

Speciation in the highlands of Mexico: genetic and phenotypic divergence in the Mexican jay (*Aphelocoma ultramarina*)

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Abstract

The pine-oak woodlands of the Mexican highlands harbour significant biological diversity, yet little is known about the evolutionary history of organisms inhabiting this region. We assessed genetic and phenotypic differentiation in 482 individuals representing 27 populations of the Mexican jay (*Aphelocoma ultramarina*) – a widespread bird species of the Mexican highlands – to test whether populations in the central and northern Mexican sierras display discrete breaks between groups, which would be consistent with a role for the different mountain chains in divergence and speciation. We found abrupt breaks in mitochondrial DNA (mtDNA; ND2 and control region) delineating four major genetic groups found in the Sierra Madre Occidental, Sierra Madre Oriental, southern Central Plateau (Bajío), and Transvolcanic Belt. These mtDNA groups were largely corroborated by data from nuclear microsatellites and phenotypic data, except that clades from the Central Plateau and Sierra Madre Oriental showed clinal change in these data sets. Uncertainty about the mutation rate for our mitochondrial markers warrants considerable caution with regard to estimating divergence times, but the major genetic groups appear to have split before the most extreme period of glacial cycling that marked the last 0.7 million years and after Mexico's period of major mountain formation. The fact that some genetic breaks do not coincide with well-known geographic barriers suggests a role for ecology in divergence and speciation, and we discuss implications for taxonomy and conservation.

Keywords: *Aphelocoma*, habitats, madre, miocene, phylogeography, pine-oak, pleistocene, Sierra, speciation

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Introduction

The Mexican highlands represent a significant focus of biodiversity in North America (Ramamoorthy *et al.* 1993; Bye 1995; Foreman *et al.* 2000; Spector 2002). Avian endemism reaches its highest levels in the sierras of western and central Mexico, especially in mixed pine-and-oak woodlands (hereafter 'pine-oak woodlands'), a signature habitat of the

Mexican highlands and the mountain isolates (sky islands) of the southwestern USA (Escalante *et al.* 1993). In recognition of their biological importance and the threats they face from logging, mining, and agriculture, these pine-oak woodlands were recently designated a globally important biodiversity hotspot by Conservation International (Mittermeier *et al.* 2005).

The high endemism in Mexican pine-oak woodlands suggests a strong role for the central Mexican sierras in divergence and speciation of birds. The fact that the most endemism-rich mountain chain, the Transvolcanic Belt, formed in the Middle Miocene (Ferrari *et al.* 1999) further

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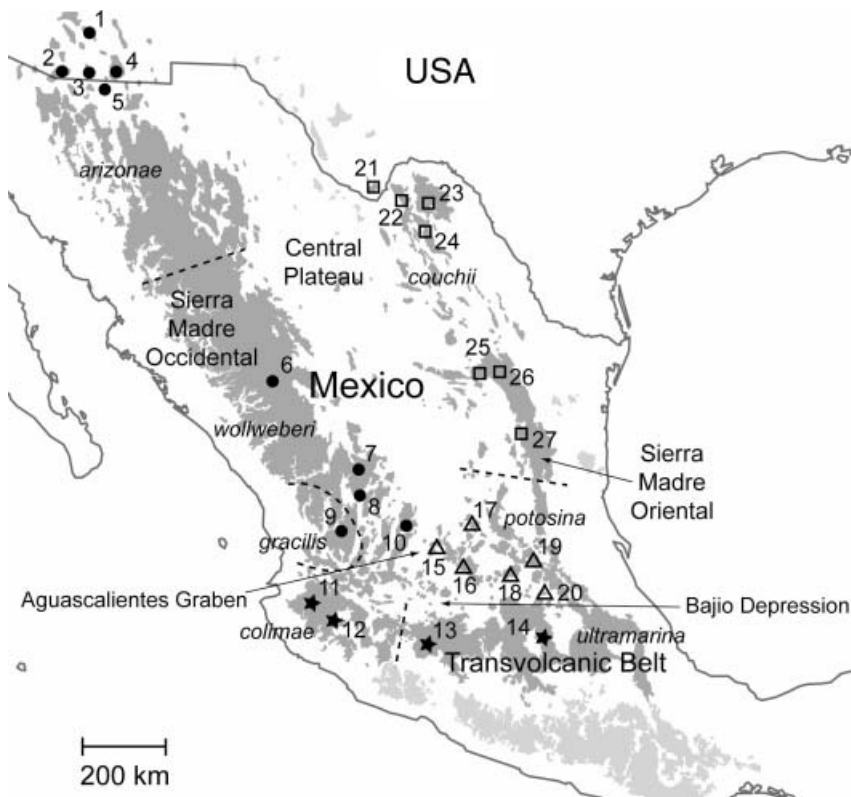


Fig. 1 Map of sampling localities and geographic features discussed in text. Approximate ranges of subspecies are delineated with dashed lines. Shaded area shows the extent of pine-oak woodlands in Mexico including areas inhabited (dark grey) and not inhabited (light grey) by Mexican jays. Symbols denote sampling localities of genetic groups identified in Fig. 2: Western (filled circle), Eastern (square), Central (triangle), and Transvolcanic (star). Site numbers correspond to locality data in Table 1.

suggests that much of this diversification was relatively recent. Despite the importance of the region, however, little is known about diversification in the Mexican highlands. While a few phylogeographic studies exist (Sullivan *et al.* 2000; García-Moreno *et al.* 2004, 2006; León-Paniagua *et al.* 2007; Puebla-Olivares *et al.* in press), there has been little focus on the northern sierras (but see Spellman & Klicka 2007). Particularly lacking is basic knowledge of the spatial and temporal scale of differentiation, which might provide insights into the roles of mountain-building, climatic shifts, and geographic isolation in divergence and speciation. Appropriate phylogeographic studies might also help discriminate between competing models of diversification for North America, such as those that emphasize geological processes such as mountain uplift (Morafka 1977; Riddle 1995; Jaeger *et al.* 2005) vs. those that focus on habitat fluctuations caused by Pleistocene climate change (Mengel 1970; Hewitt 1996; Lessa *et al.* 2003; Johnson & Cicero 2004; Weir & Schluter 2004).

The goal of this study is to examine genetic and phenotypic variation in the Mexican jay (*Aphelocoma ultramarina*) – a widespread denizen of Mexican pine-oak woodlands (Fig. 1) – to test biogeographic hypotheses for evolution in the Mexican highlands. Mexican jays are an excellent species for uncovering biogeographic patterns because they have low dispersal (Brown 1994), which has likely contributed to the evolution of marked phenotypic differ-

entiation (Peterson 1992a). Seven subspecies (Fig. 1) are recognized that vary in morphology and plumage (Pitelka 1951), ecology (Rice *et al.* 2003), and behaviour (Brown & Horvath 1989). Based on phenotypic and allozyme studies, these subspecies have been informally placed into three groups inhabiting the Sierra Madre Occidental, Sierra Madre Oriental, and the Transvolcanic Belt (Pitelka 1951; Navarro-Sigüenza & Peterson 2004). Other phenotypic studies have suggested more continuous population structure connecting the groups, particularly those of the Sierra Madre Oriental and Sierra Madre Occidental (Peterson 1991). Genetic studies, on the other hand, have indicated considerable differentiation across this region (Peterson 1990, 1992b; Rice *et al.* 2003), but limited sampling of the genome has thus far prevented more definitive conclusions about introgression and the locations of genetic breaks.

We analyzed genetic variation in two mitochondrial DNA (mtDNA) markers and 13 microsatellite loci from 27 populations throughout the species' range (Fig. 1). Additionally, we built on Pitelka's (1951) detailed monograph with a multivariate analysis of plumage spectral reflectance and morphology of museum specimens to test whether genetic and phenotypic characters show concordant patterns, and to assess whether phenotypic differentiation among allopatric Mexican jay groups is similar in magnitude to that seen between Mexican jays and a closely related, often sympatric species, the unicoloured jay (*Aphelocoma unicolor*).

