Biological Journal of the Linnean Society, 2009, **98**, 14–28. With 4 figures

Phylogeny, biogeography, and recurrent evolution of divergent bill types in the nectar-stealing flowerpiercers (Thraupini: *Diglossa* and *Diglossopis*)

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Received 27 September 2008; accepted for publication 1 March 2009

Neotropical tanagers known as flowerpiercers (*Diglossa* and *Diglossopis*) have a novel feeding adaptation, comprising a downward curved hook on the maxilla that allows these species to obtain floral nectar without pollination. Using mitochondrial DNA sequences, the phylogenetic relationships of all 18 species of flowerpiercers were studied for the first time. Strong support was found for the monophyly of flowerpiercers and for the monophyly of four superspecies within flowerpiercers. However, previously described species-groups, as well as the genus *Diglossopis*, are not monophyletic. The biogeographic origin of flowerpiercers was identified as Andean, with a single dispersal event from the northern Andes to Central America and a single dispersal event from the northern Andes to the tepuis. The first principal component, representing a contrast between hook size and bill size, was mapped onto the phylogeny to examine the evolution of relative hook size in the group. Across the phylogeny, a relatively large hook and a relatively small hook evolved multiple times in unrelated lineages, indicating lability in bill morphology. Differences in hook size among sympatric species, together with habitat partitioning and behavioural differences, can explain the coexistence of multiple species of flowerpiercers at the same locality.

ADDITIONAL KEYWORDS: adaptation – dispersal–vicariance analysis – feeding morphology – Neotropics – principal component analysis – tanager – tepuis.

INTRODUCTION

Studies of feeding adaptations have been an essential part of evolutionary and ecological studies since Darwin proposed his theory of natural selection. For example, the evolution of bill diversity in the Darwin's finches is a primary example of how natural selection can shape variation in feeding morphologies (Lack, 1947; Grant & Grant, 2007). Likewise, ecological studies of sympatric species with different feeding morphologies and behaviours have long shown how species can partition resources in their habitat (MacArthur, 1958; Schluter, 2000). Phylogenies provide an important initial framework for understanding how a particular feeding adaptation has been shaped by evolutionary and ecological factors throughout its history. In the present study, we trace the evolutionary history of a novel feeding adaptation in a group of Neotropical birds known as flowerpiercers. Flowerpiercers (genera *Diglossa* and *Diglossopis*) consist of 18 species of high elevation, nectar-feeding birds, ranging from the Mexican highlands to the Andes of northwestern Argentina (Isler & Isler, 1987; Sibley & Monroe, 1990). All flowerpiercers are nectivorous with adaptations of both the tongue and bill that facilitate nectar-feeding (Vuilleumier, 1969; Bock, 1985; Isler & Isler, 1987). The base of the tongue is flattened horizontally for the proximal two-thirds, and then narrows and splits into a bifurcation at the distal end. Each bifurcation is frilled at the tip and has a ventral groove that merges into one at the junction of the bifurcation (Bock, 1985). The bill is used in a unique method to obtain nectar from flowers. The straight

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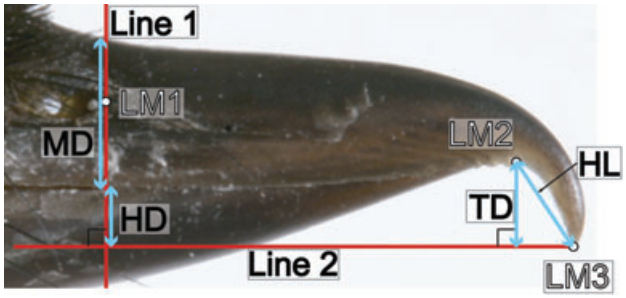

Figure 1. Measurements taken from bill photographs of museum specimens. Landmark 1 (LM1) is the middle of the anterior edge of the nostril. Landmark 2 (LM2) is the point where the first tooth projects from the ventral edge of the mandible anterior to the end of the bill. Landmark 3 (LM3) is the tip of the bill. Line 1 was made by drawing a line through LM1 that is perpendicular to the junction of the maxilla and mandible. Line 2 was made perpendicular to Line 1 and extending to LM3. Length of bill from nostril (BL; Baldwin *et al.*, 1931) was taken from LM1 to LM3. Maxilla depth (MD) was taken from the ventral to the dorsal edge of the maxilla along Line 1. Hook depth (HD), along Line 1 from the ventral edge of the maxilla to the junction of Line 2. Tooth depth (TD) was the shortest distance from LM2 to Line 2. Hook length (HL) was the shortest distance from LM2 to LM3. Concavity (CO) was calculated by taking the difference of HD and TD to account for the area generated by the upturn of the ventral edge of the maxilla posterior to the hook.

and slender maxilla terminates in a down-curved hook (Fig. 1). The specialized hook is used to hold a tubular flower at the base of the corolla (Skutch, 1954), while the attenuated mandible pierces a hole in the base of the corolla for extracting nectar (Skutch, 1954; Bock, 1985). Thus, flowerpiercers are considered nectar-stealers, as defined by Pratt (2005), because they use their unique bill shape to obtain nectar without transporting pollen or providing benefit to the plant (Skutch, 1954; but see also Irwin, 2003). Stealing nectar from the base of the corolla allows flowerpiercers to gain access to nectar sources, such as long tubular flowers adapted for hummingbird pollination that would otherwise be inaccessible (Skutch, 1954).

Although the bill and tongue of flowerpiercers are adapted to obtain nectar, they are not nectar obligates. Diets of flowerpiercers also include arthropods and fruit (Skutch, 1954; Vuilleumier, 1969; Isler & Isler, 1987). Hook size varies among the different species of flowerpiercers and is evidently correlated with the efficiency of foraging on nectar versus fruit. An experimental study conducted by Schondube & Martinez del Rio (2003) modified the hook length of *Diglossa baritula* into three discrete sizes (small, medium, and large) to determine whether variation among hook sizes impacts food accessibility for flowerpiercers. It was found that individuals with a large hook were more efficient at feeding on nectar from long tubular flowers, whereas individuals with small hooks were more efficient at feeding on fruits (Schondube & Martinez del Rio, 2003). With this model of feeding morphology, Schondube & Martinez del Rio (2003) used a phylogeny based on allozyme data (Hackett, 1995) to infer the evolution of hook length within flowerpiercers. Schondube and Martinez del Rio (2003) proposed that the ancestor for all flowerpiercers had a small hook adapted for feeding on fruit. Additionally, they hypothesized that a large hook for thieving nectar from flowers is a derived trait evolving only once. However, hook length was not quantified for all species and a complete phylogeny of all species was not available. In the present study, we quantify bill variation among all species of flowerpiercers. We then use this information in conjunction with a DNA-based phylogeny of all species to investigate how relative hook size has evolved among flowerpiercers.

The classification of flowerpiercers has been revised several times subsequent to first being described. Two synopses of flowerpiercers (Cassin, 1864; Sclater, 1875) both described two genera (*Diglossa* and *Diglossopis*) with all taxa except one belonging to one genus, *Diglossa*. The monotypic genus *Diglossopis* contained the species with the smallest hook, *Diglossopis caerulescens*. Later, Hellmayr (1935) combined all flowerpiercers in the genus *Diglossa* and reduced the number of species by merging closely-related taxa. A comprehensive monograph by Vuilleumier (1969) proposed two ranks of taxonomic classification of flowerpiercers to better represent the phylogenetic relationships among them. Within the genus *Diglossa*, Vuilleumier (1969) described four 'species-groups' and four 'superspecies' complexes within two of the species-groups (Table 1). Later, based on cranial, tongue, and mandibular morphology, Bock (1985) resurrected the genus *Diglossopis* and included *Diglossa caerulescens*, *Diglossa cyanea*, and *Diglossa glauca* in *Diglossopis*. Although Vuilleumier (1969) considered *Diglossa indigotica* to be closely related to these three species and placed all four in the same species-group, Bock (1985) disagreed and did not move *D. indigotica* to *Diglossopis*. Bock (1985) also proposed that *Diglossa* and *Diglossopis* were not closely related, but had converged on a similar bill shape for nectar feeding. Sibley & Monroe (1990) later moved *D. indigotica* to *Diglossopis*, *sensu* Vuilleumier (1969). Most current classifications, however, merge *Diglossopis* into *Diglossa* (Dickinson, 2003; Remsen *et al.*, 2008). We used our molecular phylogeny to test these hypotheses about relationships among the flowerpiercers. In particular, our phylogeny allowed us

