to allow sample pooling. We enriched samples for UCEs using a set of synthetic RNA probes that target 5060 tetrapod UCEs (MYbaits_Tetrapods-UCE-5K kit; Mycroarray), following the standard enrichment protocol (Faircloth *et al.*, 2012). After enrichment and recovery PCR, we verified the library size range with an Agilent 2100 Bioanalyzer (Palo Alto, CA, USA). We quantified the enriched pools using qPCR and combined them in equimolar ratios before sequencing on an Illumina HiSeq 2000 lane (100 bp paired-end cycle) at the University of California Santa Cruz Genome Technology Center.

After sequencing, we sorted the raw reads by sample computationally, conducted quality filtering, and trimmed reads of adaptor sequence using illumiprocessor (Faircloth, 2013). We assembled these reads into contigs with ABySS (Simpson *et al.*, 2009), built into the PHYLUCE pipeline (Faircloth, 2015), and identified UCE contigs by matching all contigs to the original probe sequences with LASTZ (Harris, 2007). We aligned UCEs for each locus using MAFFT (Katoh *et al.*, 2002) and created a concatenated matrix, retaining all loci with data for 75% of the samples. We constructed a maximum likelihood (ML) tree in RAxML v.8.0.19 (Stamatakis, 2014) under the GTRGAMMA model of evolution with 100 bootstrap searches, followed by a search for the tree with the highest likelihood.

NICHE DIFFERENTIATION

GPS locations and environmental data layers

We obtained occurrence points for each subspecies from georeferenced museum skins and from recent eBird records (as of 2012). For historical museum specimen localities that are now urban areas, we adjusted localities to nearby forested areas. We obtained environmental data using five remote-sensing layers representing vegetation variables and ten climate variables (Supporting Information, Table S1). The climate variables came from the WorldClim database (<http://www.worldclim.org>). These variables describe temperature and precipitation. We used a subset of ten of the 19 total WorldClim layers after removing nine layers owing to high correlation ($r > 0.90$) among layers. The remote-sensing layers were derived from satellite images and a radar scatterometer (Supporting Information, Table S1). Raw data were processed to generate the following five variables: (1) normalized difference vegetation index (NDVI), which describes vegetation greenness; (2) NDVI annual standard deviation (NDVI_std), which describes greenness seasonality; (3) percentage tree

cover (Tree) from MODIS; (4) canopy height from the NASA Ice, Cloud, and Land Elevation (ICESat) satellite Geoscience Laser Altimeter System (GLAS) instrument (Simard *et al.*, 2011); and (5) a variable from The Quick Scatterometer data (QSCAT), which relates to canopy structure and moisture content (Frolking *et al.*, 2006). None of the remote-sensing layers was highly correlated with one another or with the WorldClim layers.

Assessing niche divergence and conservatism

To assess niche differences, our general approach was one of comparing occupied niche space for each subspecies with respect to general differences in environmental parameters among subspecies. These kinds of comparisons help to control for the fact that environmental data will always differ for allopatric lineages, making tests for environmental differences trivial unless compared with background environmental differences (Broennimann *et al.*, 2007; Warren *et al.*, 2008, 2014; McCormack *et al.*, 2010). To carry out these tests, we first created species distribution models using the program Maxent v.3.3.3k (Phillips & Dudík, 2008). The models were created with a random 80% of the data and then tested with the remaining 20% of the data (Pearson *et al.*, 2007). We visualized species distributions in ArcMap v.10.1 and used the lowest probability value of an actual occurrence point as our lower threshold for habitat suitability. We then drew polygons enclosing each subspecies model and extracted 1000 random points from within each polygon. We then extracted data for the 15 environmental variables for each *A. unicolor* occurrence point and for all the random background points. We reduced these data to a few axes of variation (i.e. multidimensional niche space) with PCA.

We assessed differences in two ways: (1) using box plots and *t*-tests of univariate and multivariate niche traits; and (2) using a test for strong niche divergence against a null model of background divergence. To implement (2), we used the method outlined by McCormack *et al.* (2010), with modifications by Zellmer *et al.* (2012). This method compares the difference in pairwise means in multidimensional niche space with expected background means calculated from random points. Niches are considered divergent when they are more different than 98% of the null distribution, and they are considered conserved when they are more similar than 98% of the null distribution. If the observed niche difference lies within the null distribution, then it is difficult to say whether niches are divergent or whether they are merely caused by spatial autocorrelation in environmental data. We conducted these tests in the context of the phylogeny, i.e. between sister lineages and sister clades. This allowed us to determine whether there was a relationship between time and niche divergence.

RESULTS

PHENOTYPIC DIFFERENTIATION

Raw data for phenotypic and niche analyses and DNA tree files are available on Dryad at <https://doi.org/10.5061/dryad.9k58f56>. Box plots of individual morphological variables show that the three subspecies west of the Isthmus are distinct from each other, more so than the two subspecies east of the Isthmus (Fig. 2). *Aphelocoma unicolor guerrensis* is especially distinctive, with a tail that is much longer than other subspecies and a large bill more similar to subspecies on the other side of the Isthmus, although even larger. The geographically proximate subspecies *A. u. oaxacae* and *A. u. concolor* are similar to each other in many traits, but *A. u. oaxacae* can be largely distinguished by having a shorter tail (mean $\pm$ SE: *A. u. oaxacae* = 165.9 ± 1.12 mm, $N = 27$; *A. u. concolor* = 177.2 ± 1.44 mm, $N = 11$; t -test $P < 0.001$) and tarsus (mean $\pm$ SE: *A. u. oaxacae* = 38.0 ± 0.22 mm, $N = 28$; *A. u. concolor* = 40.5 ± 0.18 mm, $N = 11$; t -test $P < 0.001$). The subspecies east of the Isthmus, *A. u. unicolor* and *A. u. griscomi*, are similar in many

traits but differ in bill size, with *A. u. griscomi* having a generally longer bill (mean $\pm$ SE: *A. u. griscomi* = 21.6 ± 0.15 mm, $N = 37$; *A. u. unicolor* = 20.1 ± 0.12 mm, $N = 82$; t -test $P < 0.001$) that is also wider (mean $\pm$ SE: *A. u. griscomi* = 9.8 ± 0.07 mm, $N = 37$; *A. u. unicolor* = 9.2 ± 0.06 mm, $N = 82$; t -test $P < 0.001$) and deeper (mean $\pm$ SE: *A. u. griscomi* = 10.6 ± 0.07 mm, $N = 37$; *A. u. unicolor* = 10.1 ± 0.05 mm, $N = 81$; t -test $P < 0.001$).

The PCA on raw spectral values resulted in three PC axes that corresponded to brightness, hue and chroma (Supporting Information, Table S2). A plot of hue vs. chroma shows that subspecies east and west of the Isthmus are largely segregated, except for a handful of *A. u. griscomi* individuals (Fig. 3). For the subspecies west of the Isthmus, *A. u. concolor* is distinct from *A. u. oaxacae* and *A. u. guerrensis*, and the latter two subspecies also show some separation.

