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Chlorospingus Phylogenetics

Chlorospingus ophthalmicus Phylogeography

Phylogeography of a morphologically diverse Neotropical montane species, the

Common Bush-Tanager (Chlorospingus ophthalmicus).

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ABSTRACT

The Common Bush-Tanager (*Chlorospingus ophthalmicus*) is distributed in Neotropical cloud-forests from Mexico to Argentina and contains 25 subspecies divided into eight subspecies groups based on biogeography, eye coloration, presence of a postocular spot and chest band. All of Central America is occupied by a single subspecies group; whereas the Andes are believed to be occupied by seven additional subspecies groups. We used five mitochondrial genes to investigate the phylogeography and possible species limits of the *ophthalmicus* complex. A total of 14 monophyletic lineages were uncovered within the *ophthalmicus* complex, including three clades currently classified as separate species (*C. semifusus, inornatus* and *tacarcuna*). Divergence estimates for these clades date between 0.8 and 5.2 million years ago (Ma). Contrary to expectations based on morphological diversity, phylogeographic structure was greatest in Mexico and Central America and weakest in the Andes. Morphological and genetic divergences were not significantly correlated and most morphologically defined subspecies groups were not supported. Our evidence suggests the *ophthalmicus* complex originated in Mexico ca. 6.0 Ma (million years ago) and spread south into the Andes ca. 4.7 Ma before the completion of the Isthmus of Panama. Three genetically divergent lineages of *ophthalmicus* that formed in the Andes possess a complex checkerboard distribution, with a single lineage represented by disjunct populations from Venezuela and the southern Andes, while intervening populations in Ecuador and central Peru form two genetically and morphologically divergent lineages.
**Key Words:** Emberizidae, *Chlorospingus ophthalmicus*, phylogeography, Neotropics, Andes, Central America, leap-frog patterns.

**Introduction**

While the Neotropics possess more species than any other tropical region, the excess of species can be attributed in part to the rapid uplift of a complex system of highland regions extending from Mexico to Tierra del Fuego which promoted the formation of a distinctive highland fauna. Without this highland contingent, Neotropical species diversity would be comparable to other tropical regions that lack extensive highlands, suggesting the importance of montane diversification in promoting the excess of Neotropical diversity. A recent review of Neotropical avian phylogenetic studies (Weir 2006) suggested diversification rates in most highland genera have remained constant to the present while most lowland genera have experienced a decline in diversification rates through time. The contrast suggests ongoing speciation in the highland system may now contribute more to the buildup of biodiversity of the Neotropics than lowland faunas. However, only a handful of phylogeographic studies (Cadena et al. 2007; García-Moreno et al. 2004, 2006; Miller et al. 2007; Perez-Eman 2005) have addressed population divergence within Neotropical highland avian species. These studies demonstrate that many widespread highland species have extensive phylogeographic structure suggesting that the speciation process is being initiated extensively in the highlands.

Here we examine the phylogeographic structure within one of the most widespread and polytypic highland species of Neotropical songbirds, the Common Bush-Tanager (*Chlorospingus ophthalmicus*). This species possesses approximately 25 subspecies
(Dickinson 2003, Isler and Isler 1999) distributed in subtropical cloud-forest from Mexico to Argentina. These subspecies exhibit varying degrees of morphological divergence, with the most distinctive in the Andes and the least distinctive in Central America. Isler and Isler (1999) divided ophthalmicus subspecies into eight groups based on geography, eye-coloration (light or dark) and the presence or absence of a post-ocular spot and chest band (Fig. 5). This arrangement combined all Central American subspecies into a single group but recognized seven different groups in the Andes of South America (Fig. 1). Nevertheless, phylogenetic analysis of five ophthalmicus subspecies endemic to different highland regions in Mexico and Guatemala showed extensive genetic differentiation between subspecies in both mitochondrial DNA (García-Moreno et al. 2004) and allozymes (Peterson et al. 1992). These subspecies examined comprise only part of a single subspecies group and much additional genetic variation may occur within the ophthalmicus complex. If morphological distinctiveness results from the gradual buildup of differences through time, then Andean subspecies which are the most distinctive morphologically should exhibit even deeper phylogenetic splits than exhibited within the Central American subspecies group.

Species boundaries of ophthalmicus are poorly understood. Three taxa currently classified as distinct species, C. semifuscus, C. inornatus and C. tacarcuna, have in the past been considered subspecies (Meyer de Schauensee 1966) or allospecies (Sibley and Monroe 1990) of ophthalmicus. Alternatively, C. tacarcuna has been regarded as conspecific with C. flavigularis (Hellmayr 1936), but both coexist in sympatry in central Panama (Blake 1989) and hybridization has not been reported. In addition, ophthalmicus punctulatus from western Panama, o. cinereocephalus from central Peru, and the o.
flavopucts group of subspecies (flavopucts, trudis, exitelis, nigriceps, macarenae, phaeocephalus, hiaticolus) from Colombia, Ecuador and northern Peru (Fig. 1), have in the past been considered species due to their morphological distinctiveness. All were considered conspecific with ophthalmicus by Zimmer (1947). Recently, Sánchez-González et al. (2007) suggested several northern Mesoamerican ophthalmicus subspecies be elevated to species based on genetic and morphological considerations.