Table 1. Taxonomy of flowerpiercers following Vuilleumier (1969); Sibley & Monroe (1990)

Genus	Species-group	Superspecies complex	Species
Diglossa	1 – *major*		*major*
Diglossa	2 – *lafresnayii*	*lafresnayii*	*gloriosissima*
		lafresnayii	*lafresnayii*
		lafresnayii	*mystacalis*
		carbonaria	*gloriosa*
		carbonaria	*humeralis*
		carbonaria	*brunneiventris*
		carbonaria	*carbonaria*
			duidae
Diglossa	3 – *albilatera*	*baritula*	*baritula*
		baritula	*plumbea*
		baritula	*sittoides*
		albilatera	*venezuelensis*
		albilatera	*albilatera*
Diglossopis	4 – *caerulescens*		*caerulescens*
			cyanea
			glauca
			indigotica

Numbers correspond to species-groups in Figs 2, 3, 4.

to test the 'species-groups' and 'superspecies' of Vuilleumier (1969), as well as the monophyly of the two flowerpiercer genera (*Diglossa* and *Diglossopis*) as defined by Sibley & Monroe (1990).

Hypotheses about the historical biogeography of flowerpiercers are primarily based on 14 of the 18 species of flowerpiercers being currently distributed throughout the Andes (Isler & Isler, 1987; Sibley & Monroe, 1990). As a result of this primarily Andean distribution, Vuilleumier (1969) proposed that the Andes were likely the center of origin and important in the diversification of flowerpiercers. However, four species are not found in the Andes: two are found only in the tepuis of Venezuela (*Diglossa duidae* and *Diglossa major*) and two only in the Central America highland regions (*D. baritula* and *Diglossa plumbea*). Thus, without a complete phylogeny, the center of origin of flowerpiercers could potentially be in Central America or the tepuis. Our phylogeny allowed us to identify where flowerpiercers originated and to reconstruct patterns of dispersal and vicariance within the group.

MATERIAL AND METHODS

Taxon Sampling

All 18 species of flowerpiercers in Sibley & Monroe's (1990) taxonomy (*Diglossa* and *Diglossopis*) were included in the present study. Based on DNA–DNA hybridization (Bledsoe, 1988; Sibley & Ahlquist, 1990) and mitochondrial DNA (mtDNA; Burns, 1997; Burns, Hackett & Klein, 2003), flowerpiercers belong to a large group known as tanagers (Thraupini; Sibley & Monroe, 1990, Dickinson, 2003; Remsen *et al.*, 2008). Within tanagers, the closest relative to flowerpiercers is unknown. Thus, representatives from 23 other genera were also included for phylogenetic analysis. Eleven of these genera (*Acanthidops*, *Catamenia*, *Conirostrum*, *Embernagra*, *Haplospiza*, *Idiopsar*, *Melanodera*, *Oreomanes*, *Phrygilus*, *Sicalis*, and *Xenodacnis*) have been hypothesized to be closely related to flowerpiercers based on previous linear classifications, and molecular and morphological studies (Paynter, 1970; Bock, 1985; Yuri & Mindell, 2002; Burns *et al.*, 2003; Burns & Naoki, 2004), and twelve other genera of tanagers (*Anisognathus*, *Hemispingus*, *Hemithraupis*, *Heterospingus*, *Lanio*, *Loxigilla*, *Oryzoborus*, *Poospiza*, *Sporophila*, *Tachyphonus*, *Tangara*, and *Tiaris*) were used to explore the relationship of flowerpiercers to other tanagers. These species represent each of the major lineages of tanagers (K. J. Burns, unpubl. data). Klicka, Burns & Spellman (2007) showed that Cardinalini (i.e. cardinals and grosbeaks) is the sister taxon to tanagers. Thus, the Rose-breasted Grosbeak, *Pheucticus ludoviciana*, was included as an outgroup to root the relationships of all tanagers. Most sequences are new to this study (Genbank accession numbers EU647892–EU647960), but some additional sequences were obtained from Genbank (Burns, 1997; Klicka, Johnson & Lanyon, 2000; Sato *et al.*, 2001; Burns, Hackett & Klein, 2002; Lovette & Bermingham, 2002; Yuri & Mindell, 2002;

Burns & Naoki, 2004; Klicka *et al.*, 2007; R. Sedano & K.J. Burns, unpubl. data; Table 2).

DNA isolation and sequencing

DNA was extracted from samples using a QIAmp DNA MiniKit (Qiagen, Valencia, CA, USA). We used two mtDNA markers, cytochrome (cyt) *b* and nicotinamide dehydrogenase subunit 2 (ND2) that have been used successfully in other avian studies investigating phylogenetic relationships (Hackett, 1996; Burns & Naoki, 2004; Brumfield & Edwards, 2007). Cyt *b* was amplified using three overlapping fragments with the primer combinations; H15297/L14851, H15710/L15206, and H16058/L15656 (Groth, 1998). The gene for ND2 was amplified in two overlapping fragments with H5758/L5215 and H5766/L6316 primer combinations (Hackett, 1996; Klicka *et al.*, 2000). Polymerase chain reaction amplification and DNA sequencing was conducted in accordance with standard protocols. Sequences for the heavy and light strands and overlapping fragments were combined using SEQUENCHER (Genes Codes, Ann Arbor, MI, USA). Additionally, SEQUENCHER was used to translate each gene to proteins and to verify stop codon sites. Both gene regions were sequenced for all species, except *Diglossa gloriosa*. For this species, only cyt *b* was amplified from a museum specimen toe pad using the protocol of Mundy, Unitt & Woodruff (1997).

Phylogenetic analysis

The phylogeny was reconstructed using both Bayesian and maximum likelihood (ML) approaches. No incongruence in phylogenetic signal was found between cyt *b* and ND2 ($P > 0.05$) when the genes were compared using the incongruence length difference test (Farris *et al.*, 1995) as implemented in PAUP*, version 4.0b10, with the partitioned homogeneity test (Swofford, 2001). Thus, the data were combined in further analyses and the appropriateness of four different partitioning schemes was explored: gene and codon position, codon position, gene, and one model for the total dataset. Each partitioning strategy was assessed using the Bayes factor (Kass & Raftery, 1995) of two times the difference in the log likelihood harmonic mean between each partition strategy. Molecular models for each of the partitions were selected using the Akaike information criterion implemented in MrModelTest, version 2.2 (Nylander, 2004). All Bayesian analyses were implemented using MrBayes, version 3.1.2, with default settings (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). The initial parameters were unknown; thus, for each analysis, the variables were unlinked with uniform priors as acceptable starting conditions (Brandley *et al.*, 2006). Each analysis was performed twice for three and six million generations (sampling a tree every 500 and 1000 generations respectively) to confirm that independent analyses converged on the same phylogeny. The software TRACER, version 1.4 (Rambaut & Drummond, 2007) was used to plot the log-likelihood scores against generation time to determine when each analysis reached stationarity. All four Bayesian analyses reached stationarity by 200 000 generations and trees prior to stationarity were considered 'burn-in' and discarded. Conservatively, the first third (2000 trees) of each analysis was discarded as burn-in. The remaining two-thirds (4000 trees) from each analysis were combined for building a consensus tree. Partitioning the combined data by both gene and codon gave a significantly better likelihood score than other partitioning strategies of the total data set. Thus, we used the consensus of 16 000 post burn-in trees from the four Bayesian analyses partitioned by gene and codon to produce our Bayesian consensus tree. Nodes with ≥ 0.95 posterior probability (PP) were considered strongly supported by Bayesian analysis.

ML analysis of the entire data set and nonparametric bootstrap analysis with 1000 replicates were completed using the software GARLI, version 0.951 (Zwickl, 2006) and the GTR+I+Γ model of evolution. Bootstrapping was performed in batches of 20 with 50 bootstrap replications in each batch, which allows for random starting topologies for each batch. Nodes with $\geq 70\%$ bootstrap support were considered strongly supported.

