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Inferring speciation history in the Andes with reduced-representation sequence data: an example in the bay-backed antpittas (Aves; Grallariidae; *Grallaria hypoleuca* s. l.)

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Abstract

In the Andes, humid-forest organisms frequently exhibit pronounced genetic structure and geographic variation in phenotype, often coincident with physical barriers to dispersal. However, phylogenetic relationships of clades have often been difficult to resolve due to short internodes. Consequently, even in taxa with well-defined genetic structure, the temporal and geographic sequences of dispersal and vicariance events that led to this differentiation have remained opaque, hindering efforts to test the association between diversification and earth history and to understand the assembly of species-rich communities on Andean slopes. Here, we use mitochondrial DNA and thousands of short-read sequences generated with genotyping by sequencing (GBS) to examine the geographic history of speciation in a lineage of passerine birds found in the humid forest of the Andes, the 'bay-backed' antpitta complex (*Grallaria hypoleuca* s. l). Mitochondrial DNA genealogies documented genetic structure among clade but were poorly resolved at nodes relevant for biogeographic inference. By contrast, relationships inferred from GBS loci were highly resolved and suggested a biogeographic history in which the ancestor originated in the northern Andes and dispersed south. Our results are consistent with a scenario of vicariant speciation wherein the range of a widespread ancestor was fragmented as a result of geologic or climatic change, rather than a stepping-stone series of dispersal events across pre-existing barriers. However, our study also highlights challenges of distinguishing dispersal-mediated speciation from static vicariance. Our results further demonstrate the substantial evolutionary timescale over which the diverse biota of the Andes was assembled.

Keywords: allopatric speciation, genotyping by sequencing, introgression, leapfrog patterns, phylogenomics, tropical montane forest

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Introduction

The remarkably diverse avifauna of the humid slopes of the Andes has long been an exemplary system for investigation of the ecological processes that facilitate

high species richness along elevational gradients (Terborgh & Weske 1975; Patterson *et al.* 1998; Jankowski *et al.* 2013; Trisos *et al.* 2014). Yet the formation of new species that fuel the assembly of such species-rich communities is thought to occur via geographic isolation of populations found at similar elevations (followed by the evolution of secondary sympatry), rather than through parapatric divergence of populations along the elevational gradients on which they are apportioned today (Remsen 1984; Graves 1985; Patton & Smith 1992; Brumfield & Edwards 2007; Cadena 2007; Ribas *et al.* 2007; Guarnizo *et al.* 2009; Parra *et al.* 2009; Lavrenchenko 2011; Caro *et al.* 2013; Benham *et al.* 2014; Price *et al.* 2014; Freeman 2015). Consequently, understanding the build-up of species diversity in the Andes and other montane systems requires detailed knowledge of the geographic and temporal history of population isolation, divergence and secondary contact (Cadena 2007).

For many bird taxa of the humid forests of the tropical Andes, differentiation is thought to have occurred as a consequence of isolation across deep, broad river valleys that interrupt the continuity of the cloud forest belt (Graves 1985; Parker *et al.* 1985; Johnson 2002; Krabbe 2008; Bonaccorso 2009; Weir 2009). These valleys experience rain-shadow effects that promote the growth of arid scrub or seasonally dry tropical forest and restrict the dispersal of taxa adapted to humid forest (Weigend 2002; Killeen *et al.* 2007). The influence of these barriers on speciation in Andean birds is evident from patterns of geographic variation in phenotype, as many humid-forest taxa show pronounced differences in plumage or song on either side of arid valleys (Vuilleumier 1969; Parker *et al.* 1985; Fjeldså & Krabbe 1990; Winger & Bates 2015). However, despite the visible influence of physical dispersal barriers such as arid valleys on patterns of differentiation in Andean birds, resolving the geographic history of speciation with respect to these barriers remains difficult. Phylogeographic studies of Andean taxa have often revealed pronounced genetic structure across putative barriers, yet the phylogenetic relationships of these divergent clades are frequently poorly supported and separated by short internodes, presumably due to multiple concurrent or rapid divergence events throughout species' ranges (Pérez-Emán 2005; Miller *et al.* 2007; Gutiérrez-Pinto *et al.* 2012; Benham *et al.* 2014; Valderrama *et al.* 2014). Additionally, phenotypic and genetic breaks in Andean birds sometimes occur where no recognizable geographic barrier exists today (Graves 1982; García-Moreno *et al.* 1998, 1999; Gutiérrez-Pinto *et al.* 2012; Valderrama *et al.* 2014). Consequently, the fingerprint of differentiation is evident in many taxa, but the temporal and geographic sequence of lineage dispersal and vicariance that generated this differentiation has often remained unclear (Graves 1982; Pérez-Emán 2005; Weir *et al.* 2008; Chaves *et al.* 2011).

Further complicating historical biogeographic inference, phylogeographic studies of widespread Andean bird species have identified surprising geographic patterns of genetic relationships which suggest that, despite the linearity of geographic range in these species, neighbouring populations may not always be one another's closest relatives (Weir *et al.* 2008; Chaves & Smith 2011; Gutiérrez-Pinto *et al.* 2012). For example, Weir *et al.* (2008) found that populations of *Chlorospingus flavopectus* from the southern Andes were more closely related to populations from the Venezuelan Andes than to intervening populations. Likewise, Peruvian and Ecuadorean populations of *Basileuterus tristriatus* appeared genetically more closely related to Venezuelan populations than to neighbouring Colombian populations (Gutiérrez-Pinto *et al.* 2012). These patterns are intriguing in the light of the prevalence among Andean birds of 'leapfrog' patterns of geographic variation in plumage, wherein populations with similar plumage patterns are separated by an intervening population with a plumage pattern different than either neighbour (Chapman 1923a; Remsen 1984; Johnson 2002; Pérez-Emán 2005; Weir *et al.* 2008; Cadena *et al.* 2011). Such complex genetic and phenotypic leapfrog relationships raise the possibility that speciation in the Andes may not be as geographically simple as appears *prima facie* from species' linear, nearly one-dimensional distributions, and that idiosyncratic patterns of dispersal and extinction have been an important aspect of shaping the Andean diversity observed today. However, phylogeographic studies of humid-forest Andean organisms that have inferred genetic leapfrog patterns have been based either on single locus data (mtDNA) or on a small number of loci, and nodes suggesting leapfrog relationships have not always been well supported (e.g. Chaves & Smith 2011; Gutiérrez-Pinto *et al.* 2012). Consequently, leapfrog genetic relationships reported previously are not necessarily indicative of a complex geographic history wherein geographically adjacent populations are not sisters, but may instead be artefacts of discordance between the mitochondrial genealogy and population history (Degnan & Rosenberg 2009), or simply a lack of sufficient information to resolve the phylogeny.

Recently developed genomic methods for gathering thousands of short-read loci have proved useful for resolving phylogenies and inferring biogeographic histories (e.g. Emerson *et al.* 2010; Wagner *et al.* 2012; Eaton & Ree 2013; Harvey & Brumfield 2014; Hipp *et al.* 2014; Toews *et al.* 2016). Here, we used both Sanger sequencing of mtDNA and a reduced-representation sequencing method, genotyping by sequencing (GBS;

Davey *et al.* 2011; Elshire *et al.* 2011; Etter *et al.* 2011) to examine the phylogeographic history of a group of *Grallaria* antpittas known colloquially as the 'bay-backed' antpitta species complex (Meyer de Schauensee 1970). *Grallaria* antpittas are suboscine passerine birds with gross morphology (rotund body, long legs, short wings and tail) and behaviour (lurking, secretive habits in forest understory) that suggest low dispersal propensity, which is thought to contribute to a large number of localized, micro-endemic species throughout the Andes (Krabbe & Schulenberg 2003). The bay-backed antpitta species complex comprises a series of allopatric taxa distributed throughout mid-elevation humid cloud forest from Colombia to Bolivia. Species in this complex are distinct in plumage and voice, and have range boundaries that typically coincide with arid valleys or other geographic barriers to dispersal (Fig. 1), suggesting that these barriers influenced their differentiation (Krabbe & Schulenberg 2003).

The linear sequence of phenotypically distinct and geographically adjacent taxa in the bay-backed antpitta complex (Fig. 1) raises a classic and difficult question regarding the biogeographic history of speciation (de Queiroz 2014): Did the ancestral bay-backed antpitta disperse across existing barriers to dispersal, such that a stepping-stone series of founder events led to differentiation throughout the Andes? Or did geologic and climatic change interrupt population connectivity of a widespread ancestor (static vicariance)? South America has been a geologically and climatically dynamic region throughout the approximately 15-Myr period when much current species diversity was formed (Gregory-Wodzicki 2000; Hoorn *et al.* 2010). Such dynamism in earth history likely created many opportunities for taxa to disperse across barriers, which would lead to subsequent isolation of populations. Indeed, recent comparative phylogeographic studies of Neotropical lowland (Smith *et al.* 2014) and montane (Barber & Klicka 2010; Winger & Bates 2015) birds have demonstrated that the timing of divergence events across prominent biogeographic barriers in the tropics is often discordant among codistributed taxa, implicating a prominent role of dispersal across existing barriers in the generation of tropical avian diversity (Smith *et al.* 2014). At the same time, a dynamic period of earth history should create many opportunities for climatic and geological change to cause vicariance of widespread species after their dispersal across the landscape. Among birds, understory species with low dispersal propensity and narrow climatic niche tolerances, such as *Grallaria* antpittas, may

