

Genetic variation coincides with geographic structure in the common bush-tanager (*Chlorospingus ophthalmicus*) complex from Mexico

Jaime García-Moreno^{a,b,*}, Adolfo G. Navarro-Sigüenza^c,
A. Townsend Peterson^d, Luis A. Sánchez-González^c

^a Max Planck Research Centre for Ornithology (Vogelwarte Radolfzell) Schlossalleé 2, D-78315 Radolfzell, Germany

^b Department of Biology, University of Konstanz, D-78457 Constance, Germany

^c Museo de Zoología “Alfonso L. Herrera,” Departamento de Biología Evolutiva, Facultad de Ciencias, Universidad Nacional Autónoma de México, Apartado Postal 70-399, Mexico D.F. 04510, Mexico

^d Museum of Natural History, University of Kansas, Lawrence, KS 66045, USA

Received 5 January 2004; revised 11 May 2004

Available online 8 July 2004

Abstract

Cloud forests are distributed in the Neotropics, from northern Mexico to Argentina, under very specific ecological conditions, namely slopes with high humidity input from clouds and mist. Its distribution in Mesoamerica is highly fragmented, similar to an archipelago, and taxa are thus frequently represented as sets of isolated populations, each restricted to particular mountain ranges and often showing a high degree of divergence, both morphologically and genetically. The common bush-tanager (*Chlorospingus ophthalmicus*, Aves: Thraupidae) inhabits cloud forests from eastern and southern Mexico south to northwestern Argentina. Here we use 676 bp of mtDNA (around the ATPase 8 gene) to explore the genetic variation and phylogeographic structure of the Mexican populations of *C. ophthalmicus*. Phylogenetic analyses of mtDNA sequences indicate deep genetic structure. Five major clades, which segregate according to geographic breaks, are identified (starting from the deepest one in the phylogeny): (1) Southern Chiapas and Northern Central America, (2) Tuxtlas massif, (3) Sierra Madre del Sur, (4) Eastern Oaxaca and Northern Chiapas, and (5) Sierra Madre Oriental. The long history of isolation undergone by each clade, as suggested by the phylogeny, implies that the species status of each of them should be revised.

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Keywords: *Chlorospingus ophthalmicus*; Cloud forest; Mesoamerica; Phylogeography; mtDNA

1. Introduction

Most studies of geographic variation and species limits in Neotropical birds have addressed variation in morphological characters (e.g., Haffer and Fitzpatrick, 1985; Johnson, 2002; Marin, 1997; Navarro et al., 2001; Schuchmann et al., 2001), while studies on the corresponding genetic variation are still relatively scarce

(e.g., Aleixo, 2002; García-Moreno and Fjeldså, 2000; García-Moreno et al., 2001; Hackett, 1996; Hackett and Lehn, 1997). In species inhabiting fragmented environments, geographic variation in morphology may show clinal variation along latitudinal gradients (e.g., Benitez, 1993; García-Moreno et al., 1999a; Navarro et al., 2001), “leapfrog” patterns (García-Moreno et al., 1999b; Johnson, 2002; Maijer and Fjeldså, 1997; Rensen, 1984), or “checkerboard” patterns (García-Moreno et al., 1999a; Heindl and Schuchmann, 1998). Habitat fragmentation, limited elevational ranges, and restricted ecological breadth are among the forces that

* Corresponding author. Fax: +49-7531-884036.

E-mail address: Jaime.Garcia-Moreno@uni-konstanz.de (J. García-Moreno).

catalyze population variation and speciation (Graves, 1985, 1988).

In Mexico, complex geological processes and the confluence of two major biotic realms (Neotropical and Nearctic regions, Morrone et al., 1999; Ramamoorthy et al., 1993) have produced an astonishing biodiversity, including many endemic forms restricted to particular biomes or ecosystems (e.g., montane forests, Escalante et al., 1993). Many such taxa are distributed as series of isolated populations that correspond to islands of cloud forest, deserts, alpine habitats, etc. These strings of isolated populations show higher degrees of divergence as compared with species with broader distributions and more continuous populations (García-Moreno et al., 1999a; Navarro et al., 2001; Peterson et al., 1992).

Cloud forest, or humid montane forest (sensu Hernández-Baños et al., 1995; Terborgh, 1971), is distributed from northern Mexico to northern Argentina, between latitude 23°N and 25°S, at elevations between 600 and 3000 m (Brown and Kappelle, 1995; Webster, 1995). These forests are very diverse in terms of form, given the complex structure of tree heights and tree architecture (Foster, 2001; Gentry, 1995; Luna et al., 1994). They are also very diverse and complex floristically, with taxonomic composition reflecting the latitudinal situation: forests from Mexico to Nicaragua are dominated by taxa of Laurasian (Nearctic) origin, whereas forests from southern Central America south to Argentina are dominated by taxa of Gondwanian (Neotropical) origin, with high endemism in all regions (Foster, 2001; Gentry, 1995).

