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Long-distance gene flow and cross-Andean dispersal of lowland rainforest bees (Apidae: Euglossini) revealed by comparative mitochondrial DNA phylogeography

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Abstract

Euglossine bees (Apidae; Euglossini) exclusively pollinate hundreds of orchid species and comprise up to 25% of bee species richness in neotropical rainforests. As one of the first studies of comparative phylogeography in a neotropical insect group, we performed a mitochondrial DNA (mtDNA)-based analysis of 14 euglossine species represented by populations sampled across the Andes and/or across the Amazon basin. The mtDNA divergences within species were consistently low; across the 12 monophyletic species the mean intraspecific divergence among haplotypes was 0.9% (range of means, 0–1.9%). The cytochrome oxidase 1 (CO1) divergence among populations separated by the Andes ($N = 11$ species) averaged 1.1% (range 0.0–2.0%). The mtDNA CO1 data set displayed homogeneous rates of nucleotide substitution, permitting us to infer dispersal across the cordillera long after the final Andean uplift based on arthropod molecular clocks of 1.2–1.5% divergence per million years. Gene flow across the 3000-km breadth of the Amazon basin was inferred from identical cross-Amazon haplotypes found in five species. Although mtDNA haplotypes for 12 of the 14 euglossine species were monophyletic, a reticulate CO1 phylogeny was recovered in *Euglossa cognata* and *E. mixta*, suggesting large ancestral populations and recent speciation. Reference to closely related outgroups suggested recent speciation for the majority of species. Phylogeographical structure across a broad spatial scale is weaker in euglossine bees than in any neotropical group previously examined, and may derive from a combination of Quaternary speciation, population expansion and/or long-distance gene flow.

Keywords: cytochrome oxidase 1, molecular clock, mtDNA, neotropical biogeography, orchid bees, tropical rainforest

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Introduction

A principal aim of phylogeography is to infer biogeographical history from the genealogies of codistributed organisms (Bermingham & Avise 1986; Bermingham & Moritz 1998; Avise 2000). Because of their limited capacity for dispersal, neotropical freshwater fish (Bermingham & Martin 1998; Sivasundar *et al*. 2001; Perdices *et al*. 2002), frogs (Crawford 2003) and salamanders (Garcia-Paris *et al*. 2000) have been studied to infer patterns of regional diversification. An increasing number of studies have turned to widespread species to assess continental-scale biogeographical histories. In the neotropics, widespread species have revealed phylogeographical breaks in lowland populations separated by the northern Andes and/or the Talamanca cordilleras (Brower 1994; Zamudio & Greene 1997; Slade & Moritz 1998; Perdices *et al*. 2002; Cavers *et al*. 2003; Cortés-Ortiz *et al*. 2003; Hoffmann & Baker 2003; Novick *et al*. 2003; Eberhard & Bermingham 2004), extensive gene flow across the Amazon basin (Dick *et al*. 2003; Lemes *et al*. 2003; Eberhard & Bermingham 2004), and the existence of contact zones in Panama for populations derived from Central and South America (Bermingham *et al*. 1998; Perdices *et al*. 2002; Dick *et al*. 2003).

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In addition to revealing biogeographical histories, comparative phylogeography offers a window into the historical assembly of ecological communities. In conjunction with molecular clock analyses, the residence times of endemic species or regional populations can be inferred from molecular phylogenies (Dick *et al*. 2003). This information is important for understanding both the origin and maintenance of local species diversity (α-diversity) and the degree of variation in species composition across landscapes (β-diversity) (Hubbell 2001; Ricklefs 2004). For example, evidence for extensive gene flow across the Amazon basin would suggest a small role for endemic diversification and therefore low levels of species turnover across broad spatial scales for some taxa.

In this study, we examined the comparative phylogeography of euglossine bees (Apidae: Euglossini). Also known as orchid bees, these vagile insects exclusively pollinate ~675 species of epiphytic orchids and are the selective force behind many of the exquisite orchid floral adaptations detailed by Darwin (1888). The Euglossini comprise one of four tribes of corbiculate bees, a clade which also includes honey bees (Apini), bumblebees (Bombini) and stingless bees (Meliponini). Euglossines differ from other corbiculate bees by their long tongues, which often exceed the length of the body, by their frequently iridescent coloration, and by the morphological adaptations used by male bees for fragrance collection, which is apparently part of courtship behaviour (Dressler 1982; Eltz *et al*. 2003) and which orchids and other flowering plants have exploited for their own reproduction. While many orchids rely exclusively on euglossine bees for pollination, the bees themselves utilize a wide range of host plants representing a number of angiosperm families. Thus the geographical distributions of euglossine bees are not tied to any particular host plant species. Although restricted to tropical America, many euglossine species have broad, overlapping geographical ranges and comprise up to 25% of the local bee diversity in some lowland forests (Roubik & Hanson 2004).

Euglossine bees are classified into five genera: *Euglossa* (c. 112 described species), *Eufriesea* (c. 65 species), *Eulaema* (c. 20 species), *Exaerete* (six species) and *Aglae* (one species). The two species-poor genera *Exaerete* and *Aglae* are cleptoparasites that deposit eggs in the brood cells of *Eulaema* and/or *Eufriesea*. Although recent molecular studies have investigated the phylogeny of the group (Michel-Salzat *et al*. 2004), virtually nothing is known about the comparative phylogeography of euglossine bees, nor of any other neotropical arthropod group.