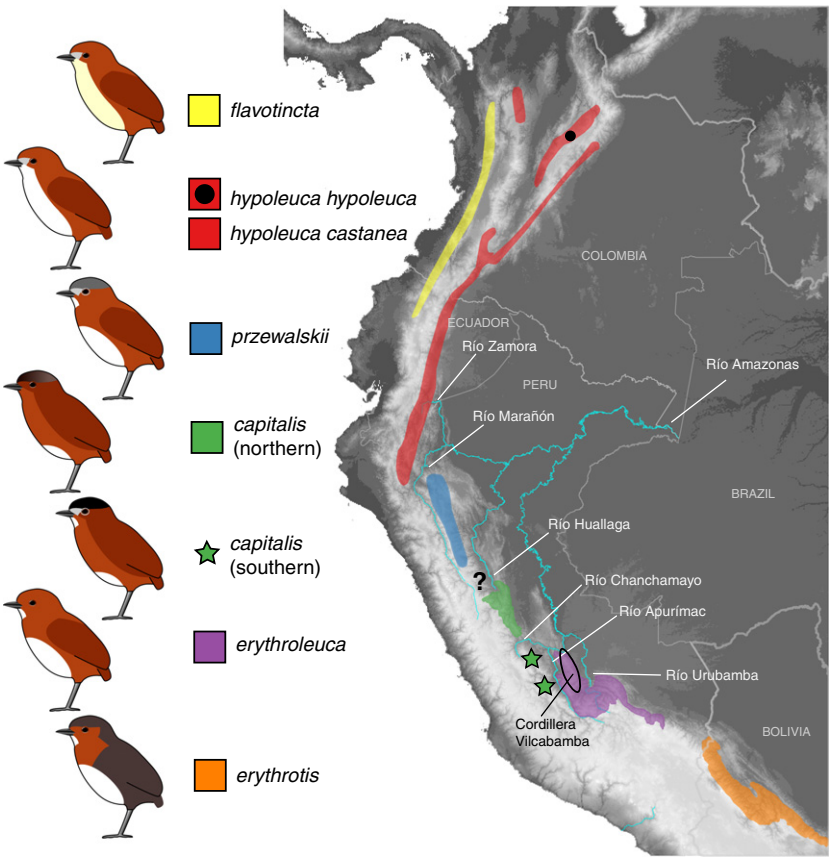

Fig. 1 The bay-backed antpitta species complex (*Grallaria hypoleuca* s. l.) traditionally contained *G. hypoleuca*, *G. flavotincta*, *G. przewalskii* and *G. erythroleuca*. Our analyses show that *G. erythrotis* is a member of the bay-backed antpitta clade, whereas *G. flavotincta* is not (Fig. 2). The southern *G. capitalis* population is a recently discovered, undescribed population distinct in plumage and song from northern *G. capitalis* (Hosner *et al.* 2015). All taxa are found in mid-elevation humid cloud forest, and the distributional limits of taxa mostly correspond to arid river valleys or other barriers (see Supporting Information). The question mark indicates a putative, but unsampled, contact zone between *G. capitalis* and *G. przewalskii*. The distributions of the closest relatives of the bay-backed antpitta clade are shown in Fig. S1 (Supporting information). Illustrations by Peter A. Hosner.

be prime candidates for taxa that have experienced geologically or climatically driven vicariance. Therefore, we use our phylogenomic results, as well as population genetic analysis of genomewide sequences, to explore the evidence for vicariance of a widespread ancestor, vs. serial dispersal events, in the differentiation of the bay-backed antpitta complex.

Methods

The bay-backed antpitta complex

Currently, five allopatric species are considered part of the bay-backed antpitta species complex based on phenotype and geographic distribution (Lowery & O'Neill 1969; Parker & O'Neill 1980; Krabbe & Schulenberg 2003): *Grallaria hypoleuca*, *G. flavotincta*, *G. przewalskii*, *G. capitalis* and *G. erythroleuca*. Meyer de Schauensee (1970) treated these taxa as conspecific, as Bay-backed Antpitta (*G. hypoleuca*). Other authors have considered the taxon *flavotincta*, which is found on the west slope of the Andes in Colombia and Ecuador, to be a subspecies of *G. hypoleuca* to the exclusion of the other species (e.g. Peters 1951; Meyer de Schauensee 1964). However, recent taxonomies treat *G. flavotincta*, *G. hypoleuca*, *G. przewalskii*, *G. capitalis* and *G. erythroleuca* as separate species and recognize *G. hypoleuca* as containing two subspecies, *G. h. hypoleuca* and *G. h. castanea* (e.g. Krabbe & Schulenberg 2003; Remsen *et al.* 2015). Recent fieldwork has revealed a population of *Grallaria* most similar to *G. capitalis*, but with differences in plumage and voice, filling a perceived distribution gap between *G. capitalis* and *G. erythroleuca* (Hosner *et al.* 2015; Supporting Information). We refer to this undescribed, southern population as 'southern *G. capitalis*', and the northern form as 'northern *G. capitalis*' (taxonomic treatment of these forms is not addressed here). Found to the south of the bay-backed antpitta complex in Bolivia is *G. erythrotis* (Fig. 1), which exhibits similarities in plumage and voice (Ridgely & Tudor 2009), but has not previously been considered part of this species complex. The boundaries between most bay-backed antpitta taxa correspond to arid river valleys or other barriers (Fig. 1); we describe these distributions in detail in the Supporting Information and Discussion.

Sampling

We sampled multiple individuals of each species in the bay-backed antpitta species complex, as well as putative close relatives (Table 1). Our sampling included one individual of *G. hypoleuca hypoleuca*, two of *G. flavotincta* and multiple individuals of *G. hypoleuca castanea*, *G. przewalskii*, northern and southern *G. capitalis*, and *G. erythroleuca*. To confirm the monophyly of the bay-backed antpitta species complex, we also included samples of several species that an ongoing comprehensive phylogenetic study of the Grallariidae (Bravo, G. A., Cuervo, A. M., Aristizábal, N., Rice. N., Carneiro, L., Aleixo, A., Pérez, J., Brumfield, R. T. & Bates, J. M., unpublished data) identified as closely related to bay-backed complex: one individual of *G. erythrotis*, one individual from each of two subspecies of *G. quitensis* (found parapatric to *G. hypoleuca* and *G. przewalskii* on the same montane slopes, but at higher elevation above treeline; Fig. S1, Supporting information), two individuals of *G. ruficapilla* (a species with a similar song to members of the bay-backed species complex, and broadly sympatric with *G. hypoleuca* and *G. przewalskii*; Fig. S1, Supporting information) and one individual of *G. milleri* (a Colombian endemic; Fig. S1, Supporting information). We also included samples of *Grallaria albigula*, an antpitta of the Andes of Peru, Bolivia and Argentina that Chapman (1923b) proposed as a close relative to *G. hypoleuca* due to plumage similarities. One sequence of *Chamaeza campanisona*, an antthrush in the suboscine family Formicariidae, was downloaded from GenBank and used as an out-group in certain mtDNA analyses.

DNA extraction

For 56 of 59 individuals sequenced for this study, genomic DNA was extracted from muscle tissue samples with an associated voucher skin or skeleton. Four samples were derived from toepads of museum specimens because no fresh samples were available (two samples of *G. flavotincta*, one sample of *G. hypoleuca hypoleuca* and one sample of *G. quitensis alticola*). DNA was extracted using standard protocols described in Winger & Bates (2015), with the addition of an extended lysis time in DTT for the toepad samples.

mtDNA sequencing

We previously sequenced the mitochondrial gene NADH dehydrogenase 2 (ND2) from 34 individuals of the bay-backed antpitta complex for a comparative phylogeographic study of Andean birds (Winger & Bates 2015). Here, we sequenced ND2 from an additional 25 individuals, for a total of 59 individuals. We also sequenced the cytochrome-*b* (cyt *b*) gene from 44 individuals of several bay-backed antpitta taxa (Table 1). We sequenced mtDNA using standard PCR and Sanger sequencing protocols described elsewhere (Brumfield *et al.* 2007; Winger & Bates 2015). Contigs were assembled in GENEIOUS v 7.1 (Biomatters, Auckland, New Zealand) and aligned using MUSCLE (Edgar 2004) called from within GENEIOUS.

Table 1 Samples used in each of three genetic data sets [NADH dehydrogenase 2 (ND2): $n = 60$, cyt *b*: $n = 44$ and genotyping by sequencing (GBS): $n = 22$]

Genus	Species	Sample	Country	Department	Locality	ND2	Cyt *b*	GBS	GenBank ND2	GenBank cyt *b*	SRA accession
Chamaeza	*campanisona*	UWBM KGB14				√	√	–	EF640009	EF639942	–
Grallaria	*albigula*	KU 21470	Peru	Puno	Above San Juan del Oro	√	√	–	KU052287	KU052284	–
Grallaria	*albigula*	KU 21616	Peru	Puno	Above San Juan del Oro	√	√	–	KU052288	KU052283	–
Grallaria	*albigula*	KU 21640	Peru	Puno	Above San Juan del Oro	√	√	–	KU052289	KU052282	–
Grallaria	*albigula*	KU 21690	Peru	Puno	Above San Juan del Oro	√	√	–	KU052290	KU052285	–
Grallaria	*albigula*	KU 9889	Argentina	Jujuy	2 km E Ocloyas	√	√	–	KU052286	KU052281	–
Grallaria	*albigula*	LSUMZ B-570	Peru	Puno	Abra de Maruncunca, 10 km SW San Juan del Oro	√	–	–	KU052291	–	–
Grallaria	*capitalis*	KU 14680	Peru	Junín	Along Río Satipo	√	–	√	KU052302	–	SRS1154433
Grallaria	*capitalis*	KU 14681	Peru	Junín	Along Río Satipo	√	√	√	KU052303	KU052267	SRS1154432
Grallaria	*capitalis*	KU 14697	Peru	Junín	along Río Satipo	√	–	√	KU052304	–	SRS1154442
Grallaria	*capitalis*	KU 14725	Peru	Junín	Along Río Satipo	√	√	–	KU052305	KU052268	–
Grallaria	*capitalis*	KU 14752	Peru	Junín	Along Río Satipo	√	√	–	KU052309	KU052265	–
Grallaria	*capitalis*	KU 14791	Peru	Junín	Along Río Satipo	√	√	–	KU052306	KU052271	–
Grallaria	*capitalis*	KU 14811	Peru	Junín	Along Río Satipo	√	√	–	KU052307	KU052270	–
Grallaria	*capitalis*	KU 14814	Peru	Junín	Along Río Satipo	√	√	–	KU052308	KU052272	–
Grallaria	*capitalis*	KU 16698	Peru	Ayacucho	Tutumbaro	√	√	–	KU052300	KU052266	–
Grallaria	*capitalis*	KU 16717	Peru	Ayacucho	Tutumbaro	√	√	–	KU052301	KU052269	–
Grallaria	*capitalis*	LSUMZ B-1849	Peru	Pasco	Cumbre de Ollon, about 12 km E Oxapampa	√	√	–	KP277611	KU052278	–
Grallaria	*capitalis*	LSUMZ B-1938	Peru	Pasco	Cumbre de Ollon, about 12 km E Oxapampa	√	√	√	KP277610	KU052279	SRS1154443
Grallaria	*capitalis*	LSUMZ B-1966	Peru	Pasco	Cumbre de Ollon, about 12 km E Oxapampa	√	√	–	KP277991	KU052277	–
Grallaria	*capitalis*	LSUMZ B-3570	Peru	Huánuco	Base of Bosque Zapatagocha above NE Acomayo	√	√	–	KP277613	KU052275	–
Grallaria	*capitalis*	LSUMZ B-3577	Peru	Huánuco	Base of Bosque Zapatagocha above NE Acomayo	√	√	√	KP277612	KU052276	SRS1154541
Grallaria	*capitalis*	LSUMZ B-7988	Peru	Pasco	Playa Pampa, 8 km NW Cushi on trail to Chaglla	√	√	–	KP277615	KU052274	–
Grallaria	*capitalis*	LSUMZ B-8057	Peru	Pasco	Playa Pampa, 8 km NW Cushi on trail to Chaglla	√	√	–	KP277609	KU052280	–
Grallaria	*capitalis*	LSUMZ B-8119	Peru	Pasco	Playa Pampa, 8 km NW Cushi on trail to Chaglla	√	√	√	KP277614	KU052273	SRS1154542
Grallaria	*erythrotis*	LSUMZ B-68092	Bolivia	Cochabamba	Prov. Scaba; Tablas Monte	√	–	√	KU052310	–	SRS1158404
Grallaria	*erythroleuca*	FMNH 390684	Peru	Junín	Cordillera Vilcabamba, headwaters Río Poyeni	√	√	–	KP277596	KU052252	–
Grallaria	*erythroleuca*	FMNH 390685	Peru	Junín		√	√	√	KP277597	KU052253	SRS1154544