Specifically, we tested whether the Sierra Madre Occidental, Sierra Madre Oriental, and Transvolcanic Belt are inhabited by divergent lineages with clear phenotypic and genetic breaks, or whether variation, particularly north of the Transvolcanic Belt, is continuous and clinal in nature. Exploring divergence times, we attempted to place splitting events within one of three broad time intervals: the Neogene [Miocene and Pliocene, 1.7–23 million years ago (Ma)], Early Pleistocene (0.7–1.7 Ma), or Late Pleistocene (< 0.7 Ma). These periods delineate distinct geological and climatic events that influenced the biotic history of North America, especially Mexico (Hafner & Riddle 2005): uplift of Mexico's major mountain chains began in the Eocene (~40 Ma), but continued well into the Neogene (Ferrusquía-Villafranca & González-Guzmán 2005); Late Pliocene cooling augured the glacial-interglacial cycling of the Early Pleistocene that intensified in the Late Pleistocene (c. 0.7 Ma; Webb & Bartlein 1992). Hence, discriminating between these time intervals allows inference regarding the relative roles of geological vs. climatic events, although both may be important. Finally, we discuss the roles of vicariance and ecological differentiation in divergence and review conservation implications for Mexican jays and other organisms in Mexican pine-oak woodlands.

Materials and methods

Sampling

Molecular analyses were based on 482 frozen blood and tissue samples from 27 populations distributed throughout the geographic range of Mexican jays (Fig. 1; Table 1). We sought to maximize geographic coverage while assuring that some populations were represented by many individuals to improve our ability to detect incomplete lineage sorting or hybridization among groups. Tissue specimens were collected in 1987–1989 (A.T.P.; deposited at the Field Museum of Natural History and the Universidad Nacional Autónoma de México) and in 1986 (B. Bowen; skeletons deposited at the Museum of Vertebrate Zoology). Later sampling sought to fill in gaps in geographic coverage: blood and feather samples were collected from jays that were captured with mist-nets and ground traps and released after processing (2002–2006, J.E.M.; Bhagabati *et al.* 2004).

Laboratory methods

We extracted genomic DNA from blood and tissue using QIAGEN DNeasy Tissue Kits following the manufacturer's protocols. For all 482 individuals, we amplified a 525 base-pair (bp) portion of the mtDNA control region (hypervariable region I) using primers JCR03 (5'-CCCCCCCATGTTTTT ACR-3'; Saunders & Edwards 2000) and H1248 (5'-CATCTT

CAGTGTGCTGCT-3'; Tarr 1995). For 47 individuals, we amplified a 636-bp portion of the NADH dehydrogenase subunit 2 (ND2) gene using primers H-6313 (5'-CTCTT ATTTAAGGCTTTGAAGGC-3'; Sorenson *et al.* 1999) and L-5758 (5'-GGCTGAATRGGMCTNAAYCARAC-3'; Sorenson *et al.* 1999). Individuals for ND2 sequencing were selected to represent the full range of genetic diversity uncovered in the control region while maintaining good geographic coverage. Polymerase chain reaction (PCR) for both genes consisted of a standard protocol (initial denaturation at 94 °C for 3 min; 40 cycles of 94 °C for 45 s, 50 °C for 45 s, and 72 °C for 30 s; and a final extension at 72 °C for 5 min). Amplification was performed in 25 µL reactions (2.5 µL of 10× PCR buffer, 2.0 µL of MgCl₂ solution, 2.0 µL of dNTPs [2.5 mM each], 1.0 µL of each primer [10 µM], 0.1 µL *Taq* polymerase, 2.0 µL of DNA template [~50–100 ng double-stranded DNA], and 14.4 µL of sterile water). To check for contamination, we ran negative controls with each reaction, adding water instead of DNA. We used the same conditions to amplify genomic DNA samples of outgroups (see below).

PCR products were purified with an UltraClean GelSpin kit (Mo Bio Laboratories). We obtained sequences from PCR products using a dideoxy-terminator cycle-sequencing reaction using a CEQ DTCS kit (Beckman Coulter). Sequencing reactions were 10 µL total volume (3.0 µL PCR product, 3.0 µL forward primer, 3.0 µL DTCS mix, and 1.0 µL sterile water), and followed the manufacturer's protocol. Products were purified with an ethanol precipitation and sequenced in a Beckman-Coulter CEQ 2000 automated sequencer.

We screened 362 individuals for genetic variation at 13 microsatellite loci (Table 1): MJG1 and MJG8 isolated from the Mexican jay (Li *et al.* 1997), and ApCo2, ApCo15, ApCo18, ApCo19, ApCo22, ApCo29, ApCo30, ApCo40, ApCo41, ApCo68, and ApCo97 isolated from the Florida scrub-jay (*Aphelocoma coerulescens*; Stenzler & Fitzpatrick 2002). Forward primers were tagged with a fluorescent dye (universal M13 forward primer) and microsatellites were amplified using a multiplex PCR kit (QIAGEN) with groups of two to three primer combinations, differentiating between loci by prior knowledge of allele size ranges obtained from test runs. We ran PCR products on an ABI3730 capillary sequencer (Applied Biosystems) and scored alleles using GENEMAPPER software (Applied Biosystems).

We aligned sequences automatically using SEQUENCHER version 4.1 (GeneCodes) and checked variable sites visually for accuracy. All sequences used in this study have been deposited in GenBank under Accession nos EU121284–EU121375. We encountered no problems suggesting amplification of nuclear pseudogenes (Sorenson & Quinn 1998) such as double peaks in sequence data; nevertheless, a subset of the samples, including all unique haplotypes identified from blood extractions, were re-extracted from feather tissue and resequenced with identical results.

Table 1 Locality data for 482 Mexican jays used to describe genetic variation. Sample sizes for control region (CR), ND2, and 13 microsatellite loci (M) are given

No.	Location	CR	ND2	M	GPS	Source*	Subspecies
	West	138	20	142			
1	Pinalaño Mountains, Arizona	15		15	32.7, – 110.0	Tissue, MVZ (170622–170636)	<i>arizonae</i>
2	Pajarito Mountains, Arizona	19	5	19	31.41, – 111.13	Tissue, FM (334257–334272, 334278–334280)	<i>arizonae</i>
3	Huachuca Mountains, Arizona	14		15	31.4, – 110.3	Tissue, MVZ (170680–170693)	<i>arizonae</i>
4	Chiricahua Mountains, Arizona						<i>arizonae</i>
	Wild Turkey Creek	14		15	31.8, – 109.3	Tissue, MVZ (170637–170651)	<i>arizonae</i>
	Horseshoe Canyon	13		15	31.8, – 109.1	Tissue, MVZ (170652–170664)	<i>arizonae</i>
	Tex Canyon	14		15	31.65, – 109.3	Tissue, MVZ (170665–170679)	<i>arizonae</i>
5	Sierra de San Jose, Sonora	5	1	5	31.25, – 110.06	Tissue, FM (343516–343517, 393726–393728)	<i>arizonae</i>
6	Sierra Madre Occidental, Durango	5	1	5	25.02, – 105.72	Blood, feather, UCLA	<i>wollweberi</i>
7	Rancho Gachupines, Zacatecas	2	2	2	23.16, – 102.88	Tissue, UNAM (15783–15784)	<i>wollweberi</i>
8	Near Valparaiso, Zacatecas	18	7	18	22.85, – 103.63	Tissue, FM (343571–343580, 393678–393680, 393743–393748)	<i>wollweberi</i>
9	Northern Jalisco						<i>wollweberi</i>
	Sierra de los Huicholes	10	3	9	21.90, – 103.85	Blood, feather, UCLA	<i>gracilis</i>
	Sierra de Bolaños	4	1	4	21.98, – 103.66	Tissue, FM (343519–343521, 393654)	<i>gracilis</i>
10	El Jaguey, Aguascalientes	5		5	22.2, – 102.6	Tissue, FM (343569–343570, 393740–393742)	<i>wollweberi</i>
	Transvolcanic	26	4	22			
11	Near Mascota, Jalisco	1		1	20.36, – 104.60	Blood, feather, UCLA	<i>colimae</i>
12	La Cañada, Jalisco	14	2	12	19.78, – 103.66	Tissue, FM (343581, 343584–343589, 393652–393653, 393669–393673)	<i>colimae</i>
13	San Nicolás, Michoacán	2			19.42, – 102.23	Feather, UCLA	<i>ultramarina</i>
14	Near Huitzilac, México	9	2	9	19.05, – 99.26	Tissue, FM (343561–343563, 343565, 343567, 393674–393677)	<i>ultramarina</i>
	Central	48	8	48			
15	Near El Puesto, Jalisco	1	1	1	21.58, – 101.98	Tissue, FM (393739)	<i>wollweberi</i>
16	Puerto Santa Rosa, Guanajuato	6	1	6	21.06, – 101.18	Tissue, FM (343545, 343547–141548, 393735–393737)	<i>potosina</i>
17	Sierra de Bledos, San Luis Potosí	20	2	20	21.86, – 101.15	Tissue, FM (343549–343560, 393663–393668, 393733–393734)	<i>potosina</i>
18	Near Chavarrías, Queretaro	1	1	1	20.83, – 99.6	Tissue, UNAM (15703)	<i>potosina</i>
19	Near Santa Inés, Queretaro	2	1	2	21.23, – 99.48	Tissue, UNAM (13126, 13118)	<i>potosina</i>
20	Near Jacala, Hidalgo	18	2	18	21.05, – 99.00	Tissue, FM (343535–343536, 343538–343544, 393655–393662, 393738)	<i>potosina</i>
	East	270	15	150			
21	Chisos Basin, Chisos Mountains, Texas	56		10	29.24, – 103.29	Blood, feather, UCLA; blood, N. Bhagabati	<i>couchii</i>
22	Sierra del Carmen, Coahuila						<i>couchii</i>
	San Isidro Canyon	37	2	27	28.90, – 102.54	Blood, feather, UCLA	<i>couchii</i>
	Agua de las Vacas Canyon	20	1	16	28.95, – 102.61	Blood, feather, UCLA	<i>couchii</i>
	Morteros Canyon	8	1	5	28.85, – 102.53	Blood, feather, UCLA	<i>couchii</i>
	Campo Dos	50	1	28	28.99, – 102.61	Blood, feather, UCLA	<i>couchii</i>
	Campo Tres	16	1	15	28.99, – 102.59	Blood, feather, UCLA	<i>couchii</i>
	Campo Cinco	4		4	28.96, – 102.57	Blood, feather, UCLA	<i>couchii</i>
	Unknown	20	1		28.93, – 102.6	Tissue, FM (343523–343529, 343531–343533, 393641–393650)	<i>couchii</i>
23	Serranías del Burro, Coahuila	24	5	15	28.89, – 101.94	Blood, feather, UCLA	<i>couchii</i>
24	Sierra Santa Rosa, Coahuila						<i>couchii</i>
	Rancho La Rosita	9	1	10	28.38, – 101.85	Blood, feather, UCLA	<i>couchii</i>
	Rancho Las Cabras	6		5	28.50, – 102.19	Blood, feather, UCLA	<i>couchii</i>
25	Taray, Sierra Arteaga, Coahuila	15	2	10	25.31, – 100.47	Blood, feather, UCLA	<i>couchii</i>
26	El Diamante Pass, Coahuila	2		2	25.36, – 100.86	Tissue, FM (343534, 393651)	<i>couchii</i>
27	Sierra Peña Nevada, Nuevo León	3		3	23.88, – 99.79	Blood, feather, UCLA	<i>couchii</i>