Approximately 25% of the euglossine species have cross-Andean distributions (Ramírez *et al*. 2002), which raises the question of the degree of evolutionary separation between populations inhabiting lowland rainforests on the eastern and western flanks of the northern Andean cordilleras. On the one hand these allopatric taxa may represent cryptic species that owe their formation to the Andean orogeny. In favour of such a vicariance hypothesis, euglossine bees are rarely collected above 2000 m (Dressler 1982) and they prefer moist forest habitats. The northern Andes rarely fall below 2000 m in elevation, and the northern tips of the cordilleras intersect with dry Caribbean coastal plains or ocean. Moreover, the tribe Euglossini is considerably older than the northern Andes. The oldest euglossine fossils, closely resembling extant *Euglossa* and *Eufriesea*, were recovered from 20- to 22-million-year-old amber from the Dominican Republic (Poinar 1998; Engel 1999). These fossils predate both the uplift of the northern Andes in Miocene/Pliocene (Gregory-Wodzicki 2000; Lundberg 1998) and the Pliocene formation of the land-bridge between Central and South America (Coates & Obando 1996).

On the other hand, the widespread euglossines are likely candidates for recent cross-Andean dispersal. The male bees are known to fly long distances to gather floral fragrances (Janzen 1971). Although relatively little is known about female euglossine bees, they are probably also capable of long-distance flights and, because they store sperm in their spermatheca for the duration of their lives, they can readily found populations following long-distance dispersal events (Michener 1979).

The objectives of this study were to (i) assess the phylogeographical structure of euglossine bees across their neotropical range, (ii) determine whether vicariance or montane dispersal best accounts for the cross-Andean distributions of several widespread species, and (iii) compare the phylogeographical patterns of euglossines with patterns found in codistributed rainforest taxa. The phylogeographical results permit us to discuss biotic and geographical causes of diversification that apply to many other neotropical taxa. Our analysis is based on intraspecific variation in the mitochondrial cytochrome oxidase 1 (CO1) gene. The 14 widespread euglossine species were sampled across the Andes and/or across the Amazon basin.

Materials and methods

Taxonomic sampling

We obtained partial mitochondrial DNA (mtDNA) CO1 sequences (~550 base pairs) from a total of 86 individuals from 14 species, representing four of the five euglossine genera: *Euglossa* ($N = 6$ species), *Eufriesea* ($N = 2$), *Eulaema* ($N = 4$) and *Exaerete* ($N = 2$) (Table 1; Appendix). The study specimens were male euglossine bees, collected at chemical baits from 22 localities in Panama (PA); Costa Rica (CR); Mexico (MX); Ecuador (EC), French Guiana (FG); and Bolivia (BO) (Fig. 1; Appendix). The specimens were stored at ambient temperature in a salt–dimethyl sulphoxide (DMSO) solution (Seutin *et al*. 1991) or in 99%

Table 1 The 14 Euglossini study species, size in mass and length (based on collections at STRI), sample size (*N*), collection sites, and maximum recorded elevation (from Ramírez *et al*. 2002; Roubik & Hanson 2004)

Study species	Mass (mg)	Size (mm)	*N*	Collection localities	Maximum altitude (m)
Euglossa allosticta Moure	35	13	5	EC-E, PA, CR	*1100*
Euglossa cognata Moure	56	13	9	EC-E, EC-W, PA, BO, FG	1100
Euglossa ignita F. Smith	48	14	10	EC-E, EC-W, FG, CR, PA	*700*
Euglossa imperialis Cockerell	74	15	7	EC-E, EC-W, PA, FG, BO	*1850*
Euglossa intersecta Latreille	100	17	3	EC-E, FG, BR	600
Euglossa mixta Friese	34	11	7	EC-E, EC-W, PA, FG, BO	1750
Eulaema bombiformis (Packard)	445	28	3	PA, CR, FG	*1700*
Eulaema cingulata (Fabricius)	270	20	8	EC-E, EC-W, PA, FG, BO	*2600*
Eulaema meriana (Oliver)	390	26	6	EC-E, PA, FG, CR, BO	*1700*
Eulaema nigrita Lepeletier	200	18	5	PA, FG, BO	*2560*
Exaerete frontalis (Guérin)	285	25	6	EC-E, FG, PA	*1100*
Exaerete smaragdina (Guérin)	175	20	9	EC-E, MX, PA, FG, BO	*2650*
Eufriesea ornata (Mocsary)	382	24	3	PA, FG	*800*
Eufriesea pulchra (F. Smith)	172	16	5	EC-E, PA, FG	*800*

The country abbreviations are Mexico (MX), Costa Rica (CR), Panama (PA), eastern Ecuador (EC-E), western Ecuador (EC-W), French Guiana (FG) and Bolivia (Bo). The elevation limits of species in our cross-Andean analysis are in italic.

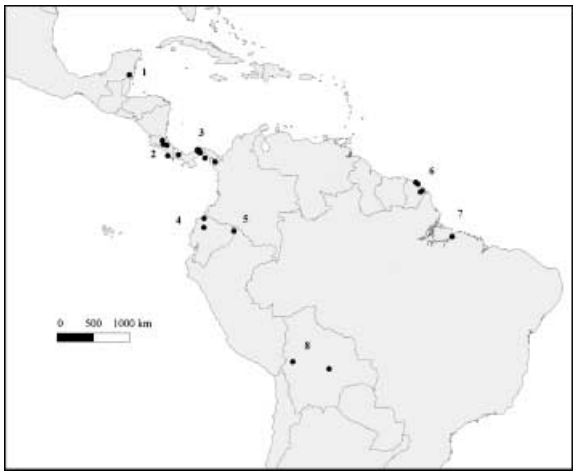

Fig. 1 Collection localities for this study, marked with black circles. Major regions or clusters marked with numbers: (1) Mexico, (2) Costa Rica, (3) Panama, (4) Coastal Ecuador, (5) Amazonian Ecuador (Yasumí), (6) French Guiana, (7) Brazil, and (8) Bolivia.

ethanol. We supplemented the field collections with DNA extracted from pinned specimens maintained at the Smithsonian Tropical Research Institute (STRI, D. W. Roubik collection). Each specimen and its corresponding DNA sample are maintained as vouchers at STRI and have been assigned a unique identification number ('STRI ID', Appendix).