Table 1 *Continued*

Genus	Species	Sample	Country	Department	Locality	ND2	Cyt *b*	GBS	GenBank ND2	GenBank cyt *b*	SRA accession
					Cordillera Vilcabamba, headwaters Río Poyeni						
Grallaria	*erythroleuca*	FMNH 390686	Peru	Junín	Cordillera Vilcabamba, headwaters Río Poyeni	√	√	√	KP277598	KU052254	SRS1154545
Grallaria	*erythroleuca*	MSB 34488	Peru	Cuzco	Abra Bella Vista	√	√	–	KP277595	KU052250	
Grallaria	*erythroleuca*	MSB 34489	Peru	Cuzco	Abra Bella Vista	√	√	√	KP277594	KU052251	SRS1154546
Grallaria	*flavotincta*	FMNH 251116	Colombia	Nariño	Ricaurte	√	–	–	KU052294	–	–
Grallaria	*flavotincta*	FMNH 251117	Colombia	Nariño	Ricaurte	√	–	–	KU052295	–	–
Grallaria	*hypoleuca castanea*	ANSP 19159	Ecuador	Zamora-Chinchipe	Panguri; ~12 km NE San Francisco del Vergel	√	–	–	KP277988	–	–
Grallaria	*hypoleuca castanea*	ANSP 19413	Ecuador	Napo	12 km NNE el Chaco; Mirador	√	–	√	KP277989	–	SRS1154557
Grallaria	*hypoleuca castanea*	FMNH 480827	Peru	Cajamarca	3.5 km W of Pueblo Libre	√	–	–	KP277990	–	–
Grallaria	*hypoleuca castanea*	LSUMZ B-33029	Peru	Cajamarca	~3 km NNE San Jose de Lourdes	√	√	√	KP277588	KU052247	SRS1154583
Grallaria	*hypoleuca castanea*	LSUMZ B-33061	Peru	Cajamarca	~3 km NNE San Jose de Lourdes	√	√	–	KP277593	KU052243	–
Grallaria	*hypoleuca castanea*	LSUMZ B-33139	Peru	Cajamarca	~3 km NNE San Jose de Lourdes	√	√	√	KP277589	KU052244	SRS1154584
Grallaria	*hypoleuca castanea*	LSUMZ B-33185	Peru	Cajamarca	~3 km NNE San Jose de Lourdes	√	√	–	KP277590	KU052242	–
Grallaria	*hypoleuca castanea*	LSUMZ B-34814	Peru	Cajamarca	Cordillera del Condor, Picorana	√	√	–	KP277592	KU052248	–
Grallaria	*hypoleuca castanea*	LSUMZ B-34833	Peru	Cajamarca	Cordillera del Condor, Picorana	√	√	√	KP277591	KU052249	SRS1154585
Grallaria	*hypoleuca castanea*	LSUMZ B-6188	Ecuador	Morona-Santiago	W slope Cordillera del Cutucú	√	√	–	KP297415	KU052245	–
Grallaria	*hypoleuca castanea*	LSUMZ B-6197	Ecuador	Morona-Santiago	W slope Cordillera del Cutucú	√	√	–	KP297414	KU052246	–
Grallaria	*hypoleuca hypoleuca*	ICN 35552	Colombia	Santander	San Isidro	√	–	√	KU052299	–	SRS1154586
Grallaria	*milleri*	IAvH-BT 4622	Colombia	Caldas	Pensilvania	√	–	√	KU052296	–	SRS1158389
Grallaria	*przewalskii*	LSUMZ B-43719	Peru	San Martín	~24 km ENE Florida	√	√	√	KP277603	KU052258	SRS1154587
Grallaria	*przewalskii*	LSUMZ B-44016	Peru	San Martín	~24 km ENE Florida	√	√	–	KP277601	KU052263	–
Grallaria	*przewalskii*	LSUMZ B-44017	Peru	San Martín	~24 km ENE Florida	√	√	–	KP277608	KU052260	–
Grallaria	*przewalskii*	LSUMZ B-44181	Peru	San Martín	~22 km ENE Florida	√	√	–	KP277599	KU052261	–
Grallaria	*przewalskii*	LSUMZ B-44369	Peru	San Martín	~22 km ENE Florida	√	√	–	KP277606	KU052262	–
Grallaria	*przewalskii*	LSUMZ B-44467	Peru	San Martín	~22 km ENE Florida	√	√	–	KP277602	KU052264	–
Grallaria	*przewalskii*	LSUMZ B-44536	Peru	San Martín	~22 km ENE Florida	√	√	–	KP277600	KU052255	–
Grallaria	*przewalskii*	LSUMZ B-5626	Peru	Amazonas	~30 km by road E Florida on road to Ríoja	√	√	–	KP277604	KU052259	–

Table 1 *Continued*

Genus	Species	Sample	Country	Department	Locality	ND2	Cyt *b*	GBS	GenBank ND2	GenBank cyt *b*	SRA accession
Grallaria	*przewalskii*	MSB 32034	Peru	Amazonas	4.5 km N Tullanya	√	√	√	KP277607	KU052257	SRS1154588
Grallaria	*przewalskii*	MSB 32286	Peru	Amazonas	4.5 km N Tullanya	√	√	√	KP277605	KU052256	SRS1154589
Grallaria	*quitensis alticola*	ICN 33381	Colombia	Cundinamarca	Boca Grande	√	–	√	KU052298	–	SRS1154590
Grallaria	*quitensis quitensis*	LSUMZ B-30042	Ecuador	Napo	5 km W Papallacta	√	–	√	KU052297	–	SRS1154591
Grallaria	*ruficapilla*	IAvH-BT 8598	Colombia	Cesar	Manaure, Serrania del Perijá	√	–	√	KU052292	–	SRS1154592
Grallaria	*ruficapilla*	LSUMZ B-31722	Peru	Cajamarca	Quebrada Lanchal, ~8 km ESE Salique	√	–	–	KU052293	–	–

ANSP, Academy of Natural Sciences of Drexel University; FMNH, Field Museum of Natural History; IAvH, Instituto Alexander von Humboldt, Colombia; ICN, Instituto de Ciencias Naturales, Universidad Nacional de Colombia; KU, Kansas University Biodiversity Institute and Natural History Museum; LSUMZ, Louisiana State University Museum of Natural Science; MSB, Museum of Southwestern Biology, University of New Mexico; UWBM, University of Washington Burke Museum. Samples with GenBank Accession numbers with prefix 'KP' are from Winger & Bates (2015).

Genotyping by sequencing

We employed GBS (Elshire *et al.* 2011; Etter *et al.* 2011) to gather genomewide data from many thousands of loci. GBS uses a restriction enzyme (in this case, *Pst*I) to cut fragments from throughout the genome. We contracted the Cornell University Institute for Genomic Diversity to prepare GBS libraries and sequence 100-base pair reads on a HiSeq 2000 (Illumina, San Diego, CA, USA) at Cornell's Biotechnology Resource Center. Guided by the results of our phylogenetic analyses of mtDNA, we choose a subset of 22 samples for GBS representing each bay-backed antpitta taxon and their close relatives (Table 1). Samples were sequenced across two lanes, each containing 95 uniquely barcoded and multiplexed samples for a larger project. The two toepad samples of *G. hypoleuca hypoleuca* and *G. quitensis alticola* were from relatively recent museum specimens (1999 and 2005, respectively) and yielded adequate DNA concentrations for GBS sequencing without enrichment. The DNA extracts from toepad samples of *G. flavotincta* were not adequate for GBS and were not included.

GBS bioinformatics and data assembly

We prepared raw reads (Illumina FASTQ files) for analyses using the software pipeline PYRAD v2.1-3.0 (Eaton 2014). PYRAD is appropriate for assembling short-read loci for species-level phylogenetic analysis, in part because it uses a clustering algorithm that allows for variation in indels, thus improving the clustering of homologous loci across divergent samples (Eaton & Ree 2013; Eaton 2014). We employed PYRAD's reverse-complement clustering method to detect and remove duplicated sequences that resulted from the overlap of very short fragments generated from GBS. When demultiplexing samples, we allowed for one base pair sequencing error in the barcode (barcodes were 5–10 base pairs). We trimmed the restriction-site and barcodes and discarded reads that contained greater than five sites with a Phred score of <20. We retained trimmed reads 70 bp or greater in length and allowed for overhanging ends in clusters containing reads of different lengths. PYRAD requires the designation of a single similarity threshold for within-sample clustering of reads to create consensus loci, as well as across-sample clustering of loci. We chose a similarity threshold of 85%, which is appropriate for analysis of species-level phylogenetic analyses on the timescale of our study (Rubin *et al.* 2012; Eaton & Ree 2013; Escudero *et al.* 2014; Hipp *et al.* 2014; Eaton *et al.* 2015). We filtered clusters by discarding those that contained a depth of coverage of

<10 reads, or more than three heterozygous sites (in exploratory phylogenetic analyses, we found consistent results when relaxing the restriction on depth of coverage to allow loci with five or more reads; Supporting Information). We discarded consensus sequences with more than four undetermined sites (Eaton & Ree 2013) and discarded loci that were heterozygous at a site across more than three samples, because loci appearing heterozygous across multiple samples may indicate clustering of paralogs with fixed differences (Eaton 2014). We then used PYRAD to assemble sequence alignments that ranged from sparse matrices with complete sampling of individuals to alignments with a reduced number of individuals but more complete coverage. The purposes of these various alignments were (i) to explore the influence of the number of loci and the sparseness of the alignment on our results, (ii) to exclude low-coverage samples from certain analyses that required more complete alignments and (iii) to produce data sets tailored to provide maximum data for different analyses.