We investigated the phylogeographic structure within Chlorospingus ophthalmicus by analyzing DNA sequences from five mitochondrial genes. Sequences were generated for all of the subspecies groups of ophthalmicus as defined by Isler and Isler (1999) and for inornatus, tacarcunae, semifuscus. Phylogenetic analyses of these sequences were used to investigate the phylogeographic history of the ophthalmicus complex, the phylogenetic placement of tacarcunae, semifuscus, and inornatus, and the concordance between morphological and phylogenetic divergence.

2. Methods

2.1 Taxon sampling

We sampled inornatus, tacarcunae, semifuscus and 17 subspecies of C. ophthalmicus with at least one representative from each subspecies group. With the exception of the morphologically variable “novicius” from western Panama, a form now believed to be of mixed ancestry between regionalis and punctulatus (Olson 1993), we follow the subspecies taxonomy of Isler and Isler (1999). All recognized subspecies from Central America and from the South American Andes south of the Colombian / Ecuadorian borders were included. In the Andes of Colombia and Venezuela, tissues
were available only for *o. jacqueti*. Tissues were obtained from vouchered museum specimens collected by the authors in Argentina, Mexico, Guatemala, Honduras, Nicaragua and Panama or from frozen-tissue collections of the Museum of Natural Sciences, Louisiana State University, the Field Museum of Natural History and American Museum of Natural History (Tables 1 and 2). Also included were ATPase 6 & 8 sequences published in a previous study of Mexican *ophthalmicus* (García-Moreno et al 2004; Table 1).

Our ongoing work (data not shown) on *Chlorospingus* systematics indicates that *C. pileatus* is the most closely related outgroup to a monophyletic clade that included *ophthalmicus, inornatus, tacarcuna* and *semifuscus*. Based on this analysis, we use *C. pileatus* as the outgroup for all the analyses presented here.

2.2 Laboratory protocols

Whole genomic DNA was isolated from muscle tissue using phenol chloroform extraction or commercially available extraction kits (Purgene). We sequenced the complete mitochondrial ATPase 6 & 8 coding region (842 base pairs [bp] including a 10 bp overlapping region between ATPase 8 and ATPase 6) for all samples using primers CO2QGL and CO3HMH (Hunt et al. 2001). We sequenced three additional mitochondrial genes for a subset of individuals representing the major lineages recovered in our analysis of the ATPase 6 & 8 dataset (Table 2). These genes were: the complete cytochrome *b* gene (* cyt b*: 1143 bp) using primers O-L14851 and O-H16065 (Weir and Schluter 2007), the complete NADH dehydrogenase subunit 2 gene (ND2: 1041 bp) using primers L5215 (Hackett 1996) and H6313 (Johnson and Sorenson 1998), and a
partial sequence of the cytochrome oxidase I gene (COI: 661 bp) using primers COIf and
COIa (Palumbi 1996). Amplification and sequencing of these genes followed standard
protocols (i.e. Hunt et al. 2001).

2.3 Phylogenetic analysis

Sequences were edited and aligned in BioEdit (Hall 1999). The 10 bp overlap
between ATPase 6 & 8 was excluded following other authors (e.g. Lovette 2004). A
partition homogeneity test (Farris et al. 1995) conducted in PAUP* v.4.0b10 (Swofford
2002) was used to determine if the phylogenetic signals in different gene regions in the
five-gene dataset were compatible and thus could be combined in phylogenetic analysis
(ATPase 6 & 8 were combined as a single gene region).

MrModeltest v2 (Nylander 2004) was used to determine the likelihoods of nested
models of sequence evolution for both our ATPase 6 & 8 and extended sequencing
datasets. A hierarchical likelihood ratio test implemented in MrModeltest v2 (Nylander
2004) was used to choose the model that best fit the data while minimizing the number of
parameters to be estimated. The GTR-Γ model best fit the ATPase 6 & 8 dataset and the
GTR-Γ-I model best fit the extended dataset.

Bayesian analyses were carried out in a parallel processing version of MrBayes
3.1.2 (Huelsenbeck and Ronquist 2001) using the appropriate model for each gene
partition. Model parameters were estimated by the program. Both the ATPase 6 & 8
dataset and extended sequencing dataset were run for 20 million generations. To
effectively sample the posterior distribution while minimizing autocorrelation between
steps, trees were sampled only every 5000 generations after an initial “burnin” period of
2 million generations. Majority-rule consensus trees were constructed from the 3600 sampled trees. Equally weighted parsimony trees were also generated for the extended sequencing dataset using a heuristic search and the branch-and-bound option in PAUP* v.4.0b10 (Swofford 2002).

We tested the validity of a global molecular clock for our extended sequencing dataset by comparing likelihoods with and without a clock assumption using a likelihood ratio test (Felsenstein 1981; for details see Weir and Schluter 2004). Likelihoods of both models were estimated in PAUP* v.4.0b10 (Swofford 2002). Because a model with a global clock assumption was not rejected ($P = 0.15$), we used both PAUP* v.4.0b10 (Swofford 2002) and BEAST v1.4.2 (Drummond and Rambaut 2006) to obtain ultrametric estimates of branch length along the Bayesian tree topology. We ran BEAST with a yule prior for 50 million generations and sampled every 1000 generations after an initial “burnin” of 2 million generations.