Although widespread, cloud forest requires specific ecological conditions for its presence, including very steep slopes, with high humidity input from clouds and mist (Foster, 2001). As a result, the geographic distribution of cloud forest is highly fragmented, analogous to an archipelago (Luna et al., 1988; Rzedowsky, 1986). Although highly fragmented in Mexico, cloud forest is distributed more continuously in Central America and along the humid slopes of the Andes through much of South America (Graves, 1985; Terborgh, 1971). In Mexico, cloud forest is present along the Sierra Madre Oriental from Tamaulipas to northern Oaxaca, in the Sierra Madre del Sur of Guerrero and Oaxaca, in the mountains of northern and southern Chiapas, and in restricted patches along the Sierra Madre Occidental and the Transvolcanic Belt (Hernández-Baños et al., 1995; Rzedowsky, 1986; Watson and Peterson, 1999). Divergence of populations has often proven extreme, both in terms of morphology and molecular characters (Navarro et al., 2001; Peterson et al., 1992), and this is frequently reflected taxonomically as endemic forms in each main mountain range (e.g., Hardy, 1969; Llorente, 1984; Sullivan et al., 1997, 2000). Hence, studies of geographic variation and phylogenetic associations of the

cloud forest biota elements are key to understand their processes of differentiation and speciation, and to devise sound conservation strategies for this highly endangered habitat.

The common bush-tanager (*Chlorospingus ophthalmicus*, Aves; Thraupidae) is a small species that inhabits cloud forests almost exclusively from eastern and southern Mexico to northwestern Argentina, with six subspecies described from cloud forests in Mexico alone (Isler and Isler, 1987; Miller et al., 1957). Geographic variation throughout the species' distribution is dramatic, with as many as 25 subspecies across the entire species range (Isler and Isler, 1987; Peters, 1951). Isler and Isler (1987) arranged the complex in eight major groups based on iris color, presence of a postocular spot, and presence of a pectoral band. Here we explore the genetic variation and phylogeographic associations within the *ophthalmicus* group, especially within Mexico (Fig. 1). As members of this complex are highly sedentary, inhabit cloud forest almost exclusively, and show at most limited altitudinal migration (Winker et al., 1997), genetic exchange among populations is expected to be minimal. In fact, previous work using allozymes, and based on a more restricted locality survey, indicates strong differentiation of at least some populations (Peterson et al., 1992). A finer locality sampling using more rapidly evolving molecular markers can help unravel which factors have been more influential in shaping a species' distribution, and add to our understanding of the evolutionary and ecological forces that are at play (Avise, 2000).

2. Methods

2.1. Sampling and outgroups

Tissue samples of 21 common bush-tanagers were selected from series collected at 13 localities across Mexico (Table 1). This sampling scheme was developed so as to represent each disjunct Mexican population by more than one sample (Fig. 1b). We also included samples from El Salvador (Cerro Pital), southern Ecuador (Zamora-Chinchiipe) and northern Peru (Amazonas). Sequences of putative related taxa, Thraupidae and Emberizidae (genera *Aimophila* GenBank Accession No. AF447312, *Calamopsiza* AF447316, *Atlapetes* AF447313, and *Junco* AF447338, and *Chlorospingus canigularis* AF447322; Yuri and Mindell, 2003), were included as outgroups with which to root the resulting trees. Nevertheless, as inclusion of these taxa had no effect on tree structure, neither with maximum parsimony (MP) nor with maximum likelihood (ML), we conducted further analyses using *C. ophthalmicus* from South America to root the tree for Mesoamerican populations.