The multivariate discriminant function analysis including all morphological and plumage variables was able to classify members of the three subspecies west of the Isthmus to their own groups with 100% accuracy (Table 2). Phenotypic traits associated with these DF axes are reported in the Supporting Information

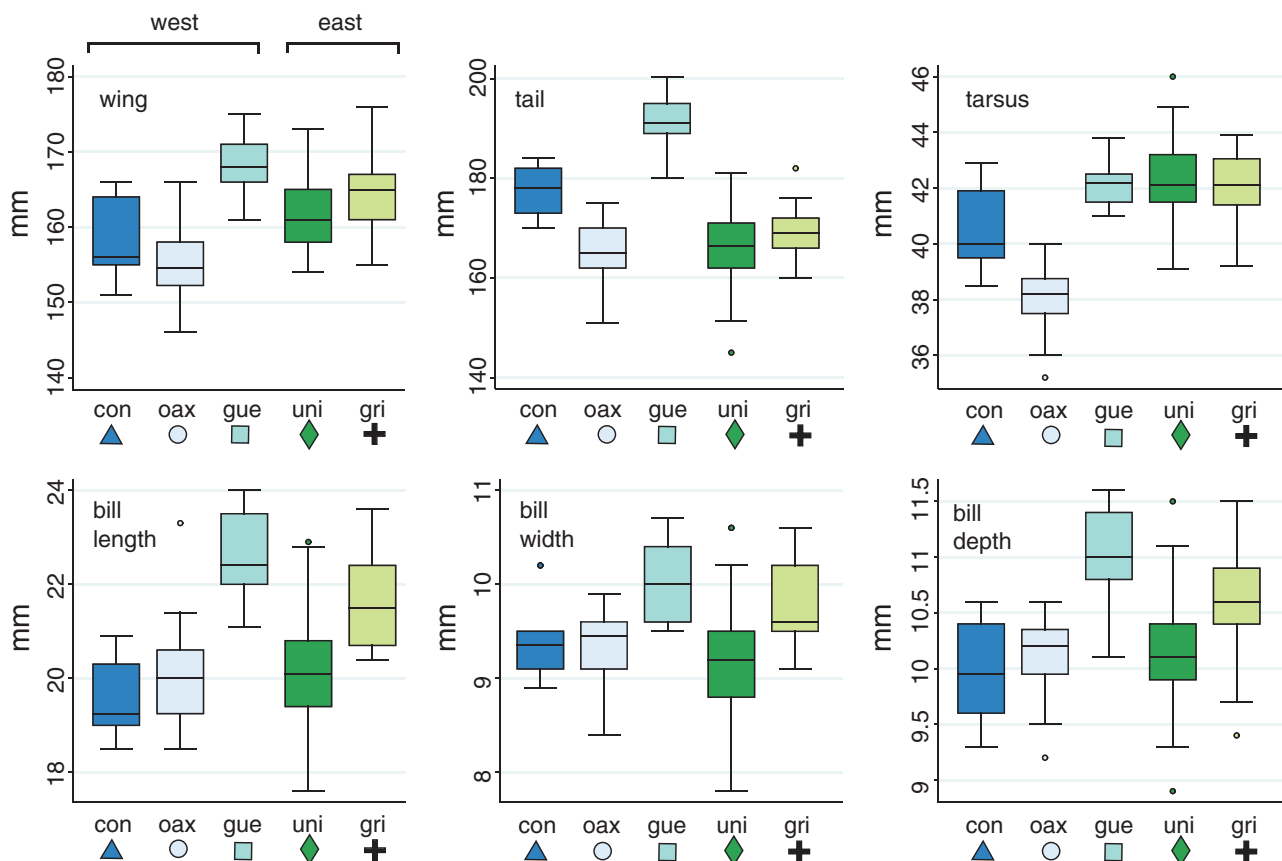


Figure 2. Differences among the five *Aphelocoma unicolor* subspecies for six morphological traits (measured in millimetres).

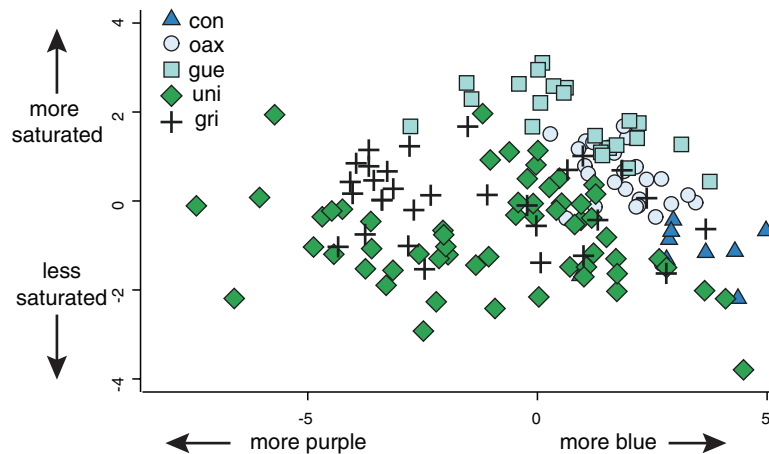


Figure 3. Scatterplot of hue and colour saturation (chroma) for the five *Aphelocoma unicolor* subspecies.

Table 2. Results of a discriminant function analysis using all morphological and plumage colour variables

Subspecies	Percentage (number) classified correctly				
	<i>A. u. concolor</i>	<i>A. u. oaxaca</i>	<i>A. u. guerrensis</i>	<i>A. u. unicolor</i>	<i>A. u. griscomi</i>
<i>A. u. concolor</i>	100 (8)	0	0	0	0
<i>A. u. oaxaca</i>	0	100 (27)	0	0	0
<i>A. u. guerrensis</i>	0	0	100 (22)	0	0
<i>A. u. unicolor</i>	0	2 (1)	0	83 (48)	16 (9)
<i>A. u. griscomi</i>	0	3 (1)	3 (1)	3 (1)	90 (29)

(Table S3). East of the Isthmus, *A. u. unicolor* and *A. u. griscomi* could be classified correctly 80–90% of the time (Table 2), with most of the misclassified *A. u. unicolor* being grouped with *A. u. griscomi*. Plotting discriminant function analysis scores shows clear separation of *A. u. concolor*, *A. u. guerrensis*, *A. u. oaxaca* and *A. u. unicolor/griscomi* in discriminant function DF1 and DF2 (Fig. 4A). Plotting DF3 and DF4, *A. u. unicolor* and *A. u. griscomi* are largely segregated, with an area of overlap (Fig. 4B). Normal mixture model results supported three morphogroups west of the Isthmus (Fig. 4C) and one east of the Isthmus (Fig. 4D), with the assignment of individuals corresponding with 98% accuracy to current subspecies boundaries west of the Isthmus.

GENETIC DIFFERENTIATION

The UCE matrix contained 2933 loci and 1549943 bp. The UCE topology was strongly supported and showed that lineages on either side of the Isthmus of Tehuantepec are reciprocally monophyletic and that *A. u. concolor*, *A. u. oaxaca* and *A. u. guerrensis* form clades (Fig. 5). Single samples of *A. u. griscomi* and *A. u. unicolor* are sister to one another. The *Cytb* mtDNA tree supports the same relationships

among subspecies (Fig. 5). For mtDNA, we were able to include two individuals of both *A. u. unicolor* and *A. u. griscomi*, which supported each as a clade. Divergence dates within unicolored jays stretched from as old as 3.9 Mya for the cross-Isthmus clades to as young as 600 000 years for the split between *A. u. guerrensis* and *A. u. oaxaca*.