Saturation plots revealed considerable saturation in uncorrected $p$-distances exceeding only 4% (Fig. 2a), thus we used the GTR-gamma-I model in all clock analysis. A large dataset of passerine molecular clocks strongly support an average molecular rate of 2% corrected sequence divergence per My$^{-1}$ (clock calibrations used GTR-G model for corrected distances; Weir and Schluter 2008) for cyt $b$. To determine the validity of applying the cyt $b$ molecular clock to our entire five-gene dataset, we compared model corrected (GTR-gamma-I model) genetic distances of cyt $b$ with those of the remaining genes (ND2, ATPase 6 & 8, COI) combined (Fig. 2b). Model corrected genetic distances of the extended dataset (excluding cyt $b$) were closely correlated ($r = 0.8$) and had a 1:1 
ratio to corrected distances for cyt b (Fig. 2b). Therefore, we used the five-gene dataset to
construct ultrametric branch lengths and applied the traditional cyt b clock to date nodes.

2.4 Morphological analysis

Isler and Isler (1999) scored subspecies of *ophthalmicus* for presence or absence
of a distinct yellow breast band, postocular spot and dark eye. These traits were used in
combination with biogeographic data to group *ophthalmicus* subspecies into groups (Isler
and Isler 1999). We confirmed their scoring of postocular spot and breast band from adult
male specimens in the American Museum of Natural History and the Field Museum of
Natural History and use their classifications here. Isler and Isler (1999) did not score
other members of the *ophthalmicus* complex for plumage traits (*tacarcuna, inornatus,*
*semifusus* and *ophthalmicus punctulatus*). Plumage traits were scored by eye for these
and for the nearest outgroup, *C. pileatus* by the lead author [(specimen voucher numbers
from American Museum of Natural History; *punctulatus* = 246543, 187968, 246548,
246542, 246549, 246547, 246541, 246546, 246550, 246552, 187969; *tacarcuna* =
736366, 136368, 136362, 136364, 136637, 136363; *inornatus* = 233689; *semifusus*
*semifusus* = 125208, 125207, 511417, 511418, 511414, 511420), (specimens from the
Field Museum of Natural History; *pileatus* = 35397, 35387, 343715, 220207, 220205,
220204, 35390, 220200, 35388, 35403)] and eye color was taken from Isler and Isler

Morphological traits were scored as 0 (trait absent, dark eye absent) or 1 (trait or
dark eye present). These plumage traits show almost no variability within subspecies with
the exception that *o. fulvigularis* is polymorphic for eye color. Breast bands were scored
as present only if yellow coloration on the breast was distinct from the belly. *C. tacarcuna* has a yellowish breast and belly and so was scored as not possessing a breast band. *C. semifuscus* and *C. o. cinereoccephalus* have slightly darker plumage on the breast but lack the bold yellow band seen in other subspecies of *ophthalmicus* and were scored as lacking bold chest bands. Isler and Isler (1999) likewise scored *o. cinereoccephalus* as lacking a breast band. Postocular spots were scored as present if one or more white feathers occurred directly behind the eye. Parsimony and maximum likelihood (one parameter model with forward and backward transition rates equal) ancestor state reconstructions were performed for each morphological trait along the ultrametric five-gene phylogeny using Mesquite v1.12 (Maddison and Maddison 2006). Clock-like branch lengths were used for maximum likelihood reconstructions. Because parsimony reconstructions in Mesquite v1.12 allow any given tree tip to possess multiple character states, *o. fulvicularis* was given both dark and pale eyes. However, separate analysis were run for pale and dark eyes in fulvicularis under maximum likelihood reconstructions because they allow only one character state for each tree tip.

### 3. Results

After deleting the 10 bp overlap, 832 bp of ATPase 6 & 8 were used for phylogenetic analysis; of these, 322 bp were variable. The partition homogeneity test showed no difference between genes (*P* = 1.0) and all gene regions were combined for phylogenetic analysis. The extended sequencing dataset, contained 3677 bp, 1029 of which were variable. Uncorrected distances for ingroup taxa in the extended sequencing
dataset ranged from 0.5 – 8.1% for ATPase 6 & 8, 0.2 – 7.4% for cyt b, 0.2 – 10.3% for COI and 0.4 – 10.3% for ND2.

Evidence that our sequences are mitochondrial in origin are: 1) DNA was extracted from muscle tissue rich in mitochondria, 2) the absence of unexpected stop codons and insertions or deletions in protein coding genes and the ease at which sequences aligned with other published sequences from Genbank, and 3) the similar tree topologies obtained from separate phylogenetic analysis of each of the protein coding genes (results not shown).

3.1 ATPase 6 & 8 analysis

All subspecies within *ophthalmicus* were reciprocally monophyletic with the following exceptions: 1) one individual of *regionalis* (from Nicaragua) grouped with *honduriatus*, but otherwise both subspecies were monophyletic, 2) *phaecephalus* and *hiaticolus* together formed a monophyletic group but were not individually monophyletic, and 3) *bolivianus, argentinus, jacqueti, fulvularis* and *peruvianus* together formed a monophyletic group but those subspecies with multiple samples were not individually monophyletic. Relationships between many subspecies groups of *ophthalmicus* were poorly resolved in the ATPase dataset.

