Supporting Information

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SI Materials and Methods

Phylogenetic Reconstruction. Species sampled for phylogenetic analyses included representative Darwin's finches and all 13 of the non-Darwin's finches belonging to Tholospiza. We included between one and four individuals of each species and used sequences from two mitochondrial and four nuclear markers. We included mitochondrial cytochrome *b* (cyt *b*) sequences for all species, and mitochondrial nicotinamide dehydrogenase subunit 2 (ND 2) sequences for all but four species. For an additional seven species (*Coereba flaveola*, *Euneornis campestris*, *Loxigilla noctis*, *Loxipasser anoxanthus*, *Melopyrrha nigra*, *Tiaris bicolor*, and *Tiaris olivacea*), we included sequences from three autosomal nuclear markers: β-fibrinogen intron 5 (FGB-I5), myoglobin intron 2 (MB-I2), and recombination-activating gene (RAG-1); and a sequence from a marker linked to the avian Z sex chromosome: aconitase 1 intron 10 (ACO1-I10). GenBank accession numbers for the sequences reported here are HQ153049–HQ153089 (new to this study) and AF108772, AF108777, AF108791, AF108792, AF108796, AF108802, AF108806-AF108808, AF281024, AF290115, AF310041-AF310043, AF382993, AF383109, AF447282, AF-447310, AF489885-AF489901, AY005219, EF567837, EU648036, EU648044–EU648046, and EU648107 (1–10). Locality and voucher information are reported in the GenBank records. For *L. noctis*, samples from Barbados, Dominica, and St. Lucia were included, because the subspecies of *L. noctis* that occurs on Barbados was recently split into a separate species (11). Sequences of the individuals from different islands were ~1% different from each other and formed a monophyletic clade; they are treated as a single branch labeled *L. noctis* on Fig. 1*A*. Methods of DNA isolation, amplification, and sequencing followed standard protocols (12). Phylogenetic analyses were performed using maximum-likelihood (ML) and Bayesian inference methods. The ML and Bayesian analysis produced largely concordant topologies, and neither analysis produced a strongly supported node that conflicted with a strongly supported node in the other analysis (ML tree is shown in Fig. 1*A*).

Phylogenetic analyses were conducted on the ABE TeraGrid, accessed via the CIPRES portal v2.2 (13). ML was implemented using RAxML v7.2.6 (14, 15), which applies the GTR + Γ model to each partition. Analyses were conducted using the concatenated data with 12 partitions: each protein-coding gene (cyt *b*, ND 2, and RAG-1) was partitioned by codon position, and the three introns (ACO1-I10, FGB-I5, and MB-I2) were each placed into a separate partition. The most likely tree was computed, and 1,000 bootstrap replicates were performed to assess support for each node. Bootstrap values greater than 70% were considered strongly supported. Bayesian analyses were implemented using MrBayes v3.1.2 (16, 17) with the 12 partitions described above, and the GTR + I + Γ model applied to each partition. The analysis was run twice independently, with each run for 5 million generations, and sampled every 500 generations. The log-likelihood values from each run were plotted against the number of generations using Tracer v1.4 (18) to determine the point at which stationarity was reached. Both runs reached stationarity well before 100,000 generations, and we thus chose a burn-in of 500,000 generations for each. Results of each analysis were compared, and the same topology and similar posterior probabilities were recovered. Thus, all of the post–burn-in trees were used to construct a consensus tree. Posterior probabilities of 0.95 and higher were considered strongly supported. We reconstructed the history of beak shape evolution on the ML and Bayesian trees using Mesquite v2.73 (19). Reconstructions were performed using both ML (under the Markov k-state 1 parameter model) and parsimony. All of these reconstructions showed that the best explanation for the pattern seen in *Loxigilla* beak shape (group D) is that it evolved convergently in the two *Loxigilla* lineages (ML reconstruction on ML tree is shown in Fig. S2).

Because we were particularly interested in the lack of monophyly of species in the genus *Loxigilla*, we conducted additional Bayesian analyses in which we enforced the monophyly of *Loxigilla*. These analyses were conducted under the same conditions as above, with the exception that we constrained *Loxigilla* to be monophyletic. Results of these constrained analyses were compared with the unconstrained analyses and evaluated using the Bayes factor test of incongruence (20). Lack of monophyly for *Loxigilla* was strongly supported by three lines of evidence. First, our trees have high posterior probabilities and bootstrap support values for nodes that would preclude *Loxigilla* monophyly. Second, *Loxigilla* was not monophyletic in any of the trees in our 95% credible set of trees in the Bayesian analysis. Last, using the Bayes Factor criterion (20), trees in which *Loxigilla* is monophyletic are a worse explanation of the data than those we recovered in our unconstrained searches. Twice the difference in harmonic means between our unconstrained searches and those constrained to have *Loxigilla* monophyly yielded a value of 93.2, well above the threshold of 20 (20) required for strong evidence against a hypothesis of *Loxigilla* monophyly.