*Vouchered museum specimens are indicated with museum numbers. Field Museum, Chicago (FM), Museum of Vertebrate Zoology, Berkeley (MVZ), Museo de Zoología 'Alfonso L. Herrera' of Universidad Nacional Autónoma de México (UNAM). Other specimens are blood and feather samples stored at the Conservation Genetics Resource Center at UCLA.

Phylogenetic analysis

Phylogenies were reconstructed using maximum parsimony (MP), maximum likelihood (ML), and Bayesian analysis (BA). MP analysis was conducted with PAUP* version 4.0b (Swofford 2000). MP trees for ND2 were obtained using branch-and-bound searches and clade support was assessed using heuristic searches [tree-bisection–reconnection (TBR) branch swapping] with 1000 bootstrap pseudoreplicates (10 random stepwise additions). Control region MP trees and bootstrap support were estimated via heuristic searches (TBR branch swapping); the best tree was obtained using 10 000 random stepwise additions and clade support estimated via 1000 bootstrap pseudoreplicates (10 random stepwise additions). We performed a partition homogeneity test on sequence data to decide if the two genes could be combined. We also used a Shimodaira–Hasegawa test (S–H test) to look for incongruity of the resulting tree topologies (Shimodaira & Hasegawa 1999). These tests indicated significant incongruities between trees based on our two genes (see Results); therefore, we did not concatenate the two genes.

Prior to ML and BA, we selected a best-fit model of nucleotide substitution for ND2 (overall gene and codon positions) and control region, using MODELTEST version 3.7 (Posada & Crandall 1998) under the Akaike information criterion. MODELTEST selected the TVM + Γ + *I* model for control region, HKY + Γ model for the overall ND2 data set, and HKY + *I*, HKY, and TVM for the first, second, and third ND2 codon positions.

For both genes, ML trees were estimated using GARLI version 0.951 (genetic algorithm for rapid likelihood inference; Zwickl 2006) available at <http://www.bio.utexas.edu/faculty/antisense/garli/Garli.html>, which offers considerable advantages over PAUP* in terms of computational efficiency. It uses a genetic algorithm that finds the tree topology, branch lengths, and model parameters that simultaneously maximize $\ln L$. Available models of nucleotide substitution include the GTR model and its more common submodels, as well as less complex models, and the program accounts for gamma-distributed rate heterogeneity (Γ) and estimation of the proportion of invariable sites (*I*). Estimation of model parameter values may be optimized or fixed, and the implementation of the model is equivalent to that in PAUP*.

GARLI analyses were conducted specifying the model ‘family’ obtained by MODELTEST, but allowing the program to estimate parameter values from the data, with gamma-rate categories set to four. Individual solutions were selected after 10 000 generations of no significant improvement in likelihood, with the significant topological improvement level set at 0.01 (first condition for termination); then, the final solution was selected when the total improvement in likelihood was < 0.05 compared with the last solution

obtained (second condition for termination). All other GARLI settings were default values, per recommendation of the developer (Zwickl 2006). Bootstrap support for both genes was assessed via 1000 pseudoreplicates; bootstrap searches ran under the same settings used for obtaining the best ML tree.

Bayesian analysis was performed using MRBAYES version 3.1.2 (Ronquist & Huelsenbeck 2003). For ND2, we partitioned data by codon position, assigning to each partition its best-fit model family of nucleotide substitution, with all parameters unlinked between partitions except topology and branch lengths. For each gene, we ran two independent analyses for 5×10^6 generations using four Markov chains and default heating values. Parameter values were estimated from the data using uniform priors. Trees were sampled every 1000 generations, resulting in 5000 trees. A majority-rule consensus tree was created from these trees after discarding the first 1000 trees as burn-in. For each analysis, we confirmed that the average standard deviation of the split frequencies approached zero.

ND2 and control region phylogenies were rooted with sequences from close and distant relatives of Mexican jays determined from a recent phylogeny of New World jays (Bonaccorso & Peterson 2007). For ND2, we used the western scrub-jay (*Aphelocoma californica*) and white-throated magpie-jay (*Calocitta formosa*). We obtained additional sequences for unicoloured jay (*Aphelocoma unicolor*; AF218920) and Steller’s jay (*Cyanocitta stelleri*; AF218923) from GenBank for use as outgroups in the control region phylogeny.

We tested ND2 for clock-like substitution rates with a likelihood ratio test implemented in PAUP*. A clock-like rate was rejected ($P < 0.001$); thus, we estimated divergence times of the major clades under a relaxed phylogenetic framework using Bayesian Markov chain Monte Carlo (MCMC) implemented in BEAST version 1.4.4 (Drummond & Rambaut 2007). This method allows substitution rates among branches to vary (Drummond *et al.* 2006). We used an HKY + Γ model and assumed stable population sizes and uncorrelated rates. The rate of each branch was drawn from a lognormal distribution. We set the mean substitution rate at 10^{-8} , as this rate is considered standard for protein-coding mtDNA (Baker & Marshall 1997; Lovette 2004), although such a generic calibration is rife with potential complications (Peterson 2006). Chain lengths were 10^7 with parameters sampled every 10^3 . Good stationary and high effective sample sizes (> 2000) were observed for all parameters. A consensus tree with divergence times was obtained from the 10 000 generated trees, after discarding the first 2000 as burn-in.