3.2 Extended dataset analysis

The extended sequencing dataset included one sample from all species and subspecies in the ATPase 6 & 8 dataset except for *o. hiaticolus* which was phylogenetically nested within *o. phaecephalus*; we included the latter to represent both
subspecies. In contrast to the ATPase 6 & 8 analysis, most nodes received strong
posterior and moderate to high bootstrap support in Bayesian and parsimony analysis of
the extended dataset (Fig. 4).

Both Bayesian (Fig. 4) and parsimony (not shown) analyses uncovered similar
tree topologies. These analysis confirm that *ophthalmicus* is not monophyletic with
respect to *C. semifusus*, *C. tacarcuna*, and *C. inornatus*. In the *ophthalmicus* complex,
three clades were strongly supported by both the Bayesian and parsimony analysis. The
first clade comprised four subspecies of *ophthalmicus* from Mexico (MEX clade
hereafter) and was basal to the remaining two clades. The second clade comprised four
Central American subspecies of *ophthalmicus* from Guatemala to western Panama (CA
clade) and the third, sister to the CA clade, comprised all Andean subspecies of
*ophthalmicus* as well as the species *C. semifusus*, *C. tacarcuna*, and *C. inornatus* (SA
clade). The monophyly of each of these clades was strongly supported. Relationships
between taxa within these clades were fully resolved in all but the SA clade.

The relationships shown in the extended sequencing dataset are in conflict with
the ATPase 6 & 8 dataset, in which all Central American and Mexican subspecies formed
a monophyletic group. Constraining the topology to that uncovered in the ATPase 6 & 8
dataset (Fig. 3) did result in a significantly worse fit to the five-gene dataset (\(p < 0.01\),
Shimodaira-Hasegawa test with significance determined using a one-tailed bootstrap test
of 1000 dataset permutations; Shimodaira and Hasegawa 1999). While we are uncertain
why these datasets differ in topology, we consider the relationships uncovered in the
extended sequencing analysis to reflect the most accurate phylogenetic hypothesis for the
genus.
Estimates of branch lengths under a global clock model were similar in PAUP and BEAST and only the results from the latter are reported here (Fig. 4).

3.3 Morphological analysis

Ancestor state reconstructions of morphological traits along the five-gene Bayesian phylogeny are presented in Figure 6. Parsimony and maximum likelihood reconstructions were congruent for all traits at each node. Ancestor state probabilities are shown only at the ancestral node to the *ophthalmicus* complex. Treating eye color as dark or light for the polymorphic *o. fulvicularis* had no effect on parsimony reconstructions and resulted in only minor changes in character probabilities at nodes in the maximum likelihood method. The ancestral lineage for the SA clade was reconstructed as uncertain in the parsimony ancestor state reconstruction for postocular spot. In the maximum likelihood ancestor state reconstruction, absence of a postocular spot received the strongest support but was not significant.

4. Discussion

Like other emberizids, *Chlorospingus ophthalmicus* is comprised of numerous morphologically distinct subspecies. However, unlike many temperate sparrows and other passerines whose subspecies rarely exhibit genetic differentiation in mitochondrial DNA (Zink 2004), many subspecies of *ophthalmicus* were highly differentiated genetically and formed reciprocally monophyletic groups. This result was paralleled by phylogeographic analysis of two other widespread Neotropical highland species of sparrow (*Buarremon brunneinucha* and *B. torquatus*; Cadena et al. 2007). Phillimore and
Owens (2006) recently estimated that approximately half of all avian subspecies distributed south of the northern hemisphere temperate and arctic zones were phylogenetically distinct. This average is exceeded by *ophthalmicus* with 60% (9 of 15 sampled subspecies) of its subspecies distinct. This high degree of genetic differentiation highlights an extended history of diversification.

4.1 Phylogenetics and Phylogeography

The phylogenetic evidence confirmed Zimmer’s (1947) hypothesis that the species *C. semifuscus* and *C. inornatus* were derived from within a paraphyletic *ophthalmicus* (Fig. 4). Together with the *o. flavopectus* subspecies group, these species formed a well-supported, monophyletic subclade within the SA clade. In addition, *C. tacarcuna* was also nested within the SA clade of *ophthalmicus*, and was not closely related to *C. flavigularis* as previously suggested (Hellmayr 1936). The *ophthalmicus* complex, expanded to include these species, forms a monophyletic group that is sister to *C. pileatus*.

The *C. ophthalmicus* complex is composed of at least 14 monophyletic and moderately to deeply diverged lineages. These lineages date between ~ 0.8 to 5.2 Ma. In addition to *semifuscus, inornatus* and *tacarcuna*, 11 genetically divergent lineages occurred within *ophthalmicus* as currently defined. With the exception of *o. hondurianus* all Mexican and Central American subspecies of *C. ophthalmicus* were reciprocally monophyletic for mitochondrial DNA haplotypes. Given that *regionalis* and *hondurianus* coalesce more than 3 Ma (Fig. 4), the placement of a single individual of *regionalis* within *hondurianus* is probably due to recent gene flow between these taxa rather than
incomplete lineage sorting (see below). In the Andes, only three reciprocally monophyletic lineages of *ophthalmicus* were recovered: 1) *peruvianus*, *fulvigularis*, *bolivianus*, *argentinus* and *jacqueti* (based on morphological similarity and biogeographic proximity to *jacqueti*, unsampled subspecies of the *venezuelensis* subspecies group probably belong to this lineage); 2) *phaecephalus* and *hiaticolus* (based on morphological similarity and biogeographic proximity to *phaecephalus*, unsampled subspecies of the *flavopectus* subspecies group probably belong to this lineage) and 3) *cinereocephalus*.

