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Testing the molecular and evolutionary causes of a 'leapfrog' pattern of geographical variation in coloration

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Abstract

Understanding the mechanisms accounting for the evolution of phenotypic diversity is central to evolutionary biology. We use molecular and phenotypic data to test hypotheses for 'leapfrog' patterns of geographical variation, in which phenotypically similar, disjunct populations are separated by distinct populations of the same species. Phylogenetic reconstructions revealed independent evolution of melanic plumage characters in different populations in the Neotropical avian genus *Arremon*. Thus, phenotypic similarities between distant populations cannot be explained by close phylogenetic affinity. Nor can they be attributed to recurring mutations in the *MC1R* gene, a locus involved in melanic pigmentation. A coalescent analysis indicates that plumage traits have become fixed at a faster rate than expected under genetic drift, suggesting that selection underlies their repeated evolution. In contrast to views that genetic drift drives phenotypic differentiation in Neotropical montane birds, our results imply that geographical variation preceding speciation may reflect the action of deterministic selective processes.

Introduction

Classic analyses of geographical variation in animal coloration played a key role in the development of central theories, hypotheses and concepts in evolutionary biology such as natural selection (Darwin, 1859), speciation (Mayr, 1942), character displacement (Brown & Wilson, 1956), mimicry (Bates, 1862; Müller, 1879), and some ecogeographic 'rules' (Zink & Remsen, 1986). More recently, studies on animal coloration have provided crucial insights into evolutionary processes operating in natural populations. For example, phylogenetic, molecular genetic and developmental work on the pigmentation of vertebrates has begun to shed light on central questions about adaptation by revealing instances of recurrent evolution of adaptive phenotypes as a result of divergent selection and by identifying genes, mutations and developmental pathways responsible for phenotypic variation (reviewed by Hoekstra, 2006; Hubbard *et al.*, 2010; Manceau *et al.*, 2010). Despite such progress, studies providing explicit evaluations of the mechanisms underlying patterns of geographical variation remain scarce and focused on a few emerging model systems.

Here, we use molecular and phenotypic data to test hypotheses posed to explain one of the most intriguing patterns of geographical variation, namely that in which morphologically similar, disjunct populations are separated from each other by morphologically distinct populations of the same species (i.e. the 'leapfrog' pattern of variation; Remsen, 1984). Such patterns exist in a variety of organisms including plants (Matsumura *et al.*, 2006), insects (Hovanitz, 1940) and amphibians (Noonan & Gaucher, 2006), and are especially pervasive in birds. Remsen (1984) demonstrated that 21% of all bird species or superspecies with three or more differentiated populations exhibit leapfrog patterns in the Andes, and new examples continue to be described (Maijer & Fjeldså, 1997; Hayes, 2001; Johnson, 2002; Salaman *et al.*, 2002;

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Donegan & Avendaño, 2010), suggesting that the prevalence of this pattern is likely still under-appreciated.

Despite nearly a century of study (Chapman, 1923), explicit tests of mechanistic explanations for leapfrog patterns are scarce. Hypotheses proposed to explain their origin can be placed in one of two categories: (1) those that evoke close phylogenetic affinities between morphologically similar but geographically distant populations and (2) those that attribute leapfrogging patterns to divergent patterns of phenotypic change among geographically proximate populations (Remsen, 1984; Chapman, 1939). Phylogenetic affinity hypotheses typically invoke demographic explanations, such as long-distance dispersal, loss of connections between currently disjunct populations, or extinction of intervening populations, to explain leapfrogging patterns. In contrast, hypotheses focusing on divergent patterns of phenotypic change posit that either convergent evolution of distant populations or accelerated divergence of central populations compared to those at the periphery of ranges causes this pattern. These hypotheses are testable by examining patterns of plumage variation in a phylogenetic context: common ancestry hypotheses predict that morphologically similar taxa are closely related, whereas hypotheses of divergent patterns of change predict no relationship between phylogeny and phenotypic traits exhibiting leapfrog patterns. Within the divergent patterns of change class of hypotheses, (2.1) convergent evolution predicts that disjunct morphologically similar taxa have evolved their plumage patterns independently, whereas (2.2) divergence of central populations predicts that similarities among disjunct populations reflect retained ancestral character states and character states of intervening populations are more recently derived.