Genetic differentiation and population structure

Because bifurcating trees may not always be the best way to represent intraspecific phylogenies (Posada & Crandall

2001), we constructed minimum-spanning networks of absolute distances between mtDNA haplotypes using the molecular variance parsimony algorithm (Excoffier & Smouse 1994) implemented in ARLEQUIN 2.0 (Schneider *et al.* 2000). To calculate corrected sequence divergence between clades, we used the number of nucleotide changes between haplotypes (D_{xy}) taking into account intraclade polymorphism (Nei 1987), so that $D_{xy} = d_{ixy} - 0.5(d_{ix} + d_{iy})$, where x and y are the groups being compared and d_i is uncorrected average genetic distance (Wilson *et al.* 1985). For mtDNA control region, we calculated a distance-based pairwise F_{ST} between populations using ARLEQUIN and assessed significance with 1000 permutations. Only control region data were used to calculate F_{ST} because all samples were sequenced for control region, whereas only a subset was sequenced for ND2. For highly variable, multilocus data sets such as microsatellites, F_{ST} does not reach its maximum at 1, but is instead constrained by population-level homozygosity levels (Hedrick 1999). Therefore, for microsatellite data, we calculated maximum F_{ST} using RECODE version 0.1 (Meirmans 2006) and present differentiation as G'_{ST} (Hedrick 2005), a standardized measure of genetic differentiation calculated by dividing obtained F_{ST} values by the maximum possible F_{ST} . We grouped populations according to clade, but also looked for differentiation within clades and among described subspecies.

We used ARLEQUIN to test microsatellite loci for linkage disequilibrium (LD) and deviations from Hardy–Weinberg equilibrium (HWE) at each site-locus combination and applied sequential Bonferroni correction to avoid type I errors (Rice 1989). We tested for population structure using STRUCTURE version 2.1 (Pritchard *et al.* 2000), a Bayesian clustering program that assigns individuals to detected clusters (K) yet does not require a priori information about the number of expected clusters or geographic locations of individuals. We assumed admixture of populations and the potential for correlated allele frequencies among populations. A preliminary analysis revealed that likelihood scores reached a plateau after $K = 5$ and decreased markedly after $K = 10$. Therefore, we conducted a detailed analysis from $K = 5$ – 10 with a burn-in of 50 000 followed by 10^6 iterations, with five runs for each value of K . We used the method of Evanno *et al.* (2005) to aid in detecting the ‘true K ’ by examining ΔK , a measure of the change in likelihood scores between runs of successive K values.

Phenotypic variation

To complement molecular data, we quantified plumage colour from museum specimens of 178 Mexican jays (96 males, 80 females, and two of unknown sex) and 17 unicoloured jays (*A. unicolor*; 15 males and two females) using an X-Rite Color Digital Swatchbook spectrometer

(X-Rite Inc.). Unicoloured jays were included to provide a comparison of differentiation among populations of Mexican jays to differentiation between them and a closely related species that sometimes occurs sympatrically. The specimens chosen were second-year birds or older collected from 1939 to 1954 representing most of the geographic distribution of the Mexican jay, as well as individuals of several subspecies of unicoloured jays. We used an online directory of cities and towns in Mexico (<http://www.fallingrain.com/world/MX>) and specific terrain features (e.g. mountain ranges) to assign specimens to genetic groups based on locality data from museum tags (Supplementary material, Appendix S1). Several specimens from eastern Mexico fell into a sampling gap between the East and Central groups (see below) and were categorized as East/Central.

We measured reflectance spectra on the crown of each specimen relative to a white standard within a 7-mm diameter. Our spectrometer measured reflectance only in the human-visible spectrum (VIS). Because Mexican jays have their peak reflectance spanning the ultraviolet (UV)–VIS range, with no secondary peaks solely in the UV, UV reflectance is expected to be highly correlated to VIS reflectance (Andersson & Prager 2006). Reflectance values were saved over 32 segments of the visible spectrum. We analyzed these 32 variables with a principal components (PC) analysis of the covariance matrix and saved PC1 and PC2 scores for each individual. Fading of coloration was not expected to pose a problem because specimens were collected within a relatively close time span and blue coloration in jays is structural, not caused by pigments.

We assessed morphological variation using the same 195 museum specimens. One of us measured unflattened wing length to the nearest 0.5 mm measured with a standard wing ruler and the following traits with digital calipers to the nearest 0.1 mm: tail length; tarsus length from the tibiotarsus joint to the distal end of the tarsometatarsus; bill length from the anterior end of the nares to the tip of the upper mandible; bill width and depth at the anterior end of the nares; and lower length from the tip of the lower mandible to the beginning of the depression where the rami meet. We added PC1 and PC2 from the plumage analysis to morphological variables and analyzed these data with a canonical discriminant function analysis (DFA) using Stata Intercooled version 10 (StataCorp 2007).

Results

Phylogenetic relationships and minimum-spanning networks

Mexican jay populations showed high levels of sequence divergence – in some cases over 9% per million years in ND2 and 2% per million years in the control region (Table 2).

	Western (n)	Western (s)	Eastern	Central	Trans
Western (north)		0.87	1.15	1.99	2.07
Western (south)	0.91		1.24	1.96	1.84
Eastern	4.43	4.37		1.22	1.65
Central	3.1	2.86	4.9		2.47
Transvolcanic	9.42	9.36	9.13	9.43	

Table 2 Pairwise corrected sequence divergence (%) between Mexican jay clades for control region (above diagonal) and ND2 (below diagonal)

Phylogenies based on the two mitochondrial segments shared many similarities, but considering the co-inheritance of the two genes on the mitochondrial genome, were surprisingly different overall (Figs 2 and 3). Although the partition homogeneity test did not suggest that the two genes represented different partitions of the data ($P = 0.33$), S-H tests indicated significant conflict between tree topologies based on control region and ND2 ($P = 0.001$). Consequently, we present the phylogenies of the genes separately.

Of 47 individuals sequenced for ND2, 102 positions were variable, identifying 28 haplotypes. MP (majority-rule consensus, four equally parsimonious trees), ML, and BA methods all supported the same topology (Fig. 2), with strong support for monophyly of the in-group and basal placement of Mexican jays from the Transvolcanic Belt ('Transvolcanic clade'). Within the rest of Mexican jays, a clade from the northern Sierra Madre Oriental ('Eastern clade') is supported as sister to a clade comprising populations from the Sierra Madre Occidental ('Western clade') and the rugged mountains of the southern Central Plateau and Bajío ('Central clade'). The latter two clades are also supported as reciprocally monophyletic. Within the Western clade, further structure between northern and southern populations – and even within Arizona – is evident.

Of 482 individuals sequenced for the control region, 57 positions were variable, identifying 64 haplotypes. MP (majority-rule consensus, 7784 equally parsimonious trees), ML, and BA methods produced mostly congruent topologies, but these topologies differed from those reconstructed from ND2, in that haplotypes from the Eastern clade failed to form a monophyletic group (Fig. 3). Bootstrap support for clades was low compared to that for ND2 clades. Also, relationships between the clades were not well supported (except for basal placement of the Transvolcanic clade). Unlike ND2, jays from Arizona were paraphyletic in the control region phylogeny.

Haplotype networks for ND2 and control region show strict geographic structuring, with Transvolcanic Belt haplotypes forming a well-defined group separated from other groups by 14 bp changes in the control region and 58 bp changes in ND2 (Fig. 4). The Western group was also distinct, separated from other groups by 2–3 bp changes in

control region and at least 17 bp changes in ND2; internal distances within this group were high – up to 5 bp changes in control region and 6 bp changes in ND2. The Eastern group was distinct from all other groups by 2–3 bp changes (control region) and 26 bp changes (ND2). Finally, the Central group showed considerable differentiation (7 bp changes in control region, 17 bp changes in ND2), with no haplotypes shared with other groups.

Analysis of ND2 with BEAST confirmed rate variation among lineages (coefficient of variation = 0.28), but overall did not reject a strict molecular clock [i.e. 95% highest probability density (HPD) for the coefficient of variation abutted zero], indicating that the likelihood ratio test for clock-like behaviour might be overly sensitive for our data set. Under a relaxed substitution rate based on the 10^{-8} substitution rate calibration, the Transvolcanic clade was estimated to have diverged from other Mexican jays around the Miocene-Pliocene boundary at 5.6 Ma (95% HPD: 4.35–9.35 Ma). The Eastern clade was estimated to have diverged in the Pliocene around 2.4 Ma (95% HPD: 2.01–4.42 Ma). The Western and Central clades were estimated to have diverged in the Pliocene or Late Pleistocene around 2.0 Ma (95% HPD: 1.13–2.73 Ma). We emphasize, however, that these estimates depend critically on the calibration employed, and as such may be imprecise or biased seriously (see Discussion).

