The tempo of trait divergence in geographic isolation: Avian speciation across the Marañon Valley of Peru

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Geographic isolation is considered essential to most speciation events, but our understanding of what controls the pace and degree of phenotypic divergence among allopatric populations remains poor. Why do some taxa exhibit phenotypic differentiation across barriers to dispersal, whereas others do not? To test factors controlling phenotypic divergence in allopatry, we employed a comparative phylogeographic approach consisting of replicates of ecologically similar Andean bird species isolated across a major biogeographic barrier, the Marañon Valley of Peru. Our study design leverages variation among codistributed taxa in their degree of plumage, morphometric, and vocal differentiation across the Marañon to examine the tempo of phenotypic evolution. We found that substantial plumage differences between populations required roughly two million years to evolve. In contrast, morphometric trait evolution showed greater idiosyncrasy and stasis. Our results demonstrate that despite a large degree of idiosyncrasy in the relationship between genetic and phenotypic divergence across taxa and environments, comparative studies within regions may reveal predictability in the pace of phenotypic divergence. Our results also suggest that social selection is important for driving differentiation of populations found in similar environments.

KEY WORDS: Allopatric speciation, Andes, mutation-order speciation, niche conservatism, plumage evolution, trait evolution.

"[T]here are still many unanswered questions about geographic speciation, and the answers may differ among taxa and among regions . . . Does geographic isolation for, say, 10,000 years usually lead to geographic variation and speciation, or is that the exception?" (Mayr and Diamond 2001).

The differentiation of populations in geographic isolation is widely recognized as a common pathway to speciation and a driving force in the generation of biodiversity (Coyne and Orr 2004). The signature of this process is written across the landscape as one of the most visible biogeographic patterns in nature: morphological differences among allopatric sister populations isolated by geographic barriers (Wallace 1852; Jordan 1908; Mayr 1942). Conceptually, the phenotypic divergence of allopatric populations is intuitive. When a barrier interrupts the gene flow between populations, they will differentiate given sufficient time or selection

pressures (Coyne and Orr 2004; Nosil 2008a; Price 2008). Yet among taxa, many geographically disjunct populations are not visibly differentiated in morphology (Mayr 1963; Endler 1977; Templeton 1980b; Allmon 1992; Mayr and Diamond 2001), and it remains difficult to explain variation in the evolutionary outcomes of allopatry (Wiens 2004b; Nosil 2008a). Why do some taxa exhibit profound geographic variation in phenotype whereas others are similar or uniform throughout their ranges?

Mayr and Diamond (2001) examined this question in their monograph on the birds of northern Melanesia, by studying patterns of geographic variation among island bird populations. Expanding on earlier assessments of geographic speciation (Mayr 1940, 1942, 1963), Mayr and Diamond (2001) considered the degree of plumage differentiation of island populations to represent "snapshots" of the stages of speciation: subtle differentiation represented the earliest stages of population divergence, whereas
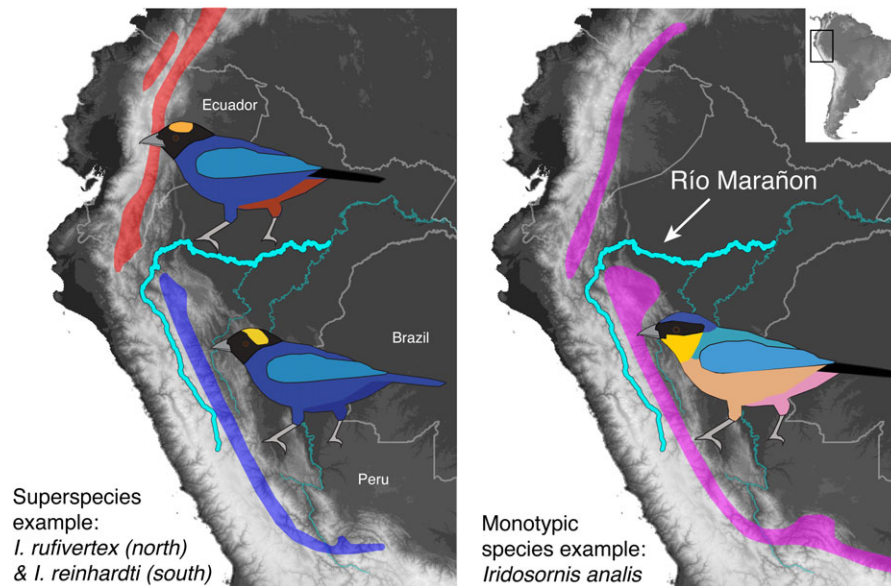
Figure 1. An example of a superspecies and a monotypic species in one of the eight genera in the study (*Iridosornis*, a montane tanager). The superspecies (comprised of the allospecies pair *I. rufivertex* and *I. reinhardti*) is differentiated in several plumage characters across the Marañon, including the color of the feathers below the tail, and the shape, location, and color of a yellow (vs. gold) patch on the head. In contrast, the monotypic species *I. analis* shows no plumage differentiation across the Marañon. Seven additional genera with superspecies and monotypic species were included in our study (Table 1, Fig. S1). Range maps provided by Robert S. Ridgely. The width of the Marañon River is exaggerated for emphasis.

marked differentiation among allopatric taxa indicated likely reproductive isolation and the achievement of speciation. Mayr and Diamond (2001) suggested several reasons why species would fail to evolve phenotypic differences in allopatry. These reasons included "recency of colonization" (the amount of time populations have occupied separate islands), homogenizing gene flow among populations (i.e., incomplete isolation across islands due to high vagility of individuals), and "phenotypic stability" (lineage-specific stasis in phenotypic evolution). Although Mayr and Diamond (2001) inferred which of these processes had led to a lack of geographic variation in particular species, they were limited at the time to studying phenotypic characters and thus lacked genetic data to independently assess the tempo of evolution.

Here, we employ a comparative phylogeographic framework to examine factors controlling the evolution of geographic variation in Andean cloud forest birds. We ask whether observed variation in phenotypic differentiation among taxa is predicted by the amount of time spent in geographic isolation, versus idiosyncratic rates of phenotypic evolution (Mayr's "phenotypic stability"). The tempo of phenotypic evolution should be highly dependent on ecological context and the strength of divergent or stabilizing selection pressures (Coyne and Orr 2004; Nosil 2008a; Price 2008). Therefore, we examine the evolution of geographic variation in multiple species that are codistributed throughout a single habitat—humid montane forest—and whose populations are isolated by a common barrier to dispersal, the arid Marañon

Valley of northern Peru. Through our comparative framework, we address Mayr and Diamond's (2001) important question in the quote beginning this manuscript: how much time is required for the evolution of phenotypic differentiation in allopatry? In particular, does a certain amount of time spent in isolation always lead to geographic variation in phenotype, or do rates of phenotypic evolution vary idiosyncratically within and across lineages?