Relationships between these genetic lineages do not conform well to the morphologically and biogeographically based subspecies groups defined by Isler and Isler (1999). The Islers arranged all Mexican and Central American subspecies of *ophthalmicus* into a single subspecies group. The phylogeny generated using only the 862 base pairs of ATPase 6 & 8 agrees with this arrangement. However, in the five-gene analysis, the MEX and CA clades were not sister to each other. Rather, the CA clade was sister to the SA clade containing all Andean subspecies of *ophthalmicus* and the species *semifusus*, *inornatus* and *tacarcunae*. The MEX clade was sister to these. We are uncertain why topologies differ between the ATPase 6 & 8 versus five-gene datasets, but given the much larger number of base pairs in the five-gene dataset, and better levels of support at nodes, we consider its topology to most closely represent the evolutionary history of this group.

Only two subspecies groups as defined by Isler and Isler (1999) correspond to reciprocally monophyletic mitochondrial clades. These include *cinereocephalus*, which is the sole member of its group, and the *flavopectus* group for which we sampled only two
subspecies, *o. phaeocephalus* and *o. hiaticolus*. Additional sampling of subspecies is necessary to confirm the monophyly of this group. Although our sample sizes for Andean subspecies were small, the Venezuelan group and the four groups from the southern Andes defined by the Islers’ shared mitochondrial haplotypes and they may not have had time to form reciprocally monophyletic groups. Despite their unique combinations of morphological characters, all appear closely related and recently diverged.

In a recent phylogenetic investigation of *ophthalmicus* subspecies from Mexico and northern Central America, García-Moreno *et al.* (2004) suggested that *ophthalmicus* originated in South America and spread northwards into Central America and finally Mexico. Our five-gene dataset rejects this hypothesis (Fig. 4). The sister relationship between the SA and CA clades with the MEX clade basal suggests that Andean forms were derived from a northern ancestor. Moreover, the sister to the entire *ophthalmicus* complex, *C. pileatus*, is also endemic to Central America lending further support for an origin outside of South America.

Phylogenetic studies are available for five other montane cloud forest inhabiting species complexes believed to have originated in northern Middle America and spread south through the Neotropics (Perez-Eman 2005, García-Moreno *et al.* 2006, Miller *et al.* 2007, Cadena *et al.* 2007; Fig. 6). Of these, *Myioborus miniatius* and *Buarremon brunneinucha* show a highly congruent pattern to *Chlorospingus ophthalmicus* with endemic clades in Mexico, the Middle American Plateau, Talamanca highlands, Darien highlands and the Andes. Area cladograms between clades inhabiting these regions were identical in *Chlorospingus* and *Myioborus* and suggest both may have experienced a similar phylogeographic history, but were slightly different in *Buarremon* (Fig. 6).
Lampornis lacks clades in the Darien and Andes but otherwise has an identical area cladogram to Chlorospingus. Myadestes has a similar pattern, but has two species distributed widely throughout Mexico and the Middle American Plateau rather than lineages endemic to each of these regions, and has a slightly different area cladogram. In all cases, the basal-most divergence events within the Neotropics occurs between Mexican / Middle American Plateau lineages and all southern lineages. These cladograms are consistent with a northern origin and southward colonization route.

Central America began as a North American peninsula. Collision of crustal plates from the Pacific with plates in the Caribbean resulted in the gradual extension of this peninsula southwards (Coates and Obando 1996). At ca. 3.1 Ma the peninsula finally joined with South America forming a contiguous landbridge between these continents (Coates and Obando 1996). The radiations of the five species complexes in Figure 6 were underway in Mexico and northern Central America before the completion of the landbridge. The interpretation of the area cladogram for the ophthalmicus complex that is most consistent with the geological history of lower Central America is as follows. At ~5.7 Ma ophthalmicus dispersed across the Isthmus of Tehuantepec from northern Mexico into the Middle American plateau. At 4.7 (95% confidence interval, 4.2 to 5.3) Ma, before the final completion of the landbridge, ophthalmicus dispersed from the Middle American Plateau into the Andes.

Highland regions in the lower part of Central America from Nicaragua to Colombia are believed to have formed no earlier than 4.5 Ma, sometime after the subduction of the Cocos plate beneath the Caribbean plate and other key processes in the formation of the Isthmus of Panama were initiated (Abratis and Worner 2001). If this
geological dating is correct, then endemic highland taxa probably did not occur in the Talamanca or Darien highlands until some time after 4.5 Ma. The area cladograms depicted in Figure 6 are consistent with taxa in these regions having colonized either from a northern or southern route after the Talamanca and Darien highlands were uplifted (Fig. 6). In Chlorospingus ophthalmicus, Myioborus, Lampornis and Buarremon, area cladograms suggest Talamanca highland endemics colonized from the north. In C. ophthalmicus, this occurred at approximately 3.2 Ma, at the final completion of the Central American landbridge.