Base composition was assessed via a chi-square test of homogeneity in PAUP*, version 4.0b10 (Swofford, 2001). PAUP*, version 4.0b10 was also used to test whether the data conformed to a molecular clock (rate homogeneity) by comparing likelihood scores with and without a molecular clock enforced.

Biogeography

We used dispersal–vicariance analysis (DIVA; Ronquist, 1997) to reconstruct the biogeographic history of flowerpiercers. Each species was assigned to one or more zoogeographic regions using the database provided by Parker, Stotz & Fitzpatrick (1996). This information, together with the phylogenies, was incorporated into DIVA to reconstruct the ancestral area for the common ancestor of all flowerpiercers. Additionally, DIVA was used to investigate dispersal events into or out of the Andes, tepuis, and Central America. DIVA uses parsimony to reconstruct dispersal, vicariance, and extinction events, given the geographical distribution of terminal taxa. Thus, DIVA assumes a constant rate across the tree and requires short branch lengths if the minimum

Table 2. Species names, GenBank numbers, voucher numbers, and locality information

Species	GenBank number	Source (museum voucher numbers, collectors, and localities)
Acanthidops bairdii	Cyt *b* (AF489878); ND2 (EU647924)	LSUMZ B16267; S. Hackett; Costa Rica: San José, Cerro de la Muerte, km 113 Pan American Highway
Anisognathus somptuosus	Cyt *b* (AY383090); ND2 (EU648011)	LSUMZ B566; T. Schulenberg; Peru: Department Puno, Abra de Maruncunca, 10 km SW San Juan del Oro
Catamenia inornata	Cyt *b* (AF310049)	Ecuador: Alto Peru; voucher not collected, see Sato *et al.* (2001)
Catamenia inornata	ND2 (EF529875)	MBM 6465; Argentina: Tucuman
Conirostrum bicolor	Cyt *b* (AF489883)	UMMZ 227715; Venezuela: Zulia, Ancon de Iturre, about 1 km northeast of town
Conirostrum bicolor	ND2 (AF383141)	STRI TRCBC1; Trinidad and Tobago: St. George County
Conirostrum margaritae	Cyt *b* (EU647892); ND2 (EU647925)	LSUMZ B7293; A. Capparella; Peru: Department Loreto, Amazonas I. Pasto 80 km NE Iquitos
Diglossa albilatera	Cyt *b* (EU647893); ND2 (EU647926)	AMNH DOT 5023; Venezuela: Aragua, km 40 on El Junquito/Col. Tovar road
Diglossa baritula	Cyt *b* (EU647894); ND2 (EU647927)	FMNH 393877; Mexico: Jalisco, Las Joyas, Sierra de Manantlan
Diglossa brunneiventris 1	Cyt *b* (EU647895); ND2 (EU647928)	FMNH 430118; Peru: Cuzco, Paucartambo, Pillahuata
Diglossa brunneiventris 2	Cyt *b* (EU647896); ND2 (EU647929)	AMNH DOT 2892; Bolivia: Department La Paz, Prov. Franz Tamayo, Parque Nacional Apolobamba
Diglossa carbonaria 1	Cyt *b* (EU647897); ND2 (EU647930)	LSUMZ B106752; D. Schmitt; Bolivia: Department Cochabamba
Diglossa carbonaria 2	ND2 (AF447275)	LSUMZ B1295; J. V. Remsen; Bolivia: Department La Paz
Diglossa duidae	Cyt *b* (EU647898); ND2 (EU647931)	AMNH DOT 9754; Venezuela: Amazonas, Cerro Yutaje
Diglossa gloriosa	Cyt *b* (EU647899)	AMNH 824762; Venezuela: Mérida, Laguna Negra
Diglossa gloriosissima	Cyt *b* (EU647900); ND2 (EU647932)	IAvH BT7531; Colombia: Antioquia, Ciudad Bolivar, Farallones del Citara
Diglossa humeralis 1	Cyt *b* (EU647901); ND2 (EU647933)	USNM B3015; M. Jacome; Ecuador
Diglossa humeralis 2	Cyt *b* (AF310050)	Ecuador: Alto Peru; voucher not collected, see Sato *et al.* (2001)
Diglossa lafresnayii	Cyt *b* (AF006229); ND2 (EU647934)	LSUMZ B351; M. Braun; Peru: Cajamarca, Cerro Chinguela, 5 km NE Sapalache
Diglossa major	Cyt *b* (AF290155); ND2 (AF290118)	FMNH 339722; Venezuela: Bolivar, Santa Elena Hwy, km 122
Diglossa mystacalis 1	Cyt *b* (EU647902); ND2 (EU647935)	FMNH 433931; Peru: Cuzco, Paucartambo, La Esperanza, 39 km (road) NE Paucartambo
Diglossa mystacalis 2	Cyt *b* (EU647903); ND2 (EU647936)	LSUMZ B7661; G. Rosenberg; Peru: Department Huánuco Unchog Pass
Diglossa plumbea	Cyt *b* (EU647904); ND2 (EU647937)	AMNH DOT 3646; Costa Rica: San Jose, Cerro de la Muerte
Diglossa sittoides	Cyt *b* (EU647905); ND2 (EU647938)	LSUMZ B22814; S. Cardiff; Bolivia: Department La Paz, B. Saavedra, 83 km by road E. Charazani, Cerro Asunta Pata
Diglossa venezuelensis 1	Cyt *b* (EU647906); ND2 (EU647939)	COP 81247; Venezuela: Anzoategui, Serranía del Turimiquire, Cerro La Launa (El Guamal)
Diglossa venezuelensis 2	Cyt *b* (EU647907); ND2 (EU647940)	COP 81246; Venezuela: Anzoategui, Serranía del Turimiquire, Cerro La Launa (El Guamal)
Diglossopis caerulescens	Cyt *b* (EU647908); ND2 (EU647941)	AMNH DOT 5022; Venezuela: Aragua, km 40 on El Junquito/Col. Tovar road
Diglossopis cyanea	Cyt *b* (EU647909); ND2 (EU647942)	FMNH 430124; Peru: Cuzco, Paucartambo, Pillahuata
Diglossopis glauca	Cyt *b* (EU647910); ND2 (EU647943)	FMNH 430121; Peru: Cuzco, Paucartambo, San Pedro
Diglossopis indigotica	Cyt *b* (EU647911); ND2 (EU647944)	IAvH BT7532; Colombia: Antioquia, Yarumal, Alto de Ventanas; Vereda El Rosario-Corcovado; Finca Villa Nueva
Embernagra platensis	Cyt *b* (EU647912); ND2 (EU647945)	FMNH 396034; Bolivia
Haplospiza rustica	Cyt *b* (EU647913); ND2 (EU647946)	FMNH 433797; Peru: Department Cuzco, Paucartambo, La Esperanza, 39 km NE Paucartambo; 13°10′S, 71°35′59″W
Haplospiza unicolor	Cyt *b* (AF290156); ND2 (AF290119)	FMNH 5186; Brazil: Sao Paulo
Hemispingus melanotis	Cyt *b* (EU647914); ND2 (EU647947)	FMNH 430079; Peru: Department Cuzco, Paucartambo, San Pedro; 13°03′23″S, 71°32′53″W
Hemithraupis flavicollis	Cyt *b* (AF006235); ND2 (EU647948)	LSUMZ B5102; S. Cardiff; Peru: Department Loreto, S Rio Amazonas, ca 10 km SSW mouth Río Napo on E bank Quebrada Vainilla, 100 m
Heterospingus xanthopygius	Cyt *b* (EU647915); ND2 (EU647949)	LSUMZ B2324; S. Lanyon; Panama: Darién, Cana on E slope Cerro Pirre
Idiopsar brachyurus	Cyt *b* (EU647916); ND2 (EU647950)	LSUMZ B22571; M. Marín; Bolivia: Department La Paz, Zongo Valley, 7 km by road N. of Summit.
Lanio fulvus	Cyt *b* (EU647917); ND2 (EU647951)	LSUMZ B2694; S. Cardiff; Peru: Department Loreto, 1 km N Rio Napo, 157 km by NNE Iquitos
Loxigilla portoricensis	Cyt *b* (AF489886); ND2 (EU648044)	LSUMZ B11351; P. Marra; Puerto Rico: Cabo Rojo, Boqueron, Penones de Melones, 1 km WNW intersection routes 301 and 303
Melanodera xanthogramma	Cyt *b* (EU647918); ND2 (EU647952)	AMNH DOT 12115; Argentina: Department Rio Negro, Bariloche
Oreomanes fraseri	Cyt *b* (AF006244); ND2 (EU647953)	LSUMZ B2069; D. Wiedenfeld; Peru: Department Lima, ca. 13 road km W Milloc
Oryzoborus maximiliani	Cyt *b* (EU647919); ND2 (EU647954)	LSUMZ B11908; Ecuador: Esmeraldas Prov., El Placer; 00°52′N, 78°33′W
Pheucticus ludoviciana	Cyt *b* (AF447373); ND2 (AF447298)	UMMZ 233649; Michigan
Phrygilus plebejus	Cyt *b* (EF529979); ND2 (EF529865)	MBM 5310; Argentina: Department Jujuy
Phrygilus unicolor	Cyt *b* (EF529980); ND2 (EF529866)	MBM 6471; Argentina: Department Tucumán
Poospiza lateralis	Cyt *b* (EU647920); ND2 (EU647955)	CUMV 50679; Uruguay: Artigas, Arroyo Mandiyu
Sicalis lutea	Cyt *b* (EU647921); ND2 (EU647956)	FMNH 391932; Peru: Department Ancash, Carhuaz, Ishinca; 09°22′49″S, 77°28′08″W
Sicalis luteola	Cyt *b* (AF489893); ND2 (EU647957)	FMNH 389274; Brazil: Roraima, Fazenda Santa Cecilia, E bank Rio Branco, Across from Boa Vista
Sporophila intermedia	Cyt *b* (EU647922); ND2 (EU647958)	FMHN 389269; Brazil: Roraima, Fazenda Santa Cecilia, E bank Rio Branco, across from Boa Vista
Tachyphonus surinamus	Cyt *b* (EU647923); ND2 (EU647959)	LSUMZ B4795; T. Davis; Peru: Department Loreto, S Río Amazonas, ca. 10 km SSW Río Napo
Tangara gyrola	Cyt *b* (AY383131); ND2 (EU648071)	LSUMZ B22850; E. Cardiff; Bolivia: Department La Paz, Prov. B. Saavedra, 83 km by road E Charazani, Cerro Asunta Pata
Tiaris fuliginosa	Cyt *b* (AF489900); ND2 (EU648107)	LSUM B12612; D. Schmitt; Bolivia: Department Santa Cruz, Prov. Velasco, 50 km ESE of Florida, Arroyo del Encanto
Xenodacnis parina	Cyt *b* (AF006257); ND2 (EU647960)	LSUMZ B7760; G. Glenn; Ecuador: Azuay, 1 km W CJS Nacional de Recreación