STUDY SYSTEM

In the humid montane forests of the Andes, many bird species have linear, geographic ranges that are on average 300 times longer than wide and correspond to elevationally narrow but latitudinally expansive bands of habitat and climate (Fig. 1; Chapman 1923; Graves 1985, 1988, 1991; Fjeldså and Krabbe 1990). Throughout these ranges, species often exhibit sharp, discrete differentiation in plumage pattern or song in accordance with geographic or ecological barriers to dispersal that intersect species' distributions, such as arid rain-shadow valleys (Vuilleumier 1969; Remsen 1984; Graves 1985, 1991; Brumfield and Remsen 1996; García-Moreno et al. 1998, 1999; Johnson 2002; Cadena et al. 2007, 2011; Ribas et al. 2007; Krabbe 2008; Bonaccorso 2009; Parra et al. 2009; Chaves and Smith 2011; Gutiérrez-Pinto et al. 2012; Isler et al. 2012; Lutz et al. 2013). Due to the presence of such marked phenotypic variation among allopatric replacements, many Andean species have long been
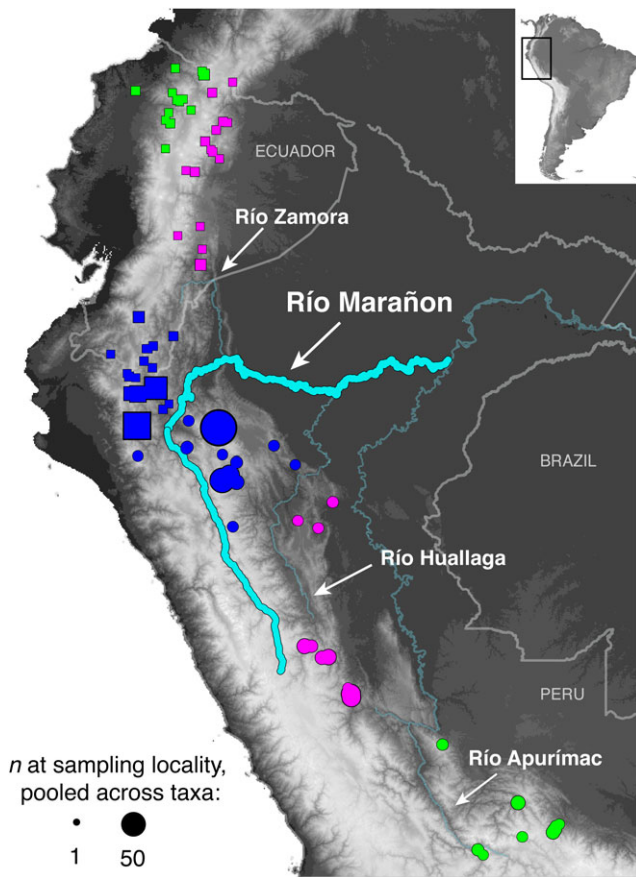
Figure 2. Collecting localities of the 522 individuals used for genetic sampling (pooled across all taxa). Trans-Marañon genetic distances were calculated using samples from the east slope of the Andes within 200 km of either side of the Marañon (blue squares and circles). For constructing broader intraspecific phylogenies, we included samples from the west slope of Ecuador (green squares), the east slope of northern Ecuador (pink squares), and from south of the rain-shadow valleys of the Huallaga (pink circles) or Apurímac rivers (green circles). The width of the Marañon River is exaggerated for emphasis.

recognized as comprising species complexes, also known as "superspecies" (Vuilleumier 1969; Remsen 1984; Fjeldså and Krabbe 1990; Weir 2009).
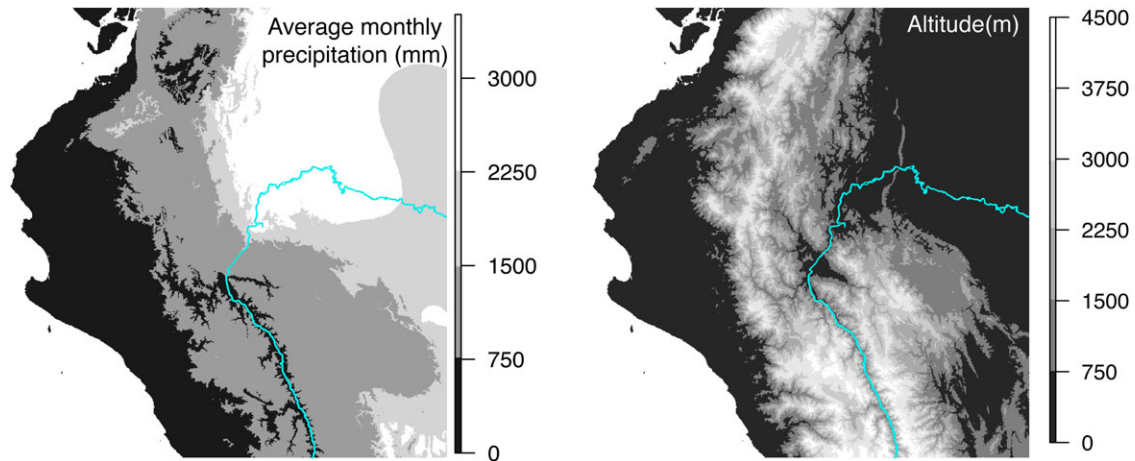
The wide, arid valley of the Marañon River in northern Peru is generally accepted as the most prominent barrier to dispersal in the Andes (Vuilleumier 1969, 1984; Parker et al. 1985; Fjeldså and Krabbe 1990; Weir 2009). The Marañon River is a tributary of the upper Amazon River and drains much of the central Andes (Fig. 2). In its upper and middle reaches, the Marañon flows north and subsequently east through a broad, rain-shadow valley supporting desert scrub and dry forest (Fig. 3; Young and Reynel 1997; Weigend 2002). This valley system is part of the Huancabamba Depression or North Peruvian Low, which includes the Porculla pass, the lowest elevation saddle between the east and

west slopes of the entire Andean chain (Fjeldså and Krabbe 1990; Young and Reynel 1997). Consequently, the arid region encompassing the upper Marañon Valley and the Huancabamba Depression (henceforth referred to here simply as "the Marañon") is a strong ecological and topographical barrier to dispersal for birds and other organisms adapted to cool, humid montane forests on the east slope of the Andes (Chapman 1926; Parker et al. 1985; Young and Reynel 1997; Johnson 2002). Among birds, the influence of this barrier is evidenced by the high number of cloud forest taxa that show plumage or other phenotypic differences on either side of the Marañon (Parker et al. 1985; Johnson 2002; Bonaccorso 2009; Schulenberg et al. 2010). However, most cloud forest bird species whose populations are isolated across the Marañon exhibit little or no differentiation in plumage or song; with respect to the Marañon, these species are widely distributed, monotypic taxa or taxa with recognized subspecies distributed on either side of the Marañon (Fjeldså et al. 1999; Weir et al. 2008; Cadena and Cuervo 2009; Ridgely and Tudor 2009; Schulenberg et al. 2010; Gutiérrez-Pinto et al. 2012; Valderrama et al. 2014). This variation in the degree of phenotypic differentiation of allopatric replacements across the Marañon provides a comparative framework with which to test hypotheses of the evolution of geographic variation.