An additional issue that is not well understood is the role that population-level microevolutionary processes (i.e. mutation, genetic drift, and selection) play in generating leapfrog patterns. The homoplasious evolution of plumage traits that seemingly underlies some leapfrog patterns (García-Moreno & Fjeldså, 1999; Norman *et al.*, 2002) raises two questions: (i) how do such traits arise independently within populations, and (ii) how do these traits become fixed over time. Genetic studies indicate that the origin of avian plumage traits may be caused by mutations within genes with large phenotypic effects (reviewed by Mundy, 2005; Price, 2002), and developmental constraints may further restrict the variety of plumage traits that can be produced (Price & Pavelka, 1996; West-Eberhard, 2003). Accordingly, repeated evolution of plumage traits leading to leapfrog patterns might result from independent mutations of conspicuous effect in the same gene (Wood *et al.*, 2005; Manceau *et al.*, 2010), with the possible array of expressed phenotypes limited by development. The fixation of alternative traits in different populations may result from genetic drift as originally proposed by Remsen (1984), but given the role of plumage in contexts such as crypsis, mate recognition and social signalling, different forms of selection could also play prominent roles (Dumbacher & Fleischer, 2001; Norman *et al.*, 2002; Mumme *et al.*, 2006; Filardi & Smith, 2008; Tibbets & Safran, 2009; Antoniazza *et al.*, 2010).

Geographical variation in the Stripe-headed Brush-finch (*Arremon torquatus*; Passeriformes, Emberizidae) represents one of the most dramatic cases of leapfrogging in birds (Chapman, 1923; Paynter, 1978; Remsen & Graves, 1995; Cadena & Cuervo, 2010). Different characters such as the presence or absence of a black pectoral band and of a white superciliary vary in leapfrog fashion but not in parallel among 14 taxa, leading to a complicated mosaic of variation in which geographically distant forms are similar and distinct from intervening ones (Fig. 1). The Chestnut-capped Brush-finch (*Arremon brunneinucha*) also exhibits leapfrog variation in the pectoral band character, which is present throughout most of its distribution but is lacking in the subspecies *apertus* from Mexico, *allinornatus* from Venezuela and *inornatus* from Ecuador (Chapman, 1923; Parkes, 1954). Here, we complement an existing molecular phylogeny of *A. torquatus* with new sequence data and use the resulting evolutionary framework together with existing phylogeographical data for *A. brunneinucha* to evaluate whether leapfrogging might be best explained by common ancestry or by divergent patterns of phenotypic change. We also evaluate whether genetic variation in a candidate gene involved in melanic coloration in birds and other vertebrates is associated with the recurrent evolution of melanic plumage traits in both species. Finally, we test whether geographical variation in *A. torquatus* and *A. brunneinucha* is caused by selection or by genetic drift using a coalescent population genetic approach. We discuss the influence that different forces might play in the evolution of geographical variation in *Arremon* and other systems, and the implications of our findings for understanding the early stages of speciation.

Materials and methods

Phylogeny reconstruction and evolution of plumage traits

Distinguishing among different hypotheses proposed to account for leapfrog patterns requires examining the evolution of plumage characters on a phylogeny. We conducted phylogenetic analyses based on sequences of the mitochondrial ND2 gene (total 1026 base pairs) for a set of lineages of *A. torquatus* selected from a comprehensive study of the complex (Cadena *et al.*, 2007). That study presented phylogenetic analyses of mtDNA sequence data for 78 individuals representing 13 of the 14 described subspecies of *A. torquatus*; sequences of two nuclear Z-linked introns were also analysed for seven of these forms. Because our interest was in examining changes in character states among subspecies (not in