Population genetic structure

None of the microsatellite loci showed consistent evidence for linkage disequilibrium or departure from HWE across populations after Bonferroni correction. Bayesian analysis of microsatellite data for 362 Mexican jays indicated considerable population structure (Fig. 5). Likelihood values [$\ln \Pr(X|K)$] reached a maximum at $K = 9$. However, as analyses indicated a plateau in likelihood scores after $K = 5$, we evaluated ΔK (Evanno *et al.* 2005), which identified a maximum at $K = 8$. Per STRUCTURE documentation (Pritchard & Wen 2004), we then looked for the smallest value of K between 5 and 8 that captured the major genetic structure. Analysis at $K = 5$ provided resolution among the genetic clades identified with mtDNA, while larger K values added subgroups of mixed assignment within the Eastern and Central clades. Therefore, we present results for $K = 5$

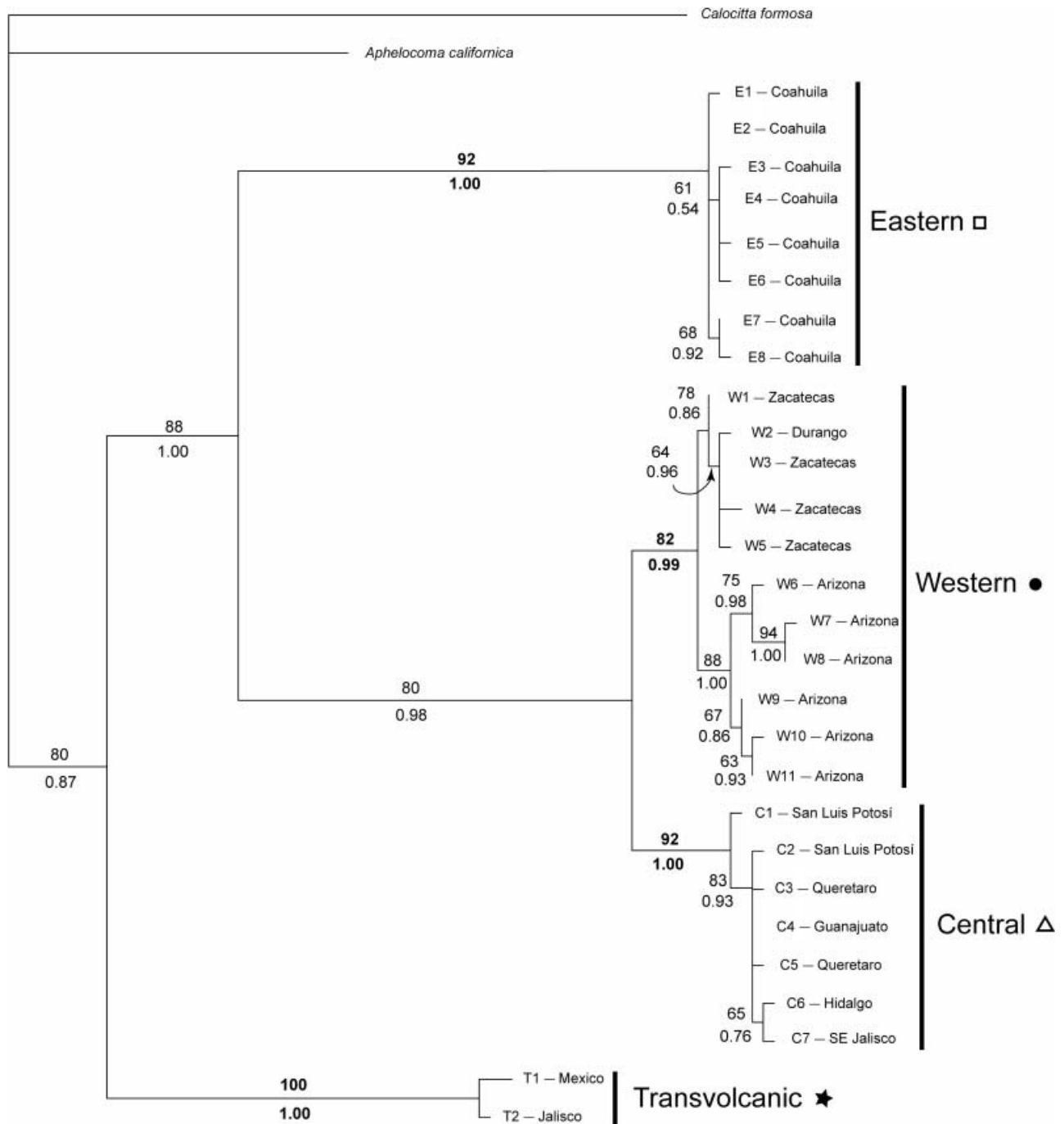


Fig. 2 Maximum-likelihood tree for Mexican jay ND2 haplotypes showing ML bootstrap scores (above) and MRBAYES posterior probabilities (below). Values in bold indicate support for the major genetic groups discussed in the paper. Symbols next to clades refer to sampling sites shown in Fig. 1.

as appropriate for the geographic scale of our study, acknowledging that additional population-level genetic structure may exist with one or more of the clades.

Most pairwise F_{ST} (control region) and G'_{ST} (microsatellites) values were large and all were significant, indicating

strong differentiation between clades and populations. The most pronounced differentiation was seen between the Transvolcanic clade and clades to the north. The lowest (although still significant) values occurred within the Western clade (between subspecies *wollweberi* and *gracilis*)

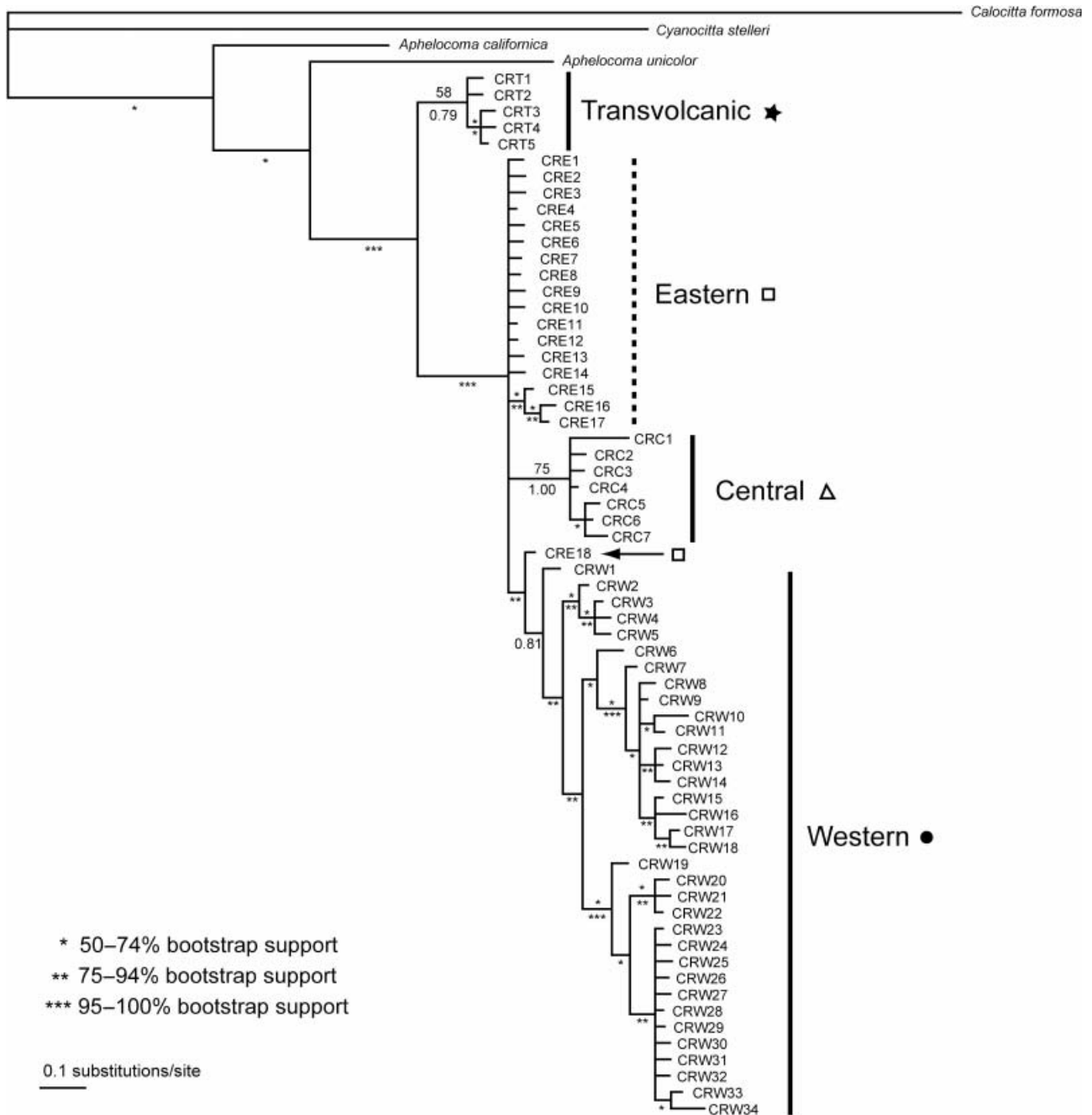


Fig. 3 Bayesian tree for Mexican jay control region haplotypes showing ML bootstrap scores (above) and MRBAYES posterior probabilities (below) for the major clades. Bootstrap scores and posterior probabilities for the other nodes are denoted with asterisks. Symbols next to clades refer to sampling sites shown in Fig. 1.

and between northern and southern populations of the Eastern clade (Table 3).