We compared taxa within eight genera of cloud forest passerine birds that have variable degrees of plumage differentiation across the Marañon Valley. Within each genus, we used genetic data to estimate divergence times between populations isolated by the Marañon. In each genus, we estimated trans-Marañon divergence times for two pairs of populations (Fig. 1): an allopatric pair of sister species that shows sharp and marked differentiation in plumage across the Marañon (which we refer to as the *superspecies*), and a species closely related to the superspecies that shows no apparent plumage differentiation despite being found on both sides of the Marañon (which we refer to as the *monotypic species*). Focusing our analysis on genera with one differentiated and one undifferentiated taxon enables us to test for commonalities in the rate of phenotypic evolution across this barrier both within and across lineages.

We ask, within each genus, do the superspecies show greater divergence times across the Marañon than the monotypic species? Further, if there is a relationship between divergence time and plumage differentiation within each genus, is there a common threshold of divergence time that predicts plumage differentiation across genera in this region? To test the relationship between plumage and genetic differentiation and other phenotypic characters, we include assessments of differentiation of size and shape characters (i.e., bill, wing, and tail dimensions, henceforth referred to as "morphometry"), as well as vocal characters.

The populations isolated by the Marañon are found in the same elevational ranges and habitats (humid montane forest) on

Figure 3. The Marañon River (blue line) flows out of the Andes through a wide valley, before turning to the east to join the Amazon River. The valley of the upper Marañon is more arid than the surrounding Andean habitats (left), as well as lower in altitude (right). Consequently, the Marañon is both an ecological and topographical barrier for species found in humid montane forest on either side. Maps drawn in R using data downloaded from http://worldclim.org (Hijmans et al. 2005).

both sides of the barrier (Stotz et al. 1996; Ridgely and Tudor 2009; Schulenberg et al. 2010). This system is therefore an example of speciation associated with niche conservatism (Wiens 2004a,b; Svensson 2012; Hua and Wiens 2013): humid forest species are unable to adapt to the arid habitats in the Marañon Valley, and consequently experience restricted gene flow across this barrier. Sharp differences in phenotype between ecologically similar allopatric replacements have long played a role in the development of speciation theory (Mayr 1942; Ricklefs and Cox 1972; Diamond 1973; Cracraft 1983; Mayr and Diamond 2001; Price 2008), and classic papers discussing speciation in humid forest Andean birds are part of this tradition (Chapman 1923; Remsen 1984). Additionally, recent theoretical work provides new perspectives from which to consider differentiation across similar environments (Mani and Clarke 1990; Schluter 2009; Nosil and Flaxman 2011; Langerhans and Riesch 2013; Mendelson et al. 2014). However, in contrast to a large body of research on the influence of divergent ecological selection pressures on differentiation (Smith 1997; Nosil 2008a,b; Schluter 2009; Sobel et al. 2009; Schemske 2010; Roesti et al. 2012), few empirical studies have examined the regulation of allopatric differentiation across similar environments (McCormack et al. 2009; Schemske 2010; Mendelson et al. 2014). Our study design enables such an examination.

Methods

STUDY SPECIES

Our study comprises trans-Marañon comparisons within 16 taxa (sister populations of a monotypic species, and a superspecies comprising a pair of sister allospecies, in each of eight genera), from four families and three major sublineages of passerine

birds (Table 1; Fig. S1). Under current avian taxonomies, both members of each allospecies pair are classified as separate species due to their substantial plumage differentiation across the Marañon (Clements 2007; Ridgely and Tudor 2009; Remsen et al. 2014). A detailed description of plumage differences is provided in the Supporting Information text and illustrated in Figure S1. All superspecies were chosen with a priori knowledge based on molecular phylogenetic studies that the pair of allospecies they contain are sisters (see Table 1). Conversely, populations isolated by the Marañon in each of eight monotypic species are considered conspecific due to their lack of phenotypic divergence (Ridgely and Tudor 2009; Schulenberg et al. 2010). These monotypic species were chosen without knowledge of trans-Marañon genetic differentiation.

GENETIC SAMPLING SCHEME

To calculate population divergence times across the Marañon, we sampled DNA from populations occupying humid montane forest in a region extending approximately 200 km from both sides of the Marañon (blue circles and squares, Fig. 2). This region extends north from the Marañon to a low elevation gap in the Andes created by the Zamora River in Ecuador (blue squares, Fig. 2), which is thought to be a barrier for some humid forest species (Krabbe 2008; Bonaccorso 2009). On the south side of the Marañon, the region encompasses the humid eastern slope of the northern Cordillera Central (Mark et al. 2008) as well as a series of ridges near the Marañon gap (blue circles, Fig. 2). Sampling for genetic divergence time calculations was from an average of 11.69 individuals per side of the Marañon per species (median sample size = 12, range = 3–20, additional details in Supporting Information text).

Table 1. Study species.

Genus (English name; Family)	Trans-Marañon "monotypic" species	Allospecies north of Marañon	Allospecies south of Marañon	Phylogenetic references	Passerine lineage
Grallaria (Antpittas; Grallariidae; Fig. S1A)	*G. squamigera*	*G. hypoleuca*	*G. przewalskii* (+ *G. capitalis and G. erythroleuca*)	This study (Fig. S1A)	Tracheophone suboscine
Thripadectes (Treehunters; Furnariidae; Fig. S1B)	*T. holostictus*	*T. flammulatus*	*T. scrutator*	(Derryberry et al. 2011)	Tracheophone suboscine
Leptopogon (Flycatchers; Tyrannidae; Fig S1C)	*L. superciliaris*	*L. rufipectus*	*L. taczanowskii*	(Bates and Zink 1994)	Suboscine
Nephelomyias (Flycatchers; Tyrannidae; Fig. S1D)	*Pyrrhomyias cinnamomeus*	*N. lintoni*	*N. ochraceiventris*	(Ohlson et al. 2008, 2009; Tello et al. 2009)	Suboscine
Ochthoeca (Chat-Tyrants; Tyrannidae; Fig. S1E)	*O. frontalis*	*O. diadema*	*O. pulchella*	(García-Moreno et al. 1998)	Suboscine
Hemispingus (Hemispingus; Thraupidae; Fig. S1F)	*H. frontalis*	*H. verticalis*	*H. xanthophthalmus*	(Shultz and Burns 2013; Burns et al. 2014)	Oscine
Iridosornis (Tanagers; Thraupidae; Figs. 1, S1G)	*I. analis*	*I. rufivertex*	*I. reinhardti*	(Sedano and Burns 2010)	Oscine
Cnemathraupis (Mountain-Tanagers; Thraupidae; Fig. S1H)	*Chlorornis riefferii*	*C. eximia*	*C. aureodorsalis*	(Sedano and Burns 2010; Burns et al. 2014)	Oscine

Our study comprises eight genera of passerine birds. In each genus, we sampled a species that is monotypic or only subtly differentiated in plumage with respect to the Marañon Valley (the "monotypic" species), and a superspecies comprised of a pair of allospecies that are divergent in plumage on either side of the Marañon. Each allospecies is classified as separate species in all modern avian taxonomic treatments. The phylogenetic references provide evidence that each allospecies pair are sister species. In two genera (*Cnemathraupis* and *Nephelomyias*), the monotypic species is classified as a different (and monotypic) genus than the superspecies, but the phylogenetic references indicate that the monotypic species is a close outgroup of the superspecies.