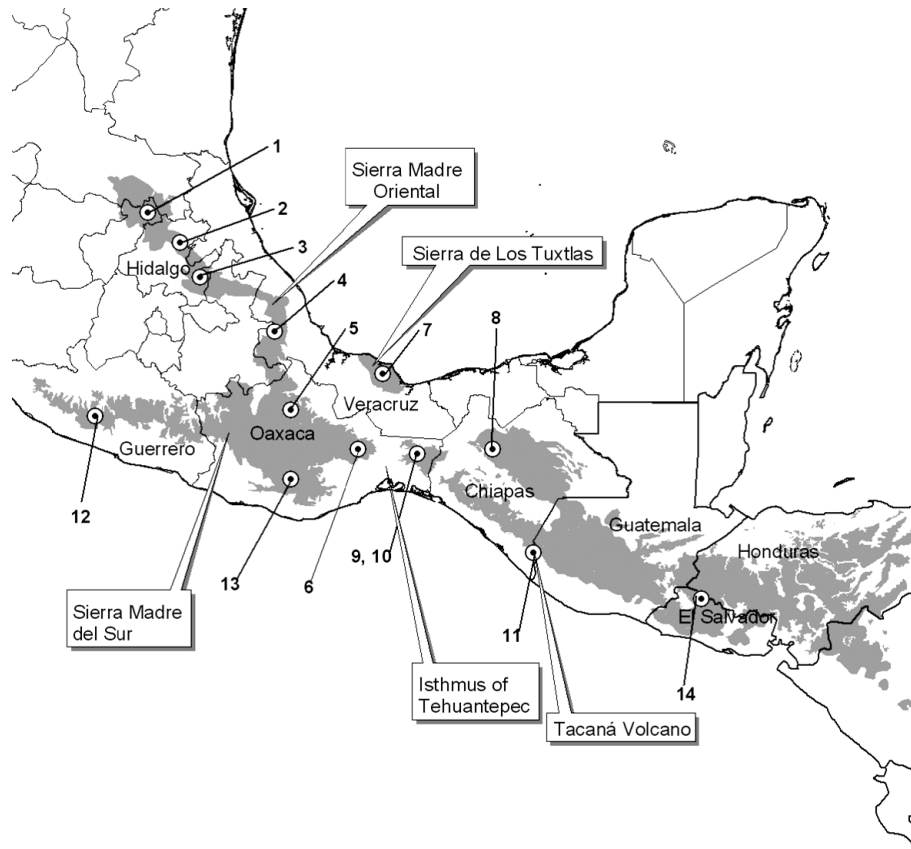


Fig. 1. Distribution of *Chlorospingus ophthalmicus* populations in Mexico and northern Central America. Dotted circles indicate collection locality of tissue samples and numbers refer to localities listed in Table 1. Some important locations mentioned in the text are highlighted in the map.

Table 1

Collection localities, GenBank Accession number, and museum catalog number for specimens of tissue samples used in the genetic analyses

Locality	Accession No.	Museum and catalog No.
1. Mexico, Querétaro, 7 km S de Tres Lagunas	AY609275	MZFC 9715
2. Mexico, Hidalgo, 5 km E Tlanchinol	AY609276	(1) MZFC BMM 085
	AY609277	(2) MZFC 10398
3. Mexico, Hidalgo, El Potrero, 5 km Tenango	AY609278	(1) MZFC 11297
	AY609279	(2) MZFC 10981
4. Mexico, Veracruz, Jardín Botánico de Xalapa	AY609280	IECO 065
5. Mexico, Oaxaca, Sierra de Huautla	AY609281	(1) MZFC 12490
	AY609282	(2) MZFC 11585
6. Mexico, Oaxaca, Cerro Zempoaltépetl, Totontepec	AY609283	MZFC MXJ 511
7. Mexico, Veracruz [Sierra de Los Tuxtlas], Volcán de Santa Marta	AY609284	(1) MZFC MX 1080
	AY609285	(2) MZFC MX 1078
8. Mexico, Chiapas, 6 km NE de Pueblo Nuevo, camino Aurora-Ermita	AY609286	(1) MZFC 9584
	AY609287	(2) MZFC 9573
9. Mexico, Oaxaca [Chimalapas] Chalchijapa, 20 km NE del campamento	AY609288	MZFC 12084
10. Mexico, Oaxaca, Chimalapas	AY609289	(1) MZFC B18089
	AY609290	(2) MZFC B18090
11. Mexico, Chiapas, Río Malá, Volcán Tacaná, x km Unión Juárez	AY609291	(1) MZFC 8826
	AY609292	(2) MZFC 8832
12. Mexico, Guerrero, El Iris, Sierra de Atoyac	AY609293	MZFC MX1437
13. Mexico, Oaxaca, Reyes Llano Grande	AY609294	(1) MZFC 12810
	AY609295	(2) MZFC 11579
14. El Salvador, Cerro El Pital	AY609296	(1) KU 4892
	AY609297	(2) KU 4885
15. Ecuador, Zamora-Chinchiipe, S. Romerillos,	AY609298	(1) ZMUC 03354
	AY609299	(2) ZMUC 03361
16. Peru, Dpt. Amazonas, Cordillera Colán,	AY609300	ZMUC JGM6-160796

2.2. DNA isolation, amplification, and sequencing

DNA was extracted from tissue samples (blood samples for South American samples) using the Qiagen extracting kit, and following manufacturer's protocols. We amplified and sequenced a fragment comprising the end of cytochrome oxidase II (63 bp), tRNA-lysine (73 bp), complete ATPase 8 (168 bp), and a fragment of ATPase 6 (382 bp). ATPase 8 and 6 have a 10 bp overlap in different reading frames, thus the total length of the sequence was 686 bp. Amplification was conducted using the primers L8929 and H9855 (Sorenson et al., 1999), and also L8950, designed specifically to match *C. ophthalmicus* sequences (5'-CCAACCACAGCTT CATGCC-3'). Position numbers are given in relation to the chicken mitochondrial genome (Desjardins and Morais, 1990). Amplified products were cleaned by gel filtration using Sephadex G50 columns (Sigma), and sequenced using dye-labelled terminators (BigDye v.2.0, Applied Biosystems). Sequencing reaction products were cleaned by gel filtration in the same way as PCR products, and resolved on an ABI 3100 automated sequencer. All sequences have been deposited in GenBank under Accession Nos. AY609275–AY609300.