Phylogenetic analyses

We built phylogenies with the ND2 alignment of 60 individuals and with a concatenated alignment of ND2 and cyt *b* from 34 individuals. The concatenated ND2 + cyt *b* phylogenies were topologically consistent with the ND2 phylogenies but had lower taxonomic coverage, so we do not discuss cyt *b* results further. We also built phylogenies from GBS data using three concatenated 'supermatrix' alignments. First, we used an alignment containing all 22 individuals for which we had GBS data, retaining loci that were present in a minimum of eight individuals, and designated *G. ruficapilla* as an out-group. This initial alignment enabled us to include several samples with lower numbers of loci while still retaining data from higher quality samples (Table 2). The remaining two alignments contained fewer representative samples but more complete coverage (Appendix S1 and Table S1, Supporting information). As described in detail in the Supporting Information, we built phylogenies using maximum-like-

Table 2 Quantity of raw reads, filtered reads, PYRAD clusters and filtered loci for all 22 *Grallaria* samples sequenced with genotyping by sequencing (GBS), and depth of coverage

Grallaria Species	Sample	GBS raw reads	Filtered reads	Total clusters	Clusters with mean depth of coverage ≥10	Mean depth of loci with depth of coverage ≥10	Consensus loci
capitalis	LSUMZ B-1938	425 637	108 217	40 595	642	23.9	402
capitalis	**LSUMZ B-3577**	**1 828 802**	**590 984**	**128 593**	**11 450**	**18.0**	**10 280**
capitalis	**KU 14680**	**2 328 813**	**834 153**	**143 691**	**21 468**	**17.5**	**19 462**
capitalis	**KU 14697**	**2 763 255**	**1 065 767**	**187 573**	**25 294**	**17.9**	**22 978**
capitalis*	**LSUMZ B-8119**	**3 507 765**	**1 192 599**	**167 024**	**34 184**	**19.5**	**31 162**
capitalis*	**KU 14681**	**4 938 498**	**1 761 662**	**200 917**	**47 295**	**23.0**	**42 808**
erythroleuca	**FMNH 390685**	**3 089 357**	**1 208 856**	**196 287**	**32 005**	**18.4**	**29 271**
erythroleuca	**MSB 34489**	**3 144 326**	**1 257 548**	**192 095**	**33 110**	**18.8**	**30 265**
erythroleuca*	**FMNH 390686**	**3 735 297**	**1 490 337**	**219 624**	**39 737**	**19.7**	**36 387**
erythrotis*	**LSUMZ B-68092**	**2 958 681**	**1 132 242**	**175 249**	**32 699**	**18.3**	**29 798**
hypoleuca	ANSP 19413	424 338	53 215	21 326	258	39.1	133
hypoleuca	LSUMZ B-34833	920 547	291 225	79 388	2521	22.6	2059
hypoleuca	**ICN 35552**	**1 434 207**	**437 690**	**108 001**	**6330**	**18.3**	**5516**
hypoleuca	**LSUMZ B-33139**	**1 453 320**	**541 142**	**120 393**	**8646**	**18.1**	**7680**
hypoleuca*	**LSUMZ B-33029**	**1 767 785**	**609 556**	**134 736**	**12 059**	**17.2**	**10 698**
milleri	IAvH-BT 4622	3 619 617	1 244 237	160 008	39 441	19.8	35 763
przewalskii	**MSB 32286**	**1 999 697**	**801 213**	**158 993**	**17 175**	**16.5**	**15 607**
przewalskii	**LSUMZ B-43719**	**2 072 212**	**704 493**	**133 134**	**18 904**	**16.5**	**17 138**
przewalskii*	**MSB 32034**	**4 820 516**	**1 812 292**	**197 943**	**50 483**	**23.2**	**46 043**
quitensis	ICN 33381	381 497	81 312	36 446	240	33.3	110
quitensis*	**LSUMZ B-30042**	**1 728 938**	**619 769**	**123 577**	**15 920**	**15.4**	**14 385**
ruficapilla	IAvH-BT 8598	3 207 953	1 223 754	175 012	31 320	20.5	28 498
	Mean	2 388 684	53 125	140 937	21 872	20.7	19 838
	Range	381 497–4 938 498	53 125–1 812 292	21 326–219 624	240–50 483	15.406–39.074	110–46 043

The samples in bold were included in the 16 sample alignment (Table S1, Fig. S2A, Supporting information) for phylogenetic analysis, and the samples in bold with an asterisk were included in the seven sample alignment (Table S1, Fig. S2B, Supporting information).

lihood (ML) analyses of all alignments in RAXML v8 (Stamatakis 2014), Bayesian analyses of mtDNA alignments in MRBAYES v3.2 (Ronquist *et al.* 2012) and Bayesian analysis of GBS alignments in EXABAYES v1.4.1 (Aberer *et al.* 2014).

We also inferred the species tree of the bay-backed antpitta complex from individual SNPs using SNAPP v1.1.10 (Bryant *et al.* 2012; Bouckaert *et al.* 2014). We used PYRAD to select a single biallelic SNP from each GBS locus, to reduce potential linkage among SNPs. If multiple SNPs are present in a locus, then PYRAD searches for the SNP with the least amount of missing data across individuals and chooses randomly if multiple SNPs have identical coverage. We used the R package phrynomics (http://github.com/bbanbury/phrynomics) to remove nonbinary SNPs, to code heterozygotes and to format input files for SNAPP. To reduce the amount of missing data for SNAPP analysis, we constructed an alignment from the representative samples of each taxon in the bay-backed antpitta complex that contained the largest number of loci (Table 2). This included *G. hypoleuca castanea* ($n = 2$), *G. przewalskii* ($n = 3$), northern *G. capitalis* ($n = 2$), southern *G. capitalis* ($n = 3$), *G. erythroleuca* ($n = 3$) and *G. erythrotis* ($n = 1$). This alignment contained 1767 biallelic SNPs with data for all 14 samples. We ran the analysis for two independent runs of 1 million generations each, sampling every 1000 generations and discarding 10% of each run as burn-in. We assessed convergence in TRACER v1.6 (Rambaut *et al.* 2014), combined independent runs with LOGCOMBINER (Bouckaert *et al.* 2014) and used TREEANNOTATOR (Drummond & Rambaut 2007) and DENSITREE (Bouckaert 2010) to view trees and determine posterior probabilities.

Molecular dating

As because rates of molecular evolution of GBS loci are poorly understood, we assessed divergence times of mtDNA gene trees in BEAST v1.8.2 (Drummond & Rambaut 2007). We chose a representative ND2 sequence from each bay-backed antpitta taxon and three outgroups (as indicated by the mtDNA and GBS phylogenetic results). We used a relaxed molecular clock with a mean rate equal to a widely used mitochondrial DNA substitution rate of 2.1%/Myr (Weir & Schluter 2008), after testing whether this rate adequately represented molecular evolution in ND2 (Supporting Information). We ran BEAST analyses in which we constrained the mtDNA topology to correspond to the evolutionary relationships indicated by the highly resolved GBS topology and in which species relationships were unconstrained. Additional details of the BEAST analysis are described in the Supporting Information.

Genetic diversity

We compared genetic diversity of bay-backed antpitta populations to assist in our inference of the geographic history of the lineage. Using the ND2 data set, we calculated haplotype and nucleotide diversity in DNASP v5 (Librado & Rozas 2009). For GBS data, we calculated SNP nucleotide diversity in VCFTOOLS (Danecek *et al.* 2011). In exploratory analyses, we found that estimates of nucleotide diversity from GBS loci were sensitive to the number of shared loci, population sample sizes, and how taxa were defined geographically. Therefore, we repeated nucleotide diversity estimates across four alignments that differed in these aspects (described in Supporting Information). For each alignment, we calculated the mean of per-site nucleotide diversity for each taxon and used the R package BOOT (Canty & Ripley 2014) to generate confidence intervals of these means with 1000 bootstrap replicates, following Lozier (2014).

Genetic differentiation

We assessed genetic differentiation among taxa in the bay-backed antpitta clade through pairwise F_{st} calculations of mtDNA (ND2) in ARLEQUIN v3.5 (Excoffier & Lischer 2010), and from GBS loci with Weir & Cockerham's (1984) weighted method implemented in VCFTOOLS (Danecek *et al.* 2011). For F_{st} calculations of GBS data, we used the 162-locus, 14-individual alignment that we describe in the Supporting Information for nucleotide diversity calculations. We used VCFTOOLS to calculate weighted F_{st} across the entire alignment (window) for every pairwise comparison (Bhatia *et al.* 2013).

Introgression

We tested for introgression between geographically adjacent populations of the bay-backed antpitta complex using the *D* statistic, or 'ABBA/BABA', tests (Durand *et al.* 2011; Eaton & Ree 2013) implemented in PYRAD (Eaton 2014). The purpose of these tests is to separate a signal of introgression from shared ancestral polymorphisms. Given a pectinate, four-taxon phylogeny [((P1, P2), P3), O], the *D* statistic identifies introgression between populations P3 and either P2 or P1, by assessing the proportion of sites in the genome with the allele patterns ABBA or BABA in populations P1, P2, P3 and O. Approximately equal numbers of ABBA or BABA are expected as a consequence of incomplete lineage sorting. In contrast, if introgression occurred between P3 and P2, an excess of ABBA sites are expected (representing introgression of the derived B allele), and if introgression occurred between P3 and P1, an excess of BABA sites are expected. The *D* statistic measures the imbalance of

ABBA and BABA sites. By assigning different taxa or populations as P1, P2, P3 and O, we designed a series of four-taxon tests to examine introgression between species and across geographic barriers (Table 3). In all tests, P1 must be differentiated from P2, but must be more closely related to P2 than either is to P3. O must be an out-group to these taxa. In each test, we designated populations on either side of a geographic barrier as P2 and P3, and tested for introgression between P3 and P2 by noting an excess of ABBA sites. By reconfiguring these assignments, we designed different four-taxon schemes, where possible, to increase the number of tests (Table 3). It was not possible to test for introgression across the Chanchamayo Valley between northern and southern forms of *G. capitalis*, because this test would require substantial population structure within either or both of the *G. capitalis* forms in order to designate a P1 population more closely related to P2 than P3. We chose the most closely related out-group (as indicated by GBS phylogenies) for each test, to capture the most shared loci as possible among the four taxa. As each population contained multiple samples, we designed four-taxon tests for every combination of individuals from each population. For all tests, we used an alignment of 16 high-quality samples used for phylogenetic analyses (Table S1, Supporting information), but retained loci shared across a minimum of four samples, to provide more loci for the four-taxon test. To assess significance of introgression, we used PYRAD to run 1000 bootstraps, which produced a *Z* score for the *D* statistic of each test. We converted *Z* scores to *P*-values, correcting for multiple comparisons using the Holm correction, following Eaton & Ree (2013).