DNA extraction and sequencing

For DNA extraction, tissue from a single middle leg or thorax was incubated at 54 °C for 6–14 h, followed by 10 min at 95 °C, in a solution comprised of 10× Perkin-Elmer TAQ Buffer (without $MgCl_2$) and 10 μL of 10 mg/mL Proteinase K. We amplified by polymerase chain reaction (PCR) 600 base pairs of the mtDNA CO1 gene using the primers CAACATTTATTTTGATTTTTTGG-3′ (CO1-F) and GATATTAATCCTAAAAAATGTTGAGG-3′ (CO1-R). PCR was performed in a 25-μL cocktail containing 1.0 μL DNA solution, 0.05 μL QiaTaq (Qiagen Corporation), 2.5 μL Qiagen buffer, 1.0 μL 25 mM $MgCl_2$, 1.25 μL each primer (10 μM stock) and 2.5 μL of 2 mM dNTPs. The thermal cycle consisted of four cycles with 95 °C for 30 s, 48 °C for 30 s, and 72 °C for 45 s, followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 45 s. This protocol resulted in strong amplification products used directly for sequencing, or occasionally it produced weak bands that were used as templates for re-amplification.

The PCR band was separated from low-melting-point agarose using GELase™ (Epicentre Technologies) and cycle-sequenced using D-Rhodamine chemistry [Applied Biosystems Incorporated (ABI)]. The sequencing products were purified in Sephadex columns, and electrophoresed on an ABI 377 DNA sequencer. All sequences were edited and aligned using the program SEQUENCHER 4.1 (Gene Codes Corporation) and deposited in GenBank (Appendix).

Data analysis

We performed a Bayesian phylogenetic analysis using MRBAYES version 3.0 (Huelsenbeck & Ronquist 2001) to represent relationships among the euglossine study species and provide estimates of statistical support for intraspecific clades. This approach provided a graphical framework for

phylogeographical comparisons, and was not intended to uncover sister taxa relationships. We used the CO1 sequence from *Apis mellifera* (Apidae: Apini) (Crozier & Crozier 1993) to root the tree, based on the phylogenetic analysis of Dick *et al*. (manuscript in preparation). Four Markov Chain Monte Carlo (MCMC) chains were run for 500 000 generations with a sampling frequency of one tree per 100 generations. The asymptote of likelihood values was consistently observed before 70 000 MCMC generations. We excluded these generations (representing 700 sampled trees) as 'burn in'. The 4300 remaining trees were used to generate a 50% majority rule consensus tree in which posterior probabilities for the internal nodes were indicated by their sample frequency. These are true probabilities given the assumptions of the general time reversible model (Huelsenbeck & Ronquist 2001). Thus, probabilities of 95% or greater were considered significant.

We performed maximum likelihood analysis in PAUP 4.04b8 to determine levels of DNA sequence divergence and to test for substitution rate heterogeneity (molecular clock analysis). When sequence divergences are low (e.g. < 5–8%) the maximum-likelihood-corrected genetic distance approximates the uncorrected distance. We used the program MODELTEST version 3.04 (Posada & Krandall 1998) to select the model of nucleotide substitution based on hierarchical likelihood ratio tests.

We tested for homogeneity in nucleotide substitution rates (molecular clock hypothesis) through a χ^2 test of maximum likelihood scores for trees obtained with and without the molecular clock constraint (Felsenstein 1988). A single representative of each species was included in this analysis ($N = 14$), with *Apis mellifera* as the outgroup.

For the divergence time analysis we applied CO1 substitution rates of 1.2–1.5% per million years (Myr^{-1}) calibrated for other insects. These are based on the estimates for rates of pairwise sequence divergence of 1.2–1.3% Myr^{-1} for cave-dwelling Corsica–Sardinian beetles tectonically separated from the Iberian peninsula ~29 million years ago (Ma) (Caccone & Sbordoni 2001), and 1.5% Myr for *Tetraopes* beetles whose origins coincide with the formation of the Sonoran desert (1 Ma) and aridification of the Southwest USA ~7 Ma (Farrell 2001). To estimate divergence times from genetic distances, we used the equation $T = K/R$, where T is the divergence time, K is the maximum-likelihood-corrected distance, and R is the published rate of pairwise sequence divergence.

Results

The CO1 data matrix consisted of 550 aligned nucleotides for 86 individuals, representing 14 euglossine species. The base frequencies were skewed toward thymine and adenosine (frequencies A = 0.3179, C = 0.1068, G = 0.1414, and T = 0.4339). The selected model was the general time reversible model with invariant sites (I = 0.2849) and gamma distribution of rates (G = 0.4493). The molecular clock hypothesis could not be rejected for the CO1 tree ($P = 0.21$; clock enforced maximum likelihood tree score — ln 2073.9009; unconstrained maximum likelihood tree score — ln 2064.8793; d.f. = 14), indicating that the application of a molecular clock is appropriate for this data set.

Species clades

The Bayesian analysis yielded high posterior probabilities ($P \leq 0.99$) in support of the monophyly of 12 of the 14 species (Fig. 2). This result is not suprising given the limited taxon sampling in this study but these 12 species were also comprised of monophyletic mtDNA haplotypes in a broader survey of the family (Dick *et al*. manuscript in preparation). Two species, *Euglossa mixta* and *Euglossa cognata*, formed a monophyletic species group ($P = 1.0$) but their CO1 haplotypes were reticulate (Fig. 3).