NICHE DIVERGENCE AND CONSERVATISM

We first looked for differences in univariate niche traits between the cross-Isthmus clades by pooling data for subspecies on either side. Comparing side-by-side plots of occupied niche space with available environmental space revealed that both cross-Isthmus clades (i.e. all unicolored jays) prefer niches with cooler temperatures, higher canopy and more tree cover than what is generally available in their geographical areas (Supporting Information, Fig. S1). This lends credence to the idea that these environmental variables are related to important aspects of the *A. unicolor* niche. We then used PCA to reduce the large number of environmental variables to a more manageable set of multivariate niche axes. Six PCs had an eigenvalue at or near one and together explained 84% of the total variance in environment (Supporting Information,

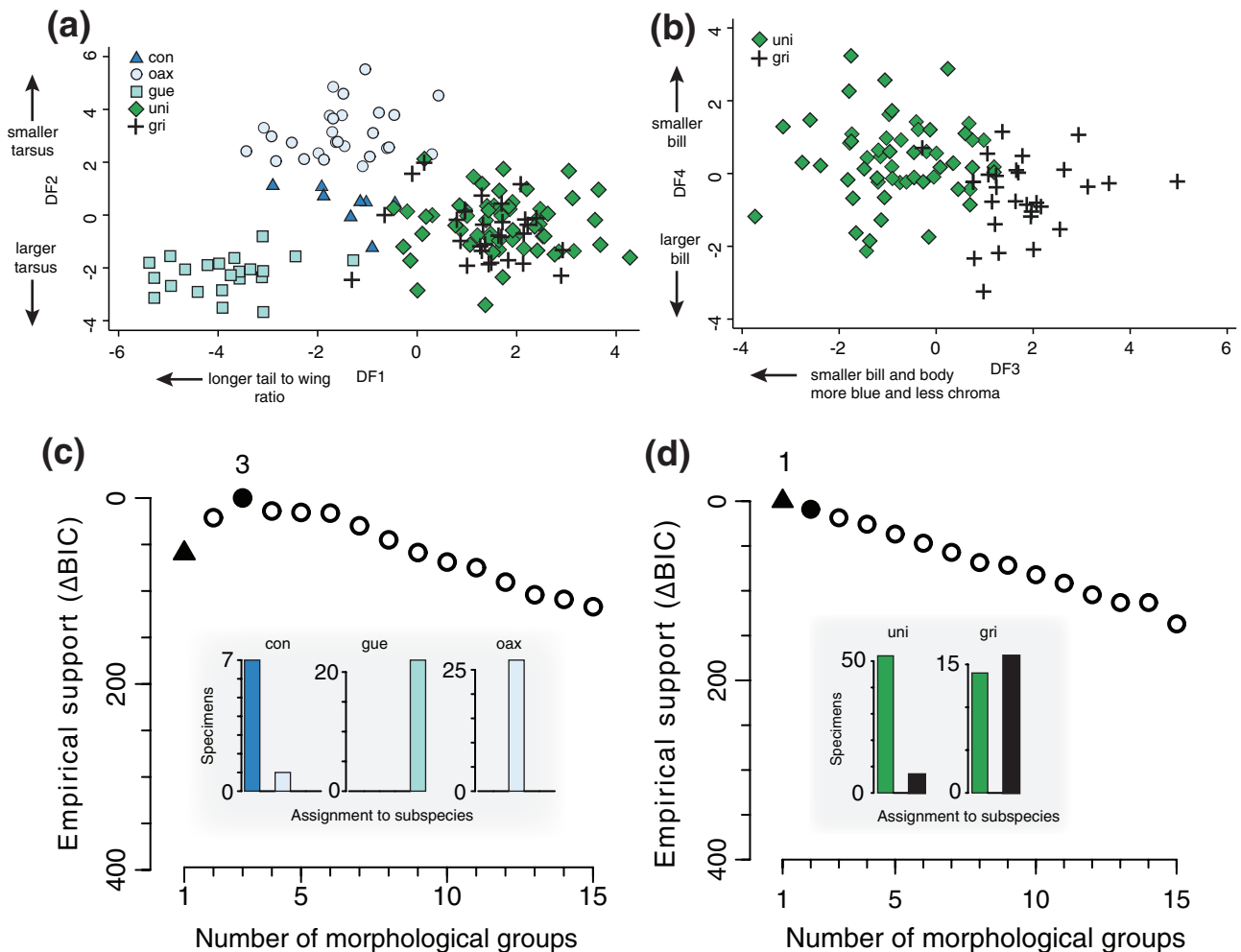


Figure 4. Results of a discriminant function (DF) analysis and normal mixture models on all morphological and plumage traits. A, differences among all five *Aphelocoma unicolor* subspecies in the first two DF axes. B, differences between only the *A. u. unicolor* and *A. u. griscomi* subspecies in the third and fourth DF axes. C, D, results of normal mixture modelling to determine the objective number of phenotypic clusters among individuals west (C) and east (D) of the Isthmus of Tehuantepec, with inset showing the assignment of individuals to each cluster with respect to their a priori subspecies assignment.

Table S4). Results of tests against a null model of background divergence on each of the six multivariate niche axes suggest increasing niche divergence with increasing phylogenetic distance (Fig. 6).

DISCUSSION

EXTRAORDINARY DIVERGENCE AND BIOGEOGRAPHICAL IMPLICATIONS

Results from integrative taxonomy show that *A. unicolor* represents a case of extraordinary divergence for a bird in the Mesoamerican cloud forests. All available genetic evidence supports monophyly for each of the five allopatric *A. unicolor* subspecies. A calibrated mtDNA tree suggests long histories of divergence for

each subspecies, dating from as recently as the mid-Pleistocene (900 000 years ago for the split between *A. u. guerrensis* and *A. u. oaxacae*) to as old as the Pliocene (2.4 Mya for the split between *A. u. concolor* and *A. u. oaxacae* + *guerrensis*). Although few individuals were sampled per lineage, the long stem branches for each lineage and the apparent lack of contact between at least four of the five lineages (*A. u. concolor*, *A. u. guerrensis*, *A. u. oaxacae* and *A. u. unicolor* + *griscomi*) suggest that monophyly will probably stand up to denser sampling.

Divergence dates help to place *A. unicolor* divergences in the context of the biogeography of the region. The deepest split in *A. unicolor*, probably ~3.3 Mya, but perhaps as old as 3.9 Mya, separates clades on either side of the Isthmus of Tehuantepec. This makes