In Ecuador, high mountain peaks restrict dispersal between humid forest found on the east and west slopes of the Andes (Weir 2009). Additionally, to the south of the Marañon in Peru, the rain-shadow valleys of the Huallaga and Apurímac rivers present significant biogeographic barriers that may subdivide east-slope Andean birds (Fig. 2; Ribas et al. 2007; Bonaccorso 2009; Weir 2009). Phylogeographic studies of other Andean bird species have sometimes revealed surprising polyphyletic intraspecific relationships with respect to this complex topography (Chaves and Smith 2011; Gutiérrez-Pinto et al. 2012). Therefore, prior to estimating trans-Marañon distances within the restricted sampling region, we constructed intraspecific phylogenies using broader geographic sampling (Figs. 2, S1) to verify that each set of putative sister populations separated by the Marañon are each other's closest relatives.

In total, we sequenced DNA from 522 individuals for this study, and added nine sequences from GenBank (see Appendix S1 for GenBank accession numbers). Five hundred of 506 samples used from Peru and 62 of 89 Ecuadorean samples were muscle tissue with an associated voucher skin or skeleton specimen deposited in a museum collection (Appendix S1). Two samples are from toe pads of museum specimens (Appendix S1). The remaining samples were unvouchered blood samples.

GENETIC METHODS

To compare relative divergence times among our study populations, we sequenced the mitochondrial gene NADH-dehydrogenase 2 (ND2), a gene commonly used in avian comparative phylogeographic studies (e.g., Barber and Klicka 2010; Naka et al. 2012). Single-locus estimates of population divergence time may be misled by variation in coalescence time of gene trees, whose divergences should predate those of species trees (Edwards and Beerli 2000; Arbogast et al. 2002; Smith and Klicka 2013). However, as is common in comparative studies, we interpret mtDNA distance as representative of relative population divergence time in a broad sense (Weir and Price 2011; Weir and Wheatcroft 2011; Naka et al. 2012; Seddon et al. 2013; Smith et al. 2014), and not indicative of nuanced events such as the time of initial population isolation. Further, due to the potential for variation in mtDNA coalescence times to obscure true population divergence times, any segregating pattern in divergence time between superspecies and monotypic species is likely to be conservative (Burney and Brumfield 2009; Naka et al. 2012).

We sequenced the ND2 gene (1041 basepairs) using standard PCR and Sanger sequencing protocols, and built phylogenies using standard maximum-likelihood methods implemented in RAxML version 7.2.8 (Stamatakis et al. 2008). To estimate relative trans-Marañon genetic divergences, we calculated the average GTR-Γ distances for each sister population pair in RAxML. To calculate node ages for each trans-Marañon comparison, we used BEAST version 1.8 (Drummond and Rambaut 2007) with a relaxed molecular clock specific to ND2 (Smith and Klicka 2010). Detailed methods for DNA sequencing and phylogenetic analyses are provided in the Supporting Information text.

PHENOTYPIC MEASUREMENTS

We quantified differences in plumage color, morphometric traits, and vocal characters across the Marañon for each superspecies and monotypic species. The first author measured plumage color variables and morphometric traits using museum skins and measured vocal characteristics from digital audio recordings. All museum specimens are from the east slope of the Andes from Ecuador south to the Huallaga Valley in central Peru. Within this region, the majority of these samples were from within the same 400 km region spanning the Marañon described above for genetic sampling (Fig. S2, Appendix S2). For song analysis, low sample sizes for some species near the Marañon required sampling from a broader region of the Andes (see below).

SPECTROPHOTOMETRIC PLUMAGE ANALYSIS

The purpose of spectrophotometric analyses was twofold: (1) to determine whether our a priori designation of plumage classes (superspecies and monotypic species) were upheld quantitatively and (2) to estimate an evolutionary distance in plumage across the Marañon for each species. We quantified plumage color divergence by using an Ocean Optics spectrophotometer (Ocean Optics, Dunedin, FL) to gather reflectance spectra and the R package *pavo* (Maia et al. 2013a) to generate and analyze color variables from these spectra. We measured approximately six specimens per population on either side of the Marañon (six specimens each for 23 populations, five specimens each for seven populations, and four specimens each for two populations; total $n = 184$ specimens; Appendix S2). All species in this study are sexually monochromatic or have only subtle sex-based differences in human-visible light. However, as some birds have been shown to have sexually dichromatic plumage in UV wavelengths visible to birds but cryptic to humans (Eaton 2005; Burns and Shultz 2012), we measured three male and three female specimens per population where possible. On each specimen, we measured 10 plumage patches that capture the major sources of plumage variation among trans-Marañon populations (Fig. S3). Detailed methods for spectrophotometric measurements are presented in the Supporting Information text.

We used *pavo* to calculate three standard colorimetric variables from the shape of spectral curves: hue (wavelength of mid-reflectance), chroma (spectral saturation), and mean brightness (Montgomerie 2006; Supporting Information text). We found similar results when we used *pavo* to analyze color in avian tetrahedral color space (Stoddard and Prum 2008); therefore, we focus here

on the colorimetric variables and present tetrahedral color space results in the Supporting Information.

Using the three colorimetric variables as multivariate data, we estimated evolutionary divergence in plumage across the Marañon by calculating the Mahalanobis distance (MD) of each patch for each trans-Marañon sister pair. MD is similar to Euclidean distance, but accounts for correlation among variables by incorporating a covariance matrix (Marcus 1993). The covariance matrices of all populations within each genus are assumed identical and estimated by pooling across populations. The pooled covariance matrix for a particular genus is

$$\hat{\sum}_{\text{Pooled}} = \frac{1}{n - \#G} \sum_{g \in G} (n_g - 1) \hat{\sum}_g$$

where n is the total number of observations, n_g the number of observations for genus g, $\#G$ the total number of genera, G the set of all genera, and $\hat{\sum}_g$ is the usual estimate of the covariance matrix of genus g. MDs were calculated with custom scripts in R (R Core Team 2014). We calculated separate MDs for each of 10 patches in each of the 16 trans-Marañon sister pairs. For each sister pair, we then determined the mean Mahalonobis distances across all 10 patches, resulting in mean trans-Marañon evolutionary distances for each of the superspecies and monotypic species. We then calculated Pearson product-moment correlations (r) of genetic distance and log mean Mahalanobis plumage distances in R (R Core Team 2014). As the variables we are correlating are independent pairwise contrasts between sister taxa, phylogenetic correction is not necessary for this analysis; however, in exploratory analyses, correlations of phylogenetic independent contrasts yielded nearly identical results to the correlations reported here.

PLUMAGE SCORING

We also estimated plumage divergence across the Marañon using a plumage scoring system developed by Tobias et al. (2010) in which patches are assessed for differences on a qualitative scale (see Supporting Information text for details). We found strong correspondence between plumage scores and MDs from spectral data, and thus present these results in the Supporting Information.