Results

mtDNA phylogeny

Phylogenies of mtDNA indicated that *Grallaria erythrotis* is sister to *G. erythroleuca*, and thus should be considered a member of this the bay-backed antpitta species complex (Fig. 2A). This relationship was not surprising given similarities in geographic and elevational distribution, plumage and song (Krabbe & Schulenberg 2003), but a sister relationship had not previously been suggested. By contrast, we found that *G. flavotincta*, which has previously been classified as a subspecies of *G. hypoleuca*, is most closely related to *G. milleri* and therefore is outside the bay-backed complex. Within the redefined bay-backed complex, three main clades were evident: a clade formed by *G. hypoleuca*, a clade containing *G. erythroleuca* and *G. erythrotis*, and a clade containing *G. przewalskii* and *G. capitalis*. However, the relationships among these clades were poorly supported. In particular, the clade containing *G. erythroleuca* and *G. erythrotis* was recovered as sister to *G. hypoleuca*, but with weak support (Fig. 2A). *G. hypoleuca* is found on the opposite end of the geographic range of the species complex as *G. erythroleuca* and *G. erythrotis*; thus, this relationship would constitute a genetic leapfrog pattern.

Grallaria quitensis quitensis was identified as the closest out-group to the bay-backed antpittas. However, the sample of *G. quitensis alticola* was recovered as sister to *G. hypoleuca* (Fig. 2A), rather than to *G. q. quitensis*, with low support. Although subspecies of *G. quitensis* differ from one another in morphology and voice (Krabbe & Schulenberg 2003), we consider a paraphyletic *G. quitensis* suspect (and the result was not supported by GBS data, below). The mtDNA phylogenies also indicated that *G. milleri* (and its closest relative, *G. flavotincta*) and *G. ruficapilla* are sisters to the bay-backed antpittas, but that *G. albigula* is only distantly related and likely a member of a different *Grallaria* clade (not shown).

The mtDNA gene tree also revealed intraspecific structure corresponding to geographic barriers. For example, we recovered a phylogeographic break between the northern and southern forms of *G. capitalis* (Hosner *et al.* 2015) across the Chanchamayo Valley (Figs 1 and 2). Additionally, *G. erythroleuca* shows a shallow division between the main Andean Cordillera and the isolated northern Cordillera Vilcabamba (Fig. 2A). The Vilcabamba population of *G. erythroleuca* has previously been suggested to represent an undescribed subspecies based on voice, plumage and morphology (Schulenberg & Servat 2001; Krabbe & Schulenberg 2003; Schulenberg & Kirwan 2012a). *G. hypoleuca* has a shallow phylogenetic break across the Zamora Valley in Ecuador (Figs 1 and 2), which has been noted as a dispersal barrier for cloud forest birds (Robbins *et al.* 1994; Krabbe 2008; Bonaccorso 2009).

GBS metrics

GBS produced an average of 2 388 685 raw reads per sample, of which 36.3% passed filtering for quality and presence of adapters in the sequence (Table 2). PYRAD recovered an average of 140 937 clusters per individual, but 84.5% of these had a stack depth of less than the minimum of 10 reads we required for loci used in downstream analyses. After removing low-coverage clusters, our data set included an average of 21 872 consensus loci per individual (Table 2). We obtained consistent phylogenetic results using a data set in which stack depth was relaxed to five or greater (Supporting Information).

GBS phylogeny

In contrast to mtDNA genealogies, phylogenies produced from the three GBS alignments were well resolved and were highly supported at all nodes. Topologies were

Table 3 Results of the four-taxon *D* tests for introgression

Barrier	P1	P2	P3	O	Number of tests	Average shared loci	Average ABBA	Average BABA	Proportion significant tests ($P < 0.05$)	Total proportion significant tests for barrier ($P < 0.05$)
Trans-Marañón 1	*hypoleuca hypoleuca*	*hypoleuca castanea*	*przewalskii*	*quitensis*	0	–	–	–	0.00	
Trans-Marañón 2	*capitalis*	*przewalksii*	*hypoleuca*	*quitensis*	41	1582.5	48.2	35	0.00	Marañón: 0
Trans-Huallaga	*capitalis* (southern)	*capitalis* (northern)	*przewalskii*	*erythroleuca* + *erythrotis*	39	6867.8	37	45.5	0.03	Huallaga: 0.03
Trans-Apurimac 1	*przewalskii*	*capitalis* (all)	*erythroleuca*	*hypoleuca*	134	2272.8	98.8	62.8	0.16	
Trans-Apurimac 2	*erythrotis*	*erythroleuca*	*capitalis* (all)	*hypoleuca*	44	2698	44	51.8	0.00	Apurímac: 0.10
Unknown barrier Southern Peru	*erythroleuca* (Vilcabamba)	*erythroleuca* (main Cordillera)	*erythrotis*	*capitalis* + *przewalskii*	15	7911.3	55	38.3	0.00	S. Peru: 0

All tests examine introgression from taxon P3 into taxon P2. Therefore, the trans-Marañón tests examine introgression between *Grallaria hypoleuca* and *G. przewalskii*, the trans-Huallaga tests examine introgression between *G. capitalis* and *G. przewalskii* (whose distributional gap or contact zone apparently lies north of the Huallaga Valley), and the trans-Apurímac tests examine introgression between *G. erythroleuca* and *capitalis*. The tests in the bottom row examine introgression between *G. erythrotis* and *G. erythroleuca*, whose barrier or contact zone is unknown (see Discussion). The tests require that the taxa P1, P2, P3 and O are related to one another according to a pectinate phylogeny [((P1, P2), P3), O]. Additionally, P1 and P2 must be somewhat differentiated from one another in order for ABBA or BABA sites to exist. This design allows two different testing schemes to examine introgression across the Marañón and Apurímac valleys. However, only one testing scheme was possible for the Huallaga Valley and the gap on the Peru–Bolivia border, as alternative testing schemes would require intrapopulation genetic structure (between a putative P1 and P2) that does not exist or was unsampled. Likewise, this constraint precluded testing of introgression between the northern and southern forms of *G. capitalis* across the Chanchamayo Valley (see text). Average shared loci are the average number of loci shared across all tests in each four-taxon arrangement (tests with <100 shared loci were excluded). The average ABBA and BABA sites are the average number of sites whose SNPs conform to the pattern ABBA or BABA across ordered samples P1, P2, P3 and O (tests where the combined number of ABBA and BABA sites was <50 were excluded). Tests were assessed for significance using bootstrapping and correction for multiple tests (see text). Number of tests refers to the number of unique four-taxon tests that follow each P1, P2, P3 and O arrangement, which depends on the number of individuals available from each of the four taxa (i.e. the more individuals sampled for a given population, the more unique arrangements are possible for a given test). The test in the first row, Trans-Marañón 1, did not have any tests that passed the filter steps of 50 combined ABBA/BABA sites (see text).

consistent across the three GBS alignments (Figs 2B and S2, Supporting information), and ML and Bayesian analyses produced identical topologies for each alignment. Therefore, we present the ML trees here, with posterior probability support from Bayesian analyses illustrated at key nodes (Figs 2B and S2, Supporting information).

The GBS phylogenies were consistent with mtDNA in recovering *G. erythrotis* as sister to *G. erythroleuca* in a well-supported clade (Figs 2B and S2, Supporting information). However, in contrast to the mtDNA gene tree, in which the placement of *G. hypoleuca* and the clade containing *G. erythroleuca* and *G. erythrotis* was uncertain, analyses of concatenated GBS loci recovered *G. hypoleuca* as sister to the remaining members of the bay-backed antpitta species complex with strong support (Figs 2B and S2, Supporting information). GBS phylogenies provide further support that *G. quitensis* is the closest out-group to the bay-backed antpitta species complex, and *G. milleri* is outside this clade. Unlike the mtDNA analyses, phylogenetic analysis of GBS loci recovered a monophyletic *G. quitensis*: the small number of high-coverage loci recovered for the toepad-derived sample of *G. q. alticola* (n = 110; Table 2) was sufficient to place this sample as sister to the sample of *G. q. quitensis* derived from fresh muscle tissue (Fig. 2B). We did not have GBS data from *G. flavotincta* and thus could not verify the sister relationship with *G. milleri* suggested by mtDNA.

The topological results of the SNAPP analysis were consistent with the ML and Bayesian phylogenetic analyses of concatenated GBS loci (Fig. 3). *G. hypoleuca* was sister to all other members of the bay-backed clade with 91.4% posterior probability support (Fig. 3).

Molecular dating

BEAST analyses (Fig. 4) imply that the ancestor of the bay-backed antpitta complex diverged from a common ancestor with *G. quitensis* in the late Miocene or early Pliocene (~5 Ma). The first divergence within the bay-backed complex (between *G. hypoleuca* and the remaining members of the clade across the Marañón Valley) was around 4.4 Ma ago and was nearly concurrent with the divergence event across the Apurímac Valley (Fig. 4). Subsequent divergence events across intervening barriers between the Marañón and Apurímac valleys occurred throughout the Pliocene and Pleistocene (Fig. 4). Analyses in which the topology was constrained to reflect the GBS tree (Fig. 4) produced nearly identical results to unconstrained analyses (Fig. S3, Supporting information).

Genetic diversity and pairwise differentiation

Haplotype and nucleotide diversity estimates from the ND2 data set indicated fairly even diversity across species, with slightly higher diversity in *G. hypoleuca*, the northernmost species (Table 4). However, *G. hypoleuca* has a larger range than the other species, as well as subspecific variation. When the northernmost samples of *G. hypoleuca* were excluded, haplotype diversity in *G. hypoleuca* decreased and was similar to diversity in the other taxa (Table 4). For the GBS analyses of nucleotide diversity, ANOVA and pairwise *t*-tests with Bonferroni corrections revealed significant differences in nucleotide diversity between each population ($P < 0.01$). These analyses generally suggested a decline in genetic diversity from north to south (Fig. S4, Supporting information). However, not every adjacent population followed this trend, depending on sample size and population definition (Fig. S4 and Appendix S1, Supporting information).

For the ND2 data set, F_{st} was high (>0.83) between all currently recognized species, and moderate (0.53) between the two forms of *G. capitalis*. For the GBS data set, F_{st} values were numerically lower (0.52–0.68 between described species and 0.23 between *G. capitalis* forms).