The following institutions provided tissues for phylogeny reconstruction: American Museum of Natural History, Field Museum of Natural History, Louisiana State University Museum of Natural Science, the Museum of Vertebrate Zoology at the University of California (Berkeley), and the Smithsonian Tropical Research Institute.

Beak Shape Analysis. The birds used for this analysis were obtained from the Museum of Comparative Zoology at Harvard University. Lateral pictures of each species were taken using a Nikon D90 camera, and the outline of the beak was determined with a feature detection program (SteerableJ in ImageJ). Three specimens were used per species. To determine whether two given (upper) beak shapes, $y_1(x)$ and $y_2(x)$, were related by a scaling transformation, we used the same methods described previously in Campàs et al. (21). Let $T_{s_l,s_d}[y_2(x)]$ denote the transformed shape (in which the length and the depth are scaled by s_l and s_d respectively: s_l and s_d being thus the scaling factors in the length and depth directions, respectively), and then consider the differences $E_s(s_l,s_d) \equiv \| y_1(x) - T_{s_l,s_d}[y_2(x)] \|$ and $E_d(s_l,s_d) \equiv \| y_1'(x) - T_{s_l,s_d}[y_2(x)]' \|$, where $y'(x)$ corresponds to the derivative of the shape along the length axis x, and $\| \cdot \|$ denotes a distance metric. Thus, E_s and E_d measure, respectively, how different the shapes and their derivatives are as a function of the scaling factors s_l and s_d. We then asked whether there exist values $s\star_l$ and $s\star_d$ for which both measures E_s and E_d have a global minimum. The distance metric used to measure the differences between shapes is defined by $\| z_1(x) - z_2(x) \| \equiv \frac{\int_0^{x_m} dx (z_1(x) - T_{s_l,s_d}[z_2(x)])^2}{\int_0^{x_m} dx (z_1(x) + T_{s_l,s_d}[z_2(x)])^2}$, where $z_1(x)$ and $z_2(x)$ are real functions. See Campàs et al. (21) for more details.

In Situ Hybridization and Immunohistochemistry. In situ hybridizations, antibody stains, alkaline phosphatase assays, and quantification of gene expression were performed as described previously (22). For immunostaining, we used anti-*TGFβIIr* (sc-400; Santa Cruz) and anti–*β-catenin* (610153; BD Transduction Laboratories) antibodies using methods described previously (22). Both of these antibodies have been previously used in

Darwin's finches (22) and are fully expected to cross-react with *Loxigilla* because they have been shown to cross-react across many different taxa (*β-catenin*: chicken, dog, mouse, and rat [BD Transduction Laboratories]; *TGFβIIr*: avians, dog, mouse, rat, pig, and sheep [Santa Cruz Biotechnology]). In situ hybridizations were carried using chicken mRNA probes as described previously (22–24). To confirm that absence of gene expression in the beak of any given species is not due to tissue degradation or to probe binding/specificity issues, we sequenced cDNA for regions corresponding to the probe-binding domains of *Bmp4*, *CaM*, *Dkk3*, and *Ihh*, and found high values of sequence conservation between *Loxigilla* (which did not vary between species) and chicken (*Bmp4*: 95%; *CaM*: 93%; *Dkk3*: 92%; *Ihh*: 95%). Thus, as shown previously with Darwin's finches (22–24), chicken probes are adequate for assessing gene expression in *Loxigilla* species. In addition, in our methodology, if a gene is not expressed in the beak region of a species, we check for its expression in other tissues within the same embryo (e.g., brain, cranial base, and the back of the head; Fig. S9).