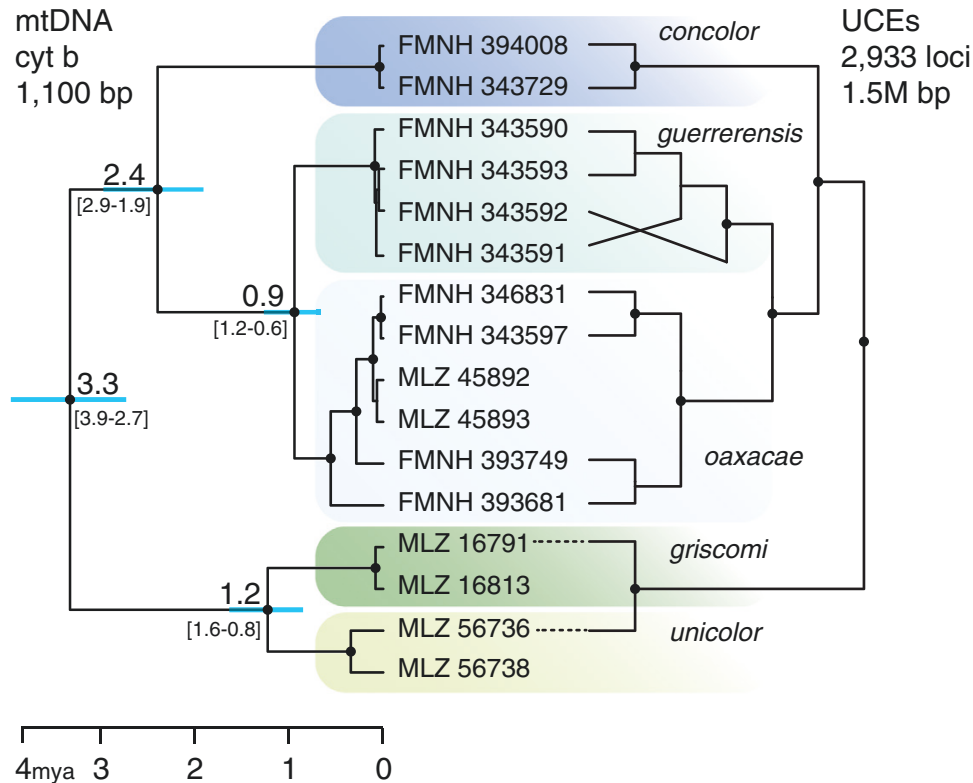


Figure 5. Phylogenies of *Aphelocoma unicolor* based on mitochondrial DNA and ultraconserved elements (UCEs). For the Bayesian time-calibrated mitochondrial DNA phylogeny generated in BEAST, the mean estimated split dates are provided on the nodes, with the 95% highest probability density shown below in square brackets. For both phylogenies, nodes with perfect support are shown with black dots.

A. unicolor the bird with the oldest known divergence time across the Isthmus, comparable to but older than those found in the hepatic tanager (*Piranga flava*) and slate-throated redstart (*Myioborus miniatus*) (Barber & Klicka, 2010). Divergence across the Isthmus in *A. unicolor* was on the very early side of the first of two ‘pulses’ of diversification (*sensu* Barber & Klicka, 2010) resulting either from the original formation of this geographical barrier by tectonic uplift in the Miocene (Barrier *et al.*, 1998) or from somewhat later marine incursions in the Pliocene. The unusually old divergence times found in *A. unicolor* almost certainly relate to its low dispersal rates, which follow from its cooperative breeding behaviour, in which young stay close to their natal territories (Webber & Brown, 1994).

Similar to plant and reptile divergences (Castoe *et al.*, 2009; Gutiérrez-Rodríguez *et al.*, 2011; Ornelas *et al.*, 2013), where a lack of gene flow means that the original divergence history stands a better chance of being preserved, *A. unicolor* can potentially provide a window into the original vicariance history of Mesoamerican cloud forests. As discussed above, the phylogenetic history within *A. unicolor* suggests that a single Pliocene divergence event, either related to

uplift or to marine incursion, led to the cross-Isthmus distribution. On the western side, populations in the Sierra Madre Oriental split from those in the Oaxacan Highlands and Sierra Madre del Sur at the onset of glacial cycling in the late Pliocene or early Pleistocene (2.4 ± 0.5 Mya), with the Oaxacan Highlands and Sierra Madre del Sur populations remaining connected until the mid-Pleistocene (0.9 ± 0.3 Mya). On the eastern side, a split between populations in mountainous areas on either side of the Río Motagua (Chiapas and Guatemala vs. Honduras, also known as the Motagua-Polochic Faults) also occurred during the period of glacial cycles in the mid-Pleistocene (1.2 ± 0.4 Mya), much more recently than various snakes species, which diverged across this barrier between 3 and 8 Mya (Castoe *et al.*, 2009; Daza *et al.*, 2010). In sum, divergences in *A. unicolor* seem to have been influenced largely by habitat redistribution during glacial cycles, but also possibly by tectonic uplift for the earliest split.

Few bird species have been well sampled across multiple cloud forest patches in the region to compare with *A. unicolor*, but among those that have, there is little concordance in the timing of divergences or the

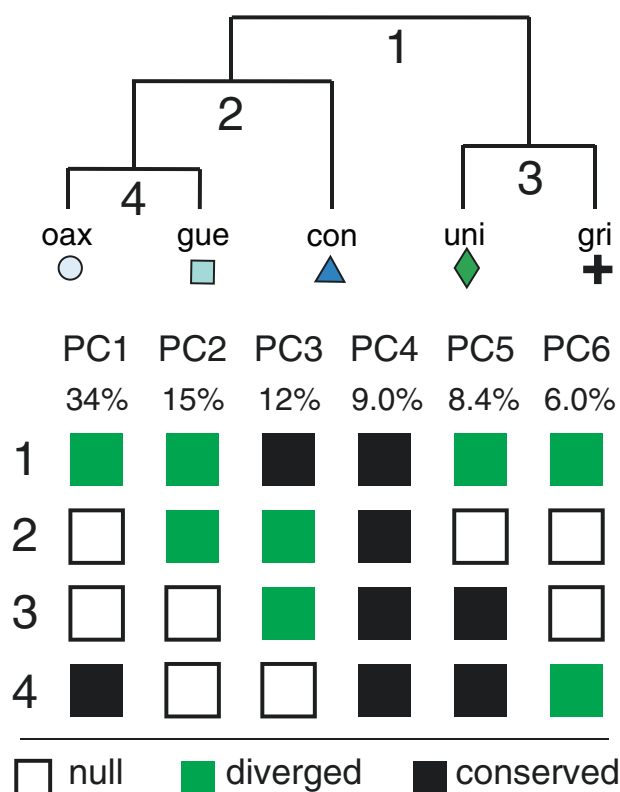


Figure 6. Results of tests for strong niche divergence on multivariate niche axis in relationship to the phylogeny. Boxes show whether each niche axis was more divergent than background divergence (diverged), more similar than background divergence (conserved), or was similar to background divergence and therefore failed to reject the null hypothesis (null). Percentages indicate the amount of variation explained by that axis.

relationships of the geographical areas. The common chlorospingus, formerly the common bush-tanager (*Chlorospingus flavopectus*, formerly *Chlorospingus ophthalmicus*), has monophyletic lineages in the same cloud forest patches (García-Moreno *et al.*, 2004; Bonaccorso *et al.*, 2008; Maldonado-Sánchez *et al.*, 2016); however, the relationships among these lineages are not well resolved, and the divergence times are mostly younger, dating to the Pleistocene (Maldonado-Sánchez *et al.*, 2016). The northern emerald toucanet (*Aulacorhynchus prasinus*) also has monophyletic lineages in similar cloud forest patches (Navarro-Sigüenza *et al.*, 2001; Puebla-Olivares *et al.*, 2008; Bonaccorso *et al.*, 2011). There is no calibrated tree for this species, but sequence divergence ranges up to ~3.7%, meaning that divergences are unlikely to be much older than the Pleistocene. In the northern emerald toucanet, there is evidence for more than one divergence event across the Isthmus and a leapfrog pattern among forest patches. Whatever the original scenario was for the

break-up of Mesoamerican cloud forests, species, even low-dispersing ones, appear to have responded to it idiosyncratically, and dispersal appears to have played at least as large a role as strict vicariance. This pattern has also been found in South American species as they responded to the uplift of the Andes and formation of the Amazon River Basin (Burney & Brumfield, 2009; Smith *et al.*, 2014).