Introgression

D statistics from four-taxon tests revealed little evidence for introgression between taxa and across barriers (Table 3). We disregarded tests that contained fewer than 50 combined ABBA or BABA sites (Streicher *et al.* 2014). With these filters applied, tests contained an average of 4487.6 total loci, with an average of 53.5 ABBA and 45.0 BABA loci across all tests (Table 3). Among these tests, only the tests between *G. capitalis* into *G. erythroleuca* (across the Apurímac Valley) showed any evidence of introgression, with 10% of tests showing significant introgression at $P < 0.05$ (Table 3). This comparison also had the highest number of total tests (n = 178), which may suggest that the tests for introgression between other taxon pairs are underpowered. However, the numbers of ABBA/BABA sites reported here are comparable to other recent studies of nonmodel organisms (Eaton & Ree 2013; Streicher *et al.* 2014).

Discussion

The influence of geographic barriers on the speciation of Neotropical organisms is well established (e.g. Wallace 1852; Cracraft & Prum 1988; Knowlton *et al.* 1993; Bates *et al.* 1998). However, inferring the history of dispersal and vicariance across these barriers has been more elusive (Smith *et al.* 2014). Here, we show that a lineage of humid-forest Andean birds has pronounced genetic and phenotypic differentiation throughout its range, mostly concordant with a series of intervening arid valleys. mtDNA genealogies served well to (i) redefine the study

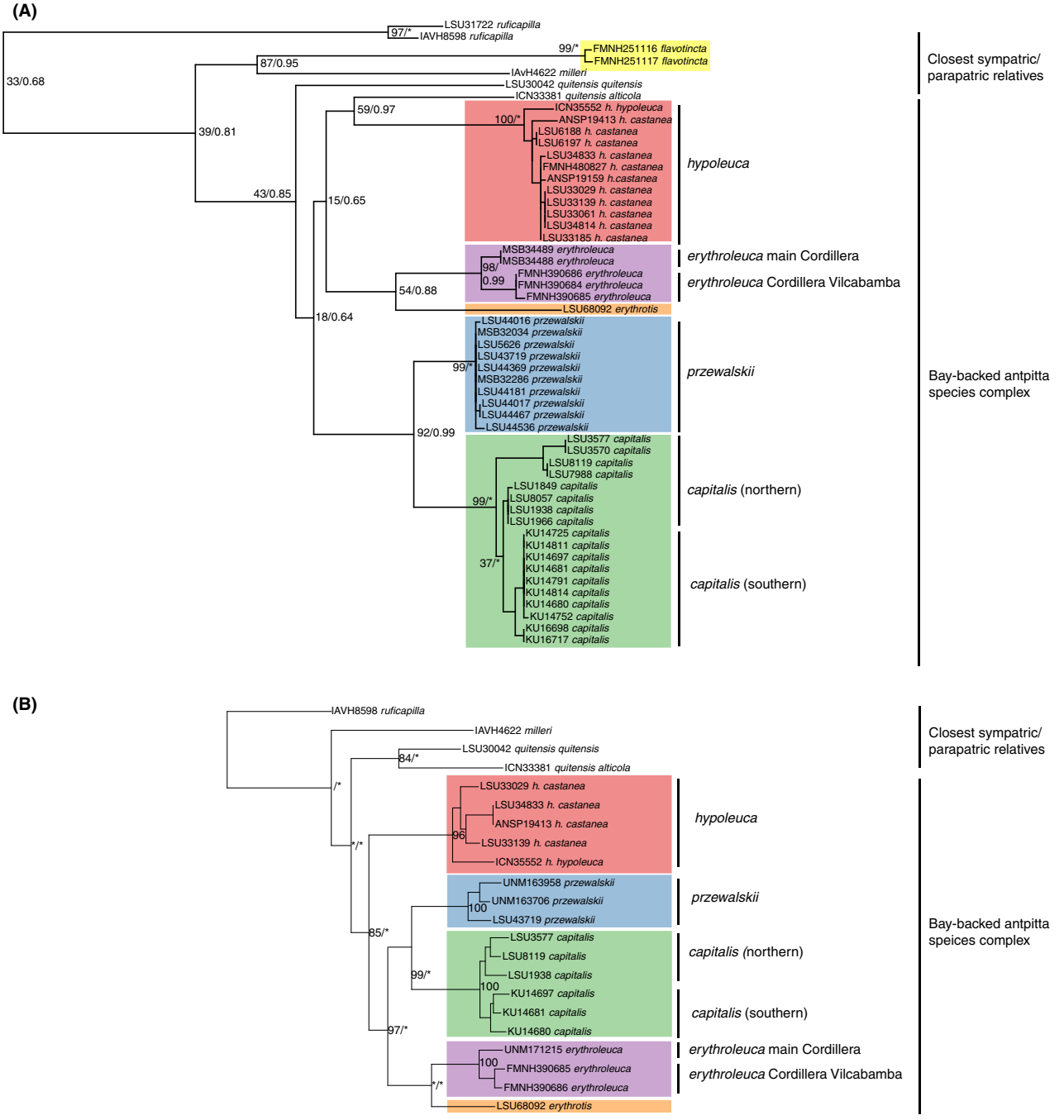

Fig. 2 Maximum-likelihood (ML) phylogenies of the bay-backed antpittas and their relatives. Colours of clades correspond to the taxa labelled in Figs 1 and 4. Values at nodes are ML bootstrap values/Bayesian posterior probabilities, with an asterisk indicating 100% bootstrap or posterior probability support. (A) Phylogeny of all 60 individuals in the study built from ML analysis of the mtDNA gene ND2. The out-group *Chamaeza campanisona*, as well as samples of the distantly related *Grallaria albigula*, were included in the analysis but pruned for visual simplicity (see text). *Grallaria erythrotis* is recovered as sister to *G. erythroleuca*. Together, *G. erythroleuca* and *G. erythrotis* are recovered as sister to *G. hypoleuca* with weak support in both ML and Bayesian analyses. The mtDNA analysis did not recover a monophyletic *G. quitensis* (see text). Notably, *G. flavotincta* is recovered as sister to *G. milleri*, rather than to its previously hypothesized sister *G. hypoleuca*. (B) Maximum-likelihood phylogenies built from a matrix of 23 639 genotyping by sequencing (GBS) loci across 22 individuals. This alignment included the most individuals, but the sparsest coverage, of the three GBS alignments used for concatenated phylogenetic analysis (Table S1, Supporting information). The topology is identical to those produced from more complete alignments with fewer individuals, shown in Fig. S2 (Supporting information). In all GBS phylogenies, *G. hypoleuca* is sister to the remaining member of the bay-backed antpitta complex. *G. ruficapilla* and *G. milleri* were designated as out-groups in the ML analysis and therefore did not receive bootstrap support values. GBS data were not available for *G. flavotincta*.

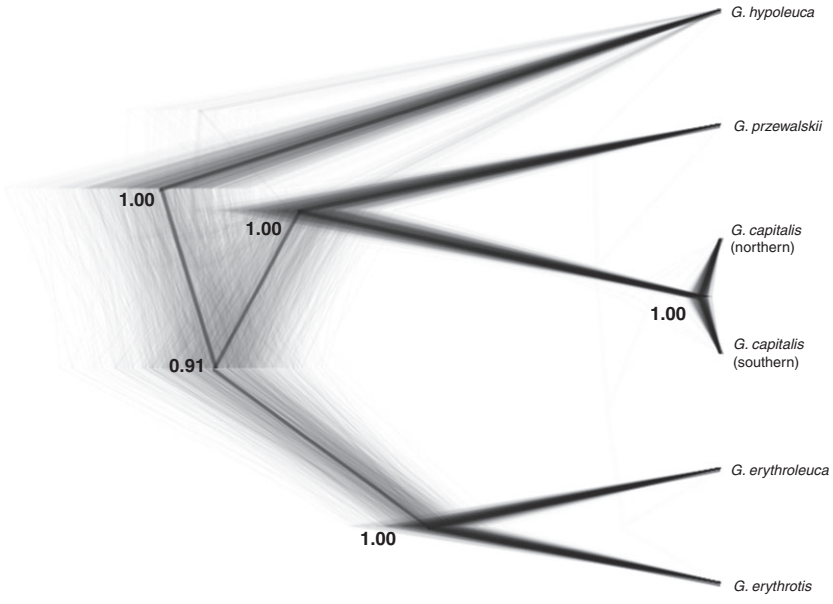

Fig. 3 DENSITREE visualization of 1980 post burn-in SNAPP trees produced from 1767 unlinked, biallelic SNPs. The maximum clade credibility tree is in bold, and the posterior probabilities of nodes in this topology are listed on the branches subtending clades. The MCC topology, in which the clade containing *Grallaria erythrotis* and *G. erythroleuca* is sister to the clade containing *G. capitalis* and *G. przewalskii*, received 91% posterior probability support, whereas alternative topologies received negligible support.