AMNH, American Museum of Natural History; CUMV, Cornell University Museum of Vertebrates; COP, Colección Ornitológica Phelps; FMNH, Field Museum of Natural History; IAvH, Instituto de Investigación de recursos Biológicos Alexander von Humboldt; LSUMZ, Louisiana State University Museum of Natural Science Collection of Genetic Resources; MBM, University of Nevada Las Vegas, Marjorie Barrick Museum of Natural History; STRI, Smithsonian Tropical Research Institute; UMMZ, University of Michigan Museum of Zoology; USNM, National Museum of Natural History.

number of historical events is assumed (Ree *et al.*, 2005). Each species of flowerpiercer occurs in only one or two zoogeographic areas. Thus, ancestral nodes were constrained such that the maximum number of areas that could be reconstructed for each node was two (Bremer, 1992; Bremer, 1995).

To estimate dates of speciation and dispersal events and their associated error, we used BEAST, version 1.4.7 (Drummond & Rambaut, 2007). For birds, a variety of mtDNA sequence divergence rates have been estimated, but the most widely used calibration is 2% sequence divergence per Myr, first estimated from restriction fragment length polymorphism data in geese (Shields & Wilson, 1987). Subsequently, this rate has continued to be identified in a variety of bird studies. In a recent study, Weir & Schluter (2008) confirmed the generality of this rate for cyt *b* for avian divergences younger than 12 Myr, which corresponds to the age of diversification of the clade that we studied. Thus, we employed the rate reported by Weir & Schluter (2008) (2.1% = 0.0105 mean substitution rate) when examining the divergence times of flowerpiercers. BEAST analyses were run on the cyt *b* data for 20 million generations with data partitioned by codon and employing the GTR+I+Γ model of nucleotide substitution. We used TRACER, version 1.4 to explore our results and calculate mean ages of each node and the 95% highest posterior density interval associated with each node.

Morphological measurements

Measurements were taken from 757 specimens representing the 18 recognized species. Males and females were measured and, when possible, multiple populations throughout the distribution of each species were sampled. Eight measurements were taken to quantify different aspects of bill and hook shape (Fig. 1). Two measurements of bill size were taken with calipers, width of bill at base (Baldwin, Oberholser & Worley, 1931) and width of bill at nares. Bill depth was not measured due to the large number of specimens that did not have mandibles that were properly closed. Other bill measurements were taken using digital photographs (Griffith & Sheldons, 2001) of the bill. A Canon 20D digital camera with a 60-mm EF-S macro lens was mounted and leveled onto a copy stand or tripod. Individual birds were carefully placed in clamps that were then attached to an independent stand for stability. Each specimen was aligned by eye to an elevated and leveled ruler to ensure that the bill was horizontal with the ruler and perpendicular under the camera. Photographs were digitally downloaded to a computer and analysed using ImageJ (Abromoff & Magelhaes, 2004). Six measurements were taken from each photograph using three landmarks that were easily distinguished in each photograph (Fig. 1).

Measurement error was assessed by re-measuring a total of 100 specimens consisting of males and females for each species. A model II analysis of variance was used to partition the total variability and covariability of a set of measurements for a given set of individuals into within and among components (Bailey & Byrnes, 1990). Thus, the variability between measurements of the same individual is compared with the total variability found among all individuals. The 100 resampled individuals were measured a second time with calipers and rephotographed within a minimum of 2 days from when the original measurement was taken.

The mean for each species of each character was graphed to determine whether the species could be easily separated into the previous hook size classification of small, medium, and large, as proposed by Schondube & Martinez del Rio (2003). However, no treatment of the data resulted in the three discrete hook size categories but, instead, the data formed a continuum. Thus, a principal component analysis (PCA) was used to summarize the patterns of variation in bill and hook shape. The mean of each log-transformed character for each species was used for the PCA. One of the measurements (concavity) is negative in individuals for which the tooth extends below the ventral surface of the maxilla. Thus, because the log of a negative number is undefined, ten was added to concavity values prior to log transformation. Summary statistics and the PCA were implemented using SYSTAT, version 10 (Systat Software Inc., San Jose, CA, USA).

Analysis of character evolution

To assess how bill shape has changed on the phylogeny, we mapped the continuous first principal component (PC) score for each species and both sexes using two methods of ancestral character reconstruction: weighted squared-change parsimony (Maddison, 1991) as implemented in MESQUITE, version 2.0 (Maddison & Maddison, 2007) and a Bayesian approach with the software BayesTraits, beta 1.1 (Pagel, 1999; Pagel, Venditti & Meade, 2006).

The parsimony approach for mapping searches for values that minimize the sum of squares of change along the tree, whereas the squares are weighted inversely by the length of the branch (Felsenstein, 2004). Thus, branch length is taken into account for reconstruction. Although incorporating branch lengths is an improvement over linear parsimony, other assumptions, such as Brownian motion, uncertainty in branch lengths, and uncertainty in phylogeny, are not taken into account (Schluter, Price &

Mooers, 1997). To address these assumptions, the software BayesTraits was also used. Phylogenetic and branch length uncertainty were taken into account by sampling every sixteenth tree (1000 trees total) from the 16 000 post burn-in trees from the Bayesian phylogenetic analysis and loading them into BayesTraits for the Metropolis Hastings algorithm to sample from. The rate deviation and data deviation settings for the Metropolis Hastings algorithm were set such that the acceptance rate of alternate topologies and parameter settings was in the range 20–40%. The Bayesian analysis of character evolution was run for 10.05 million generations, with uniform parameter priors and the burn-in set to 50 000 generations (which always reached stationarity). At each iteration, a new tree is selected and a new combination of rate parameters is estimated. The Bayesian analysis was sampled at every 100 generations, allowing the mean ± SD for each node to be generated.