Aphelocoma unicolor also seems extraordinary in its level of niche divergence. A classic comparative study by Peterson *et al.* (1999) found that niche conservatism is the general rule between taxa found on either side of the Isthmus. It is no surprise that we detected broad aspects of niche conservatism in our environmental data, in the sense that all *A. unicolor* lineages preferred areas within their ranges associated with cloud forests, e.g. cooler temperatures, higher canopy and more tree cover (Supporting Information, Fig. S1). More detailed analyses across multidimensional niche axes, however, suggest that cross-Isthmus *A. unicolor* clades have diverged ecologically over ~3 Myr and that niche divergence has increased with time and with increasing phylogenetic distance (Fig. 6). We cannot say at the moment how these broad-scale niche differences might translate to differential selection pressures, but the situation could be similar to other cases of niche differentiation in *Aphelocoma*. For example, the California scrub jay (*Aphelocoma californica*) and Woodhouse's scrub jay (*Aphelocoma woodhouseii*) occupy different types of a more general 'scrub' habitat that has resulted in divergent selection pressures based on food resources and associated bill adaptations (Peterson, 1993; Bardwell *et al.*, 2001; Gowen *et al.*, 2014). Field studies assessing resource abundance and use would be needed to test whether the detected niche divergence might translate to selective pressures and how these might relate to observed phenotypic variation in *A. unicolor*.

NEW INSIGHTS INTO PHENOTYPIC VARIATION

Pitelka (1951) conducted a major phenotypic analysis of the genus *Aphelocoma*, but he was limited by lack of specimens for some *A. unicolor* groups (e.g. Pitelka, 1946) and by the methodological toolkit available to him. Although he noted several of the same patterns we found in univariate traits (e.g. the long tail of *A. u. guerrensis*), at the time of his monograph multivariate statistics were not well developed and incorporated into zoological studies, and analysis of plumage colour was still carried out qualitatively using the colour nomenclature of Ridgway (1912).

New specimens and objective multivariate clustering methods extend Pitelka's work and suggest that there are at least four diagnosable phenotypic groups

within *A. unicolor*. The most distinctive of these is *A. u. guerrensis*, with its long tail and purplish plumage. Each of the three lineages west of the Isthmus is markedly different from one another: *A. u. guerrensis* mentioned above; *A. u. concolor* with its pale blue plumage; and *A. u. oaxacae* with its darker blue plumage and relatively short wing, tail and tarsus. The least diagnosable subspecies (*A. u. unicolor* and *A. u. griscomi*) are still correctly classified between 80 and 90% of the time. The reduced diagnosability could be attributable to their higher variance in phenotypic traits compared with other subspecies. This higher variance was not caused by low sample size, because *A. u. unicolor* had the largest sample size of any subspecies, but instead appeared to relate to significant geographical variation within each group or greater measurement error in plumage colour.

Within *A. u. griscomi*, geographical differentiation was especially pronounced, with 100% of individuals being classifiable to four sampling regions in Honduras and El Salvador (Supporting Information, Fig. S2). This is impressive considering that our sampling of *A. u. griscomi* covers barely half of its range, because it is likely to occur to the north and east of where Pitelka set its range limits (Chavarría & Batchelder, 2012). Future work should focus on sampling from these areas for both phenotypic and genetic diversity, and in other areas that might be inhabitable by unicolored jays, but where they are not currently documented.

Our phenotypic results also show how the consideration of colour as a multivariate trait involving both hue and saturation can lead to greater insight than what the eye alone can discern (Cuthill, 2006). Pitelka (1951) was vexed by a leapfrog pattern in plumage coloration, clear to the naked eye (Fig. 1B), where subspecies (*A. u. concolor* and *A. u. unicolor*) on different sides of the Isthmus appear more similar in plumage colour. Yet, when colour is considered on multivariate rather than univariate axes, as in Fig. 3, the leapfrog pattern disappears. One way to group the individuals plotted for colour hue and colour saturation might be as Pitelka did, grouping individuals in the upper left (more purple, more saturated) and lower right (more blue, less saturated) of the plot in Fig. 3. The split across the Isthmus, however, occurs along an orthogonal axis, with similarities between cross-Isthmus lineages likely to be the result of later convergence into similar parts of colour space.

INTEGRATIVE TAXONOMY AND SPECIES LIMITS

Taxonomy is most challenging when lineages are on the speciation continuum (Roux *et al.*, 2016), i.e. in the process of speciation. There is still wide disagreement on exactly when a species becomes a species and what

criteria we should use to identify species. Although our species concepts might not have improved much through time, our ability to collect data has. One benefit of integrative taxonomy is that it approaches cases in the speciation continuum from multiple perspectives, assembling data that can address criteria key to any number of species concepts (Dayrat, 2005; Padial *et al.*, 2010; Schlick-Steiner *et al.*, 2010). In this way, integrative taxonomy is in conceptual harmony with the evolutionary (or lineage) species concept (De Queiroz, 2007), which considers species as lineages on independent evolutionary trajectories, with operational criteria of other species concepts being the mileposts these lineages pass along the way to speciation.

One early milepost that all five *A. unicolor* subspecies seem to have passed is genetic monophyly, the key criterion of the phylogenetic species concept. They have been isolated long enough, with apparently no gene flow, to have developed a large number of unique substitutions. Addressing a later milepost on the speciation continuum, it is not clear to what extent these lineages have achieved reproductive isolation, the key criterion of the biological species concept. The ability to interbreed is impossible to test among allospecies, in vertebrates anyway. Reproductive isolation can arise from prezygotic and postzygotic mechanisms, and both can involve divergence in the ecological niche, morphology and plumage colour (Coyne & Orr, 2004; Edwards *et al.*, 2005; Price, 2008). Prior research suggests that premating mechanisms are key to reproductive isolation in birds, which retain an ability to hybridize across species limits long after species in other taxonomic groups (Price & Bouvier, 2002). Thus, although we cannot determine reproductive isolation directly, we can assess phenotypic and niche divergence as a proxy for the plausibility of premating isolation.

In assessing species limits in *A. unicolor*, a convenient yardstick with which to compare divergence is other cases of recently recognized species in *Aphelocoma*. The trans-volcanic jay (*Aphelocoma ultramarina*) was recently split from the Mexican jay (*Aphelocoma wollweberi*) based on ~5 Myr divergence, phenotypic diagnosability, evidence for ancient (not modern) gene flow, but little evidence for niche divergence (McCormack *et al.*, 2008, 2010, 2011; McCormack & Venkatraman, 2013). The California scrub jay (*A. californica*) and Woodhouse's scrub jay (*A. woodhousei*) were also recently split based on more recent divergence (~2 Myr), phenotypic diagnosability, significant niche divergence (with a plausible adaptive corollary), and evidence for reduced (but non-trivial) gene exchange over a narrow contact zone (McCormack *et al.*, 2011; Gowen *et al.*, 2014).

Based on these comparisons, the cross-Isthmus clades of *A. unicolor* are clear candidates for species

rank because they are as old as other species-level splits within *Aphelocoma*, phenotypically distinct and divergent in their niches. Within the west clade, *A. u. concolor* makes a strong case for species rank with its ~2 Myr of divergence, phenotypic differentiation from its sister clade (*A. u. oaxacae* + *guerrerensis*) and moderate evidence for niche divergence. At ~900 000 years old, *A. u. guerrenensis* is not as divergent from its sister lineage *A. u. oaxacae* as other recent *Aphelocoma* species-level splits, but its striking phenotypic differentiation is not only evolutionarily interesting (perhaps related to its small population size), but also makes a compelling case for the plausibility of premating isolation. Finally, the apparent overlap in some phenotypic traits, lack of firm knowledge about the full extent of variation within lineages, and the lack of evidence for niche divergence weighs towards keeping *A. u. unicolor* and *A. u. griscomi* within a single species. Divergence in vocalizations would be an excellent addition to this integrative data set, but there are currently not enough samples in existing repositories to address this question quantitatively. We recommend four species as below.