MORPHOMETRIC ANALYSIS

We measured seven standard morphometric features on museum specimens (culmen length from nares to tip, culmen width, culmen depth, wing length, tail length, tarsus length, middle toe length) using a digital calipers. We did not measure tail length in *Grallaria* antpittas because the tail is so short it is difficult to accurately measure. We measured 10–15 adult specimens on either side of the Marañon from every superspecies and monotypic species (i.e., 20–30 specimens per species) for a total of 339 specimens (Appendix S2). Sample sizes of *Grallaria squamigera* and

Thripadectes spp. were lower due to scarcity of specimens in museum collections. Where possible, we measured equal numbers of males and females from each population. We log-transformed data for analysis and interpolated missing values (resulting from broken or misprepared features in a small number of specimens) using a multivariate linear regression of nonmissing data within each genus following Seeholzer et al. (2012). We conducted analyses in which we excluded outliers that failed tests of univariate normality, but this did not affect any of the results so we present analyses of the full dataset. To determine whether populations that are divergent in plumage across the Marañon are also divergent in morphometric traits, we conducted MANOVA and linear discriminant analyses in R (R Core Team 2014) for each superspecies and monotypic species, using population (north or south of the Marañon) as the classifying factor. To estimate evolutionary distance in morphometric traits across the Marañon, we also calculated trans-Marañon MDs for each superspecies and monotypic species using the methodology described above for spectrophotometric analysis.

BIOACOUSTIC ANALYSIS

To assess evolutionary distance in song across the Marañon, we analyzed digital audio recordings that were made in the field by the first author, provided by the Macaulay Library (Cornell Lab of Ornithology, Ithaca, NY), downloaded from the Xeno-canto database (http://www.xeno-canto.org), and accessed from published collections (Moore et al. 2013). Song analyses suffered from several complications, most importantly the difficulty of identifying homologous vocalizations across lineages. Our analysis includes genera with different mechanisms of song inheritance (learned song in oscines versus genetically inherited in suboscines) as well as different syringeal morphology (Table 1). Additionally, in some species, it was not clear which vocalization type represented a "song" versus a "call." To account for these issues, we always measured the same vocalization type in each trans-Marañon comparison, such that each evolutionary distance represents measurement of a homologous feature within that species. Adequate sample sizes of high-quality recordings for potentially homologous vocalizations within the study region were not available for several populations, necessitating the inclusion of a small number of additional recordings from outside the sampling region (Fig. S2, Appendix S3). Our total sampling included recordings of 163 individuals (median sample size = 5 individuals per population on either side of the Marañon, range = 1–10).

We analyzed recordings in Raven Pro 1.4 (Cornell Lab of Ornithology, Ithaca, NY), using default settings (window size = 512 samples). We drew boxes around songs to measure first and third quartile frequencies, center frequency, and bandwidth (difference between first and third quartile frequencies). We
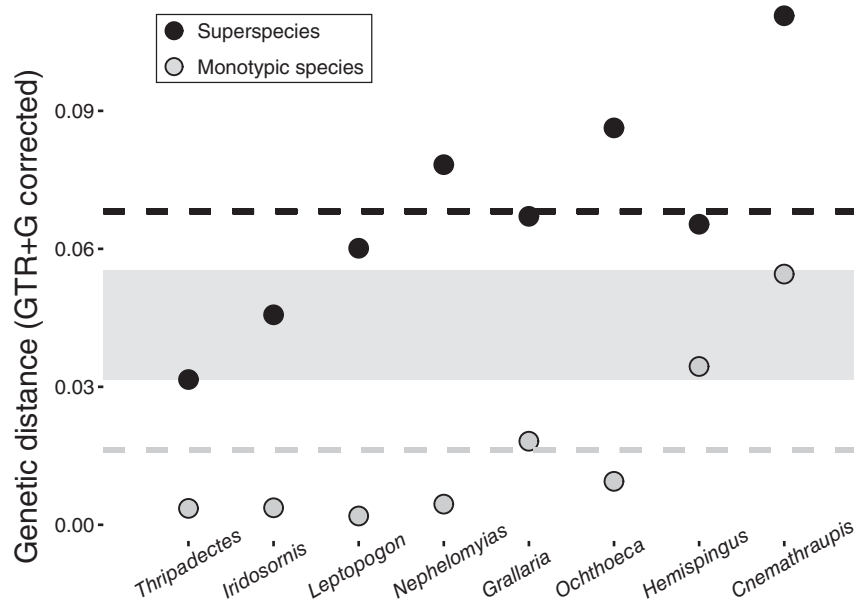
Figure 4. Mean genetic distances (GTR-Γ corrected) across the Marañon for each of eight superspecies and monotypic species. Within each genus, the genetic distance of the superspecies across the Marañon (black circles) was always higher than that of the monotypic species (gray circles). Across genera, the mean trans-Marañon genetic distances of the superspecies were significantly higher than the mean monotypic species distance (see Results). Below 3.2% GTR distance, species were always nondifferentiated in plumage, and above 5.4%, every species was differentiated in plumage; between these values (gray bar), two taxa were differentiated in plumage (*Thripadectes* and *Iridosornis*), one species was undifferentiated in plumage (*Hemispingus*), and one "monotypic" species showed subtle plumage differentiation (*Cnemathraupis* = *Chlorornis riefferii*; see Supporting Information text).

concluded that additional variables that are sometimes used in avian song analysis, such as syllable type, note quantity or song duration (e.g., Weir and Wheatcroft 2011; Mason et al. 2014), would be idiosyncratic for each genus and could introduce further elements of nonhomology. We measured at least three songs per individual and used the mean measurements of these songs as per-individual measurements. These measurements were used to calculate trans-Marañon MDs. As outlined above for morphometric traits, we performed MANOVA and linear discriminant analyses of vocal traits for species with sample sizes greater than six individuals (resulting in the exclusion of two species), as MANOVA tests could not be performed with smaller sample sizes.

COMPARISONS TO ADDITIONAL TAXA

Many phylogenetic studies have been conducted of additional avian taxa found in the humid montane forest in the Andes, and for 16 of these taxa, published ND2 sequences are available from both sides of the Marañon within our sampling region (Supporting Information text). Although these taxa do not conform to our study design of a genus containing both a superspecies and a monotypic species, they provide additional insight into the tempo of plumage evolution in this region. Therefore, we used published sequence data to calculate GTR-Γ genetic distances for these taxa across the Marañon, and used the plumage scoring method (Tobias et al. 2010) to estimate plumage differentiation across the

Marañon. Detailed methods and results are presented in the Supporting Information.

Results

GENETIC RESULTS

Phylogeny construction using the broad geographic sampling indicated that for all study taxa, the populations close to the Marañon on either side (blue circles and squares, Fig. 2) are each other's closest relatives (Fig. S1). GTR-Γ distances and node ages estimated in BEAST for these trans-Marañon sister populations were highly correlated ($r > 0.98$) and illustrate the same general result; therefore, we use mean GTR-Γ genetic distances as a proxy for divergence time.