RESULTS

Sequence variation

Sequences for both cyt *b* and ND2 were aligned without gaps or insertions. The total data set of cyt *b* and ND2 contained 878 (40%) parsimony informative sites; cyt *b* was responsible for 383, whereas ND2 was responsible for 495 sites. Including outgroups, the uncorrected sequence divergence (*p* distance of Nei (1987)) of cyt *b* was in the range 0–13.4% and 0–20.7% in ND2. Within flowerpiercers, *p* distance for cyt *b* was in the range 0–11.3% and 0–16.6% for ND2. Base frequencies for both cyt *b* and ND2 were disparate in guanine, which is common in other avian studies including these mitochondrial markers (Burns *et al.*, 2003; Brumfield & Edwards, 2007; Klicka *et al.*, 2007). A likelihood ratio test failed to reject the hypothesis of rate homogeneity among lineages ($\chi^2 = 29.62$, d.f. = 20, $P > 0.05$).

Phylogenetics

Flowerpiercers form a monophyletic clade with representatives of ten other genera of tanagers with 0.97 PP and 61% bootstrap support (*Acanthidops*, *Catamenia*, *Conirostrum*, *Haplospiza*, *Idiopsar*, *Melanodera*, *Oreomanes*, *Phrygilus*, *Sicalis*, and *Xenodacnis*; tree not shown). Of these ten genera, representatives of six of these (*Acanthidops*, *Catamenia*, *Haplospiza*, *Idiopsar*, *Phrygilus*, and *Xenodacnis*) form a clade that is sister to flowerpiercers; however, support for a sister relationship between this clade and flowerpiercers was not strong (0.58 PP and 52% bootstrap).

Both the ML and Bayesian analyses gave strong support for the monophyly of flowerpiercers (PP 1.0, 98%), and almost all the other nodes showed very strong support among flowerpiercers (Fig. 2). The only difference in topology between the ML and Bayesian analyses is the position of *D. gloriosa*. On the Bayesian consensus tree, *D. gloriosa* was sister to a clade containing all other members of the *carbonaria* superspecies, whereas the likelihood analyses place this species sister to *Diglossa humeralis* (Figs 2, 3). However, strong support for relationships within the *carbonaria* superspecies was lacking in all analyses. Most other nodes were strongly supported by both Bayesian and likelihood analyses, although the two analyses did differ in their degree of support for some nodes.

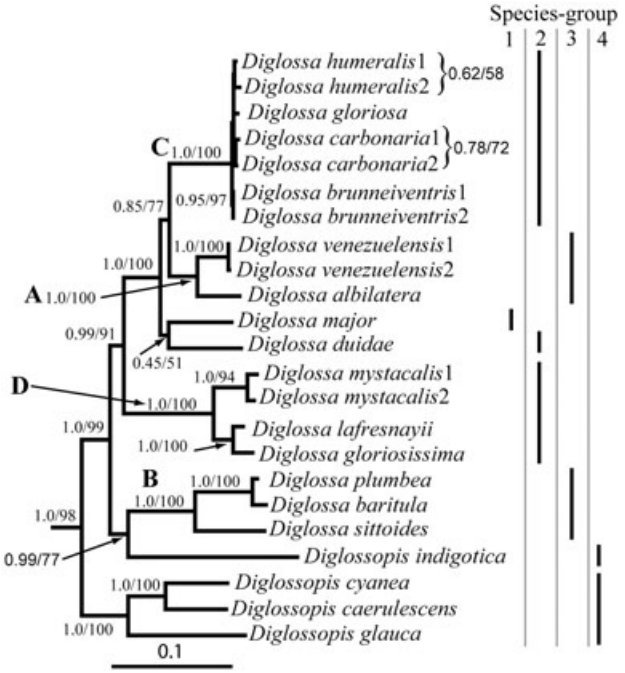

Figure 2. Phylogram of the maximum likelihood analysis (–ln *L* 25004.44). Support values for each node are indicated with posterior probabilities followed by maximum likelihood bootstrap percent support values. Species-groups are numbered and designated by black vertical lines in the same column: 1, *major*; 2, *lafresnayii*; 3, *albilatera*; 4, *caerulescens* (= *Diglossopis*). Bold indicates superspecies designated by Vuilleumier (1969): A, *albilatera*; B, *baritula*; C, *carbonaria*; D, *lafresnayii*.

We used the strong support found for the majority of nodes in the phylogeny of flowerpiercers to test the classification proposed by Vuilleumier (1969). Vuilleumier (1969) grouped all flowerpiercer species into one of four different species-groups (Table 1). One species-group, *major*, is monotypic. The remaining three species-groups *caerulescens* (= the genus *Diglossopis*), *albilatera*, and *lafresnayii* are not monophyletic (Fig. 2). *Diglossopis indigotica* is more closely related to the *baritula* superspecies, making the *cae-*

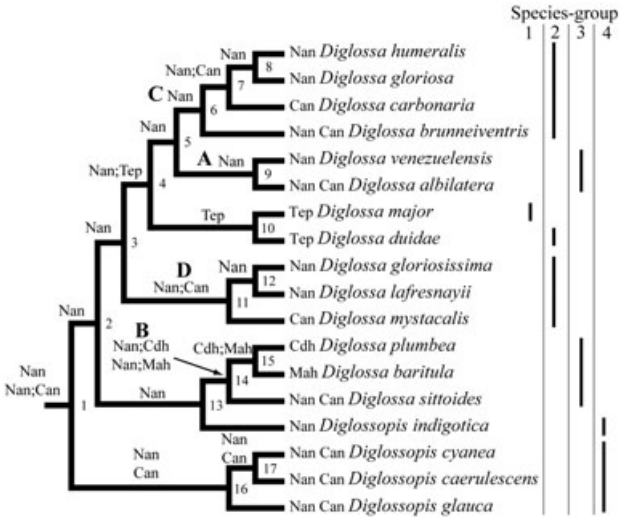

Figure 3. Dispersal–vicariance analyses of flowerpiercer distributions. Phylogeny shown is the topology of the maximum likelihood analysis. Areas separated by a semicolon represent that taxon existing in both areas (i.e. node 7), whereas areas listed above one another represent equally optimal reconstructions (i.e. node 16). Nan, Northern Andes; Can, Central Andes; Tep, tepuis; Mah, Madrean Highlands; Cdh, Chiriquí-Darién Highlands (Parker *et al.*, 1996). Species-groups are numbered and designated by black vertical lines in the same column: 1, *major*; 2, *lafresnayii*; 3, *albilatera*; 4, *caerulescens* (= *Diglossopis*). Bold indicates superspecies designated by Vuilleumier (1969): A, *albilatera*; B, *baritula*; C, *carbonaria*; D, *lafresnayii*.

rulescens species-group and thus both the genus *Diglossa* and genus *Diglossopis* paraphyletic.

In contrast to the species-group classification, strong support was found for Vuilleumier's (1969) superspecies classification. The four superspecies Vuilleumier (1969) described (*carbonaria*, *lafresnayii*, *albilatera*, and *baritula*; Table 1) were strongly supported in all analyses (Fig. 2). Species within the *carbonaria* superspecies were weakly differentiated ($p = 0.5\%$) compared to the other superspecies ($p = 4.4\%$–5.6%). Indeed, interspecific variation within the *carbonaria* superspecies was similar to the amount of variation found within most avian species (Avise & Walker, 1998; Ditchfield & Burns, 1998). For species in which more than one individual was sampled, the results obtained in the present study showed congruence to the species-level taxonomy of flowerpiercers.