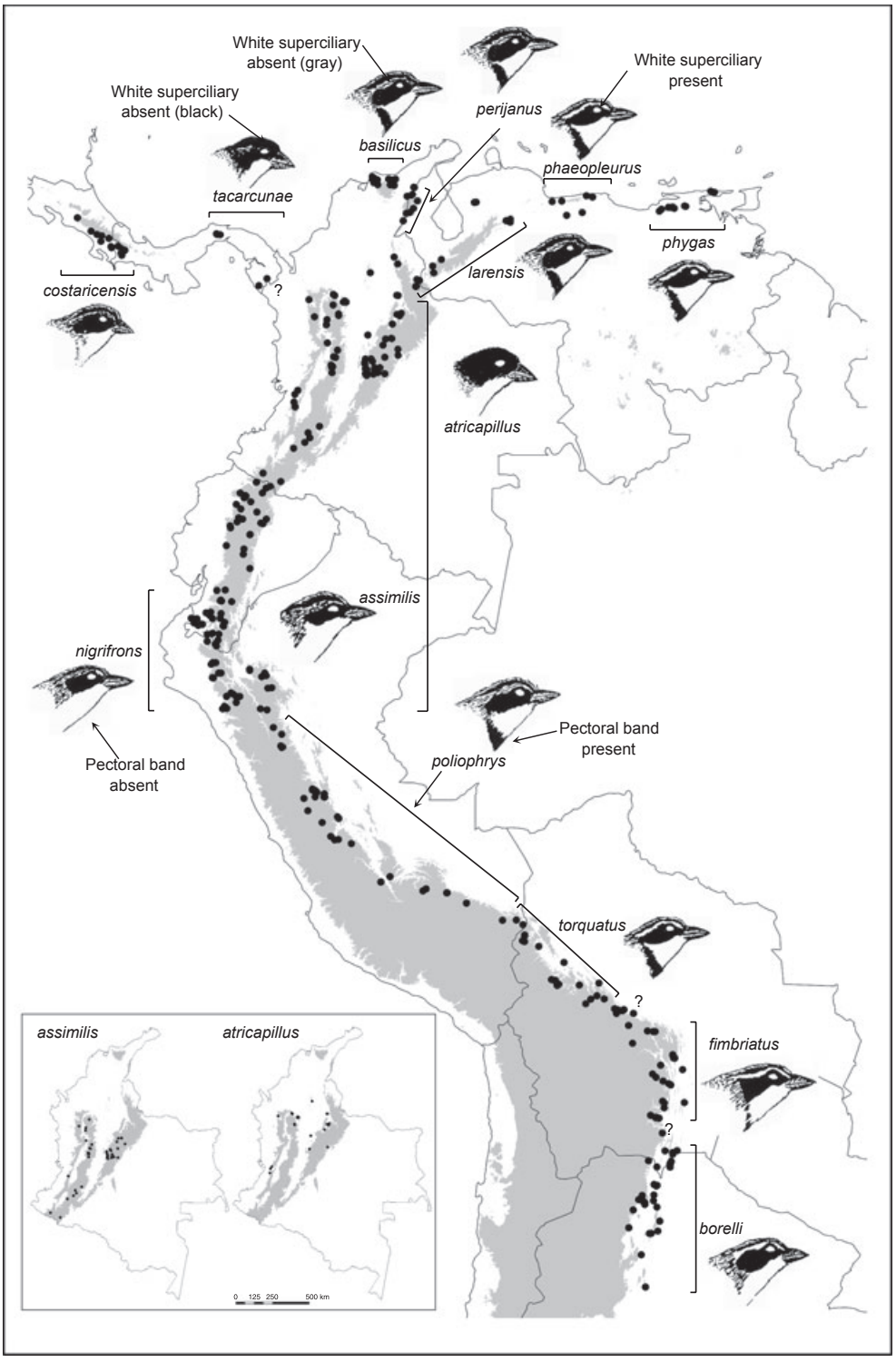

Fig. 1 Distribution of and plumage variation among populations of *Arremon torquatus*. Because *atricapillus* and *assimilis* are intermingled in Colombia, their distributions are shown separately in the inset. Note the nonconcordant geographical patterns in the presence/absence of a black pectoral band and of a white superciliary, and the phenotypic resemblance of geographically distant populations (e.g. *phygas* and *torquatus*) contrasting with intervening forms (e.g. *atricapillus*, *assimilis*). Illustrations from Paynter (1978) reproduced with permission of the Museum of Comparative Zoology, Harvard University.