Phylogeographical structure

Phylogeographical mtDNA differentiation was weak or absent in the 12 monophyletic euglossine species (mean haplotype divergence = 0.9%, range of means 0.0–1.9%, Table 2). For four species, *Euglossa intersecta*, *Eufriesea pulchra*, *Exaerete frontalis* and *Exaerete smaragdina* (Fig. 2), identical conspecific CO1 haplotypes were observed in French Guiana and eastern Ecuador, a distance of 2500 km across the Amazon basin, and a fifth species, *Eulaema cingulata*, had identical mtDNA haplotypes in French Guiana and Bolivia (3000 km apart). West of the Andes, *Euglossa ignita* harboured identical CO1 haplotypes in western Ecuador and Panama (1250 km apart).

We also observed identical mtDNA haplotypes in conspecific cross-Andean populations of two species: *Eulaema nigrita* (Panama and French Guiana) and *Eulaema cingulata* (both slopes of the Ecuadorian Andes and Bolivia). Generally speaking, however, the Andean cordillera marked the deepest genetic break for the euglossine species examined, some of which exhibited patterns of reciprocal monophyly across this barrier (Table 2): *Eufriesea ornata* and *Eufriesea pulchra*; *Exaerete frontalis* and *Exaerete smaragdina*; and *Euglossa imperialis* and *Euglossa allosticta*. However, even the cross-Andean divergences were low (mean = 1.1%, range 0–2.0%, $N = 10$ species).

Low levels (≤ 2 base pairs) of within-site polymorphism were observed in *Eufriesea ornata* (FG), *Eulaema meriana* (FG, PA), *Eulaema cingulata* (FG), *Exaerete frontalis* (PA) and *Euglossa imperialis* (PA), representing genetic distances of 0.2–0.3%. Divergent haplotypes were observed in *Euglossa ignita* from individuals sampled within Ecuador (Y46 vs. Y47; 1.4%) and within French Guiana (F32 vs. F45; 2.7%).

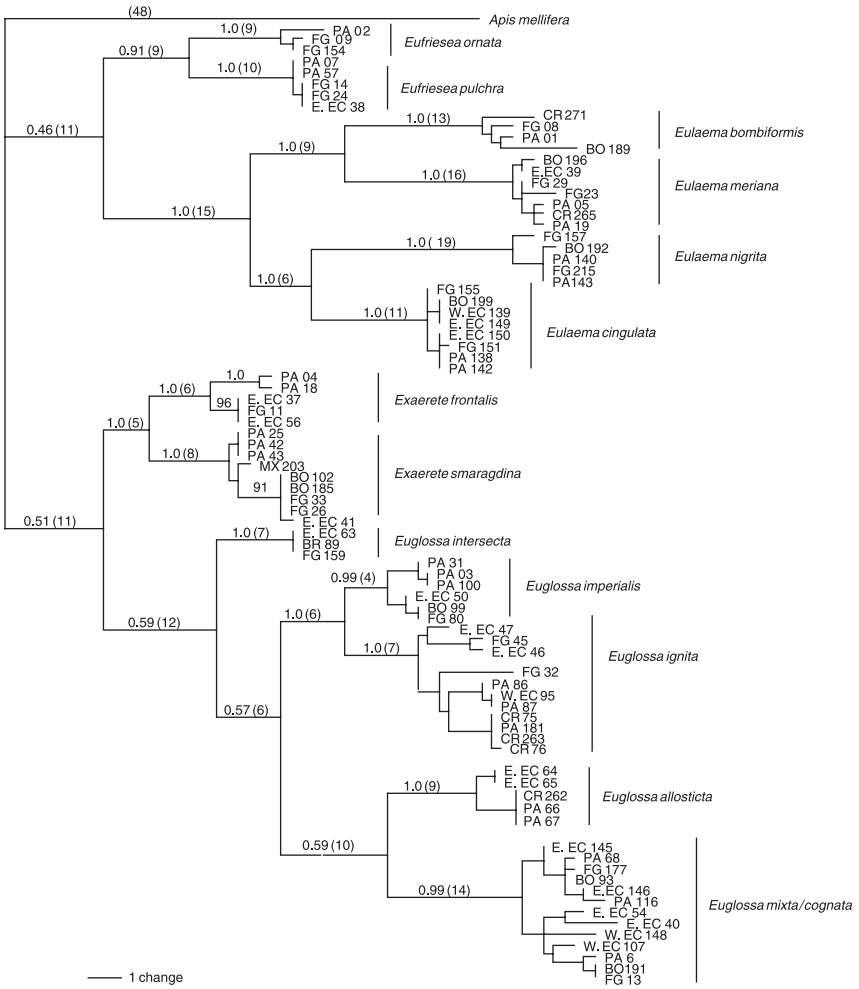

Fig. 2 Bayesian analysis of the phylogenetic relationships among euglossine species and populations. Based on partial sequences (~550) of the CO1 gene. Branch support values are Bayesian posterior probabilities. The absolute number of base changes is provided in parentheses, The taxa are represented by the collection location and STRI ID numbers (Appendix). Sites are Mexico (MX), Costa Rica (CR), Panama (PA), western Ecuador (W.EC), eastern Ecuador (E.EC), Brazil, (BR), French Guiana and Bolivia (BO).

Discussion

Despite the broad geographical coverage of our sampling of euglossine bees, the mtDNA results show the weakest phylogeographical structure yet reported for any group of widespread neotropical organisms, and demonstrate high levels of gene flow, past or present, across the neotropical lowlands for representative species in all four euglossine genera investigated. Here we discuss the interplay between euglossine population structure and neotropical landscape history. We compare our results with patterns found in other widespread neotropical species, and we discuss the potential geographical and biotic causes of diversification in this species-rich group.