BIOGEOGRAPHY

Four equally optimal reconstructions were found using DIVA (Fig. 3). The earliest node and most other nodes include the northern Andes as part of their distribution. Depending on the reconstruction, 12–14 of the 17 nodes include the northern Andes in their distributions. Each of the four reconstructions identified 11 dispersal events. The majority of these dispersal events involve movement out of the northern Andes into other geographic regions. All four reconstructions identify the following eight branches as involving dispersal events out of the northern Andes: between nodes 6 and 7, between nodes 3 and 4, between nodes 3 and 11, between nodes 13 and 14, between nodes 14 and 15, on the branch leading to *Diglossa brunneiventris*, on the branch leading to *Diglossa albilatera*, and on the branch leading to *Diglossa sittoides*. Two of the reconstructions identify the remaining three dispersal events as also involving movement out of the northern Andes (on the branch leading to *Diglossopis glauca*, on the branch leading to *D. caerulescens*, and on the branch leading to *Diglossopis cyanea*). Alternatively, the other two reconstructions identify the dispersals on these three branches as movements out of the central Andes into the northern Andes. Inferred vicariant events include the diversification of flowerpiercers in Central America at node 15, a split between the northern and central Andes at node 11, and a split between the northern Andes and the tepuis at node 4. In addition, two of the reconstructions identified a vicariant event at the earliest split within flowerpiercers at node 1. In agreement with Hackett (1995), one dispersal event (between node 13 and 14) can explain the presence of flowerpiercers in Central America. At this point in the history of flowerpiercers, there was a dispersal from the northern Andes to either the Madrean Highlands or Chiriquí-Darién Highlands, both of which are regions in Central America. Similarly, a single dispersal event (between nodes 3 and 4) can account for the distribution of flowerpiercers in the tepuis.

Because of the low support for the relationships among members of the *carbonaria* superspecies (Fig. 2), all possible reconstructions among these species were explored. Regardless of how these species are arranged, only one or two dispersal events from the northern Andes into the central Andes were reconstructed in this part of the tree. Similarly, because of the weak support for node 10 (Fig. 2), we also explored alternative reconstructions of the position of these two taxa. These reconstructions indicated that an additional dispersal event may have occurred from the northern Andes into the tepuis. However, none of our reconstructions show movement out of the tepuis into other areas.

BEAST analyses (Table 3) indicate that the timing of speciation events for flowerpiercers span several Myr, from approximately 15 Mya to as recently as 500 000 years ago. Five speciation events (nodes 6, 7,

Table 3. Age of nodes in the flowerpiercer phylogeny as inferred by BEAST

Node	Age (Myr)	95% highest posterior density interval (Myr)
1	15.1	10.4–20.5
2	14.3	9.2–20.1
3	10.1	7.2–13.4
4	6.2	4.3–8.5
5	5.0	3.1–7.0
6	0.5	0.4–0.1
7	0.5	0.4–0.1
8	0.4	0.2–0.7
9	2.6	1.4–3.8
10	6.0	3.6–8.6
11	3.4	2.1–4.8
12	1.5	0.8–2.4
13	12.4	6.4–20.0
14	5.0	2.7–7.7
15	0.8	0.3–1.3
16	9.1	5.3–13.5
17	4.7	2.6–7.1

Node numbers correspond to labels in Figs 3, 4.

8, 12, and 15) date to the Pleistocene, but other speciation events are much older. Five nodes (5, 9, 11, 14, and 17) fall within the range of the Pliocene and seven more (1, 2, 3, 4, 10, 13, and 16) date as far back as the middle Miocene.

Bill evolution

All characters had low measurement error (< 10%) except for hook depth, which was therefore excluded from the PCA so that the unstructured variation due to error did not affect the loadings (Lougheed, Arnold & Bailey, 1991). PCs with eigenvectors ≥ 1 were retained for mapping the ancestral character state and to summarize variation. PC1 accounted for 62.1% and 60.3% of male and female variance, respectively (Table 4). Three variables associated with hook size (concavity, hook length, and tooth depth) had strong negative loadings, whereas four variables associated with bill size (maxilla depth, bill length, bill width, and bill width at nares) had strong positive loadings (Table 4). Thus, PC1 represents a contrast between hook size and bill size. Species with large factor scores for PC1 have small hooks relative to a larger bill, whereas species with smaller factor scores for PC1 have large hooks relative to a small bill size. PC2 accounted for 24.1% and 23.9% of total variance between males and females, respectively. PC2 loaded positively for all variables (Table 4), indicating that PC2 may be capturing variation associated with overall size.

Table 4. Factor loadings for the eight characters of bill and hook size

	Male		Female	
Variable	PC1	PC2	PC1	PC2
Concave	–0.858	0.317	–0.848	0.317
Hook length	–0.701	0.585	–0.725	0.548
Tooth depth	–0.669	0.717	–0.708	0.679
Maxilla depth	0.584	0.648	0.533	0.686
Bill length	0.871	0.398	0.823	0.454
Bill width	0.885	0.115	0.863	0.128
Bill width at nares	0.886	0.376	0.876	0.339
Eigenvalues	4.344	1.689	4.219	1.670
Variance	62.060	24.130	60.280	23.860

Eigenvalues and variance associated with each principal component (PC) are shown.

The PC1 value for each species was used to reconstruct the evolution of relative hook size using weighted squared-change parsimony. Character states for males and females of each species and for each reconstructed node were almost identical. Thus, we only present the reconstruction for male relative hook size (Fig. 4). Although Mesquite maps continuous data, for graphical representation, the software generates ten character states by default, which represent the range of PC1 values divided equally and traced onto the phylogeny (Fig. 4). Character state 1 corresponds to a larger hook relative to a smaller bill (more negative PC 1 values), whereas character state 10 corresponds to a smaller hook relative to a larger bill (larger PC 1 values). Both parsimony and Bayesian character state reconstruction analyses produced largely congruent results (Fig. 4). The ancestral node of all flowerpiercers (node 1) was reconstructed as relatively intermediate in terms of size of hook relative to size of bill. Although an intermediate hook size is common at many nodes in the radiation of flowerpiercers, relative hook size has diverged multiple times during the evolution of flowerpiercers. At the earliest speciation event within flowerpiercers (node 1), relative hook size begins to diverge. The ancestor at node 16 was reconstructed as having a smaller hook relative to a larger bill, whereas the ancestor at node 2 was reconstructed with a larger relative hook. Thus, both analyses show a major divergence in an important morphological characteristic at the first speciation event in flowerpiercers. Within two independent lineages, a relatively larger hook size has evolved (nodes 5 and 13). An inverse pattern, the evolution of a proportionately smaller hook from a relatively intermediate-sized hook, was also shown in the evolution of *D. major* and in the evolution of the