estimating ancestral states for multiple nodes within subspecies in which genealogical relationships among individuals need not be tree-like), and to reduce computational constraints for analyses of character evolution, we selected a single individual per subspecies from the existing data set at random and supplemented these with one new sequence of the Venezuelan form *A. t. phaeopleurus* (GenBank accession HQ342165), the only subspecies missing in the previous study. The selection of individuals was trivial, because most subspecies formed reciprocally monophyletic groups (Cadena *et al.*, 2007; i.e. selecting any individual from a subspecies would not influence results) and when they did not (*borelli-fimbriatus* and *assimilis-nigrifrons*), the random selection of individuals was unlikely to produce any biases because in these cases sequence divergence between all individuals within and among the taxa involved was minimal, because we are confident that plumage character states are fixed within subspecies, and because in one case (*assimilis-nigrifrons*) character states for the traits being examined in this analysis do not differ between subspecies (Chapman, 1923; Paynter, 1978; Cadena *et al.*, 2007; Cadena & Cuervo, 2010). The resulting matrix was used to infer relationships among lineages using the Bayesian relaxed clock approach implemented in the program BEAST (Drummond & Rambaut, 2007) using a Yule process tree prior and the TrN+G model of sequence evolution, which was selected as the best-fit to the data according to the Akaike Information Criterion implemented in MODELTEST 3.7 (Posada & Crandall, 1998). Analyses consisted of 30 million generations of sampling, of which the first 15 million were discarded as burnin; the 15 000 trees obtained in the remaining generations were used in subsequent analyses of character evolution. To determine whether our reduction of the data matrix to one individual per subspecies had any influence on phylogeny reconstruction, we also ran a phylogenetic analysis with a data matrix including all available sequences (i.e. 82 individuals, because three new sequences of *A. t. larensis* and *A. t. perijanus* have been added to the data set lately) in BEAST under the GTR+I+G model for 1×10^8 generations (1×10^7 discarded as burn-in); the topology of the maximum clade credibility tree in this analysis did not differ from that of the reduced data set. Our earlier phylogenetic study (Cadena *et al.*, 2007) indicated that phylogenetic signal in ND2 data is consistent with signal in two nuclear DNA introns. This, in combination with the fact that the ND2 tree was consistent with one obtained from another nuclear locus which we sequenced for this study (phylogenetic analyses not shown), validates the use of ND2 sequences for phylogeny reconstruction.

To examine the evolution of plumage coloration in *A. torquatus*, we focused on two characters that vary in leapfrog fashion among populations: a black pectoral band and a white superciliary (Fig. 1). We scored these two characters as present or absent for each of the 14 described subspecies based on literature accounts (Chapman, 1923; Paynter, 1978) and on examination of large samples of museum specimens (Cadena & Loiselle, 2007; Cadena & Cuervo, 2010). Using the maximum-likelihood approach implemented in MESQUITE 2.6 (Maddison & Maddison, 2009) based on the Markov k-state 1 parameter model (Lewis, 2001), we reconstructed ancestral states for each character on the maximum clade credibility tree obtained from Bayesian analysis. Likelihood ancestral state reconstruction for the pectoral band character was ambiguous owing to high homoplasy and state changes along short branches, so we also conducted ancestral reconstruction for this character using parsimony as a simple heuristic. To consider the effect of phylogenetic uncertainty on inferences of trait evolution, we also estimated ancestral states for both characters using parsimony across the 15 000 post-burn-in trees, and summarized patterns of change across all these trees in MESQUITE. This procedure provided us with a rough assessment of whether plumage traits have changed repeatedly in our study species, and allowed us to determine whether the observation that phenotypically similar forms are often not close relatives (see Results) is robust regardless of the uncertainty inherent to reconstructing the phylogeny. Note that because inference of ancestral character states is often unreliable, we did not seek to reconstruct the states of particular nodes in the tree. Therefore, pointing to particular evolutionary scenarios (e.g. whether plumage similarities in distant relatives reflect convergence or parallel evolution) is beyond the scope of our analyses.

As an alternative way to examine trait evolution not requiring the inference of ancestral character states, we used the *K* statistic of Blomberg *et al.* (2003) to estimate the phylogenetic signal of the two plumage characters involved in the leapfrog pattern in *A. torquatus* relative to signal expected for traits evolving under a Brownian motion model. If *K* equals one (the statistic ranges from zero to infinitum), then differences in traits between terminals on a phylogeny (subspecies in our study) are proportional to the length of the branches separating them. When *K* is lower than one, traits are evolutionarily labile because close relatives are less similar than expected under Brownian motion evolution; when *K* is greater than one, traits are evolutionarily conserved. For binary traits like those we studied, convergence is expected when the rates of evolution are high, as indexed by low levels of phylogenetic signal (Ackerly, 2009). These analyses were conducted separately for each trait based on the maximum clade credibility tree that included the 82 individuals of *A. torquatus* and also on the maximum clade credibility tree resulting from the analyses of sequences of a single, randomly selected individual of each subspecies. To conduct the analyses, we employed the package Picante for R (Kembel *et al.*, 2010).