Quantitative PCR. Quantitative PCR (qPCR) in chicken embryos was performed using methods and primers described previously (22). For qPCR expression assays in *Loxigilla*, we used the frontal nasal primordia from *L. noctis* and *Loxigilla violacea* embryos corresponding to the Hamburger–Hamilton stage 26 ($n = 3$ individuals per species). RNA was treated with TURBO DNase (Applied Biosystems); cDNA was generated using the high-capacity RNA-to-cDNA Kit (Applied Biosystems); and qPCR was performed using the SYBR green protocol (Kapa Biosystems). Forty cycles of amplification were used, and data acquisition was carried out with an Eppendorf Mastercycler. We assayed expression of *Bmp4*, *CaM*, and *Col2a1* using primers designed against regions conserved between *Loxigilla* species (see below). Because our embryo collection quotas are very strict, we did not have embryos from later stages to assess genes expressed at stage 30 (i.e., *TGFβIIr*, *β-catenin*, *Dkk3*, and *Ihh*) through qPCR. In addition, due to insufficient samples, we were not able to include *Loxigilla portoricensis* in the analysis; however, we believe this is not critical because *L. noctis* and *L. violacea* are representative of the two different developmental programs reported in the article. Gene expression was assayed in triplicate for each sample and normalized for GAPDH. Data from all qPCR experiments were analyzed using the comparative threshold cycle (CT) method (25), and statistical significance of expression differences was established using a standard two-tailed Student *t* test. Primers used for qPCR in *Loxigilla* include *Bmp4* [*Bmp4*-F1 (5′- GATTCCTGGTAACCGAATGCT-3′), *Bmp4*-R1 (5′- CCACCTTGTCATACTCATCCA-'3′)]; calmodulin [*CaM*-F1 (5′- GAGGCAAATCGTGCCATAAGCAGA-3′), *CaM*-R1 (5′- GACGATTGACAGTCAACAATATGA-3′)]; and collagen 2a1 [*Col2a1*-F1 (5′-AAAGGACAGACGGGCGAACC-3′), *Col2a1*-R1 (5′- GCTCTCCGGGACGGCCAGGGT-3′)].

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Fig. S1. Shape comparisons between the different morphological groups identified in Tholospiza. Visualization of morphological group shapes in comparison with group A. For each group a representative beak profile is uniformly rescaled so that both landmarks (beak tip and end of upper beak profile) match exactly with a representative profile for group A. This figure is meant to illustrate the degree to which the shapes of morphological groups differ from one another.

Fig. S2. Ancestral character reconstruction in Tholospiza. ML phylogeny of Tholospiza with ML reconstruction of beak shape evolution, using the Mk1 model incorporating an equal rate of change between any particular beak shape. Colored areas of pie charts indicate relative likelihood of support for particular beak shapes for each ancestral node. Note the relatively low level of support for group D at ancestral nodes connecting *L. noctis* with the clade containing *L. violacea* and *L. portoricensis*, indicating convergence in beak shape evolution.

Fig. S3. qPCR assays of *Col2a1* (a cartilage marker), *Bmp4*, and *CaM* in the developing beaks of stage 26 *L. noctis* and *L. violacea* embryos. Expression levels of *L. noctis* are shown relative to those of *L. violacea*. Similar to what was found using in situ hybridization, *L. noctis* has high levels of *Col2a1* expression, in agreement with the large prenasal cartilage of this species. Similarly, *Bmp4* and *CaM* are expressed at higher levels in *L. noctis* than in *L. violacea*, because these two molecules have been shown to cause the expansion of the prenasal cartilage in chicken embryos at this stage. Two-tailed t tests were performed for comparisons between *L. violacea* ($n = 3$) and *L. noctis* ($n = 3$). Asterisks denote significance at $P < 0.05$: $P_{Col2a1} = 0.021$, $P_{Bmp4} = 0.038$, and $P_{CaM} = 0.019$. Bars represent SE measurements.

Fig. S4. Quantification of gene expression area in the developing beaks of *Loxigilla* species. The percentage of the beak where the different genes analyzed were expressed was calculated using methodologies described previously (21). We quantified expression of (*A*) *Bmp4* and *CaM* (stage 27); (*B*) *TGFβIIr*, *β-catenin*, and *Dkk3* (stage 30); and (*C*) *Bmp4* and *Ihh* (stage 30). Plotted values represent averages (and SE measurements) from three individuals per species.

Fig. S5. (*A–D*) Positive controls for the genes examined in this study. We did not detect expression of *CaM* in the developing beaks of *L. violacea* and *L. portoricensis*. Similarly, *TGFβIIr*, *β-catenin*, and *Dkk3* were expressed at very low levels in the beaks of *L. violacea*, *TGFβIIr* was not expressed in the beaks of *L. portoricensis*, and *Ihh* was not expressed in the beaks of *L. noctis*. However, species in which genes were not detected in the beak region showed expression in other craniofacial regions (within the same embryo), as indicated with arrowheads. These results indicate that absence of gene expression in the developing beak cannot be explained by species-specific differences in probe/antibody reactivity, by differences in probe/antibody specificity across species, or by tissue degradation of the samples. Data for *Bmp4* are not shown because this gene was not expressed in other craniofacial regions. However, our qPCR assays confirm that *Bmp4* is expressed at very low, almost undetectable, levels in *L. violacea* relative to *L. noctis* (Fig. S3), similar to what is seen in the in situ hybridization data. For *CaM*, *Dkk3*, and *Ihh*, whole-head images are shown, and for *TGFβIIr* and *β-catenin*, close-up images of the anterior part of the face are shown to highlight fluorescence signal. See refs. 1 and 2 for more details on the use of positive controls in our studies.