2.3. Phylogenetic analyses

Sequences were aligned by eye using Se-Al v.2.11 (Rambaut, 2002). Because the majority of the fragment sequenced is protein-coding (with the exception of tRNA-Lys), and has no introns or gaps, alignment of sequences was straightforward. We conducted phylogenetic analyses under three optimality criteria: minimum evolution, maximum parsimony, and maximum likelihood, all using PAUP* v. 4.0b10 (Swofford, 2002), except where otherwise stated. Alternative topologies were compared statistically using the test of Shimodaira and Hasegawa (1999) as implemented in PAUP*.

Model parameters for likelihood searches were estimated using Mr. Modeltest (Nylander, 2002), which compares goodness of fit of models using the likelihood ratio test statistic (Huelsenbeck and Rannala, 1997). The best fitting model for the *Chlorospingus* sequences was HKY85+ Γ , with a ts/tv ratio=14.7043 and a gamma distribution shape parameter=0.1064. We used the base frequency values suggested by the model (pA=0.3093, pC=0.3517, pG=0.1015), although the empirical values were very similar to those. Subsequent heuristic searches under the likelihood optimality criterion were performed with these parameters starting from a Neighbour-joining tree and swapping branches with the TBR algorithm. We used bootstrap analyses (Felsenstein, 1985) to estimate branch support for parsimony and minimum evolution trees. A haplotype network was constructed using the TCS package (Clement et al., 2000).

We also developed tree hypotheses using Bayesian inference approaches. These analyses were conducted using Mr. Bayes v.3.0b4 (Huelsenbeck and Ronquist, 2002). We ran simultaneously four Markov chains initiated from random trees for 500,000 generations, and sampling every 100 generations. We used again the HKY+ Γ model of nucleotide substitutions, with four different partitions experiencing different rates of evolution: first, second, and third codon position, and tRNA; variation within each partition followed a gamma distribution. The first 4000 trees of the chain were discarded and the posterior probabilities for each node, as well as the consensus phylogeny, were estimated based on the last 1000 trees in the chain, all generated in the stationary phase.

For lack of any geological event or fossil information that could be unambiguously linked to a node in our phylogeny, we turned to a coalescence based approach to estimate divergence times (Nielsen and Wakeley, 2001; Palsbøll et al., 2004), as implemented in the program Mdiv (Nielsen, 2001). In this method, the coalescent model is established with three parameters: θ (equivalent to $2N_e\mu$; N_e =effective population size, μ =mutation rate), T (divergence time between two populations), and M (migration rate between two populations). After exploring values ranging through 5 orders of magnitude, we empirically set the upper value of M to 1, and of T to 20, and let the simulations run for 1,000,000 cycles. The first 100,000 cycles were discarded, and the parameters θ , M , and T were sampled after each cycle, as implemented in the program Mdiv (Nielsen, 2001). We complemented this approach with a standard method based on net genetic divergence between clades (Avise and Walker, 1998), and using the commonly accepted rate of evolution of 2% sequence divergence per million years (Klicka and Zink, 1997 and references therein).

3. Results

3.1. Genetic analyses

We detected ample genetic variation within and among common bush-tanager populations from Mexico and Northern Central America. Informative sites were distributed as follows: 9 in COII (out of 63 bp), two in tRNA-Lys (73 bp), 24 in ATPase8 (168 bp), and 55 in ATPase6 (372 bp). Uncorrected genetic distances among Mesoamerican ingroup haplotypes ranged 0.3–7.3%; overall 100 characters were variable, 90 of which were phylogenetically informative. Uncorrected distances between Mesoamerican samples and South American samples ranged 4.7–8.0% (Table 2). Distribution of the variation among codon positions was as expected in a coding section, with 19 informative first codon