Cross-Andean phylogeography

The Andean cordilleras form a major barrier between the rainforests of Middle America/Chocó (trans-Andean region) and the Amazon basin (cis-Andean). Lowland rainforests climb to approximately 1000 m in elevation, while the flanking Andean cordilleras are at least 2000 m in

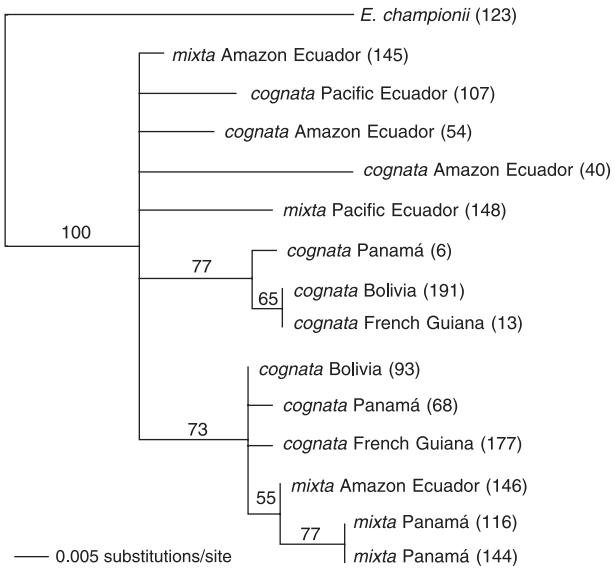

Fig. 3 Maximum likelihood CO1 phylogeny of the *Euglossa cognata*/*Euglossa mixta* species group, rooted with *Euglossa championi*. Node support values are derived from 100 maximum likelihood bootstrap replicates.

Table 2 Phylogeographical patterns in the euglossine study species excluding *Euglosssa cognata/mixta*

Study species	*N*	*K*	Mean *D* among haplotypes	Mean *D* cross-Amazon	Amazon sites	Mean *D* cross-Andes	West-of-Andes sites
Euglossa allosticta	5	2	0.012	—	EC	0.012	CR, PA, EC
Euglossa ignita	11	8	0.019	0.017	EC, FG	0.020	CR, PA, EC
Euglossa imperialis	7	4	0.010	0.0	EC, FG, BO	0.012	PA, EC
Euglossa intersecta	3	1	0.0	0.0	EC, FG, BR	—	—
Eulaema bombiformis	3	4	0.017	—	FG	0.017	PA, CR
Eulaema cingulata	8	4	0.002	0.003	EC, FG, BO	0.003	PA, EC
Eulaema meriana	7	6	0.007	0.006	EC, FG, BO	0.008	PA, CR
Eulaema nigrita	5	3	0.004	0.007	FG, BO	0.004	PA
Exaerete frontalis	6	3	0.012	0.0	EC, FG	0.017	PA
Exaerete smaragdina	9	4	0.009	0.005	EC, FG, BO	0.012	MX, PA
Eufriesea ornata	3	3	0.011	—	FG	0.011	PA
Eufriesea pulchra	5	2	0.001	0.0	EC, FG	0.002	PA
Mean	6	3.7	0.009	0.004		0.011	

The columns indicate the number of conspecific bees sequenced (*N*), total number of CO1 haplotypes (*K*), the mean of maximum likelihood corrected pairwise divergences (*D*), the mean value of cross-Amazon divergences, and the mean value of cross-Andean divergences. See Table 1 for country codes.

elevation. Nevertheless, 433 of 714 lowland tree species (c. 60%) found in central Panama also occur in the Amazon basin (Dick *et al*. in press), and 1431 species (c. 30%) of Ecuador's lowland vascular plants occur east and west of the Andes (Jørgensen & León-Yánez 1999; Raven 1999). A quarter (46 species) of all euglossine species also have cross-Andean distributions (Ramírez *et al*. 2002). Molecular studies of conspecific populations on either side of the Andean divide, including freshwater fish (Perdices *et al*. 2002), rainforest trees (Dick *et al*. 2003), *Heliconius* butterflies (Brower 1994), toads (Slade *et al*. 1998) and bats (Ditchfield 2000; Hoffmann & Baker 2003) report levels of cross-Andean genetic divergence consistent with the timing of the final, Pliocene uplift of the northern cordilleras or earlier.

The CO1 divergence among the 11 cross-Andean euglossine species was consistently low and included identical cross-Andean conspecific CO1 haplotypes in two *Eulaema* species. The divergence time estimates, ranging from 0 to 1.42 million years ago based on arthropod molecular clock calibrations, provide compelling evidence for cross-Andean dispersal well after the final phase of uplift. Genetic evidence for cross-Andean dispersal has been reported for some bats (Ditchfield 2000; Hoffmann & Baker 2003) which reach the same elevations as euglossine bees (Table 1). Our results are unusual, however, in that the entire group of euglossines investigated appears to have taken part in recent cross-Andean dispersal. This raises the question of how lowland euglossine bees have managed to cross the world's most extensive tropical mountain range.

The segments of Andean cordilleras that flank lowland rainforests generally rise high above the tree line. However, there is a narrow pass (approximately 20 km wide), 2000 m in elevation, at the portal of the Magdalena river valley in Colombia, and several other passes that are somewhat higher. Euglossine bees are rarely collected at 2000 m, even as transients (Dressler 1982), but *Eulaema cingulata* (2600 m), *Eulaema nigrita* (2600 m) and *Exaerete smaragdina* (2650 m) in our study have been collected above this elevation (Table 1). The other eight species in our cross-Andean group have been recorded at maximum elevations ranging from 700 m (*Euglossa ignita*) to 1850 m (*Euglossa imperialis*). The passes may render the Andes a filter, rather than an absolute barrier, for the movement of high-elevation euglossine species.

Alternatively, euglossine bees may circumvent the Andes by clinging along the forested, mid-elevation slopes of the northern cordilleras as they intersect with ocean or dry Caribbean lowlands. Some euglossine bees are capable of long forays into xeric habitats. Minckley & Reyes (1996) found a lone individual of *Eulaema polychroma* in the Sonora desert of Arizona 550 km from its northern range limit. The authors suggest that euglossine bees climb in elevation when passing through deserts, citing the example of *Eufriesea* aff. *caerulescens* collected in high-elevation xeric grasslands in Chihuahua, Mexico. These mid-elevation forests, now largely converted to pastures, may have provided a corridor for biotic interchange.