Darien highland endemics were phylogenetically embedded within Andean clades in both the Chlorospingus and Myioborus complexes suggesting they colonized from South America. Myadestes may have also colonized the Darien from the Andes though other interpretations of its area cladogram (Fig. 6) are possible. In contrast, Buarremon appears to have colonized the Darien from the north. In Chlorospingus, two separate colonization events of the Darien highlands from South America probably resulted in the two Darien endemics, C. inornatus and C. tacarcunae. These occurred at approximately 3.3 and 3.0 Ma, at the completion of the Central American landbridge. Alternatively, a single colonization of the Darien followed by a back colonization into the Andes is possible.

In the SA clade, Andean lineages of the ophthalmicus complex diverged between 2.4 and 3.5 Ma and form a phylogenetic leapfrog pattern (Figs 1,4,6). The lack of genetic differentiation between populations from Venezuelan and southern Peru to Argentina (Figs 3 and 4) is surprising given that intervening regions in Central Peru to northern Colombia are occupied by the genetically and morphologically divergent lineages C.
semifuscus, o. cinereoccephalus and the o. flavopectus subspecies group (represented in Fig. 4 by o. phaeocephalus). Given the deep divergence events between these related lineages, semifuscus, cinereoccephalus and the o. flavopectus subspecies group have probably occupied the Central Andes since shortly after the Andes were colonized (Fig. 4). A similar pattern was thought to occur in Myioborus redstarts with M. brunniceps from the southern Andes similar morphologically to north Andean and Teput taxa despite intervening morphologically divergent species in the central Andes. However, molecular phylogenetic analysis demonstrated that these Myioborus taxa are not closely related (Perez-Eman 2005). We know of no other taxon that exhibits the genetic leapfrog pattern observed in Chlorospingus. The lack of strong genetic differentiation between the Venezuelan samples and south Andean subspecies suggests that a range expansion must have occurred fairly recently within the last one million years of the Pleistocene. Whether this involved a continuous expansion up the eastern edge of the Andes or a long distance dispersal event is unknown.

Along the eastern edge of the Andes, major river valleys are believed to form geographic barriers to dispersal in many Andean birds. Phylogenetic breaks in ophthalamicus only correlated with one such river valley barrier. The Apurimac valley of Peru separated the ranges of o. cinereoccephalus and o. peruvianus, which last shared a common ancestor ca. 3.5 Ma. Surprisingly, a phylogenetic break did not coincide with the Marañon river valley of northern Peru, a barrier known to have caused such breaks in other cloud-forest specialists (Myadestes ralloides, Miller et al. 2007; Ochthoeca cinnamomeiventris, García-Moreno et al. 1998). Rather, a phylogenetic break occurs between C. o. hiaticolus and C. o. cinereoccephalus. These subspecies are distributed
north and south of the Rio Apurimac valley region in Huanuco. A similar phylogenetic
break occurs in this region in *Ochthoea frontalis* (García-Moreno et al. 1998).

4.2 Morphological Evolution

All subspecies from Central America and Mexico were phylogenetically
distinctive despite their morphological uniformity in eye color, presence of breast band
and postocular spot (Figs 4 and 5). The deepest phylogenetic split within *ophthalmicus*
occurred in Central America and Mexico, separating morphologically similar subspecies
into the deeply diverged MEX and CA clades (Fig. 4). By contrast, only one Andean
subspecies was phylogenetically distinctive (*o. cinereacephalus*) despite the greater
morphological variability of Andean subspecies. The lack of genetic differentiation
between five of the seven morphologically defined subspecies groups in the Andes (Isler
and Isler 1999) suggests rapid race formation following a recent range expansion in the
mid to late Pleistocene. The rapid formation of boldly patterned races is reported in high
latitude sparrows (Mila et al. 2007a,b; Fry and Zink 1998; Klicka et al. 1999) and a
number of other species (e.g. Odeen and Bjorklund 2003; Pavlova et al. 2005; Zink et al
2002a, 2002b), but has not been reported at this scale in a Neotropical species. In Andean
*ophthalmicus*, the origin of many subspecies may be related to intense climatic cycles of
the late Pleistocene in the Andes that are thought to have promoted rapid divergence of
some avian groups (Weir 2006).

Ancestor state reconstructions based on parsimony (Fig. 5) suggest the immediate
common ancestor to the *ophthalmicus* clade possessed a dark eye, postocular spot, and
breast band. This ancestral morphotype is retained in all subspecies of the MEX and CA
clades but in the SA clade it is present only in the three southern most races *o.
argentinus*, *o. bolivianus* and *o. fulvicularis*. Maximum likelihood reconstructions gave
similar conclusions.

Each of these characters exhibits leapfrog patterns in which populations sharing a
class character state are geographically bisected by populations exhibiting an alternative state
(Fig. 5). Such leapfrog patterns characterize more than 20% of Andean species
complexes and are thought to play an important role in diversification and speciation
(Remsen 1984). Whether geographically separated populations exhibiting similar color
patterns are more closely related to each other than to intervening populations (and thus
represent both morphological and phylogenetic leapfrog patterns) has not previously been
tested.