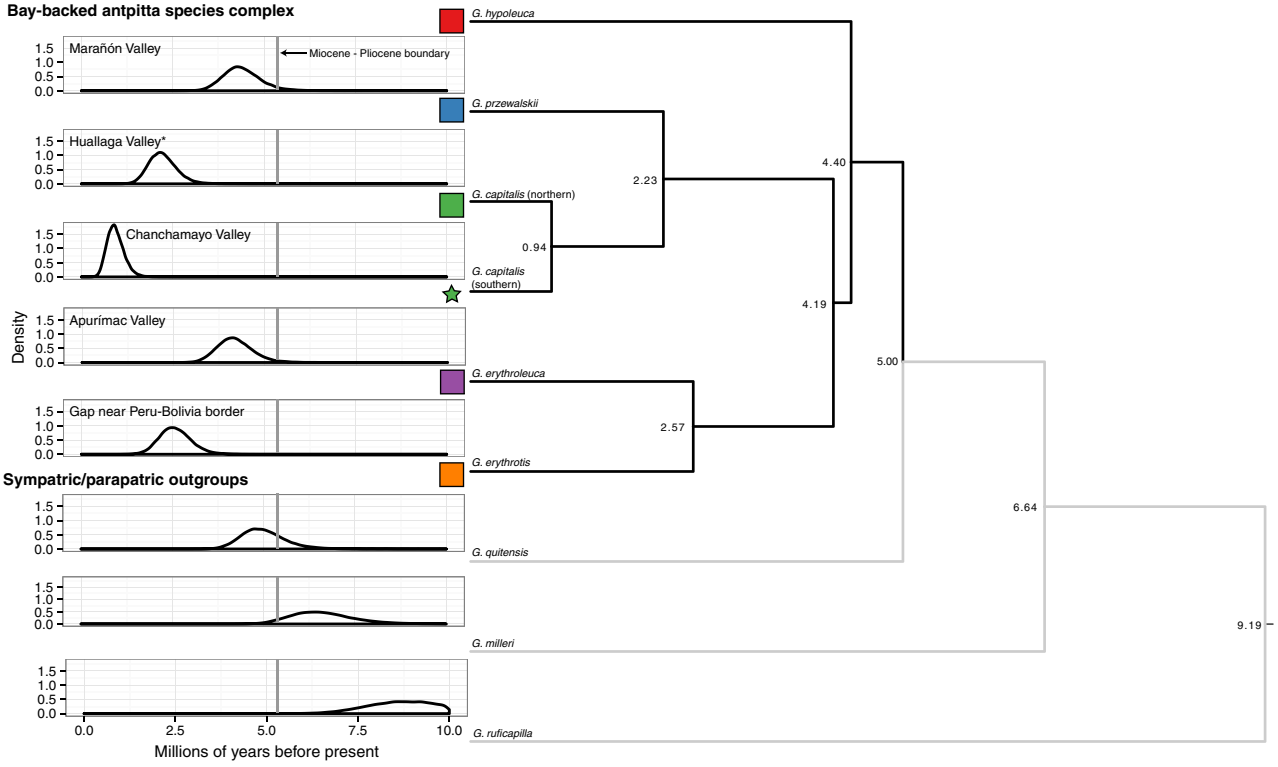

Fig. 4 Divergence time analyses conducted in BEAST. Numbers at nodes are mean node ages in millions of years, and density plots are of the estimated range of node ages from an MCMC chain of 50 million steps (with 25% of steps discarded at burn-in). The density plot of each divergence is pictured between the lineages that it divides; each divergence corresponds to a geographic barrier (Fig. 1). The Huallaga Valley is indicated with an asterisk because the distributional gap between *Grallaria przewalskii* and *G. capitalis* is found slightly north of the valley (see Discussion). In this analysis, the ND2 gene tree was constrained to reflect the species tree, and the node ages were similar to those estimated in an unconstrained analysis (Fig. S3, Supporting information).

group as consisting of *Grallaria hypoleuca*, *G. przewalskii*, *G. capitalis*, *G. erythroleuca* and *G. erythrotis* (but not *G. flavotincta*), and to (ii) recover clades and document spatial patterns of genetic structure. However, mtDNA genealogies were not sufficiently resolved to infer the geographic history of speciation. In particular, mtDNA

Table 4 mtDNA haplotype and nucleotide diversity for each taxon (calculated in DNASP)

Grallaria Taxon	Sample size	Haplotype diversity	Nucleotide diversity
hypoleuca (all)	12	0.879	0.00401
hypoleuca castanea near Marañon	7	0.667	0.00318
przewalskii	10	0.644	0.00092
capitalis (all)	18	0.824	0.00831
capitalis (northern)	8	0.821	0.00988
capitalis (southern)	10	0.511	0.00156
erythroleuca	4	0.667	0.00768
erythrotis	1	–	–

Statistics were not computed for *Grallaria erythrotis*, as only one sample was available.

trees suggested that the southernmost species (*G. erythroleuca* and *G. erythrotis*) are more closely related to the northernmost species (*G. hypoleuca*) rather than to their geographic neighbours (Fig. 2A), but this relationship had weak support. By gathering thousands of short-read loci with GBS, we resolved the phylogeny using both concatenated and species-tree approaches to phylogenetic analysis. The resulting highly resolved GBS phylogenies consistently revealed a more straightforward biogeographic history wherein neighbouring populations along the east slope of the Andean cordillera are each other's closest relatives (Figs 2 and 3), implying that the genetic leapfrog pattern suggested in the mtDNA gene tree was an artefact of poor phylogenetic resolution. Genetic leapfrogs have been suggested in other Andean taxa (Pérez-Emán 2005; Weir *et al.* 2008; Gutiérrez-Pinto *et al.* 2012), but with the possible exception of leapfrog patterns in *C. flavopectus* (Weir *et al.* 2008), these relationships have also not received strong phylogenetic support and thus the historical biogeographic implications of these patterns have remained unclear.

Phylogenies of mtDNA suggested a somewhat surprising sister relationship of *G. flavotincta* with *G. milleri* (as opposed to *G. hypoleuca*), but we were not able test this with genomewide DNA sampling. The relationships among subspecies of *G. quitensis* also deserve further attention. Field collection of fresh material (Moyle *et al.* 2014) of several populations—notably *G. flavotincta*, *G. quitensis alticola* (sampled here with a toepad), and an unsampled subspecies of *G. quitensis* found south of the Marañón Valley (*atuensis*)—are needed to clarify the relationships of these taxa.

Biogeographic history

Our molecular dating analysis indicates that the bay-backed antpitta species originated in the early Pliocene or late Miocene and continued to diversify throughout the Pleistocene (Fig. 4). These divergence times are similar to the dates estimated in numerous other genus- and species-level phylogenetic studies of Andean birds and other taxa (e.g. Weir 2006, 2009; Ribas *et al.* 2007; Elias *et al.* 2009; Guarnizo *et al.* 2009; Sedano & Burns 2010; Chaves *et al.* 2011; Luebert *et al.* 2011; Quintero *et al.* 2012; Kieswetter & Schneider 2013; Lutz *et al.* 2013; Benham *et al.* 2014). In these studies, both Andean uplift, which occurred throughout the last 10 Myr, and Plio-Pleistocene glacial cycles have been implicated as important factors in biotic diversification in the Andes. The exact processes, however, by which orogeny and climate change have promoted speciation in the Andes remain difficult to demonstrate, but are relevant to disentangling the role of dispersal and vicariance in the speciation of Neotropical birds. For example, some authors have argued that montane uplift has passively isolated populations throughout the Andes (Ribas *et al.* 2007; Quintero *et al.* 2012), whereas others have suggested that the uplift of the Andes created a continuous band of montane habitat that promoted lineage dispersal and subsequent isolation across a topographically complex and geographically extensive region (Chaves *et al.* 2011; Benham *et al.* 2014; Valderrama *et al.* 2014). Likewise, alternative scenarios have been proposed for how Plio-Pleistocene glacial cycles have influenced speciation. The advance of ice sheets and paramo during glacial maxima may have forced humid montane species into refugia (Ramírez-Barahona & Eguiarte 2013). Alternatively, down-slope shifts of humid montane forest could have increased connectivity across low-elevation barriers, thus promoting dispersal and gene flow in humid-forest organisms (Vuilleumier 1969; Graves 1982; Benham *et al.* 2014); in this scenario, upslope shifts during interglacials would promote divergence (Hooghiemstra *et al.* 2006; Ramírez-Barahona & Eguiarte 2013). Likely, each of these processes has played some role in different taxa and in different regions.

How did orogeny influence the diversification of the bay-backed antpittas? Our results suggest that the bay-backed antpitta clade originated in the northern Andes and dispersed southward during the late Miocene or early Pliocene. This evidence comes first from our phylogenetic results. The out-groups to the bay-backed antpitta complex—*G. ruficapilla, G. milleri, G. flavotincta* and *G. quitensis*—are northern Andean species of Colombia, Ecuador and far northern Peru (Fig. S1, Supporting information), and *G. hypoleuca*, the northernmost member of the bay-backed complex, is out-group to the remainder of the bay-backed complex (Fig. 2). These results suggest that the ancestor of the bay-backed antpitta complex likely diverged from its most recent com-

mon ancestor in the northern Andes. Our genetic diversity results lend support for a northern ancestor, by demonstrating first that *G. hypoleuca* has higher genetic diversity in the northern part of its range (Table 4), and second that genetic diversity tends to be lower in the southernmost species in the bay-backed complex (Fig. S4, Supporting information). However, the sample sizes available to estimate genetic diversity with GBS data in this study were small. Additionally, there was variation in the north to south trend of declining diversity (Fig. S4, Supporting information), which may reflect a more complex history of colonization and demographic change.

According to fossil, geologic and palaeofloristic evidence, the Bolivian Andes had uplifted sufficiently to support humid montane forest by the late Miocene (6–8 Ma), prior to the time that we estimate the ancestor of the bay-backed antpitta clade originated in the northern Andes (Graham *et al.* 2001; Bershaw *et al.* 2010; Mulch *et al.* 2010). Thus, given its likely northern origins, it seems that the bay-backed antpitta clade is too young to have diversified as a consequence of orogenic surface uplift, and probably invaded the central Andes after montane forest had already existed there for some time. The influence of orogenic uplift on the origins of the bay-backed antpittas in the northern Andes is less clear. Although the Eastern Cordillera of the Colombian Andes experienced a period of recent and rapid orogeny between 2 and 5 Myr (Gregory-Wodzicki 2000; Garzione *et al.* 2008; Hoorn *et al.* 2010), the Central and Western Cordilleras of Colombia were likely formed earlier in the Oligocene and Miocene (Hoorn *et al.* 2010; Mora *et al.* 2010) and therefore could have played a role in the divergence of the ancestral bay-backed antpitta from its nearest relatives.

If the ancestral bay-backed antpitta invaded the central Andes from the north, then the question remains, did a series of founder events across existing barriers lead to genetic and phenotypic divergence of each taxon, or was the range of the ancestor fragmented by vicariance after it became established in the central Andes? Or is a signature of both processes evident? A major difficulty in testing dispersal vs. vicariance in humid-forest Andean organisms is that although estimates exist for the timing of uplift in different regions of the Andes, we lack a robust understanding of the relationship between Andean uplift and the incision of the deep canyons that isolate Andean humid-forest taxa today (Jeffery *et al.* 2013). For example, canyons may form during orogeny, but they may also be produced later as a consequence of erosion, and the rate of erosion can depend on climatic conditions (Gregory-Wodzicki 2000; Jeffery *et al.* 2013). Therefore, although some biologists have postulated that the low-elevation area surrounding the Marañón Valley, known as the North Peruvian Low, has existed as a topographic feature for millions of years prior to the earliest avian divergence in this region (Johnson 2002; Miller *et al.* 2007), it remains difficult to translate general knowledge of the timing of Andean uplift into certainty of when valleys such as those of the Marañón and Apurímac rivers became prominent dispersal barriers for humid-forest birds (Bates & Zink 1994; Weir 2009).