The dispersal abilities of some Euglossini seem to be positively correlated with body size. *Eulaema* — the only genus in our study with identical cross-Andean haplotypes — and *Exaerete* can be 10 times larger than *Euglossa* (Table 1; Roubik & Hanson 2004). *Eufriesea* is intermediate in size. The two largest genera have the broadest geographical distributions, with c. 58% (n = 11; *Eulaema*) and 67% (n = 4;

Exaerete) of the species distributions crossing the Andes, in contrast to 17% (10 species) and 22% (22 species), respectively, for *Eufriesea* and *Euglossa* (Dick *et al*. manuscript in preparation). Larger body size may provide *Eulaema* and *Exaerete* with a dispersal advantage in terms of exposure and thermal tolerance (Roubik 1993), or energy for long-distance dispersal and founder events.

Amazon basin populations

The Amazon basin and Guiana collection sites cover a distance of 2500 km between French Guiana and eastern Ecuador and 3000 km between French Guiana and Bolivia. Despite the extreme distances between our collecting sites, we found little evidence of mtDNA differentiation for any of nine euglossine species collected from opposite sides of the Amazon basin (*Euglossa cognata* and *Euglossa mixta* are not counted as they were not included in these comparisons; see Table 2). The mean divergence among the cross-Amazon samples was 0.4% (range of means, 0–1.7%), and five species harboured identical CO1 haplotypes across the sampling area. The lack of mtDNA phylogeographical structure in orchid bees collected on opposite sides of the Amazon basin contrasts with studies of echymid and murid rodents and marsupials sampled at a smaller scale along a 1000-km transect along the Rio Juruá in the western Amazon, in which medium to strong phylogeographical structure [4.0–19% mtDNA cytochrome *b* (cyt *b*) divergence] was reported for 19 of the 29 species (Patton & da Silva 2004). The euglossine bees not only showed no mtDNA differentiation across the Amazon basin, but also very few nucleotide differences within populations sampled across this region in all cases except *Euglossa ignita*. Mean haplotype divergence within *Euglossa ignita* collected from either side of the basin was 1.7%, with a range of 0.4–2.7%, reminiscent of the high levels of haplotype diversity and pairwise sequence divergence of mtDNA cyt *b* ranging from 1.0 to 3.3% in widespread bats from the Amazon basin (Ditchfield 2000). The absence of geographical structure in Amazon euglossine bees suggests high levels of long-distance gene flow, and is in accordance with field observations of long-distance flights by the larger species (Janzen 1981).

Some differences in the degree of divergences found in euglossine bees vs. mammals may derive from differences in the mtDNA substitution rates. The mtDNA cyt *b* gene is the marker of choice for comparative phylogeography in vertebrates (Ditchfield 2000), while CO1 is the most commonly sequenced mtDNA gene for arthropods (Simon *et al*. 1994). The mtDNA of arthropods differs from vertebrate mtDNA in its high content of A and T nucleotides (Crozier & Crozier 1993; Simon *et al*. 1994), which made up 75% of the bases in the euglossine mtDNA. Most third-position substitutions are A and T transversions, which occur less frequently than transitions. Thus, base composition may constrain the rate of nucleotide substitution, and partly explain the low mtDNA divergences within euglossine species. However, the slower substitution rates for arthropod CO1 (1.2–1.5% Myr^{-1}) compared to vertebrate cyt *b* (~2% Myr^{-1}), do not adequately explain levels of euglossine CO1 divergence that are several times lower than the levels found in cyt *b*.

Phylogeographic study of other corbiculate bees also suggests that slow substitution rates alone are not sufficient to explain the euglossine mtDNA results. The mtDNA of major geographic races of *Apis mellifera* differs by ≥ 2.0% (Smith 1991). *Apis koschevnikovi* is deeply geographically structured in Borneo, with intraspecific CO1 divergence ≤ 8% (Tanaka *et al*. 2001a), and *Apis cerana* from the same locations shows CO1 divergence ≤ 5.2% (Tanaka *et al*. 2001b). However, weak phylogeographic structure (and low mtDNA diversity) have been reported for *Apis dorsata* in Borneo (Tanaka *et al*. 2001b) and for *Bombus terrestris* (Estoup *et al*. 1996) across Europe. The weak phylogeographic pattern in *Bombus terrestris* is probably the result of its recent population expansion across the post-glaciated landscape of continental Europe.

The absence of geographical structure in Amazon euglossines may also derive from Quaternary population expansion. However, unlike Europe (Taberlet *et al*. 1998; Hewitt 1999), there is no convincing evidence for a major change in forest cover in the Amazon during the Pleistocene glacial periods (Colinvaux *et al*. 2000; Moritz *et al*. 2000), which might explain recent population expansion in the euglossine taxa. Recent population expansion may result from recent speciation. Limited support for this hypothesis comes from a molecular systematic analysis of the Euglossini using CO1 (and the nuclear gene EF-1α; Dick *et al*. manuscript in preparation) demonstrating mtDNA divergences < 1% for seven species pairs, including *Euglossa mixta/cognata*, and *Euglossa ignita/orellana*. The model of population expansion across the neotropics following recent speciation merits further investigation using fast-evolving population genetic markers such as microsatellites.