***APHELOCOMA UNICOLOR* DU BUS, 1847**

Type. An unsexed specimen in the Brussels Museum labelled from Tabasco; however, this species does not occur in the state of Tabasco (van Rossem, 1942). The type locality was later designated as Ciudad de las Casas, Chiapas (Brodkorb, 1944). The nominate subspecies, *A. u. unicolor*, occurs in the highlands of Chiapas, Mexico and Guatemala. The larger-billed and generally more purplish *A. u. griscomi* is found in the highlands of Honduras, El Salvador, and northern Nicaragua (Pitelka, 1946).

***APHELOCOMA CONCOLOR* CASSIN, 1848**

Type. An unsexed specimen, ANSP:Bird:3039 labelled S. America, later determined probably to have originated near Xalapa, Veracruz (Phillips, 1986). This species is brighter blue than *A. u. oaxacae* and *A. u. guerrenensis* and has a larger wing and tail than *A. u. oaxacae*. It is found in cloud forests from Hidalgo south to Veracruz, Mexico (Pitelka 1946).

***APHELOCOMA OAXACAE* PITELKA, 1946**

Type. Adult female, MLZ:Bird:39121, collected by M. del Toro Avilés from Moctum, Oaxaca, Mexico on 18 October 1941. This species is smaller and is restricted to the state of Oaxaca, Mexico (Pitelka, 1946).

***APHELOCOMA GUERRERENSIS* NELSON, 1903**

Type. Adult male, USNM:Bird:185539 collected by E. W. Nelson and E. A. Goldman from Omiltemi, Guerrero on 19 May 1903. This species is a saturated, purplish blue and has a very long tail and large bill. It is endemic to the cloud forests of Guerrero, Mexico (Pitelka, 1946).

CONSERVATION IMPLICATIONS

Cloud forests are among the most biodiverse habitats in the Americas, especially considering the small land area they cover. Our study highlights the impressive biodiversity of Mesoamerican cloud forests, especially in their capacity to generate divergence at or near the species level across a relatively small geographical area. Given that *A. unicolor* is probably more similar to reptiles and amphibians in its dispersal patterns, it would be fruitful to re-evaluate widely distributed, low-dispersing cloud forest species for genetic and phenotypic divergence (e.g. Rovito *et al.*, 2015; Hofmann & Townsend, 2017; Solano-Zavaleta & de Oca, 2018). *Aphelocoma unicolor*, like many cloud forest species, appears to be dwindling in parts of its former range, especially in Veracruz in the heavily degraded forests around Xalapa (*A. u. concolor*) and in the eastern part of the range of *A. u. guerrenensis* around the former collecting site of Omiltemi, Guerrero. The entire known geographical range of *A. u. guerrenensis* occurs over a very small portion of the Sierra Madre del Sur, perhaps only 100 km × 40 km. Although there will never be perfect agreement over species limits for cases on the speciation continuum, integrative taxonomy should prove useful in Mesoamerican cloud forests, where the allopatric arrangement of populations frequently defies easy application of any one species concept or delimitation criterion. Even with lots of data, decisions made on the speciation continuum will always be controversial, but integrative taxonomy at least allows us to approach greater harmony between biodiversity and taxonomy.

ACKNOWLEDGEMENTS

For funding, we thank the Occidental College Undergraduate Research Center, Richter-ASP Fellowship, the Kurata Award, the Hayes Fund, the Moore Laboratory Endowment and the Borestone Foundation. National Science Foundation Grant NSF-DEB-1652979 supported J.E.M. during the writing of this manuscript. The following museums provided access to specimens: National Museum of Natural History (Jacob Saucier, Chris Milensky and Mercedes

Foster), Museum of Comparative Zoology (Jeremiah Trimble and Scott Edwards), Western Foundation of Vertebrate Zoology (Linnea Hall and René Corado), University of Kansas Biodiversity Institute (Town Peterson and Mark Robbins), University of Michigan Museum of Zoology (Janet Hinshaw), The Field Museum (Ben Marks and David Willard) and the American Museum of Natural History (Paul Sweet, Tom Trombone and Peter Capainolo). We also thank Brant Faircloth, Uma Dandekar from the sadly defunct UCLA Core Sequencing Facility, Josh Fisher (NASA-JPL), Fiona Gowen, Alex Broad, Jessie Salter, Chuck Oravec, Octavio Rojas and Claudio Mota (INECOL), Tom Webber, Daniel Aldana and Roberto Carlos Aleman. Dan Pondella, Gretchen North and two anonymous reviewers provided comments on the manuscript.