Within each genus, the trans-Marañon genetic distance for the superspecies was always higher than that of its congeneric monotypic species (Fig. 4). Across genera, the trans-Marañon GTR-Γ genetic distances for all superspecies (mean = 6.81%) were significantly greater than the GTR-Γ distances for all monotypic species (mean = 1.62%; $P < 0.001$, $n = 16$, Student's t-test; Fig. 4). There was little variation in trans-Marañon genetic distances among the multiple individuals sampled within each population: the average SD of trans-Marañon genetic distances among individuals within each superspecies was 0.002%, and was 0.005% for individuals within each monotypic species. For phylogeny construction, this genetic structure resulted in
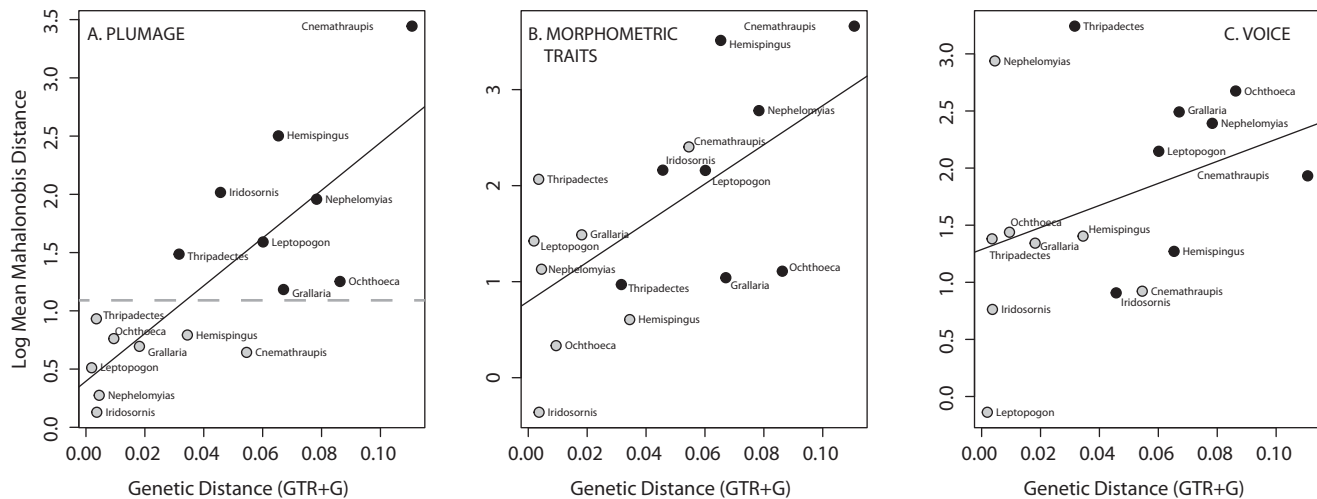
Figure 5. Phenotypic distances (log mean Mahalanobis distance) in plumage (A), morphometric traits (B), and voice (C) versus genetic distance between sister pairs isolated by the Marañon. Black circles indicate superspecies and gray circles monotypic species. (A) The distance in colorimetric variables (hue, chroma, and brightness) of 10 plumage patches in each trans-Marañon comparison. Mean plumage distance is positively correlated with genetic distance ($r = 0.79$). Moreover, every superspecies' plumage distance is higher than every monotypic species' plumage distance (indicated by the dashed line separating gray and black circles), supporting the a priori plumage designations in the study. (B) Distance in morphometric (size and shape) characters. Although morphometric distance is positively correlated with genetic distance ($r = 0.63$), change in morphometric traits is idiosyncratic with respect to the plumage designations: some populations isolated for millions of years lack morphometric trait differentiation. (C) Distance in vocal frequency characters is weakly correlated with genetic distance ($r = 0.45$). High distances in the *Thripadectes* superspecies and *Nephelomyias* monotypic species may be due to sampling artifacts (see text for details).

reciprocal monophyly with respect to the Marañon for every superspecies (Fig. S1). In contrast, only one monotypic species contained populations that were reciprocally monophyletic with respect to the Marañon (Fig. S1H).

SPECTROPHOTOMETRY RESULTS

Across all 16 trans-Marañon comparisons, log mean MD of colorimetric variables was positively correlated with GTR-Γ genetic distance ($r = 0.79$, Fig. 5A). Within each genus, the mean MD of the 10 plumage patches was always higher for the superspecies than for the monotypic species (Fig. 5A). Across genera, every superspecies' mean MD was higher than every monotypic species' mean MD (Fig. 5A). Thus, the a priori qualitative classifications of superspecies and monotypic species are supported by quantitative analysis of spectrophotometry.

MORPHOMETRIC RESULTS

There is a positive relationship between genetic distance and log mean MD of morphometric traits across the Marañon, but it was slightly weaker than the relationship of genetic distance and plumage MD ($r = 0.63$; Fig. 5B). Moreover, morphometric trait divergence was idiosyncratic with respect to plumage and genetic divergence: some superspecies with high genetic and plumage divergence had low morphometric distances, and vice versa (Fig. 5B). Consequently, morphometric distances for the

superspecies were marginally nonsignificantly greater than for the monotypic species ($P = 0.06$, df $= 14$, $n = 16$, Student's *t*-test).

MANOVA tests were consistent with these results. MANOVA revealed significant differences ($P < 0.05$) in morphometric traits across the Marañon in six superspecies and three monotypic species. This result was also recovered for main population effects when sex was included as a factor. Linear discriminant analyses predicted 100% of individuals to the correct side of the Marañon based on morphometry in only three superspecies and two monotypic species, but predicted at least 85% of individuals to the correct side of the Marañon in seven superspecies and three monotypic species. Thus, morphometric trait differentiation occurred in some, but not all, superspecies, but morphometric trait differentiation was also found in some plumage-monotypic species.

BIOACOUSTIC RESULTS

Overall, superspecies had significantly greater trans-Marañon MD in vocal frequency characters than monotypic species ($P = 0.02$, df $= 14$, $n = 16$, Student's *t*-test). MD of vocal characters was not strongly correlated with genetic distance ($r = 0.45$, Fig. 5C). However, when we estimated a single covariance matrix of all individuals pooled together for calculating MD, the correlation of MD and genetic distance improved ($r = .65$). Fourteen of

16 species had sufficient sample sizes to perform MANOVA on vocal characters (one superspecies and one monotypic species were excluded). Of these tests, five of seven superspecies and one of seven monotypic species were significantly different in voice across the Marañon ($P < 0.05$). Linear discriminant analyses identified at least 90% of individuals to the correct population for the species that had significant MANOVA distances.

Discussion

Understanding the factors that govern the tempo of speciation is a major theme of contemporary evolutionary biology (Ricklefs 2007; Rabosky 2013). Speciation is often conceptualized as a series of stages, each of which may be a rate-limiting factor in the tempo of speciation (Mayr 1942; Allmon 1992; Nosil 2008a; Price 2010). Recent studies have proposed that speciation rate is not predicted by the rate at which species evolve reproductive isolation (Rabosky 2013; Rabosky and Matute 2013), and that phenotypic diversity may frequently be a result of trait divergence in allopatry, as opposed to species interactions during secondary contact (Tobias et al. 2014; Zink 2014). These insights suggest that the dynamics of the earlier stages of speciation, such as the tempo and degree of phenotypic evolution in geographic isolation (Price 2010), may be important for regulating speciation rate as well as the evolution of phenotypic diversity.