Ancestral polymorphism

The lack of resolution of *Euglossa cognata* and *Euglossa mixta* haplotypes into monophyletic species lineages (Fig. 3) may be explained by hybridization, or by incomplete sorting of ancestral alleles. *Euglossa mixta* and *Euglossa cognata* are broadly codistributed throughout the neotropics, and morphologically distinguished by size (Table 1) and by the shape of the mid-tibial brush used for fragrance manipulation by the males. The strongest evidence for hybridization — which we have not found — would be shared and geographically codistributed haplotypes. We believe that the reticulate gene phylogeny is more simply explained by the persistence of ancestral alleles, a pattern that occurs

when the derived and progenitor species have large effective population sizes (Moore 1997). *Euglossa cognata* and *Euglossa mixta* are two of the most abundant and wide-ranging euglossine species (Table 1), and at least the contemporary populations probably do have large effective populations. The genetic distances among the *cognata* + *mixta* haplotypes are high (≤ 2.2%) compared to the other taxa reported here, and the relatively long persistence of mtDNA haplotypes suggests the moderate age of these species in addition to stable and large effective female population size through the Pleistocene.

Euglossa ignita contained similarly divergent and geographically unstructured CO1 haplotypes. In a larger phylogenetic analysis (Dick *et al*. manuscript in preparation), three intraspecific *Euglossa ignita* clades formed an unresolved polytomy with *Euglossa flammea*, *Euglossa orellana* and *Euglossa chalybeata*, which suggests recent speciation and the retention of ancestral alleles in *Euglossa ignita*.

Causes of diversification

Under Hubbell's neutral theory (Hubbell 2001), the rate of expansion of a founder population is analogous to the rate of spread of a neutral allele; a population increase of *n* individuals requires approximately *n* generations (Kimura & Ohta 1973). Thus, in the absence of competitive superiority, widespread species in species-rich habitats should be relatively old. The results presented here, together with the sister taxa analysis of Dick *et al*. (manuscript in preparation), suggest that the widespread euglossine species are recent in origin and have experienced rapid population expansion relative to the rate of mtDNA substitution. The euglossine species are young compared to geographically restricted taxa such as neotropical dirt frogs (Crawford 2003), and widespread trees such as *Symphonia globulifera*, whose conspecific populations diverged in the Tertiary (Dick *et al*. 2003). In any event, the broad geographic distribution of young lineages of euglossine bees is at odds with Hubbell's neutral theory of biogeography and biodiversity. It may reflect competitive superiority over displaced species, or it may indicate that lowland forests are not ecologically saturated.

The recent origins and high genetic connectivity of the study populations — across geographical barriers and large distances — would also seem to discount the importance of vicariant speciation in the group. This view may result in part from our focus on species with broad geographical distributions. For example, 15 named, and thus putatively monophyletic, species groups amongst the euglossine genera have species found on both sides of the Andes, with geographical distributions that are either entirely *cis*- or *trans*-Andean. In the absence of phylogenetic analysis it is not possible to infer with certainty the sister group relationship of species on either side of the Andes, but the overall pattern suggests that the mountain chain may have played an important role in the diversification of euglossine bees.

Refuges have played an important intellectual role in models of Amazonian speciation, although strong empirical support for Pleistocene refuges has not been forthcoming (Moritz *et al*. 2000; Bermingham & Dick 2001). The occurrence of widespread and undifferentiated euglossine bees across the Amazon basin provides another line of evidence against the importance of Pleistocene refuges. Again, however, access to the broader database of euglossine bees suggests that speciation in Amazonia is complex. The *Euglossa 'analis'* group, containing widespread species *mixta* and *cognata*, and the *'piliventris'* group in the subgenus *Glossura*, with widespread *imperialis* and *ignita*, each have one Mesoamerican and four Amazonian endemics (Nemésio 2004). In both groups, most species are sympatric in lowland Amazonia. Although sympatric *Glossura* have notably different tongue lengths (Roubik 2004), no differences in orchid or floral resource use have been documented, and ecological differentiation has not been investigated. Thus, the possibility of initial divergence in Pleistocene refugia cannot be discounted.

Heliconius butterflies provide insights into biotic factors that may have led to speciation in the Euglossini. Like the widespread euglossines, *Heliconius* speciation is recent, and widespread Amazon populations exhibit little mtDNA geographical structure (Brower 1996; Flanagan *et al*. 2004). Nevertheless, geographical populations have differentiated morphologically through Müllerian mimicry. The unrelated and unpalatable species *H. erato* and *H. melpomene* covary geographically in aposematic coloration, leading to reproductive isolation of geographical races through assortative mating (Jiggins *et al*. 2001). Müllerian mimicry has been documented in bumblebee-like *Eulaema bombiformis*, *Eulaema meriana*, *Eulaema seabrai* and *Eufriesea ornata*, whose coloration signals the jolting sting of the female (Dressler 1979). The colour patterns also covary geographically, but no studies have investigated reproductive isolation among the geographical races. Roubik & Hanson (2004) suggest that mimicry is widespread in the Euglossini, and may account for morphological similarities of 14 euglossine groups in Panama alone. This hypothesis needs to be assessed with species-level phylogeny, however, to determine if putative mimics are morphologically similar owing to common ancestry.

Speciation could involve other aspects of euglossine biology, such as divergence in habitat choice, or in the male display and mating odours associated with floral fragrance hosts (Williams 1982). Unfortunately, little is known about the ecology of euglossine bees. In fact, the courtship function of the fragrance 'bouquet' has never been demonstrated (Cameron 2004). Even less is known of the biology of elusive female euglossines, which are not drawn to chemical

baits. Advances in phylogeny, comparative phylogeography, and population genetics of euglossines will help to uncover the role of geography in the genesis of population structure and speciation, while providing insights into processes that affect many other neotropical organisms. However, more basic field research is needed, and it will need to be guided by an evolutionary focus, if we hope to understand the biotic factors that have led to speciation in this diverse group of pollinators.

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Appendix

List of euglossine specimens used in this study. The STRI ID number applies to the specimen and DNA voucher (see Materials and methods). Abbreviations are as follows: Ecuador (EC), French Guiana (FG), Panama (PA), Brazil (BR), Mexico (MX), Bolivia (BO), and Costa Rica (CR); and Barro Colorado Island (BCI).