Table 2
Genetic distances

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1. Queretaro	—	2/0	0/1	4/0	8/0	7/0	43/2	27/2	28/1	28/1	40/1	41/2	30/0	31/0	34/1	43/3
2. Tlanchinol 1	0.003	—	2/1	4/0	9/0	7/0	43/2	27/2	28/1	30/1	41/1	42/2	31/0	32/0	34/1	43/3
3. El Potrero 1	0.002	0.005	—	3/1	9/1	7/1	43/3	27/3	28/2	28/2	41/2	42/3	31/1	32/1	34/1	43/4
4. Jalapa	0.007	0.006	0.007	—	11/0	9/0	45/2	27/2	28/1	30/1	43/1	44/2	33/0	34/0	34/1	45/3
5. Huautla	0.014	0.016	0.018	0.020	—	4/0	46/2	28/2	29/1	31/1	42/1	39/2	36/0	37/0	34/1	41/3
6. Zempoaltepetl	0.012	0.012	0.014	0.016	0.006	—	42/2	24/2	25/1	27/1	40/1	37/2	32/0	33/0	32/1	38/3
7. Tuxtla 1	0.147	0.136	0.151	0.148	0.153	0.131	—	40/4	39/3	41/3	36/3	39/4	36/2	35/2	30/2	36/4
8. Pueblo Nuevo 1	0.076	0.071	0.079	0.072	0.076	0.061	0.141	—	3/1	5/1	35/3	37/4	29/2	29/2	30/2	35/5
9. Chalchijapa	0.074	0.069	0.077	0.070	0.074	0.059	0.128	0.006	—	4/0	35/2	36/3	28/1	28/1	26/2	33/4
10. Chimalapas 2	0.074	0.077	0.077	0.078	0.081	0.066	0.138	0.010	0.006	—	37/2	38/3	31/1	30/1	28/2	33/4
11. Tacaná	0.123	0.119	0.132	0.130	0.126	0.115	0.112	0.109	0.102	0.111	—	11/1	40/1	37/1	22/2	30/4
12. El Salvador 1	0.137	0.132	0.146	0.143	0.121	0.110	0.135	0.126	0.114	0.124	0.022	—	43/2	40/2	21/3	27/5
13. Atoyac	0.075	0.074	0.082	0.081	0.093	0.077	0.105	0.074	0.072	0.080	0.115	0.137	—	5/0	38/1	44/3
14. Reyes Llano Grande 2	0.079	0.077	0.086	0.086	0.098	0.081	0.101	0.078	0.070	0.077	0.103	0.123	0.008	—	38/1	44/3
15. Amazonas ^a	0.152	0.138	0.144	0.140	0.141	0.126	0.126	0.109	0.104	0.115	0.080	0.084	0.164	0.151	—	3/4
16. Ecuador 2 ^a	0.178	0.178	0.192	0.191	0.167	0.149	0.148	0.154	0.132	0.132	0.114	0.108	0.185	0.166	0.020	—

Above diagonal: number of transitions/transversions; below diagonal: ML distance using the model described in the text.

^a Taxa missing 200 bp (Amazonas) and 70 bp (Ecuador 2) of sequence.

positions, 6 in second positions, 67 in third positions, and 2 in non-coding positions. Sequences easily aligned with other avian mitochondrial genomes (e.g., Desjardins and Morais, 1990; Mindell et al., 1999) and translated to amino acids with no frameshift mutations or unexpected stop codons, suggesting that our sequences are indeed mtDNA and not nuclear pseudogenes.

Maximum-likelihood (ML), minimum evolution (ME), and maximum parsimony (MP) analyses all converged to the same tree topology presented in Fig. 2, differing only in small rearrangements within the major clades (ML: $-\ln$ likelihood = 1719.03381; ME score = 0.21472 (p -distance); MP (equal weights): length = 142 steps, consistency index = 0.7113 excluding uninformative sites, re-scaled consistency index = 0.6431). The tree includes 5 well-supported clades (abbreviations in parenthesis refer to Fig. 1): (1) SMO—Sierra Madre Oriental from northern Oaxaca north to Hidalgo, (2) EONC—Oaxaca east of the Isthmus of Tehuantepec and northern Chiapas, (3) SMS—Sierra Madre del Sur, (4) Stux—the Tuxtla region, and (5) NCam—southern Chiapas (SChs) and El Salvador. The samples from South America, which were used as outgroup, also formed a well-supported monophyletic clade basal to the Mesoamerican samples. In general, branch support was strong for the deep nodes and much weaker for short terminal nodes within each of the clades outlined above.

Results from Bayesian analyses were similar to the ME/MP/ML tree (Fig. 2B). The only difference between the Bayesian tree and the likelihood tree was the position of the EONC clade, which was placed as sister to the SMO clade in the likelihood analysis, and sister to the SMS clade in the Bayesian tree (and in the Bayesian ML tree). In any case, the branch linking the EONC clade either to the SMO clade (ML) or to SMS (Bayesian analysis) has low bootstrap support (unresolved in minimal evolution analyses, 59% in parsimony analyses) or a low posterior probability (63%). The two topologies are not significantly different according to the Shimodaira and Hasegawa test (1999) (diff. $-\ln$ likelihood = 0.59369, $p = 0.292$). The same test could not detect significant differences between trees estimated with and without the molecular clock enforced (diff. $-\ln$ likelihood = 1.53617, $p = 0.21$), suggesting that these sequences are evolving in a clock-like fashion within this group.

A minimum spanning network was consistent with the phylogenetic analyses: the network consisted of seven groups of sequences, each more than 11 steps (95% connection limit) from its closest relative. These groups coincided with clades found in the phylogenetic analyses, except that southern Chiapas and El Salvador were split into separate groups more than 11 steps apart.