In *Chlorospingus ophthalmicus* most leap-frog patterns arose from either multiple
transitions to a single state along the phylogeny (absence of breast band and postocular
spot) or from back transitions to the ancestral character state (eye-color; Fig. 5). Eye
color is dark in Central America (excluding eastern Panama) and the southern Andes with
intervening populations possessing light colored eyes. A transition to light colored eyes
occurred in the ancestor of the SA clade, and then switched back to dark colored eyes in
populations of the southern Andes. The absence of a breast band occurs in three
populations: the Darien highlands of Panama, the western slope of the Andes of southern
Colombia and Ecuador and in Central Peru with intervening populations possessing
breast bands (Fig. 5). Although uncertainty exists in the reconstruction of breast bands, it
is probable that breast bands were lost at least twice. These results suggest that some
leap-frog patterns are not mirrored by concordant phylogenetic patterns but resulted from
a complex history of character transition. The presence or absence of a white postocular
spot was, however, partially concordant with phylogeography. Populations in Central
America, Venezuela and the southern Andes all possess a postocular spot while
intervening populations from eastern Panama to Central Peru do not. The loss of this spot
by populations in the Central Andes dates to about 3.5 Ma. Venezuelan and southern
Andean subspecies are not genetically differentiated, which suggests a recent dispersal
event of birds with postocular spots around the Central Andean populations that lack
them. This represents the only Andean case we are aware of where two geographically
disjunct populations, that share a morphological trait, are more closely related to each
other than to the intervening populations that lack the trait. The absence of a postocular
spot in *o. peruvianus* might represent a second loss of this character state. Alternatively,
it may have resulted from past introgression with Andean forms to the north.

None of these morphological traits unambiguously characterize the major clades
(MEX, CA or SA clades) of the *ophthalmicus* complex. No traits characterize the
separation of the MEX and CA clades and only pale eye coloration characterizes most,
but not all members of the SA clade. Instead, the multiple transitions between character
states at different points along the phylogeny have resulted in a geographic patch-work of
morphological traits which bears little resemblance to phylogeny or geography and helps
explain the high subspecies diversity in this species. Analysis of other plumage traits
(crown color, throat color) that vary between populations is needed.

4.3 Taxonomic Considerations
C. ophthalmicus as currently defined is paraphyletic with respect to three taxa currently recognized as species: C. semifuscus, C. inornatus and C. tacarcunae. Though all three of these latter taxa have been considered conspecific within ophthalmicus (Meyer de Schauensee 1966), each possesses a number of unique plumage features and on these grounds are generally afforded species status (Remsen et al. 2007; American Ornithologists' Union 1998). In addition, semifuscus possesses a unique social system in which males form singing assemblages resembling leks (Bohorquez and Stiles 2002). The exact role of these singing assemblages is uncertain and it is not apparent whether they would render this taxon reproductively isolated from other ophthalmicus taxa.

Under the phylogenetic or evolutionary species concepts semifuscus, inornatus and tacarcunae along with the 11 additional genetically divergent lineages in the ophthalmicus complex (Figures 3 and 4) appear to represent separate species. Under the biological species concept, species boundaries are difficult to judge in this complex, as most forms are completely allopatric. In several cases where genetically divergent subspecies come in geographic contact, morphologically intermediate populations are reported to occur, suggesting gene flow.

Morphological evidence of gene flow is best documented by populations which are morphologically and geographically intermediate between dwighti and postocularis in Guatemala (Zimmer 1947), and regionalis and punctulatus in western Panama (Olson 1993). Our study found no genetic evidence of introgression between dwighti and postocularis but we lacked samples from populations believed to be of mixed origin. Further analysis is necessary to confirm the mixed ancestry of morphologically intermediate populations. If such analysis confirm gene flow between dwighti and
postocularis, it would suggest that lineages descended from the basal most split within
ophthalmicus have incomplete reproductive barriers despite approximately 5.7 million
years of evolutionary divergence. In the case of the more recently diverged regionalis and
punctulatus, our samples come from pure populations of regionalis and from a
population morphologically most similar to punctulatus but showing some signs of
morphological introgression with regionalis (Olson 1993). Mitochondrial haplotypes of
our punctulatus-like samples were genetically divergent from regionalis and showed no
signs of mixed ancestry, but analysis of multiple unlinked loci are needed for verification.
The only genetic evidence of gene flow uncovered by our study occurred between
regionalis and honduratus which last shared a common ancestor about 3.2 Ma. We only
sampled three individuals of regionalis from Nicaragua, yet one of these individuals
possessed a haplotype belonging to the geographically proximate honduratus (Figure 3).
Additional study of contact zones is necessary to assess the extent of gene flow and the
strength of reproductive barriers. In the absence of such information, we refrain from
making taxonomic recommendations under the biological species concept.

Three phylogenetic splits occurred within ophthalmicus subspecies. Populations
of the Mexican o. ophthalmicus from Hidalgo / Querétaro and Oaxaca / Veracruz formed
two genetically diverged, reciprocally monophyletic lineages. More than one taxon was
originally described from the range of o. ophthalmicus (i.e. sumichrasti; Ridgway 1901).
Likewise our samples of o. albifrons from Guerrero and Oaxaca formed two reciprocally
monophyletic groups. Populations of albifrons in Oaxaca are often considered a distinct
subspecies (persimilis; Phillips 1966), a conclusion supported by our findings. Although
sample sizes were low, Nicaraguan and Costa Rican populations of o. regionalis were
also genetically divergent from each other. Slight morphological differences have
previously been described between the Nicaraguan and Costa Rican populations (Zimmer
1947) and when combined with genetic data, suggest that two taxa may be involved.
However, these morphological differences could have resulted from introgression
between *regionalis* and *honduriatus* in Nicaragua as suggested by the placement of one
of our Nicaraguan samples of *regionalis* within *honduriatus*. Further molecular and
morphological analysis is necessary to determine the taxonomic status for each of these
populations.