Species	Location	STRI ID no.	GenBank accession no.	Species	Location	STRI ID no.	GenBank accession no.
Eulaema cingulata	Santa Rita, PA	138	AY506449	*Euglossa ignita*	Cana, PA	87	AY506382
Eulaema cingulata	Santo Domingo, EC	139	AY506445	*Euglossa ignita*	AltoTambo, EC	95	AY506381
Eulaema cingulata	BCI, PA	142	AY506451	*Euglossa ignita*	Rio Caimito, PA	181	AY506380
Eulaema cingulata	Yasuní, EC	149	AY506447	*Euglossa ignita*	La Virgen, CR	263	AY506377
Eulaema cingulata	Santo Domingo, EC	150	AY506448	*Euglossa imperialis*	Metropolitan Park, PA	31	AY506389
Eulaema cingulata	Kourou, FG	151	AY506450	*Euglossa imperialis*	Yasuní, EC	50	AY506392
Eulaema cingulata	Kourou, FG	155	AY506446	*Euglossa imperialis*	Kourou, FG	80	AY506391
Eulaema cingulata	Ixiamas, BO	199	AY506444	*Euglossa imperialis*	Rurenabaque, BO	99	AY506390
Eulaema nigrita	BCI, PA	140	AY506454	*Euglossa imperialis*	Metropolitan Park, PA	3	AY506387
Eulaema nigrita	BCI, PA	143	AY506452	*Euglossa imperialis*	BCI, PA	100	AY506388
Eulaema nigrita	Kourou, FG	157	AY506456	*Euglossa imperialis*	AltoTambo, EC	94	AY506386
Eulaema nigrita	Ixiamas, BO	192	AY506455	*Euglossa allosticta*	Yasuní, EC	64	AY506406
Eulaema nigrita	Kourou, FG	215	AY506453	*Euglossa allosticta*	Yasuní, EC	65	AY506407
Eulaema bombiformis	Soberania NatPk, PA	1	AY506467	*Euglossa allosticta*	Santa Rita, PA	66	AY506404
Eulaema bombiformis	Montagne Tortue, FG	8	AY506466	*Euglossa allosticta*	Santa Rita, PA	67	AY506405
Eulaema bombiformis	La Virgen, CR	271	AY506469	*Euglossa allosticta*	La Virgen, CR	262	AY506393
Eulaema meriana	La Virgen, CR	265	AY506459	*Euglossa intersecta*	Yasuní, EC	63	AY506399
Eulaema meriana	Pipeline Road, PA	5	AY506461	*Euglossa intersecta*	Belem, BR	89	AY506400
Eulaema meriana	Yasuní, EC	39	AY506464	*Euglossa intersecta*	Kourou, FG	159	AY506401
Eulaema meriana	Ixiamas, BO	196	AY506463	*Eufriesea ornata*	Cerro Campana, PA	2	AY506365
Eulaema meriana	Pipeline Road, PA	19	AY506462	*Eufriesea ornata*	Montagne Tortue, FG	9	AY506363
Eulaema meriana	Kourou, FG	23	AY506465	*Eufriesea ornata*	Kourou, FG	154	AY506364
Euglossa cognata	Yasuní, EC	40	AY506418	*Eufriesea pulchra*	Yasuní, EC	38	AY506362
Euglossa cognata	Napo, EC	54	AY506417	*Eufriesea pulchra*	Soberania NatPk, PA	57	AY506360
Euglossa cognata	Soberania Park, PA	68	AY506420	*Eufriesea pulchra*	Soberania NatPk, PA	7	AY506361
Euglossa cognata	Rurenabaque, BO	93	AY506419	*Eufriesea pulchra*	Kourou, FG	14	AY506358
Euglossa cognata	Alto Tambo, EC	107	AY506416	*Eufriesea pulchra*	Sinnamary, FG	24	AY506359
Euglossa cognata	Montagne Tortue, FG	177	AY506421	*Exaerete frontalis*	BCI, PA	4	AY506482
Euglossa cognata	BCI, PA	6	AY506415	*Exaerete frontalis*	Montagne Tortue, FG	11	AY506479
Euglossa cognata	Kourou, FG	13	AY506414	*Exaerete frontalis*	Pipeline Road, PA	18	AY506483
Euglossa mixta	BCI, PA	116	AY506425	*Exaerete frontalis*	Yasuní, EC	37	AY506480
Euglossa mixta	BCI, PA	144	AY506426	*Exaerete frontalis*	Yasuní, EC	56	AY506481
Euglossa mixta	Yasuní, EC	145	AY506423	*Exaerete frontalis*	Montagne Tortue, FG	29	AY506460
Euglossa mixta	Yasuní, EC	146	AY506424	*Exaerete smaragdina*	Las Perlas, PA	25	AY506474
Euglossa mixta	Santo Domingo, EC	148	AY506422	*Exaerete smaragdina*	Montagne Tortue, FG	26	AY506472
Euglossa mixta	Ixiamas, BO	191	AY506413	*Exaerete smaragdina*	Montagne Tortue, FG	33	AY506473
Euglossa ignita	French Guiana	45	AY506383	*Exaerete smaragdina*	Yasuní, EC	41	AY506478
Euglossa ignita	Yasuní, EC	46	AY506384	*Exaerete smaragdina*	Las Perlas, PA	42	AY506475
Euglossa ignita	Yasuní, EC	47	AY506376	*Exaerete smaragdina*	Las Perlas, PA	43	AY506477
Euglossa ignita	Rio San Juan, CR	75	AY506378	*Exaerete smaragdina*	Buena Vista, BO	102	AY506470
Euglossa ignita	Rio San Juan, CR	76	AY506379	*Exaerete smaragdina*	Ixiamas, BO	185	AY506471
Euglossa ignita	Cana, PA	86	AY506385	*Exaerete smaragdina*	Chetumal, MX	203	AY506476