To calculate the time to the most recent ancestor between the two groups that make up the SMO clade (Fig. 2), we estimated $\theta \sim 4$. The migration rate, M , with a

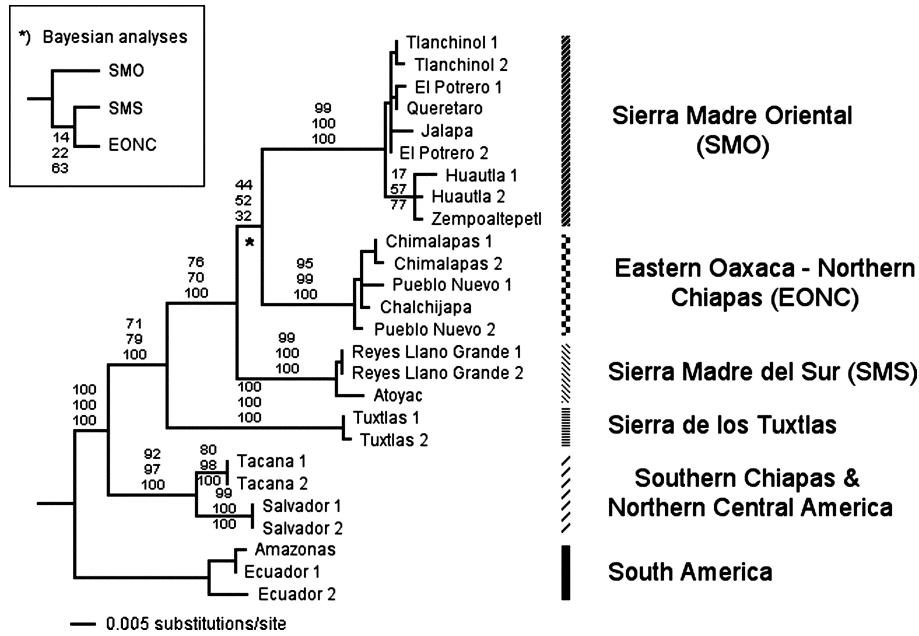


Fig. 2. Best tree recovered with maximum likelihood using the HKY+ Γ model (see text for details). All methods and analyses recovered the main clades highlighted in the figure. The same branch order of the ingroup was recovered when external outgroups were included and with different parsimony and minimum evolution analyses. Support figures above branches correspond to ME(ML distance)/MP(equal weights)/ML(Bayesian credibility value). The node marked with a star is the only one that showed a different arrangement in the Bayesian analysis (inset), and all methods gave poor support to it. Three smaller clades were consistently recovered and reasonably well supported, and are thus shown in the figure: Northern Oaxaca, Southern Chiapas (Tacana), and El Salvador.

designated upper bound of 1 (found empirically), reached a maximum around 0.1, suggesting that migration between the two groups is very reduced (1 individual every 10 generations). T peaked around 1.5, although other values (except very small ones) are compatible with the data; the 95% confidence interval of T (scaled by the population size) is given by (0.8 and 19.4). Assuming a generation time of one year, and a mutation rate one-third that of d-loop (i.e., 8×10^{-6} substitutions per site per generation in Fringillid finches; Marshall and Baker, 1997), we estimate T , the time to the most recent common ancestor, at about 750,000 years. Our results suggest that these populations likely diverged more than 0.8 times $2N_e$ generations ago, or 400,000 years ago using our assumptions. Given that *Chlorospingus* sequences appear to be evolving in a clock-like fashion, we could extrapolate these age estimates to other nodes in our tree. We find that sequences within the SMS and EONC clades also coalesce at a similar time, and populations from SChs and NCAM are estimated to have diverged a bit less than 1.5mya ($1.86 \times T$). The clades SMO, SMS, and EONC diverged from each other almost 4mya ($5.04 \times T$), and the STux clade split from the others about 5.5mya ($7.33 \times T$), not long after the split from the Southern Chiapas and El Salvador clades (6mya— $8.12 \times T$). We reach similar results using more conventional means of age estimation. Using a standard mtDNA clock (2% sequence divergence per million years; Klicka and Zink, 1997), and correcting for

intra-clade variation (Avise and Walker, 1998), the split between northern and southern SMO populations is timed at 450,000 years ago, a figure similar to our coalescent estimate. Other estimates are also of similar magnitude (e.g., separation of SMO, SMS, and EONC between 3.1 and 3.7mya, separation between STux and these previous three clades around 5.5mya).

4. Discussion

MtDNA sequences of Mesoamerican *C. ophthalmicus* populations reveal extreme levels of genetic differentiation. This result is in agreement with previous studies (Peterson et al., 1992), which also identified strong differentiation among four *Chlorospingus* populations based on 29 allozyme loci. Four distinct means of reconstructing phylogenetic relationships converged on a single solution: five well-differentiated clades in Mexico, with a clear hierarchical pattern of relationships, segregated from one another along clear geographic boundaries. Each of these lineages has apparently been isolated genetically from all others for significant periods of time (Fig. 2, Table 2). If this result is corroborated with other evidence, such as morphological or nuclear DNA data, different lineages of common bush-tanagers in Mexico may in fact represent different species (Helbig et al., 2002; Irwin, 2002; Puerto et al., 2001; Sánchez-Gonzalez et al., submitted; Wiens and Penkrot, 2002).