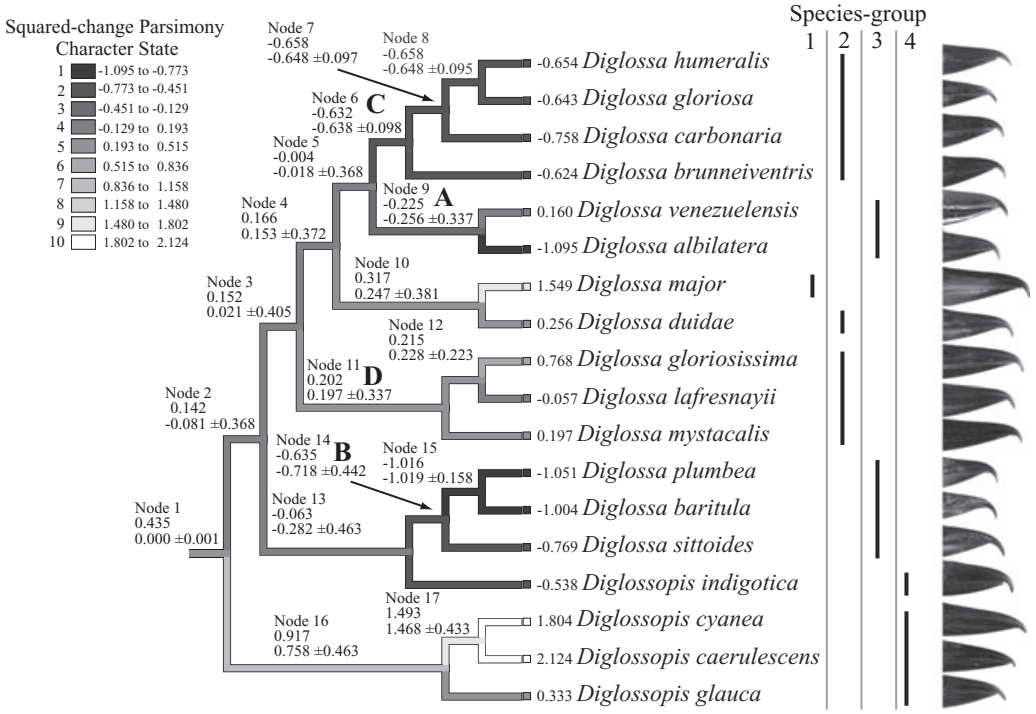

Figure 4. Ancestral character state reconstruction of principal component 1 (PC1) for male flowerpiercers. The mean PC1 value for each species is listed to the left of each species name. At each node, the weighted squared-change parsimony estimation is shown above the Bayesian estimate and standard deviation. Larger values indicate species with smaller hooks relative to bill size and more negative values indicate birds with larger hooks relative to bill size. Shaded branches and character states (1–10) are for illustrative purposes and show changes in relative bill size from the parsimony reconstruction. Phylogeny shown is the topology of the maximum likelihood analysis. Species-groups are numbered and designated by black vertical lines in the same column: 1, *major*; 2, *lafresnayii*; 3, *albilatera*; 4, *caerulescens* (= *Diglossopis*). Bold indicates superspecies designated by Vuilleumier (1969): A, *albilatera*; B, *baritula*; C, *carbonaria*; D, *lafresnayii*.

common ancestor of *Diglossopis caerulescens* and *Diglossopis cyanea* (node 17; Fig. 4).

DISCUSSION

Diversification of flowerpiercers

The complete molecular phylogeny of all currently recognized species of flowerpiercers enables the assessment of previously proposed relationships (Vuilleumier, 1969; Bock, 1985; Sibley & Monroe, 1990). The four superspecies described by Vuilleumier (1969) are monophyletic with strong support (Fig. 2) and the relationships of members of the *baritula* superspecies group are congruent with those described by Vuilleumier (1969) and Hackett (1995). The amount of sequence divergence among the species within the *carbonaria* superspecies is less than that found in other flowerpiercer superspecies. However, the plumage patterns found within the *carbonaria* superspecies are the most variable of all flowerpiercer superspecies (Vuilleumier, 1969; Isler & Isler, 1987; Restall, Rodner & Lentino, 2006). In the northeastern Andes of western Venezuela, the black-backed and rufus-bellied *Diglossa gloriosa* is replaced by the black-backed and bellied *D. humeralis* in the eastern and southern Andes of Colombia, Ecuador, and northern Peru. The dark form of *D. humeralis* is then replaced to the south in the Andes of Peru by the dark-backed and rufus-bellied *D. brunneiventris*. Finally, in the Andes of Bolivia the black-backed and gray-bellied *Diglossa carbonaria* replaces *D. brunneiventris* with a narrow area of contact and hybridization. Additionally, *D. humeralis* has three subspecies (*nocticolor*, *humeralis*, and *aterrima*) that are allopatrically distributed and vary in the presence and absence of grey feather patches on the shoulders and rump. A small disjunct population of *D. brunneiventris* (*Diglossa brunneiventris vuilleumieri*) exists in the northern part of the central and western Cordillera of Colombia and differs from the Peruvian population only by having a smaller body size and a larger black throat patch (Graves, 1980). Thus, the *carbonaria* superspecies represent a relatively recent radiation in which rapid change in plumage morphol-

ogy has occurred. Our BEAST analyses indicate that the earliest node of the *carbonaria* superspecies group dates to 0.475 Mya (0.207–0.785 Mya). Our findings with the *carbonaria* superspecies group add to the growing number of cases that have identified rapid plumage divergence with little mtDNA divergence (Zink *et al.*, 2003; Kondo, Baker & Omland, 2004; Milá *et al.*, 2007).

The 'leapfrog' plumage pattern (Remsen, 1984) of a dark back and lighter-bellied species separated by an all dark plumage species found in the *carbonaria* superspecies (above) mirrors the pattern found in the *lafresnayii* superspecies. However, the *lafresnayii* superspecies group diverged much earlier than the *carbonaria* group. Our BEAST analyses showed divergence within the *lafresnayii* superspecies began approximately 3.3 Mya (2.13–4.80 Mya). The parallel evolution of this leapfrog plumage pattern in two lineages of flowerpiercers that have speciated at different times can potentially shed light on the processes of vicariance, ecology, and genome evolution that has generated these unique plumage patterns found in multiple species of birds in the Andes (Remsen, 1984).

Although our phylogeny showed concordance with the superspecies taxonomy of Vuilleumier (1969), our analyses disagreed with his species-groups. Our trees showed that the *albilatera*, *lafresnayii*, and *caerulescens* species-groups were not monophyletic. Using allozyme data and mtDNA, Hackett (1995) reached the same conclusion regarding the *albilatera* and *lafresnayii* species-groups, even though her taxon-sampling was incomplete. Vuilleumier (1969) united members of these species groups based on shared plumage characteristics such as degree of sexual dichromatism and the presence or absence of 'scaly' forehead feathers. The nonmonophyly of the *albilatera* and *lafresnayii* species-groups suggests that these characteristics have evolved multiple times during the evolutionary history of flowerpiercers. Vuilleumier also grouped members of the *caerulescens* species-group (=the genus *Diglossopis*) based on plumage characters such as overall blue plumage, weak sexual dimorphism, and nonscaly forehead feathers. Although Bock (1985) concluded that one of these species *D. indigotica* did not belong in this group based on features of its bill, subsequent taxonomies elevated this species-group to genus level and included *D. indigotica* (e.g. Sibley & Monroe, 1990). Our phylogenies clearly show that *Diglossopis indigotica* is more closely related to *D. sittoides*, *D. baritula*, and *D. plumbea* than it is to other members of *Diglossopis*. Thus, the phylogeny of this study does not support the further use of *Diglossopis*, and we recommend that all flowerpiercers be considered members of the genus *Diglossa*.

Biogeography

The Andes clearly played an important role in the biogeographic history of flowerpiercers. The biogeographic reconstruction of the ancestor for all flowerpiercers was inferred as inhabiting the northern Andes or northern and central Andes, as originally hypothesized by Vuilleumier (1969). In addition, most ancestral and extant species include the northern Andes in their distribution. A variety of events within the Andes such as uplift, habitat changes, climatic cycles, and tectonic activity were likely important in flowerpiercer diversification. Both the northern and central Andes reached half of their current elevation approximately 10 Myr ago (Gregory-Wodzicki, 2000). However, the central Andes completed its uplift prior to the northern Andes, and underwent a final, rapid uplift between 10 and 6 Myr ago (Garzione *et al.*, 2008). The main uplift of the northern Andes took place more recently, between 7 and 4 Myr ago (Hooghiemstra & Van der Hammen, 2004), with some parts experiencing a rapid, final uplift between 5 and 2 Myr ago (Gregory-Wodzicki, 2000). These dates correspond well with the timing of many flowerpiercer speciation events (Table 3) and those of other Andean birds (García-Moreno & Fjeldså, 2000). However, some nodes in the flowerpiercer tree date more recently than these events. Vicariance caused by habitat shifts occurring during late Pleistocene glacial cycles has been hypothesized as an important mechanism for generating current levels of species diversity in the Neotropics (Haffer, 1974). Within flowerpiercers, several speciation events occurred within the last 800 000 years, when Pleistocene glacial cycles were extreme and thus these events are consistent with this hypothesis. In agreement with Graves (1982), the *carbonaria* superspecies represents a recent radiation that likely was strongly influenced by Pleistocene glaciation cycles. In addition, diversification of the two Central American species (*D. plumbea* and *D. baritula*) also dates to the late Pleistocene.

Two dispersal events occurred out of the Andes during the evolution of flowerpiercers, one to Central America and one to the tepuis. Dispersal to Central America occurred between nodes 13 and 14. The timing of this event (approximately 5–12 Mya; Table 3) is earlier than the final closure of the Panamanian isthmus connecting Central and South America (3.5–2.5 Myr ago; Coates & Obando, 1996). Molecular studies comparing avian taxa distributed in Central and South America are only now beginning to be reported. No general pattern has emerged; some studies report correspondence with landbridge formation, whereas others show dispersal preceded the final formation (Pérez-Emán, 2005; Barker, 2007; DaCosta & Klicka, 2008; Weir *et al.*, 2008).