We examined the tempo of the first stage of allopatric speciation by asking whether the degree of phenotypic differentiation among allopatric populations is a result of time spent in isolation versus idiosyncratic rates of phenotypic evolution. We found that within eight genera of Andean birds, taxa that were differentiated in plumage across the Marañon Valley had deeper genetic divergences across the Marañon—often by an order of magnitude— than did their closely related monotypic congeners (Fig. 4). This result suggests that the observed variation in plumage differentiation across the Marañon is related to the amount of time populations have been isolated by this barrier, rather than variation in rate of phenotypic evolution among closely related taxa.

Across the study genera, the relative divergence times of sister populations of superspecies and monotypic species isolated by the Marañon are suggestive of a threshold of isolation time— roughly two million years—required for substantial plumage divergence: below 3.2% GTR-Γ genetic distance (1.56 million years of divergence in our BEAST analysis of node ages), plumage differentiation was never observed, and above 5.4% GTR-Γ genetic distance (2.49 million years), plumage differentiation was always observed (Fig. 4). Among phylogeographic studies, including those of Andean humid forest species, numerous examples exist both of deep genetic divergences among phenotypically similar, allopatric populations (Omland et al. 2000; Smith et al. 2011; d'Horta et al. 2013; Valderrama et al. 2014), as well

as marked phenotypic differentiation among recently diverged populations (Weir and Schluter 2004; Milá et al. 2007; Mauck and Burns 2009; Campagna et al. 2012; Seeholzer et al. 2012; Burns et al. 2014). Our comparison of ecologically similar, codistributed taxa that are isolated across the same barrier suggests that despite idiosyncrasy in the tempo of phenotypic evolution across taxa, regions, and environments, phenotypic differences at the growing tips of the tree of life may nevertheless evolve predictably within a particular geographic and ecological context.

In contrast to plumage evolution, we found greater idiosyncrasy in change of morphometric traits over the course of millions of years of geographic isolation, with some deeply divergent populations lacking detectable morphometric differences. This is not surprising, as the superspecies in our study—even those with statistically significant differences in morphometric traits—are morphometrically similar sister taxa that differ principally in plumage. Morphometric trait differentiation in allopatry is generally thought to reflect ecological adaptation (Ricklefs and Bermingham 2007; Martin and Mendelson 2014) and the generally conserved morphometric traits among the sister pairs in our study reflect the ecological similarities populations isolated by the Marañon (Chaves and Smith 2011).

For vocal characters, some of the idiosyncrasy in relationship with divergence time (such as the surprisingly high MDs in the *Thripadectes* superspecies and the *Nephelomyias* monotypic species; Fig. 5C) may be due to artifacts of sample size, recording quality and conditions, or the crudeness of measuring frequency characters from visual examination of spectrograms. Our field experience suggests that most superspecies have subtle yet distinct vocal differences on either side of the Marañon, whereas these differences are not present in the monotypic species—an impression shared by other field workers (e.g., Schulenberg et al. 2010) but not supported by our quantitative analysis.

DRIVERS OF PHENOTYPIC DIFFERENTIATION IN THE ANDES

The importance of environmental gradients and ecological differences for driving the evolution of geographic variation has been firmly established (Endler 1977; Sobel et al. 2009). Yet for students of geographic variation in Andean birds, a central question has been, why do populations differ abruptly and dramatically in phenotype when they are found in similar habitats and environments (Chapman 1923; Remsen 1984; Johnson 2002; Parra 2010; Cadena et al. 2011)?

Chapman (1923) and Remsen (1984) noted that geographically isolated populations of Andean cloud forest birds show discrete plumage differences that are difficult to attribute to differences in environment throughout their ranges. Although these authors were specifically concerned with "leapfrog" patterns of geographic variation throughout the Andes, their work has

implications for the factors driving phenotypic evolution among Andean taxa more generally. Most notably, Remsen (1984) suggested that among Andean humid forest taxa, "many phenotypic changes may appear at random with respect to geography and are not induced by the environment in any predictable way . . . If this hypothesis is correct, much of the phenotypic differentiation involved in the speciation process may be due to stochastic factors, absence of gene flow, and transilience [Templeton 1980a] rather than to more predictable, environmentally induced factors." These statements were interpreted as an argument for genetic drift driving plumage divergence by Cadena et al. (2011), who provided molecular evidence that genetic drift was not responsible for the evolution of plumage differences in an Andean cloud forest bird species. Although genetic drift is one possible explanation for stochasticity in phenotypic evolution among ecologically similar populations, drift is not the only process by which populations can diverge when selection pressures are uniform (Templeton 1980a; Mani and Clarke 1990; Unckless and Orr 2009). In particular, we suggest it is worth examining Remsen's (1984) emphasis on the stochasticity of plumage evolution from the perspective of mutation-order theory (Mani and Clarke 1990), which has seen increased attention in recent work on speciation (Schluter 2009; Schemske 2010; Nosil and Flaxman 2011; Langerhans and Riesch 2013; Mendelson et al. 2014).

Mutation-order theory proposes that when allopatric populations exist in uniform environments, differentiation will occur not because mutations are inherently more favorable in one population versus the others, but rather because mutations only have the opportunity to be favored in the population in which they appear (Nosil and Flaxman 2011). For example, a mutation that leads to a change in plumage color may potentially be favorable throughout a bird species' entire range, either due to direct ecological adaptation or sexual selection on mate preference (Price 2008). However if gene flow between populations is prevented by extrinsic barriers, the favorable mutation will not have the opportunity to spread, and the mutation will only become fixed in the population in which it arose (Price et al. 2010). In this scenario, the stochastic order at which mutations appear drives divergence (Mani and Clarke 1990; Schluter 2009; Nosil and Flaxman 2011; Langerhans and Riesch 2013; Mendelson et al. 2014). In contrast to drift, mutation-order theory does not diminish the roles of natural or social selection in driving the fixation of mutations within populations (Price et al. 2010; Mendelson et al. 2014). Additionally, although mutation-order theory is conceptually similar to "nonecological speciation," ecology may still play a role in mutation-order speciation (Nosil and Flaxman 2011). Rather, mutation-order theory argues that when selection pressures are uniform or similar across populations, the order at which mutations appear is more important than differences in ecological selection pressures in predicting their ultimate fixation; that

is, if mutations appear in a different order among populations, phenotypes may evolve differently despite uniform selection pressures (Unckless and Orr 2009; Schemske 2010; Mendelson et al. 2014).

We suggest that mutation-order theory provides a useful framework for considering the seemingly random patterns of plumage divergence across similar environments long observed by students of avian speciation in the Andes (Chapman 1923, Remsen 1984), because it reconciles Remsen's (1984) emphasis on stochasticity and disassociation from environmental conditions with Cadena et al.'s (2011) evidence for selection. However, we caution that mutation-order speciation is difficult to demonstrate to the exclusion of other processes, and further work is necessary to establish its role in our study system. Most importantly, demonstrating that two natural environments have identical selection pressures is difficult, if not impossible (Langerhans and Riesch 2013), and in the absence of gene flow, even weakly divergent selection pressures will eventually lead to differentiation (Wright 1931; Templeton 1980a; Coyne and Orr 2004). Indeed, other studies have suggested that allospecies of Andean birds are geographically isolated along different portions of latitudinal environmental gradients (Fjeldså et al. 1999; Jiguet et al. 2010), and that isolation in these subtly different portions of climatic space may drive differentiation (Graves 1991; Chaves and Smith 2011).