4.1. Phylogeny

The deepest branch in the tree corresponds to the southernmost samples included in the study (southern Chiapas and El Salvador); although these populations form a clade in our results, they are quite different from one another and they may prove to be distinct from each other once more Central American populations are analyzed. Also deep in the phylogeny is the population of the Tuxtlas massif of southern Veracruz, which were the most genetically differentiated samples in the study (Table 2). This mountain massif arose from volcanic activity in the Oligocene (Martin-del Pozzo, 1997) and has always been isolated from other mountain masses. Its avifauna (and perhaps other components of its biota) represents an often-neglected local focus of endemism (e.g., Winker, 1997). Other bird endemics in the region include *Buarremon "brunneinucha" apertus* (Peterson et al., 1992), *Campylopterus excellens*, and *Geotrygon carrikeri* (Peterson, 1993). Howell and Webb (1995) suggested that the Tuxtlas *Chlorospingus* populations are closely related to populations in Chiapas; our results however, suggest that relationships in this region are more complex than currently appreciated, as the populations from Chiapas fall into two strongly differentiated clades.

We consider relationships among the three clades in the Sierra Madre del Sur (SMS), Sierra Madre Oriental (SMO), and eastern Oaxaca—northern Chiapas (EONC) to remain unresolved. Some analyses suggest a closer relationship between SMO and EONC, while others indicate sister—group relationships between SMS and EONC; still, all of these nodes have low support and relationships cannot be confidently resolved based on data at hand. These three clades clearly have a long history of isolation (Fig. 2). That the populations of EONC would be more closely related to those of SMO and SMS than to Central American populations is consistent with the geology and physiography of the region. Ferrusquía (1993) considered the Tacaná Volcano as the northernmost section of the Central American Cordilleras volcanic system, whereas the areas corresponding to northern Chiapas and western Sierra Madre de Chiapas belong to a different, younger folding process from the Cenozoic. This pattern of differentiation coincides with distributions of other species complexes, such as *Atlapetes albinucha*–*Atlapetes gutturalis* and *Basileuterus rufifrons*–*Basileuterus delatitrii*, although none of these have been studied at the molecular level. Whether populations were established and isolated by geological events or not is a complex question, but coincident phylogeographic patterns are clear.

Cloud forest is patchy along the Sierra Madre del Sur, and our samples represent the extremes of *Chlorospingus* distributional area in the region. Within the Sierra Madre del Sur, the sequence from central Guerrero

(Atoyac) differs by almost 1% from samples taken just 250 km away in southern Oaxaca. Some cloud forest islands in the Sierra Madre del Sur may have remained isolated from one another for long periods of time (compare, e.g., with *Eupherusa poliocerca* and *E. cyanophrys*, which show such a pattern). However, more thorough sampling is required to assess the degree of genetic differentiation and discontinuity in these populations, and to corroborate whether genetic variation there is spatially structured. The Sierra Madre Oriental clade appears to be further subdivided into two clades, with samples from northern Oaxaca and samples from farther north (San Luis Potosí south to central Veracruz) segregating into distinct subclades. Our preliminary estimates (given the small sample sizes) suggest that the migration rate between these two clades is low, and that these clades have been effectively isolated for thousands of years.

4.2. Historical biogeography

When rooted with its closest Fringillid relatives (Yuri and Mindell, 2003), the basal clades among common bush-tanagers are the southernmost populations, including first those of South America, then those of Central America, and finally those of southern Mexico. This topology agrees roughly with a scenario of range expansion from South America northward into Northern Central America and Mexico, with a great amount of local differentiation and speciation along the way. The oldest nodes of our phylogeny predate the completion of the Panamanian land bridge (around 3–5 mya; Keigwin, 1982; Marko, 2002), but coincide with a time when there was an important faunal exchange between North and South America (Marshall et al., 1979). Several other molecular studies have also reported ages preceding the completion of the Central American land bridge in a wide variety of Central American organisms (e.g., fresh water fishes, Bermingham and Martin, 1998; trees, Cavers et al., 2003; frogs, Crawford, 2003; birds, Outlaw et al., 2003; arthropods, Zeh et al., 2003). This is consistent with a model in which a temporary land corridor allowed an early surge of colonization from South America in the late Miocene (Bermingham and Martin, 1998; Zeh et al., 2003). Other possibilities, however, involve inaccuracies in the present dating of the uplift of the Isthmus or errors inherent to time estimation from molecular data (Arbogast et al., 2002; Graur and Martin, 2004; Hillis et al., 1996; Shaul and Graur, 2002)—see the large confidence limits associated with our appraisals in the results section.

Within continuous land areas, although cloud forests presently occur exclusively at relatively high elevations, evidence indicates that they occupied different altitudinal strata under climatic conditions that differ from the present ones (Foster, 2001; Graham, 1993; Toledo,

1982). Bush-tanagers and other habitat-restricted species, could expand their distributions in periods of cooler and more humid climatic conditions (e.g., during some Pleistocene glaciations, in which now-fragmented cloud forest became established at lower elevations), followed by subsequent isolation of populations as the cloud forest withdrew to higher elevations during interglacial periods (Foster, 2001; Vuilleumier and Simberloff, 1980). The common bush-tanager is very sedentary and almost exclusively confined to cloud forest, and it must have tracked the forest's expansion and contraction cycles. There is ample evidence that organisms respond to climate change and that each species responds in a different manner (e.g., Hughes, 2000; McCarthy, 2001; Peterson et al., 2002; Vrba, 1985; Walter, 1993; Walther et al., 2002). Although the response to eco-climatic changes may involve quick speciation through directional selection (Walter and Paterson, 1994), in the majority of cases the response is conservative, with each species tracking its own niche and niche conservatism between allopatric sister species (Peterson et al., 1999).