Because our DIVA analyses showed a dispersal from the Andes to the tepuis, a scenario in which the tepuis were a source of origin for the *carbonaria* superspecies (Graves, 1982) can be rejected. Instead, a single dispersal event from the Andes to the tepuis (between nodes 3 and 4) can explain the presence of flowerpiercers in the tepuis. This dispersal event occurred between 6 and 10 Mya, and the two flowerpiercers in the tepuis speciated from each other approximately 6 Mya (Table 3). Because the two tepui taxa do not form a strong monophyletic group, it is possible that the tepuis were colonized by two independent dispersals from the northern Andes, as suggested by Mayr & Phelps (1967). The timing of these two dispersals would still be in the range 6–10 Mya. The tepuis originated in the Cretaceous and subsequent erosion into the Tertiary resulted in the table-like structure of these highland formations (Maguire, 1970). Thus, the dispersal of flowerpiercers to the tepuis is old enough to have occurred before much of this erosion, when the area occupied by the modern Andes had a greater connection to the area occupied by the modern tepuis. Although not many avian species have been studied in this area using molecular data, two species of antbirds from separate lineages (Braun *et al.*, 2005; Brumfield & Edwards, 2007) also have sequence divergence values corresponding to a date old enough for these species to be descended from relictual taxa that were once more widespread across northern South America. Several studies (Chapman, 1931; Cook, 1974; Haffer, 1974) have hypothesized that dispersal between the Andes and tepuis occurred more recently and was facilitated by Pleistocene climate change temporarily connecting habitat between the two regions. Dispersal may have occurred through a continuous habitat connection (e.g. cool climate theory; Chapman, 1931) or by island hopping across low mountains with temporarily favorable habitat for highland birds (e.g. modified cool climate theory; Haffer, 1974). Our analyses show that, if calibrations provide a reasonable estimate of divergence times, then Pleistocene glacial cycling is too recent to explain flowerpiercer distributions in the tepuis.

Evolution of hook shape

The weighted squared-change parsimony and the Bayesian method of ancestral state reconstruction both estimated the ancestor of all flowerpiercers as having an intermediate bill and hook shape (Fig. 4). The first descendents of flowerpiercers (nodes 2 and 16; Fig. 4) represent an early divergence in morphology among flowerpiercers. A large bill with a relatively small hook is inferred for the ancestral species represented by node 16, whereas ancestral species represented by node 2 has a larger hook relative to a smaller bill. This split between these sister taxa is supported by concordant divergence in other morphological characters of the cranium, tongue, and mandible found among the descendents of these two lineages (Bock, 1985). The evolution of a relatively larger hook at node 2 (Fig. 4) may have been a key innovation for feeding on nectar from flowers with long corollas. This interpretation is supported by the larger number of species that have descended from node 2 (15 species) compared to relatively few that have descended from node 16 (three species; Fig. 4). Two of the four DIVA reconstructions also show that this split correlates with a geographic separation, with the ancestral distribution of node 2 being restricted to the northern Andes and the ancestral distribution of node 16 being restricted to the central Andes. Subsequently, descendents of these species re-colonized the other areas. Thus, these two reconstructions suggest that smaller hooks may have evolved in the central Andes and, more recently, these species with relatively smaller hooks colonized the northern Andes.

After this initial divergence, relative hook size continued to evolve, with some species evolving larger relative hooks from smaller hooks and some species evolving smaller relative hooks from larger hooks. Thus, our complete-species phylogeny and quantification of bill variation shows that the evolution of hook size in flowerpiercers was not a simple progression from a small relative hook to a large relative hook, as presented by Schondube & Martinez del Rio (2003). Instead, our phylogeny showed that relative hook size changed frequently throughout the history of the group, with large relative hooks and small relative hooks each evolving multiple times. The evolution of this diversity in relative hook size may have allowed different species of flowerpiercers to coexist in the Andes. Many flowerpiercers have some overlap in distribution, with up to eight flowerpiercer species occurring together in some regions (Isler & Isler, 1987; Sibley & Monroe, 1990). The majority of species that overlap in distribution differ in magnitude of relative hook size by four or more character states (e.g. 1 versus 5, 3 versus 7, 6 versus 10, etc.). Thus, most flowerpiercer species that overlap in distribution have dramatically different relative hook sizes. Because species with different relative hook sizes have different degrees of nectar and fruit foraging abilities (Schondube & Martinez del Rio, 2003), these different hook sizes could facilitate the coexistence of flowerpiercers in the same location. Previous studies have noted other factors that vary among species of flowerpiercers that overlap in distribution (Lyon & Chadek, 1971; Moynihan, 1979; Isler & Isler, 1987; Rojas-Nossa, 2007). Overlapping species also display differences in microhabitat preference, levels of aggressiveness, and degree of sociality. For example, when aggressive competitors are present, such as some species of hum-

mingbirds or other *Diglossa*, species in the *baritula* and *albilatera* superspecies avoid confrontation and retreat to thick vegetation while surreptitiously stealing nectar from flowers (Lyon & Chadek, 1971; Moynihan, 1979; Isler & Isler, 1987). Members of the *carbonaria* superspecies are aggressive and defend flower patches or territories from both hummingbirds and congeners (Moynihan, 1979; Isler & Isler, 1987). Thus, in areas of overlapping habitat and flower choice (Moynihan, 1979: 59), the difference in behavioural strategies may enable the coexistence of more efficient nectar thieving species. Where species of the reticent *baritula* and *albilatera* superspecies coexist (predominantly in the northern Andes: Moynihan, 1979; Isler & Isler, 1987), they are restricted to different microhabitats. *Diglossa sittoides* is found more often in open areas at the forest edges, whereas *D. albilatera* is generally found in denser vegetation and the understory of second growth forest (Moynihan, 1979; Isler & Isler, 1987). Additionally, *Diglossopis indigotica* inhabits the canopy forest layer and joins mixed-species flocks (Isler & Isler, 1987). This habitat partitioning may limit the interaction and competition *Diglossopis indigotica* experiences from other species that also have large relative hook sizes, which are more sedentary and found primarily in the understory (Isler & Isler, 1987; Parker *et al.*, 1996). Thus, a combination of both behavioural and selective habitat partitioning occurs among many species of flowerpiercers. Taken together, these behavioural differences and the bill shape differences that we identified in the present study can account for 94% of the overlapping species, suggesting a role for competitive exclusion and character displacement in the evolution of the flowerpiercer radiation. Future behavioural studies that focus on foraging efficiency of different bill types on particularly sized flowers and fruits in areas of overlap would help clarify the significance of the repeated evolution of diverse bill types within flowerpiercers. The present study provides the phylogenetic framework and bill classification scheme that will encourage this type of research.

ACKNOWLEDGEMENTS

We thank the scientific collectors, collection managers and curators at the following institutions for providing the tissues used in the study: American Museum of Natural History (AMNH), Field Museum of Natural History, National Museum of Natural History, Louisiana State University Museum of Natural Science Collection of Genetic Resources, Cornell University Museum of Vertebrates, Marjorie Barrick Museum of Natural History at the University of Nevada Las Vegas, Colección Ornitológica Phelps, Venezuela (COP), Instituto De Investigación de Recursos Biológicos Alexander von Humboldt (IAvH), and the International Center for Tropical Agriculture, Colombia. Additionally, we thank the following museums for access to specimens in their collections: AMNH, COP, San Diego Natural History Museum, and the Natural History Museum of Los Angeles County. We thank R. Sedano, J. Pérez-Emán, P. Pulgarin, and F. K. Barker for their assistance in allocating tissues and for sequencing some of the samples. For assistance in the laboratory, we thank R. Sedano and, for comments on the manuscript, we thank J. V. Remsen, R. Bowie, T. Reeder, P. Pryde, R. Mauck III, R. Racicot, and R. Sedano. Funds for this project were provided by the Chapman Fund (AMNH), Collection Study Grant (AMNH), Ralph W. Schreiber Ornithological Research Award by the Los Angeles Audubon Society, Mabel Myers Memorial Scholarship (SDSU), and the National Science Foundation (IBN-0217817 and DEB-0315416).

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