REFERENCES

- Aguilar C, Wood PL Jr, Belk MC, Duff MH, Sites JW Jr. 2016.** Different roads lead to Rome: integrative taxonomic approaches lead to the discovery of two new lizard lineages in the *Liolaemus montanus* group (Squamata: Liolaemidae). *Biological Journal of the Linnean Society* **120**: 448–467.
- Amadon D. 1966.** The superspecies concept. *Systematic Zoology* **15**: 245–249.
- Andersson S, Prager M. 2006.** Quantifying colors. In: Hill G, McGraw K, eds. *Bird coloration*, Vol. 1. Cambridge: Harvard University Press, 41–89.
- Andújar C, Pérez-González S, Arribas P, Zaballos JP, Vogler AP, Ribera I. 2017.** Speciation below ground: tempo and mode of diversification in a radiation of endogean ground beetles. *Molecular Ecology* **26**: 6053–6070.
- Barber B, Klicka J. 2010.** Two pulses of diversification across the Isthmus of Tehuantepec in a montane Mexican bird fauna. *Proceedings of the Royal Society B: Biological Sciences* **277**: 2675–2681.
- Bardwell E, Benkman CW, Gould WR. 2001.** Adaptive geographic variation in western scrub-jays. *Ecology* **82**: 2617–2627.
- Barrier E, Velasquillo L, Chavez M, Gaulon R. 1998.** Neotectonic evolution of the Isthmus of Tehuantepec (Southeastern Mexico). *Tectonophysics* **287**: 77–96.
- Bonaccorso E, Guayasamin JM, Peterson AT, Navarro-Sigüenza AG. 2011.** Molecular phylogeny and systematics of Neotropical toucanets in the genus *Aulacorhynchus* (Aves, Ramphastidae). *Zoologica Scripta* **40**: 336–349.
- Bonaccorso E, Navarro-Sigüenza AG, Sánchez-González LA, Townsend Peterson A, García-Moreno J. 2008.** Genetic differentiation of the *Chlorospingus ophthalmicus* complex in Mexico and Central America. *Journal of Avian Biology* **39**: 311–321.
- Brodkorb P. 1944.** The type localities of some Mexican birds of the genera *Aphelocoma*, *Cyanocitta*, and *Peucedramus*. *The Auk* **61**: 400–404.
- Broennimann O, Treier UA, Müller-Schärer H, Thuiller W, Peterson A, Guisan A. 2007.** Evidence of climatic niche shift during biological invasion. *Ecology Letters* **10**: 701–709.
- Burney CW, Brumfield RT. 2009.** Ecology predicts levels of genetic differentiation in Neotropical birds. *The American Naturalist* **174**: 358–368.
- Cadena CD, Zapata F, Jiménez I. 2017.** Issues and perspectives in species delimitation using phenotypic data: Atlantean evolution in Darwin's finches. *Systematic Biology* **67**: 181–194.
- Castoe TA, Daza JM, Smith EN, Sasa MM, Kuch U, Campbell JA, Chippindale PT, Parkinson CL. 2009.** Comparative phylogeography of pitvipers suggests a consensus of ancient Middle American highland biogeography. *Journal of Biogeography* **36**: 88–103.
- Caviedes-Solis IW, Leaché AD. 2018.** Leapfrogging the Mexican highlands: influence of biogeographical and ecological factors on the diversification of highland species. *Biological Journal of the Linnean Society* **123**: 767–781.
- Chavarría L, Batchelder RL. 2012.** Seven new records from Nicaragua and range extensions for two additional species. *Cotinga* **34**: 28–32.
- Coyne JA, Orr HA. 2004.** *Speciation*. Sunderland: Sinauer Associates.
- Cuthill IC. 2006.** Color perception. In: Hill G, McGraw K, eds. *Bird coloration*, Vol. 1. Cambridge: Harvard University Press, 3–40.
- Dayrat B. 2005.** Towards integrative taxonomy. *Biological Journal of the Linnean Society* **85**: 407–415.
- Daza JM, Castoe TA, Parkinson CL. 2010.** Using regional comparative phylogeographic data from snake lineages to infer historical processes in Middle America. *Ecography* **33**: 343–354.
- De Queiroz K. 2007.** Species concepts and species delimitation. *Systematic Biology* **56**: 879–886.
- Edwards SV, Kingan SB, Calkins JD, Balakrishnan CN, Jennings WB, Swanson WJ, Sorenson MD. 2005.** Speciation in birds: genes, geography, and sexual selection. *Proceedings of the National Academy of Sciences of the United States of America* **102**: 6550–6557.
- Faircloth B. 2013.** *Illumiprocessor: a Trimmomatic wrapper for parallel adapter and quality trimming*. Available at: <http://dx.doi.org/10.6079/J9ILL>
- Faircloth BC. 2015.** PHYLUC is a software package for the analysis of conserved genomic loci. *Bioinformatics* **32**: 786–788.
- Faircloth BC, Glenn TC. 2012.** Not all sequence tags are created equal: designing and validating sequence identification tags robust to indels. *PLoS One* **7**: e42543.
- Faircloth BC, McCormack JE, Crawford NG, Harvey MG, Brumfield RT, Glenn TC. 2012.** Ultraconserved elements anchor thousands of genetic markers spanning multiple evolutionary timescales. *Systematic Biology* **61**: 717–726.
- Fišer C, Robinson CT, Malaré F. 2018.** Cryptic species as a window into the paradigm shift of the species concept. *Molecular Ecology* **27**: 613–635.
- Frolking S, Milliman T, McDonald K, Kimball J, Zhao M, Fahnestock M. 2006.** Evaluation of the SeaWinds scatterometer for regional monitoring of vegetation phenology. *Journal of Geophysical Research: Atmospheres* **111**: D17302.

- García-Moreno J, Navarro-Sigüenza AG, Peterson AT, Sánchez-González LA. 2004. Genetic variation coincides with geographic structure in the common bush-tanager (*Chlorospingus ophthalmicus*) complex from Mexico. *Molecular Phylogenetics and Evolution* **33**: 186–196.
- Gowen FC, Maley JM, Cicero C, Peterson AT, Faircloth BC, Warr TC, McCormack JE. 2014. Speciation in Western Scrub-Jays, Haldane's rule, and genetic clines in secondary contact. *BMC Evolutionary Biology* **14**: 135.
- Gutiérrez-Rodríguez C, Ornelas JF, Rodríguez-Gómez F. 2011. Chloroplast DNA phylogeography of a distylous shrub (*Palicourea padifolia*, Rubiaceae) reveals past fragmentation and demographic expansion in Mexican cloud forests. *Molecular Phylogenetics and Evolution* **61**: 603–615.
- Harris RS. 2007. *Improved pairwise alignment of genomic DNA*. PhD Thesis. The Pennsylvania State University.
- Hofmann EP, Townsend JH. 2017. Origins and biogeography of the *Anolis crassulus* subgroup (Squamata: Dactyloidae) in the highlands of nuclear Central America. *BMC Evolutionary Biology* **17**: 267.
- Katoh K, Misawa K, Kuma Ki, Miyata T. 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Research* **30**: 3059–3066.
- Maldonado-Sánchez D, Gutiérrez-Rodríguez C, Ornelas JF. 2016. Genetic divergence in the common bush-tanager *Chlorospingus ophthalmicus* (Aves: Emberizidae) throughout Mexican cloud forests: the role of geography, ecology and Pleistocene climatic fluctuations. *Molecular Phylogenetics and Evolution* **99**: 76–88.
- Mastretta-Yanes A, Moreno-Letelier A, Piñero D, Jorgensen TH, Emerson BC. 2015. Biodiversity in the Mexican highlands and the interaction of geology, geography and climate within the Trans-Mexican Volcanic Belt. *Journal of Biogeography* **42**: 1586–1600.
- Mayr E. 1963. *Animal species and evolution*. Harvard University: Belknap Press.
- McCormack JE, Heled J, Delaney KS, Peterson AT, Knowles LL. 2011. Calibrating divergence times on species trees versus gene trees: implications for speciation history of *Aphelocoma* jays. *Evolution* **65**: 184–202.
- McCormack JE, Peterson A, Bonaccorso E, Smith TB. 2008. Speciation in the highlands of Mexico: genetic and phenotypic divergence in the Mexican jay (*Aphelocoma ultramarina*). *Molecular Ecology* **17**: 2505–2521.
- McCormack JE, Tsai WL, Faircloth BC. 2016. Sequence capture of ultraconserved elements from bird museum specimens. *Molecular Ecology Resources* **16**: 1189–1203.
- McCormack JE, Venkatraman MX. 2013. A distinctive genetic footprint of ancient hybridization. *The Auk* **130**: 469–475.
- McCormack JE, Zellmer AJ, Knowles LL. 2010. Does niche divergence accompany allopatric divergence in *Aphelocoma* jays as predicted under ecological speciation?: insights from tests with niche models. *Evolution* **64**: 1231–1244.
- McLachlan G, Peel D. 2000. *Finite mixture models*. Hoboken: John Wiley & Sons.
- Myers N, Mittermeier RA, Mittermeier CG, da Fonseca GAB, Kent J. 2000. Biodiversity hotspots for conservation priorities. *Nature* **403**: 853–858.
- Navarro-Sigüenza AG, Peterson AT, López-Medrano E, Benítez-Díaz H. 2001. Species limits in Mesoamerican *Aulacorhynchus* toucanets. *The Wilson Bulletin* **113**: 363–372.
- Noguerales V, Cordero PJ, Ortego J. 2018. Integrating genomic and phenotypic data to evaluate alternative phylogenetic and species delimitation hypotheses in a recent evolutionary radiation of grasshoppers. *Molecular Ecology* **27**: 1229–1244.
- Ornelas JF, Sosa V, Soltis DE, Daza JM, González C, Soltis PS, Gutiérrez-Rodríguez C, de los Monteros AE, Castoe TA, Bell C. 2013. Comparative phylogeographic analyses illustrate the complex evolutionary history of threatened cloud forests of northern Mesoamerica. *PLoS One* **8**: e56283.
- Padial JM, Miralles A, De la Riva I, Vences M. 2010. The integrative future of taxonomy. *Frontiers in Zoology* **7**: 16.
- Pearson RG, Raxworthy CJ, Nakamura M, Townsend Peterson A. 2007. Predicting species distributions from small numbers of occurrence records: a test case using cryptic geckos in Madagascar. *Journal of Biogeography* **34**: 102–117.
- Peterson A, Soberón J, Sánchez-Cordero V. 1999. Conservatism of ecological niches in evolutionary time. *Science* **285**: 1265–1267.
- Peterson AT. 1992. Phylogeny and rates of molecular evolution in the *Aphelocoma* jays (Corvidae). *The Auk* **109**: 133–147.
- Peterson AT. 1993. Adaptive geographical variation in bill shape of scrub jays (*Aphelocoma coerulescens*). *The American Naturalist* **142**: 508–527.
- Phillips AR. 1986. *The known birds of North and Middle America: part 1*. Denver, CO: Denver Museum of Natural History.
- Phillips SJ, Dudík M. 2008. Modeling of species distributions with Maxent: new extensions and a comprehensive evaluation. *Ecography* **31**: 161–175.
- Pitelka F. 1946. A new race of *Aphelocoma unicolor* from southern Mexico. *Condor* **48**: 44–45.
- Pitelka F. 1951. Speciation and ecologic distribution in American jays of the genus *Aphelocoma*. *University of California Publications in Zoology* **50**: 195–464.
- Pokrant F, Kindler C, Ivanov M, Cheylan M, Geniez P, Böhme W, Fritz U. 2016. Integrative taxonomy provides evidence for the species status of the Ibero-Maghrebian grass snake *Natrix astreptophora*. *Biological Journal of the Linnean Society* **118**: 873–888.
- Ponce-Reyes R, Reynoso-Rosales V-H, Watson JE, VanDerWal J, Fuller RA, Pressey RL, Possingham HP. 2012. Vulnerability of cloud forest reserves in Mexico to climate change. *Nature Climate Change* **2**: 448–452.
- Price T. 2008. *Speciation in birds*. Greenwood Village: Roberts and Co. Publishers.
- Price TD, Bouvier MM. 2002. The evolution of F1 postzygotic incompatibilities in birds. *Evolution* **56**: 2083–2089.
- Puebla-Olivares F, Bonaccorso E, De Los Monteros AE, Omland KE, Llorente-Bousquets JE, Peterson AT, Navarro-Sigüenza AG. 2008. Speciation in the emerald toucanet (*Aulacorhynchus prasinus*) complex. *The Auk* **125**: 39–50.
- Ridgway R. 1912. *Color standards and color nomenclature*. Baltimore: A. Hoen and Company.