Regardless of whether mutation-order processes act exclusively to drive plumage differentiation among our study species, or whether selection pressures facing populations isolated by the Marañon differ in subtle ways, the principal feature of geographic variation in plumage among Andean cloud forest birds is one of discrete differences despite similar ecology and conversed morphometric traits (Parra 2010). In other systems, stasis in morphometric traits and relatively faster divergence of traits associated with social signaling has been interpreted as indicative of social selection, as opposed to divergent ecological adaptation, driving divergence (Arnegard et al. 2010; Martin and Mendelson 2012; Safran et al. 2013). Therefore, consistent with previous assessments of speciation in Andean birds (Parra 2010; Cadena et al. 2011), our study suggests that social selection plays an important role in driving phenotypic differentiation across the Marañon.

When allopatric populations exist in environments with similar selection pressures, the pace of genetic and phenotypic divergence is not accelerated by divergent selection. Thus, the limiting factor for the tempo of trait divergence is the waiting time for novel, favorable mutations to appear (Price 2002; Price et al. 2010; Maia et al. 2013b; Mendelson et al. 2014)—and for divergence to occur, the same mutation cannot become fixed among different populations (Unckless and Orr 2009). Due to the waiting time for these mutations, it has been suggested that divergence in similar environments may take a long time (Price 2008, 2010). This waiting time may explain the approximately two million year

isolation threshold for plumage differentiation observed among our study taxa. Our results are therefore consistent with Price (2010), who argued that time spent in isolation should be the primary factor predicting phenotypic differentiation of ecologically similar allopatric populations. The influence of demographic variables, such as effective population size, on the relationship between divergence time and population differentiation awaits further investigation (Price et al. 2010; Smith and Klicka 2013).

ECOLOGICAL PREDICTORS OF ISOLATION TIME

Our result that isolation time predicts phenotypic differentiation compels us to ask, why have some taxa experienced longer periods of geographic isolation than others? The shallow genetic differentiation of monotypic species in our study may reflect Mayr's "recency of colonization:" that is, monotypic species may have dispersed more recently across the Marañon than their congeneric superspecies, and simply have not been geographically isolated for long enough for phenotypic differences to arise. However, it is also possible that the ranges of monotypic species have been distributed across the Marañon for long enough to diverge, but the monotypic species have experienced greater homogenizing gene flow across the Marañon. Such gene flow could have occurred in pulses. For example, montane habitats in the Andes are thought to have shifted to lower elevations during cooler glacial periods, which could have created greater habitat connectivity across the Marañon (Vuilleumier 1969; Ribas et al. 2007; Weng et al. 2007; Ramírez-Barahona and Eguiarte 2013). Given the similar environments on either side of the Marañon and thus the potential for plumage mutations to be favorable on both sides of the barrier, even small amounts of gene flow might erase incipient phenotypic differences, and this gene flow could have served to push estimates of mitochondrial divergence times towards the present (Rheindt and Edwards 2011).

Distinguishing initial divergence time from post-divergence gene flow is not possible with single-locus data alone (Nielsen and Wakeley 2001; Durand et al. 2011). However, ecology and natural history may provide supporting evidence to explain the lack of differentiation in the monotypic species. For example, elevation has been shown to predict levels of phenotypic differentiation in Andean birds, with taxa occupying lower montane forest generally showing less geographic variation than high elevation taxa (Graves 1985, 1988). Among our study genera, the superspecies occupy higher elevation forest than the monotypic species in five of eight genera, but in two genera (*Grallaria* and *Thripadectes*), the monotypic species has broad elevational overlap with the superspecies, and in the remaining genus (*Ochthoeca*), the monotypic species is found at higher elevation than the superspecies (Schulenberg et al. 2010; B. Winger, pers. obs.). Thus, elevation predicts phenotypic and genetic differentiation in some, but not all, of our study taxa. A relationship between elevation and differ-

entiation in the Andes is likely a consequence of greater habitat connectivity at lower elevations. However, greater habitat connectivity could result in either increased gene flow among populations separated by barriers, or greater opportunity for colonizing taxa to disperse across and become established on both sides of barriers (Chaves et al. 2007). Thus, a correlation between elevation and differentiation does not reveal whether the undifferentiated taxa in our study represent recent colonization events across the Marañon or taxa that have experienced greater homogenizing gene flow.

Differences in vagility associated with foraging ecology have also been shown to predict levels of genetic differentiation and speciation rate among tropical birds (Burney and Brumfield 2009; Claramunt et al. 2012). Among our study taxa, dispersal ability ranges from poor (understory-dwelling antpittas and foliage-gleaners) to high (canopy-foraging tanagers), but the foraging ecology of taxa in our study does not appear to predict genetic differentiation. Rather, our results reveal substantial variation in genetic differentiation across the Marañon among congeners that do not have pronounced differences in foraging ecology. Although there may be subtle differences in dispersal ability within genera not revealed by general foraging ecology, vagility does not broadly explain the patterns of genetic or phenotypic differentiation reported here. We suggest this pattern reflects the strength of the Marañon as a topographical and ecological isolating barrier — even for species with relatively high dispersal.

Conclusions

Our study of the Marañon revealed that substantial differentiation in plumage required approximately two million years to evolve in allopatry, and always evolved after this length of isolation; this result was consistent across a taxonomically diverse set of passerine birds. Although the tempo of evolution is highly variable across regions and systems, we suggest that focused comparative studies within regions may reveal more predictability in the pace of allopatric differentiation than is evident by comparing across studies of individual taxa. Given the ecological similarity and low morphometric trait differentiation between allopatric populations, we consider social selection a likely promoter of plumage evolution in this system, and we suggest that mutation-order processes are involved in driving the evolution of geographic variation of Andean humid forest taxa.

DATA ARCHIVING

The doi for our data is 10.5061/dryad.p0v93.

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Associate Editor: R. Brumfield
Handling Editor: J. Conner

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Supporting Information Text. Supporting methodology and results.

Appendix S1. Samples used in genetic analysis.

Appendix S2. Specimens used in plumage and morphometric analyses.

Appendix S3. Recordings used in vocal analysis.

Appendix S4. GenBank accession numbers or sample numbers of 15 additional taxa included from published studies.

Figure S1. Illustrations, phylogenies, and sampling maps of each species.

Figure S2. Sampling locality maps for spectrophotometric, morphometric and vocal analyses.

Figure S3. Plumage patches used for spectrophotometric analysis.

Figure S4. Plumage distance in tetrahedral color space versus genetic distance across the Marañon.

Figure S5. Plumage scores versus genetic (patristic) distance across the Marañon for the main study taxa and 15 additional taxa from GenBank.

Table S1. Total scores from plumage scoring analysis (main study taxa).