Phenotypic variation

Factor loadings from our PC analysis of plumage reflectance revealed that the first principal component (PC1; 80.8% of

the total variance) described brightness (positive loading on all variables). The second principal component (PC2; 18.6% of the total variance) described chroma — the amount of grey/white mixed with blue, resulting in a ‘washed-out’ look at low chroma (negative loadings in blue wavelengths and positive loadings in other wavelengths resulting in a flatter reflectance curve). Previous research has validated

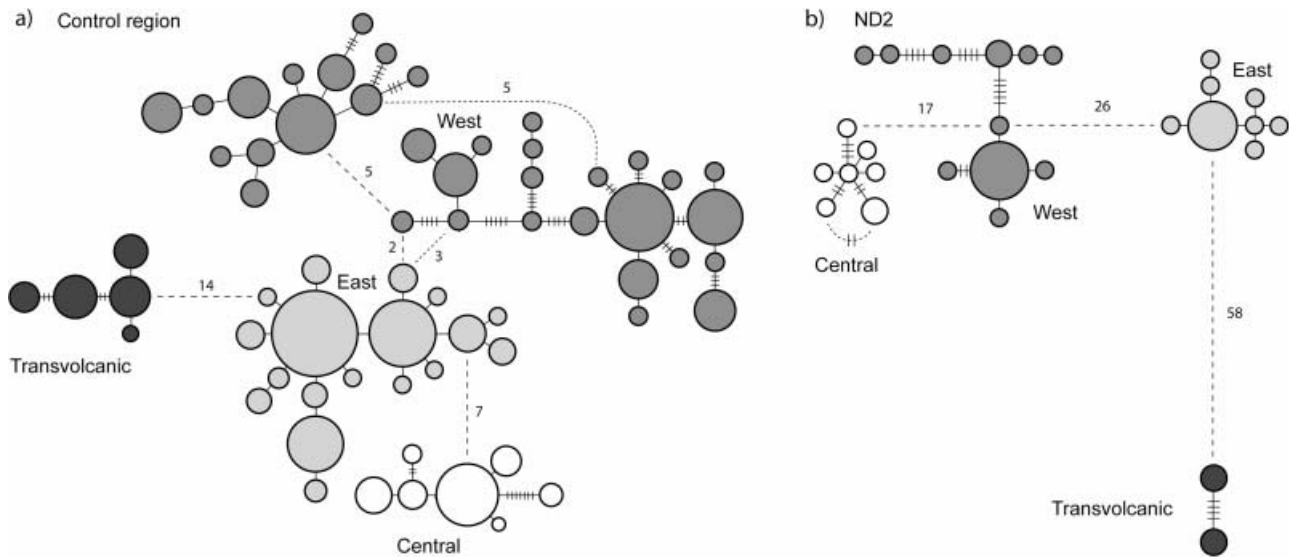


Fig. 4 Minimum-spanning networks of absolute genetic distances between mitochondrial DNA haplotypes of (a) control region, and (b) ND2. Each circle represents a haplotype, with size proportional to the haplotype's frequency in the population (only proportional within groups). Different shadings indicate the four main genetic groups. Unmarked network branches represent a single nucleotide substitution; hatch marks along branches represent additional substitutions. Dotted lines represent alternative pathways of equal parsimony. Dashed lines represent connections among the four groups (accompanied by number of substitutions).

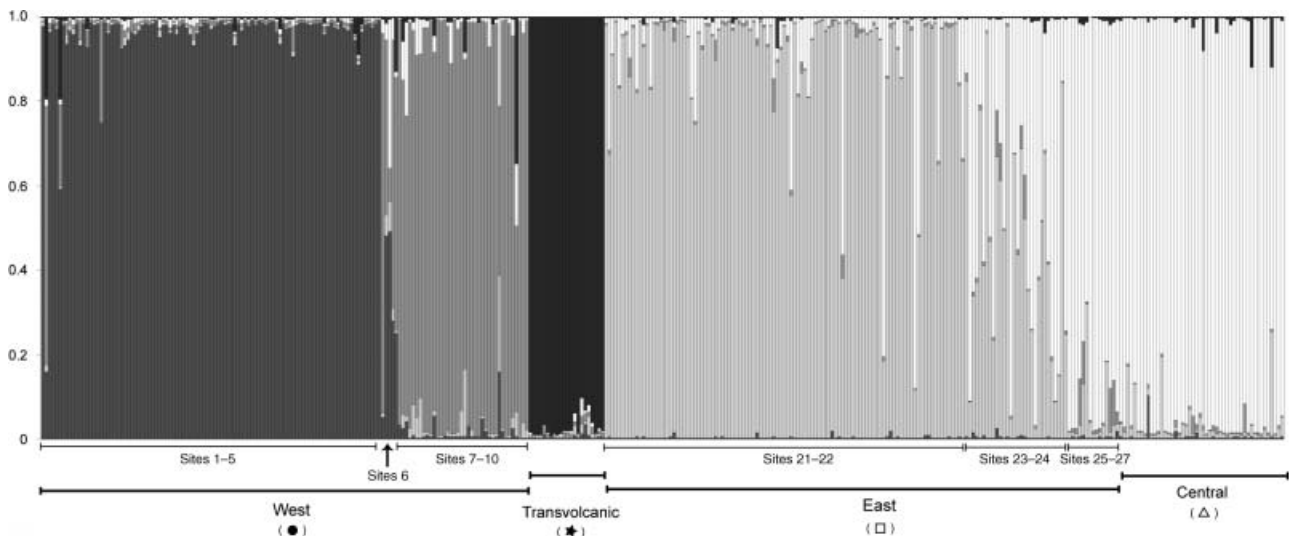


Fig. 5 Plot of posterior probability of assignment for 362 Mexican jays (vertical lines) to five genetic clusters based on Bayesian analysis of variation at 13 microsatellite loci. Symbols below localities refer to site locations from Fig. 1.

the generality of PC1 and PC2 as descriptors of brightness and chroma, respectively (Grill & Rush 2000). DFA using PC1 and PC2 scores and morphological traits reduced nine traits to three functions that described 98.5% of the total variation. Plumage traits had the highest coefficients for function 1 (df1), although wing–tail ratio was also important (Table 4). Function 2 (df2) was predominately a bill length–wing length ratio.

The major genetic groups of Mexican jays showed considerable separation when df1 and df2 scores were plotted in phenotype space (Fig. 6). Indeed, the Transvolcanic clade differed from other Mexican jays nearly as much as unicoloured jays did; the Western clade was also completely distinct in phenotype space. These results are highly robust because males, females, and second-year birds were included in the analysis, increasing within-group variation. Mexican

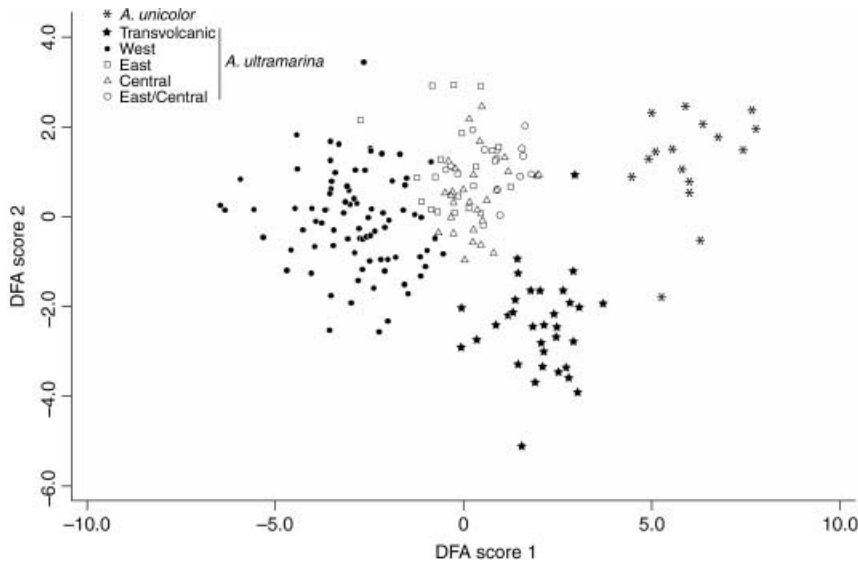


Fig. 6 Scatterplot of canonical DFA scores for Mexican jays and unicoloured jays in phenotypic space. DFA coefficients for the phenotypic traits used in the analysis are found in Table 4.