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Fig. S6. The prenasal cartilage (pnc) throughout beak development. At stage 27, the pnc, labeled with *Col2a1*, represents a very small portion of the developing beak of *L. violacea* and *L. portoricensis*. By stage 30, the pnc ceases its expansion and the premaxillary bone has already formed from a separate condensation. By late developmental stages (stage 35; embryonic day 9), the pnc remains a thin rod (data shown for *L. violacea*), ruling out the possibility that this tissue can pattern the beak later in development (i.e., that this species has a heterochronic shift in cartilage expansion). nc, nasal cartilage. Arrowheads indicate the location of the developing pnc. (Scale bars: stage 27 embryo, 0.1 mm; stage 30 embryo, 0.2 mm; and stage 35 embryo, 0.4 mm.)

Fig. S7. *β-catenin* expression in the developing premaxillary bone (pmx) of stage 30 *L. portoricensis* and *Geospiza fortis* embryos. (*A*) Confocal microscopy image shows that in the developing beak of *L. portoricensis*, *β-catenin* (green) is localized in the cytoplasm and not in the nucleus (blue; DAPI staining), suggesting that this molecule is not involved in generating the pmx tissue in this species. (*B*) In contrast, *β-catenin* plays an active role in promoting osteogenesis of the pmx in *G. fortis*, because this molecule is primarily localized in the nucleus. Magnification for both panels is the same. pnc, prenasal cartilage.

Fig. S8. Functional experiments in chicken. (*A*) Comparison of wild-type embryos and embryos injected with RCAS::*Bmp4*, RCAS::*Ihh*, RCAS::*Bmp4* + *Ihh*, RCAS::*Hip1*, and RCAS::*Noggin*. Dorsal views of embryos stained for alizarin red (bone) and alcian blue (cartilage). In addition to the *Osteopontin* probe (Fig. 4), we used *PTHrP-Rec* on sagittal sections of stage 39 (embryonic day 13) chicken embryos to reveal early osteoblasts. Arrowheads indicate the location of the pmx (premaxillary bone) and the signal from the mRNA probe used. (*B*) Functional experiments in the frontal bone confirming that the effect of RCAS::*Bmp4* + *Ihh* infection is due to an expansion of dermal bone tissue. Dorsal head views of stage 41 (embryonic day 15) embryos stained for alizarin red (bone) and alcian blue (cartilage). The left side of the head is infected with RCAS::*Ihh* + RCAS::*Bmp4*, and the right side is uninfected (control). We used *RSCH* and *bsp II* probes on sagittal sections of stage 39 (embryonic day 13) chicken embryos to reveal RCAS infection (*RSCH*) and osteoblasts (*bsp II)*.

Fig. S9. In chicken and in *Loxigilla* embryos, *Ihh* and *Bmp4* cause increases in the depth and width of the beak, but do not cause changes in the length. (*A*) Histogram showing beak variation in stage 39 (embryonic day 13) wild-type and chicken embryos infected with RCAS::*Ihh* + RCAS::*Bmp4*. Infected embryos showed a significant change in their depth and their width relative to wild-type controls, whereas the length remained unchanged. Two-tailed *t* tests were performed [RCAS::*Ihh* + RCAS::*Bmp4*: $n = 9$; $\mu_{depth} = 107.13 \pm 16.78$ (± SD); $\mu_{length} = 72.03 \pm 9.54$; $\mu_{width} = 58.2 \pm 10.3$; wild type: $n = 10$; $\mu_{depth} = 66.27 \pm 5.72$; $\mu_{length} = 66.59 \pm 5.11$; $\mu_{width} = 34.03 \pm 3.68$; $P_{depth} = 4.05 \times 10^{-5}$; $P_{length} = 0.1905$; $P_{width} = 5.58 \times 10^{-5}$; bars represent SE measurements; double asterisks indicate significance at the 0.01 level; ns, not significant]. (*B*) Adult beaks of *L. portoricensis* and of *L. violacea* are proportionally deeper and wider than the beaks of *L. noctis*, but they are not longer. Because *Bmp4* and *Ihh* produce changes in depth and width, they are involved in the scaling changes seen in *Loxigilla* species. Beak measurements in *B* are taken from museum specimens ($n = 5$ per species) and corrected for body weight following procedures established previously (1).

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