- Rojas-Soto OR, Sosa V, Ornelas JF. 2012.** Forecasting cloud forest in eastern and southern Mexico: conservation insights under future climate change scenarios. *Biodiversity and Conservation* **21**: 2671–2690.
- Roux C, Fraisse C, Romiguier J, Anciaux Y, Galtier N, Bierne N. 2016.** Shedding light on the grey zone of speciation along a continuum of genomic divergence. *PLoS Biology* **14**: e2000234.
- Rovito SM, Vásquez-Almazán CR, Papenfuss TJ, Parra-Olea G, Wake DB. 2015.** Biogeography and evolution of Central American cloud forest salamanders (Caudata: Plethodontidae: Cryptotriton), with the description of a new species. *Zoological Journal of the Linnean Society* **175**: 150–166.
- Schlick-Steiner BC, Steiner FM, Seifert B, Stauffer C, Christian E, Crozier RH. 2010.** Integrative taxonomy: a multisource approach to exploring biodiversity. *Annual Review of Entomology* **55**: 421–438.
- Scrucca L, Fop M, Murphy TB, Raftery AE. 2016.** mclust 5: clustering, classification and density estimation using gaussian finite mixture models. *The R Journal* **8**: 289–317.
- Simard M, Pinto N, Fisher JB, Baccini A. 2011.** Mapping forest canopy height globally with spaceborne lidar. *Journal of Geophysical Research: Biogeosciences* **116**: G04021.
- Simpson JT, Wong K, Jackman SD, Schein JE, Jones SJ, Birol I. 2009.** ABySS: a parallel assembler for short read sequence data. *Genome Research* **19**: 1117–1123.
- Smith BT, McCormack JE, Cuervo AM, Hickerson MJ, Aleixo A, Cadena CD, Pérez-Emán J, Burney CW, Xie X, Harvey MG. 2014.** The drivers of tropical speciation. *Nature* **515**: 406–409.
- Solano-Zavaleta I, de Oca AN-M. 2018.** Species limits in the Morelet's Alligator lizard (Anguidae: Gerrhonotinae). *Molecular Phylogenetics and Evolution* **120**: 16–27.
- Stamatakis A. 2014.** RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* **30**: 1312–1313.
- van Rossem AJ. 1942.** Du Bus' types of *Cyanocorax unicolor* and *Sylvia taeniata*. *Wilson Bulletin* **54**: 212–213.
- Warren DL, Cardillo M, Rosauer DF, Bolnick DI. 2014.** Mistaking geography for biology: inferring processes from species distributions. *Trends in Ecology & Evolution* **29**: 572–580.
- Warren DL, Glor RE, Turelli M. 2008.** Environmental niche equivalency versus conservatism: quantitative approaches to niche evolution. *Evolution* **62**: 2868–2883.
- Webber T, Brown JL. 1994.** Natural history of the unicolored jay in Chiapas, Mexico. *Proceedings of the Western Foundation of Vertebrate Zoology* **5**: 135–160.
- Webster MS. 2017.** *The extended specimen: emerging frontiers in collections-based ornithological research*. Boca Raton, FL: CRC Press/Taylor & Francis Group.
- Weir J, Schluter D. 2008.** Calibrating the avian molecular clock. *Molecular Ecology* **17**: 2321–2328.
- Winker K. 2016.** An examination of species limits in the *Aulacorhynchus "prasinus"* toucanet complex (Aves: Ramphastidae). *PeerJ* **4**: e2381.
- Younger JL, Strozier L, Maddox JD, Nyári ÁS, Bonfitto MT, Raherilalao MJ, Goodman SM, Reddy S. 2018.** Hidden diversity of forest birds in Madagascar revealed using integrative taxonomy. *Molecular Phylogenetics and Evolution* **124**: 16–26.
- Zellmer AJ, Hanes MM, Hird SM, Carstens BC. 2012.** Deep phylogeographic structure and environmental differentiation in the carnivorous plant *Sarracenia alata*. *Systematic Biology* **61**: 763–777.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. WordClim and remote-sensing layers used for niche analysis.

Table S2. Results of a principal components analysis on binned spectral data taken from the nape plumage.

Table S3. Results of a discriminant function analysis on all morphological and plumage variables.

Table S4. Results of a principal components analysis on niche variables.

Figure S1. Occupied niche space compared with available environmental niche space for cross-Isthmus clades showing preference for cooler temperatures (A), higher canopy (B) and more tree cover (C) for both clades. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure S2. Results of a discriminant function analysis including morphology and plumage traits, showing differentiation within subspecies *Aphelocoma unicolor unicolor* (A) and within subspecies *Aphelocoma unicolor griscomi* (B).

Figure S3. Occupied niche (coloured boxes) compared with background environment (grey boxes) on each multivariate niche axis for individual subspecies and cross-Isthmus clades.