A compelling source of information on the timing of arid valley formation comes from seasonally dry tropical forest plants, which exhibit high levels of endemism within the inter-Andean valleys that isolate humid-forest taxa. Dry forest has existed in and around the Andes for 10–15 Myr (Särkinen *et al.* 2012), but phylogenetic studies indicate that the seasonally dry floras endemic to the Marañón and Apurímac valleys diverged from one another approximately 5 Ma (Pennington *et al.* 2010; Särkinen *et al.* 2012). Recent geologic studies have revealed evidence of orogenic activity during the Pliocene along the eastern slope of the central Andes, which likely contributed both to increased orographic precipitation on Andean slopes and the development of arid conditions in Andean valleys (Spikings & Crowhurst 2004; Pfiffner & Gonzalez 2013; Pingel *et al.* 2014). The timing of this geologic activity, and the estimated divergence times between the Marañón and Apurímac dry forest floras, is close to the range of divergence times of bay-backed antpitta species across both the Marañón and Apurímac valleys (Fig. 4). Although molecular dating from single loci carries assumptions on the validity of a molecular clock, these results may indicate that the climatic and geologic events that led to the isolation of dry forest floras in the broad canyons of the eastern Andean cordillera also caused range fragmentation of humid-forest taxa across these same valleys (Killeen *et al.* 2007).

Further support for a vicariant history of speciation in the bay-backed antpittas may come from the topological shape of their phylogeny. If the group originated in the north and spread to the south via a series of dispersal events across existing barriers, a pectinate topology that reflects the history of this dispersal would be expected [*hypoleuca*, (*przewalskii*, (*capitalis*, (*erythroleuca*, *erythrotis*)))]. Instead, the GBS topology implicates early and nearly concurrent divergence across the Marañón and Apurímac valleys (Fig. 4), followed by more recent divergences of intervening populations during the Plio-Pleistocene [*hypoleuca*, ((*G. capitalis*, *przewalskii*), (*erythroleuca*, *erythrotis*))]; this pattern is more consistent with vicariance. However, introgression between neighbouring populations, coupled with isolation by distance between non-neighbours, could potentially erase an historical signature of dispersal in the topology of the tree

(Eaton & Ree 2013). For example, even if *G. przewalskii* and *G. capitalis* were historically paraphyletic with respect to *G. erythroleuca* and *G. erythrotis* (as predicted by the topology consistent with serial dispersal), subsequent introgression between the neighbouring *G. przewalskii* and *G. capitalis* could produce the observed topology wherein these taxa are monophyletic sisters. Using the ABBA/BABA test, we did not find evidence that introgression within this group has obscured historical phylogenetic relationships, suggesting that the observed topology represents historical patterns of divergence [although we note that we lack sampling within a potential contact zone between *G. przewalskii* and *G. capitalis* (see below)]. Although serial dispersal seems unsupported by the topology, we caution that other scenarios besides static vicariance are potentially plausible given the topology, including origination in the central Andes and dispersal to the north and south.

If a series of dispersal events led to the geographic isolation of each incipient bay-backed antpitta species, then a signature of bottlenecking might be expected from north to south in each population (Austerlitz *et al.* 1997; DeGiorgio *et al.* 2011; Slatkin & Excoffier 2012). Using summary statistics of genetic diversity, we found that species further south tend to have lower genetic diversity than their neighbours to the north, although the consistency of this pattern depended on sample size and how populations were defined (Fig. S4, Supporting information). However, even in the absence of strong physical barriers, serial founder effects are predicted to occur at the leading edge of a population during range expansions, and summary statistics are not sufficient to distinguish this process from founder effects that occurred specifically via bottlenecking across a geographic barrier (DeGiorgio *et al.* 2011; Slatkin & Excoffier 2012). Therefore, more nuanced demographic modelling and greater sample sizes are required to strengthen our confidence in testing vicariance vs. dispersal scenarios using population genetic methods. These demographic models will need to incorporate the real complexities of evolution in the Andes. For example, Andean birds have likely undergone repeated population size changes during glacial oscillations (Ramírez-Barahona & Eguiarte 2013), which could obscure the signal of earlier events and complicate interpretation of population genetic parameters (Peter & Slatkin 2014; Shafer *et al.* 2015).

Previous studies of Andean organisms have demonstrated that dispersal throughout the topographically complex Andes has been an important factor in their diversification (Weir *et al.* 2008; Weir 2009; Chaves *et al.* 2011; Gutiérrez-Pinto *et al.* 2012; Benham *et al.* 2014; Winger & Bates 2015). Therefore, our goal in posing dispersal vs. vicariance in the bay-backed antpittas is not to argue for the broader influence of one process to the exclusion of the other in shaping Andean diversity. Furthermore, dispersal and vicariance likely work in conjunction in the Andes in a cyclical manner, with barriers becoming more permeable (facilitating dispersal) or impassable (facilitating vicariance) at different times, and it may not always be possible to separate these processes. Thus, we cannot eliminate the possibility of dispersal across barriers after their formation in all cases, or more complex patterns of extinction and recolonization. Nevertheless, the topological shape of the bay-backed antpitta phylogeny, the closely timed early divergence events across the Marañón and Apurímac valleys, the coincidence of these divergence events to the isolation of dry forest floras endemic to these valleys and the subsequent occurrence of more recent Plio-Pleistocene divergences among geographically intervening populations collectively suggest that vicariant sundering of an ancestral range is a plausible explanation for the differentiation of the bay-backed antpitta clade.

Maintenance of allopatry and the evolution of secondary sympatry

In some pairs of neighbouring bay-backed antpittas, allopatry is maintained despite the absence of obvious physical barriers. For example, the boundary between *G. przewalskii* and *G. capitalis* is near to, but apparently not congruent with, the arid upper Huallaga Valley. *G. capitalis*, although principally found south of the Huallaga, is also found just north of this valley, and *G. przewalskii* is known from only 150 km to the north of *G. capitalis*, with no apparent biogeographic boundaries separating the two species (Fig. 1; Schulenberg & Kirwan 2012b; M. Harvey & G. Seeholzer, personal communication). Also, no major topographical barrier separates *G. erythroleuca* and *G. erythrotis* (Fig. 1), but neither taxon has been found in an approximately 150-km segment of the Peruvian Andes (Robbins *et al.* 2013). A distributional gap or phylogeographic break has been observed in similar locations among other Andean taxa (Graves 1982; Cadena & Cuervo 2010; Gutiérrez-Pinto *et al.* 2012; Isler *et al.* 2012; Valderrama *et al.* 2014).

Fjeldså *et al.* (1999) proposed that certain areas of the eastern Andean slopes have been persistently humid due to profound orographic precipitation, even during glacial maxima, whereas other areas are more susceptible to climatic fluctuations. Fjeldså *et al.* (1999) further suggested that this spatial variation in climatic stability —as opposed to physical fragmentation of ranges by deep valleys—promoted differentiation in humid-forest Andean organisms. Although there are no deep can-

yons or other rain shadows near the Peru–Bolivia border where the gap occurs between *G. erythroleuca* and *G. erythrotis*, patches of dry forest nevertheless persist in the foothills here at lower elevations (Kessler & Helme 1999; Herzog & Kessler 2002; Killeen *et al.* 2007). These somewhat mysteriously located dry forests (Killeen *et al.* 2007) could be a vestigial indication that the upper montane regions inhabited by bay-backed antpittas today have been more climatically stable than the regions where they are not found. In other words, the high niche conservatism of bay-backed antpittas to humid forest may have promoted their divergence, and serve to maintain their present allopatry, even in the absence of deep canyons or other topographical barriers. However, further fieldwork is required to determine whether contact between bay-backed antpittas occurs in these apparent distributional gaps, which would help reveal whether allopatry in these cases is maintained by niche conservatism in persistently humid areas, vs. by competition or reinforcement among neighbouring taxa.

The factors that maintain allopatry in these antpittas will also help explain the relatively slow rate of evolution of secondary sympatry in *Grallaria*. Our results suggest that secondary sympatry takes several million years to evolve in this lineage, which is consistent with the upper bound of macroevolutionary assessments of the evolution of secondary sympatry in birds (Weir & Price 2011; Pigot & Tobias 2015) and contrasts with recent demonstrations of rapid secondary sympatry in other avian taxa (Campagna *et al.* 2012; Andersen *et al.* 2015). The most closely related parapatric species to the bay-backed group is *G. quitensis*, which diverged from the bay-backed ancestor at least 5 Ma and is found on the same montane slopes as *G. hypoleuca* and *G. przewalskii*, but above treeline in open paramo or humid grasslands (Fig. S1, Supporting information). The most closely related fully sympatric species to the bay-backed group is *G. ruficapilla*, which diverged from the ancestor to the bay-backed complex ~9 Ma (Fig. S1, Supporting information). Other sympatric species include *G. squamigera*, which is at least 22 Myr divergent from the bay-backed group (Winger & Bates 2015). Thus, our results highlight that the community assembly of Andean *Grallaria* that exist sympatrically or parapatrically on the same Andean slopes—which at some locales involves as many as 10 species—has likely required substantial amounts of evolutionary time, perhaps as a consequence of high niche conservatism. Thus, studies of the ecological factors that mediate elevational gradients in diversity should consider the historical context and tempo of allopatric divergence and secondary sympatry (Freeman 2015; Mittelbach & Schemske 2015).

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B.M.W. conceived the study; B.M.W., J.M.B., G.A.B., A.M.C. and P.A.H. designed the research; B.M.W., N.A., L.E.C., G.A.B., A.M.C., P.A.H. and A.M.C. gathered the data; B.M.W. analysed the data; and B.M.W. wrote the manuscript with input from J.M.B., G.A.B., A.M.C. and P.A.H.

Data accessibility

mtDNA sequences produced for this study are deposited in GenBank (Table 1) and Illumina reads in NCBI Sequence Read Archive (SRP065881, Table 1). Assembled GBS alignments, tree files and other data are deposited in Dryad (doi:10.5061/dryad.q268m).

Supporting information

Additional supporting information may be found in the online version of this article.

Appendix. S1 Additional details on the distribution of bay-backed antpitta taxa, additional methodological details, and details of nucleotide diversity analyses.

Table. S1 The number of loci and SNPs for each of the three alignments used for maximumlikelihood (RAXML) and Bayesian (EXABAYES) phylogenetic analyses of GBS data.

Fig. S1 Geographic ranges of the bay-backed antpitta species and parapatric and sympatric *Grallaria* species that are the closest relatives of the bay-backed complex.

Fig. S2 Maximum-likelihood GBS phylogenies constructed from A) the intermediate coverage matrix representing 3019 loci across 16 individuals, and B) the complete coverage matrix of 1846 loci across 7 individuals (Table S1).

Fig. S3 BEAST analysis of ND2 sequences with the topology unconstrained.

Fig. S4 Analysis of nucleotide diversity of four alignments.