Table 3 Pairwise F_{ST} between Mexican jay populations for mtDNA (control region; above diagonal) and pairwise G'_{ST} (Meirns 2006) for microsatellites (below diagonal). Populations within clades have been subdivided to show differentiation within clades and among subspecies

Clade (sites; subspecies)	West1	West2	West3	Central	Trans1	Trans2	East1	East2
Western1 – north (1–5; <i>arizonae</i>)		0.57	0.58	0.78	0.82	0.82	0.77	0.64
Western2 – south (6–8, 10; <i>wollweberi</i>)	0.4		0.05	0.85	0.9	0.9	0.84	0.82
Western3 – south (9; <i>gracilis</i>)	0.48	0.07		0.86	0.91	0.91	0.84	0.85
Central (15–20; <i>potosina</i>)	0.55	0.26	0.3		0.92	0.92	0.84	0.83
Transvolcanic1 – west (11–12; <i>colimae</i>)	0.69	0.58	0.63	0.65		0.55	0.91	0.95
Transvolcanic2 – east (13–14; <i>ultramarina</i>)	0.52	0.4	0.48	0.52	0.56		0.92	0.96
Eastern1 – north (21–24; <i>couchii</i>)	0.55	0.27	0.32	0.12	0.6	0.44		0.11
Eastern2 – south (25–27; <i>couchii</i>)	0.57	0.24	0.27	0.11	0.69	0.58	0.2	

Table 4 DFA coefficients for phenotypic traits and proportion of variance explained for each function

Trait	Function 1	Function 2	Function 3
Plumage PC 1	-1.193	-0.153	-0.127
Plumage PC 2	-0.901	-0.214	-0.788
Wing	-0.518	-0.83	0.478
Tail	0.591	-0.223	-0.694
Tarsus	-0.042	-0.007	0
Bill length	-0.208	0.526	-0.042
Lower length	0.198	-0.123	0.026
Bill depth	0.385	0.049	-0.299
Bill width	0.065	0.231	-0.195
Variance Explained	0.714	0.154	0.117

jays from the Eastern and Central clades were not distinct from one another in phenotype space, but the DFA did allow for higher-than-chance assignment to their respective groups (Central, 64%; Eastern, 58%), indicating some degree

of phenotypic differentiation (Table 5). When Central and Eastern clades were analyzed separately in a DFA, correct assignment increased to 78.57% and 83.33%, respectively.

Discussion

Genetic differentiation

Our study uncovers remarkable genetic differentiation among Mexican jay populations characterized by high sequence divergence, high F_{ST} values, and abrupt breaks between groups. Both mtDNA markers indicated four genetic clades that coincide with the Sierra Madre Occidental (Western), Sierra Madre Oriental (Eastern), southern Central Plateau (Central), and Transvolcanic Belt (Transvolcanic). At least three of these groups were also supported by an independent nuclear DNA data set. These results indicate that the central and northern Mexican mountain chains have had an important role in differentiation of Mexican jays.

Table 5 Discriminant function analysis results. Per cent and number (in parentheses) assigned

Group	Predicted group membership					
	West	Central	East	Transvolcanic	East/Central	Unicoloured
Western	91.57 (76)	3.61 (3)	4.82 (4)	0	0	0
Central	0	64.29 (18)	21.43 (6)	0	14.29 (4)	0
Eastern	4.17 (1)	12.50 (3)	58.33 (14)	0	20.83 (5)	0
Transvolcanic	0	3.03 (1)	0	96.97 (32)	0	0
Eastern/Central	0	8.33 (1)	16.67 (2)	0	75.00 (9)	0
<i>A. unicolor</i>	0	0	0	5.88 (1)	0	94.12 (16)

Substitution rates in the control region were low compared to those in ND2, which was unexpected given that the part of the control region that was sequenced is thought to be noncoding and subject to high mutation rates. Slow substitution rates in the so-called hypervariable portion of the control region have also been documented in gulls (Crochet & Desmarais 2000) and partridges (Randi & Lucchini 1998), suggesting constraints caused by stable secondary structures and possible homoplasmy due to repeated mutation at a few hypervariable sites. This effect could explain why our control region data had weak phylogenetic signal and why the minimum-spanning network showed complicated relationships among haplotypes including multiple equally parsimonious connections. Despite some incongruities, control region haplotypes showed the same strict geographic structuring as ND2, with no shared haplotypes among clades.

Nuclear markers showed similar patterns, with unambiguous assignment of individuals from the Western and Transvolcanic clades to different genetic clusters based on Bayesian analysis of microsatellite variation. In contrast to mtDNA data, individuals from the Eastern and Central clades showed gradual genetic change from north to south, with some mixed assignment for geographically intermediate populations (Fig. 5). Clinal patterns of change are also supported by phenotypic data (see below) and prior morphological (Pitelka 1951; Peterson 1991) and molecular (allozyme) studies (Peterson 1990, 1992b).

Disagreement between patterns of nuclear/phenotypic data and mtDNA for Central and Eastern groups suggests several possibilities. First, male-biased dispersal could result in low mtDNA introgression due to the matrilineal inheritance of mtDNA, but dispersal in Mexican jays seems to be approximately equal between the sexes (Brown 1994). Second, nuclear genes sort themselves four times more slowly than mtDNA; thus, recently differentiated populations will usually share more ancestral polymorphisms in nuclear genes (Moore 1995). This interpretation is challenged by our ND2 phylogeny, which rejects a sister-taxon relationship between the Eastern and Central clades. Finally, a zone of contact could exist in the sampling gap between the two clades, possibly representing secondary contact between

the groups; but if a clinal transition or zone of mixing exists, it would have to be quite narrow to result in the strict geographic structuring of mtDNA haplotypes seen in our data, and it could not by itself explain the discord between nuclear and mtDNA data. However, such a pattern is consistent with Haldane's (1922) rule, which observes that the heterogametic sex (in birds, the female) is usually the first to show hybrid sterility (e.g. Tegelström & Gelter 1990). As the only explanation not currently contradicted by other data, we tentatively consider it to be the most likely possibility. Further investigation — including expanded sampling and behavioural observations within areas of contact — is required to fully evaluate alternate hypotheses.

Nuclear and mtDNA data also point to significant differentiation within clades, especially in the Western clade, which showed geographic structuring from north to south, agreeing with previous allozyme results (Peterson 1990). However, five individuals from a geographically intermediate population (site 6; Fig. 5) showed mixed assignment based on nuclear data, and two control region haplotypes were shared between north and south, suggesting clinal variation and gene flow, respectively. Based on this evidence and the gap in sampling between sites 5 and 6 (Fig. 1), the strongly supported phylogenetic split between northern and southern populations in the Sierra Madre Occidental (Figs 2 and 3) could be a sampling artefact. Observed structure among the sky islands of Arizona (Fig. 1, sites 1–5), however, is not a sampling artefact and may reflect Late Pleistocene divergence followed by mixing of these lineages during glacial maxima when forests were more widespread (McCormack 2007).