One scenario that can be derived from our phylogeny is a northward expansion of populations from northern Central America, which eventually became 'trapped' in the Tuxtla mountains. This ancestral population may have been split by vicariant events such as the spontaneous fracture of the cloud forest as the climate changed and/or the uplift of the Northern Central American highlands (Rogers et al., 2002), and the population on the Mexican side eventually seeded the other Mexican clades as the cloud forest moved from lowland back into higher elevation. Similar vicariant mechanism has been suggested by studies in other habitat-restricted taxa of different ages and vagility, like plethodontid salamanders (Wake, 1987), various amphibians (Campbell, 1999), mammals (Sullivan et al., 1997, 2000), beetles (Lieberr, 1991; Marshall and Lieberr, 2000), and vascular plants (Luna-Vega et al., 1999, 2001). Luna-Vega et al. (2001) also noted the long isolation of the montane vegetation of Los Tuxtlas, and its close relation to northern Central America.

If current conditions are typical of the history of the group, presently isolated *Chlorospingus* populations are unlikely to experience genetic exchange of a magnitude sufficient to overcome the homogenizing effects of genetic drift within populations, which in turn are likely to cause differentiation between populations (Hartl and Clark, 1989). The relatively long branches leading to each clade in the phylogeny (Fig. 2) suggest a long history of isolation for each clade after its split from the ancestral population. Interestingly, *Chlorospingus* evolution in Mesoamerica appears to predate the marked climatic oscillations that characterized the Pleistocene—if our dating scheme is reasonable. Indeed, to fit the bush-tanager phylogeny into Pleistocene climatic

fluctuations, *Chlorospingus* mtDNA sequences would have to have evolved 5–7 times faster than the rate estimates we used. The split between northern and southern SMO populations is timed at $4.5\text{--}7.5 \times 10^5$ years ago (with a large error), a figure coincident with Pleistocene origins, but that still sets the origin of the different Mesoamerican clades well before the Pleistocene glaciations (Fig. 2 and Table 2).

The apparent old age of the group highlights the importance of history, along with ecological factors, to try to disentangle the processes that have given rise to its current disjunct distribution (Vuilleumier and Simberloff, 1980). The geographic setting in which bush-tanagers have evolved—clearly fragmented and with widespread isolation of populations—has undoubtedly promoted their diversification. The existence of multiple diagnostic morphological features and marked genetic differentiation suggest that species limits in this complex should be reassessed. Many challenges still remain, however, in our understanding of the diversification processes in Mesoamerica. For *C. ophthalmicus* we still have to integrate our results into an understanding of the broader picture of bush-tanager evolution and phylogeny. Central and South American populations are probably equally diverse and subdivided, and thus we have analyzed only a truly minor portion of the diversity in the genus *Chlorospingus*. For Mesoamerica as a whole there is a need for additional molecular-based case studies in many organisms that, taken together, will help us understand the processes that have given rise to the rich biota of the area, as has been the case in other areas of the world (Avise, 1992; Taberlet et al., 1998). And in order to get a full picture of Mesoamerican dynamics, we will have to complement the information provided by molecular tools with additional data from a broad array of disciplines within biology and geology, including studies on morphological and phenological variation, climate and niche modeling and paleo-ecology to name a few. Only a multidisciplinary approach will give us a comprehensive picture of the mechanisms behind the diversification processes in the tropics.

Acknowledgments

We thank the curators of the collections surveyed for access to specimens. Tissue samples were kindly supplied by John Bates (Field Museum of Natural History), Mark Robbins (University of Kansas Natural History Museum), Alejandro Espinosa (Instituto de Ecología A.C.), and Jon Fjeldså (Zoological Museum, University of Copenhagen). Assistance in the field collection of specimens deposited in the skin and tissue collections of the Museo de Zoología since 1983 were obtained from Blanca Hernández, Griselda Escalona, Miriam Torres, Octavio Rojas, Hesiquio Benítez, Alejandro

Gordillo, D. Scott Baker, Noé Vargas, Javier Sahagún, Samuel López, Fanny Rebón, Adam Fry, Laura and Fernando Villaseñor, and many students and colleagues from UNAM. Comments to earlier drafts of the manuscript were obtained from Jorge Llorente, Octavio Rojas, Isolda Luna, Juan José Morrone, Livia León, and two anonymous reviewers. Funding for developing diverse stages of the project in the field and laboratory work were obtained from DGAPA-UNAM (IN 214200), CONACyT (R27961), CONABIO (E018, V009), a Max Planck postdoctoral fellowship (to J.G.M.), and the US National Science Foundation. Collecting and export permits were obtained from the Instituto Nacional de Ecología (SEMARNAT).

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