**Conclusion**

The molecular phylogenetic hypothesis for the *ophthalmicus* complex suggests a
Mexican origin with subsequent colonization and diversification throughout the
Neotropical highland system, a conclusion reached by several other phyogeographic
studies of widespread Neotropical highland species (Perez-Eman 2005, García-Moreno et
al. 2006, Miller et al. 2007, Cadena et al 2007). Together, these studies suggest that a
North American cloud-forest fauna may have served as a source for many currently
widespread highland Neotropical taxa. The chain of isolated highland regions of Mexico
and Central America produced the most genetically distinctive but least morphologically
distinctive populations of *ophthalmicus*, while Andean populations were composed of
few genetically differentiated but multiple morphologically differentiated forms.
Morphological analysis of other widespread cloud-forest species complexes is necessary
to determine the generality of these patterns.
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References


Figures

Figure 1

Geographic distribution and sampling localities of the *Chlorospingus ophthalmicus* complex. Subspecies groups (names highlighted in gray) follow those defined by Isler and Isler (1999). Lines between populations delineate approximate subspecies boundaries. Boundaries are less well defined in Colombia and Venezuela. Inset panel shows distributions of three species (*semifuscus*, *inornatus* and *tacarcuna*) closely allied to *ophthalmicus*. The question mark shows the possible occurrence of *o. phaeocephalus* along the western Andes of north-western Ecuador (see text). Numbered dots refer to sampling localities listed in Table 1.

Figure 2

(a) Saturation plot for the five-gene dataset; (b) relationship between maximum likelihood model corrected distances for cytochrome b only and the five-gene dataset. The slope of the least squares regression line is 1.09, \( r^2 = 0.64; P < 0.0001 \).

Figure 3

Bayesian phylogeny of the *Chlorospingus ophthalmicus* complex for the ATPase 6 & 8 genes rooted to *C. pileatus* (not shown). Tip numbers refer to sampling localities in Table 1. Posterior probabilities are only shown for nodes connecting major lineages with support greater than 0.5 and for clarity are not shown within subspecies. Asterisks indicate a probability of 1.0. Country abbreviations as follows: Mexico (Mex), Guatemala
(Gua), Honduras (Hon), El Salvador (ES), Nicaragua (Nic), Costa Rica (CR), Panama (Pan), Venezuela (Ven), Ecuador (Ecu).

Figure 4
Bayesian phylogeny of the *Chlorospingus ophthalmicus* complex for the five-gene dataset. Right, branch lengths without clock-like assumption and posterior probability shown above nodes as in Figure 3 and parsimony bootstrap values (percentage of 1000 bootstrap replicates) shown below nodes. Left, branch lengths (and 95% confidence intervals) estimated with maximum likelihood under a global molecular clock. Scale bars show branch length in percent sequence divergence and in millions of years ago. Three main clades in the *ophthalmicus* complex are labeled MEX (Mexican), CA (Central American) and SA (South American). Country abbreviations as in Figure 3 and as follows: Arg (Argentina), Bol (Bolivia).

Figure 5
Ancestor state reconstructions of morphological traits along the five-gene phylogeny for the *Chlorospingus ophthalmicus* complex. Character states for each taxon are shown at tree tips. The most parsimonious reconstruction is plotted using different shading along tree branches. Pie diagrams show maximum likelihood support for character states in the ancestral *ophthalmicus*. *Ophthalmicus fulvigularis* is polymorphic for eye-color. Treating eye color as dark or pale did not change reconstructions. Geographic distribution of
character states are shown on maps. Presence of trait shown by gray (hatching) and absence of trait shown by black on trees and maps.

**Figure 6**

Comparative phylogeography of five species complexes with an inferred north to south colonization route. A) Neotropical highland regions, B) *Chlorospingus ophthalmicus* complex, C) *Myioborus miniatus* complex (Perez-Eman 2005), D) *Lampornis* (García-Moreno *et al.* 2006) and E) *Myadestes* (Miller *et al.* 2007), E) *Buarremon brunneinucha* complex (Cadena *et al.* 2007). Branch lengths do not represent time or genetic distance. Arrows on maps depict one possible interpretation of the phylogeographic history for each area cladogram below. Other interpretations are possible but conflict with the known geological history of the Central American landbridge (see text).
Table 1

List of *Chlorospingus* samples used in this study. Genbank Accession numbers for ATPase 6 & 8 are given. LSUMZ, Louisiana Museum of Natural History; AMNH, American Museum of Natural History; NMNH, National Museum of Natural History; MBM, Marjorie Barrick Museum of Natural History; FMNH, Field Museum of Natural History; STRI, Smithsonian Tropical Research Institute; MZFC, Museo de Zoologia, Facultad de Ciencias; UKNH, University of Kansas Natural History; ZMUC, Zoological Museum, University of Copenhagen; ANSP, Academy of Natural Sciences, Philadelphia; MVUP, Museo de Vertebrados de la Universidad de Panamá

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<td>Bolivia: La Paz, Cerro Asunta Pata</td>
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**Table 2**
Genbank accession numbers for the extended five-gene dataset.

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* samples previously published in Garcia-Moreno et al. 2004
Chlorospingus Phylogenetics

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Fig. 2
Fig. 4