Timing of divergence and biogeography

Biological diversification in North America has been understood through models that emphasize Neogene geological events (Morafka 1977; Riddle 1995; Jaeger *et al.* 2005) and/or habitat fluctuations resulting from Pleistocene glacial cycles (Mengel 1970; Johnson & Cicero 2004; Weir & Schluter 2004). The uplift of Mexico's major mountain chains beginning in the Tertiary produced geographic barriers and established high-altitude habitats, offering new

ecological opportunities for diversification. This effect is exemplified by the youngest of Mexico's mountain chains, the Transvolcanic Belt, whose formation was largely completed in the Middle Miocene (~15 Ma; Ferrari *et al.* 1999) and whose high levels of endemism suggest *in situ* diversification since that time. The northern mountain chains are older, with the origin of the Sierra Madre Occidental dating to approximately 30–35 Ma and the Sierra Madre Oriental dating to the Early Tertiary (Ferrusquía-Villafranca & González-Guzmán 2005); thus, uplift of these mountain chains is probably too old to have been responsible for diversification events between extant sister taxa. On the other hand, habitat fluctuations during the Pleistocene were dramatic (Dansgaard *et al.* 1993) with well-documented genetic consequences for plant and animal taxa (Hewitt 1996; Avise & Walker 1998; Lessa *et al.* 2003). Among birds, isolation in refugia during glacial maxima is thought to have driven the speciation of many extant sister taxa (Johnson & Cicero 2004; Weir & Schluter 2004), although the importance of the Pleistocene compared to older periods has been debated (Klicka & Zink 1997).

We offer some broad speculation on the timing of divergence in Mexican jays in relation to these diversification models, acknowledging the potential for error associated with assuming a particular divergence rate, which we discuss below. Timing estimates under a relaxed phylogenetic framework suggest that divergence of the Transvolcanic clade occurred in the Miocene or Early Pliocene (4.35–9.35 Ma), while divergence of the Eastern clade occurred in the Pliocene (2.01–4.42 Ma). Western and Central clades are estimated to have diverged later in the Pliocene or Early Pleistocene (1.13–2.73 Ma). These timing estimates would seem to reject the hypothesis that formation of the Mexican highlands played a role in diversification of Mexican jays, although uplift of the Transvolcanic Belt might have continued into the Pliocene and Pleistocene (Ferrusquía-Villafranca & González-Guzmán 2005) and therefore might have played a role in differentiation of the Transvolcanic clade. By the same token, these results suggest that divergence likely occurred prior to the extreme glacial–interglacial cycles that characterized the last 0.7 Ma of the Pleistocene and that are thought to have been the major contributor to biological diversification during this period (Webb & Bartlein 1992).

Although our dating methods allowed for rate heterogeneity among lineages, several assumptions are still inherent in choosing particular divergence rates for protein-coding mtDNA; previous estimates have clustered around 2% per million years, but have shown some heterogeneity among taxa (Lovette 2004). Importantly, most calibrations come from the mtDNA cytochrome *b* (*cyt b*) gene, while our study used the ND2 gene; although both are protein-coding genes, some evidence points to elevated divergence rates in ND2 compared to *cyt b* (Roy 1997). If so, this would generate

more recent dates for lineage-splitting, which would still result in rejection of geological hypotheses for divergence, but would make discriminating between Pliocene and Pleistocene hypotheses more difficult. Considering that our estimates of divergence time in Mexican jays are surprisingly old, we therefore feel it appropriate to offer these divergence estimates as hypotheses to be confirmed or rejected by analyses using more genes; most importantly, application of more taxon-specific calibrations of molecular divergence rates will be critical in clarifying these points.

The role of ecology

Speciation facilitated by differentiation in ecological dimensions is often posed as an alternative to speciation through vicariance, although the two may work in concert (Schluter 2001). As such, an alternate hypothesis is that ecological factors might have driven divergence in Mexican jays with or without strong vicariant barriers. Indeed, a niche-modelling study using three of the four clades described here (minus Central) demonstrated significant ecological differentiation and abrupt niche shifts among Mexican jay lineages (Rice *et al.* 2003), similar to patterns seen in some adaptive radiations (Losos *et al.* 2003) in which the role of ecology is paramount (Schluter 2000). In contrast, conservatism of ecological niches over evolutionary time is often observed when vicariance has played a dominant role in speciation (Peterson *et al.* 1999; Wiens 2004).

Two lines of evidence from our data suggest that vicariance might not fully explain divergence in Mexican jays and point to a role for ecology in differentiation. First, genetic breaks largely coincide with breaks in phenotypic characters that describe shape differences (e.g. DFA1, wing–tail ratio; DFA2, bill length–wing length ratio) that are often linked to ecology in birds, specifically in *Aphelocoma* jays where bill shape varies adaptively with local resources (Peterson 1993; McCormack 2007). Second, whereas some of the genetic breaks in Mexican jays occur across well-known vicariant barriers, others do not. As in Mexican jays, many other species show differentiation of Transvolcanic Belt populations, which are isolated from northern populations by the Bajío Depression (Marshall & Liebherr 2000; Sullivan *et al.* 2000; León-Paniagua *et al.* 2007; but see several bird studies that show little or no differentiation of Transvolcanic Belt populations, e.g. *Spizella passerina*, Milá *et al.* 2006; *Junco* spp., Milá *et al.* 2007; *Sitta carolinensis*, Spellman & Klicka 2007). However, separation of the Eastern and Central clades and, to a lesser extent, the Western and Central clades do not occur in well-known zones of turnover between sister taxa (Ramamoorthy *et al.* 1993). The Western–Central break corresponds to a geological feature known as the Aguascalientes Graben, which cuts a low-lying valley through highland forest in Aguascalientes (Fig. 1); this feature could pose a barrier to dispersal, but other com-

parable habitat breaks have not resulted in similar levels of genetic divergence. Considering the paucity of phylogeographic studies of the Mexican highlands, strong conclusions about the role of vicariance and ecology based on comparative phylogeography must await further research. Our observed genetic breaks could very well denote important, but overlooked vicariant barriers. The vicariance hypothesis would be strengthened if codistributed taxa with similar dispersal patterns but conserved ecological niches also show concordant genetic breaks to those seen in Mexican jays.

Implications for taxonomy and conservation

The genetic clades described here bear some resemblance to previous subspecies classification (Pitelka 1951) and, especially, to the three groups into which these subspecies have often been organized (Pitelka 1951; Navarro-Sigüenza & Peterson 2004). The Western clade comprises the subspecies (from north to south) *arizonae*, *wollweberi*, and *gracilis*. Available evidence suggests clinal change in genetics and phenotype among these groups. Interestingly, there is no evidence for substantial genetic differentiation of *gracilis*, which lies at the extreme southern end of a cline of decreasing size and has often been considered distinctive in phenotype, habitat, and vocalizations (Brown & Horvath 1989). The Transvolcanic clade comprises subspecies (west to east) *colimae* and *ultramarina*, and although considerable genetic differentiation occurs among populations of the two subspecies (Table 3), they also share mtDNA haplotypes, indicating gene flow or insufficient time since divergence for completion of gene sorting. The major surprising result was the strong differentiation in mtDNA of the Central clade, which corresponds approximately to the subspecies *potosina*, formally grouped with the more northern subspecies of the Sierra Madre Oriental, *couchii*. According to the ND2 phylogeny, the two may not even be sister taxa.

Genetic, phenotypic, and ecological differences among these allopatric groups suggest barriers to reproduction. On the basis of sequence divergence, lack of haplotype sharing, and diagnostic phenotypic differences comparable in magnitude to those found between Mexican jays and an occasionally sympatric congener (*Aphelocoma unicolor*), the Transvolcanic and Western clades provide relatively uncontroversial candidates for species status no matter what species concept is employed. Reproductive isolation between the Eastern and Central clades is supported by the lack of haplotype sharing without a clear geographic barrier to dispersal and the robust ND2 phylogeny, which indicates that the two are not sister taxa; however, nuclear DNA and phenotypic data suggest ongoing gene flow, perhaps in secondary contact. Detailed study of the genetics, ecology, and vocalizations in potential areas of contact (see Pitelka 1951) are needed to fully evaluate the degree of reproductive isolation among these newly delimited groups.

Mexican jays are currently considered a species of least concern by the International Union for the Conservation of Nature and Natural Resources (BirdLife International 2007). Taxonomic splitting would have several implications for conservation of Mexican pine-oak woodlands. Under a revised taxonomy, Mexico's pine-oak woodlands would gain at least two new endemic or near-endemic species, some (e.g. the Transvolcanic clade) with restricted, patchy ranges in areas of dense and increasing human habitation. This study and other recent investigations (Navarro-Sigüenza & Peterson 2004) demonstrate that much remains to be learned about diversity and speciation in Mexico, particularly in the highlands. Further work is needed for basic phylogeographic assessments so that evolutionarily distinct taxa may be recognized before they are lost to extinction.

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Supplementary material

The following supplementary material is available for this article:

Appendix S1 Locality data for specimens of 178 Mexican jays and 17 unicolored jay used in phenotypic analysis.

This material is available as part of the online article from:

<http://www.blackwell-synergy.com/doi/abs/10.1111/j.1365-294X.2008.03776.x>

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