Molecular basis of phenotypic variation

The *melanocortin-1 receptor* gene (*MC1R*) controls melanin deposition in the skin, hair and feathers in several vertebrates (Mundy, 2005; Hoekstra, 2006). Point substitutions in its coding region correlate with differences in plumage pigmentation within (Theron *et al.*, 2001; Doucet *et al.*, 2004; Mundy *et al.*, 2004; Baiao *et al.*, 2007; Uy *et al.*, 2009) and presumably among (Pointer & Mundy, 2008) multiple avian species, and the rate of amino acid change at this locus correlates with the degree of sexual dichromatism in plumage across some species (Nadeau *et al.*, 2007). Thus, *MC1R* represents an appropriate candidate locus to test for a common genetic basis as the cause of parallel evolution of melanic plumage traits.

We tested whether alternative phenotypes in *A. torquatus* and *A. brunneinucha* were associated with shared mutations in *MC1R* by sequencing 859 of the 945 base pairs encompassing its coding region for a representative set of specimens following methods described by Cheviron *et al.* (2006). We generated sequences for 14 individuals (including both *A. torquatus* and *A. brunneinucha*) representing eight taxa exhibiting a black pectoral band and six taxa lacking such band (Table 1). This sampling scheme also allowed consideration of representatives of *A. torquatus* exhibiting (four taxa) and lacking (six taxa) a white (i.e. unmelanized) superciliary. Although we did not sequence the entire coding region of the gene, we considered all of the sites that have been implicated in plumage variation in other birds. Nucleotide sequences were translated into aminoacid sequences to test for the occurence of nonsynonymous substitutions associated with alternative phenotypes.

Testing the role of drift and selection

To test whether the evolution of leapfrogging in plumage traits in *Arremon* was a result of genetic drift or of some form of selection, we employed a coalescent approach based on mtDNA gene trees (Masta & Maddison, 2002; see also Brown *et al.*, 2010; Boul *et al.*, 2007). Specifically, we sought to determine whether the rate of fixation of plumage characters within subspecies was more rapid than the rate of fixation of neutral mtDNA (ND2) sequences, thereby rejecting the hypothesis of genetic drift and implying selection. This method assumes that one or more nuclear genes code for phenotypic traits (presence/absence of a black pectoral band in both *A. torquatus* and *A. brunneinucha* and presence/absence of a white superciliary in *A. torquatus*) and asks whether the degree of fixation of phenotypes observed is likely to occur under neutrality.

First, *s* of Slatkin & Maddison (1989), a measure of incomplete lineage sorting among populations (i.e. subspecies in this study), was calculated for maximum likelihood (ML) trees inferred from the analysis of mtDNA sequences using Garli 0.951 (Zwickl, 2006). We used a GTR substitution model and the default settings for run termination in each analysis (10 000 generations, lnL increase for significantly better topology = 0.01, score improvement threshold = 0.0500). The mtDNA data sets used were those described in our earlier

Table 1 *MC1R* polymorphism data for 14 *Arremon* taxa. Only nonsynonymous substitutions are shown. Asterisks indicate agreement with the consensus sequence, and heterozygous sites are denoted with standard IUPAC codes (R = A or G). Sites are numbered in reference to the chicken *MC1R* sequence (GenBank accession AB201628). See acknowledgements for museum acronyms.

		Nucleotide site															
		0	0	0	0	0	3	3	4	4	4	5	6	6			
		6	7	7	8	8	4	7	1	2	9	8	2	6			
Taxon	Voucher	4	0	9	5	9	6	6	9	4	6	3	8	6	Pectoral band	Superciliary	Accession
Consensus		G	G	G	A	G	A	G	G	A	A	C	G	G			
A. t. basilicus	IAvH BT 463	*	*	*	*	*	*	*	*	*	*	*	*	*	Present	Absent	HQ342152
A. t. phygas	COP JLP 363	*	*	*	*	*	*	*	*	*	*	T	*	*	Present	Present	HQ342153
A. t. poliophrys	LSUMZ B1844	*	*	*	*	*	*	*	*	*	*	*	*	C	Present	Absent	HQ342154
A. t. torquatus	LSUMZ B1284	*	*	*	*	*	G	*	*	*	*	*	*	*	Present	Present	HQ342155
A. t. larensis	ICN FGS 3906	*	*	*	*	*	*	*	*	*	*	*	*	*	Present	Absent	HQ342156
A. t. fimbriatus	ZMUC 120843	*	*	*	*	*	G	R	*	C	*	*	*	*	Present	Present	HQ342157
A. t. assimilis	LSUMZ B31948	*	*	*	*	*	*	*	*	*	*	*	*	*	Absent	Absent	HQ342162
A. t. borelli	MBM 4989	*	A	A	*	*	G	*	*	*	*	*	*	*	Absent	Present	HQ342159
A. t. nigrifrons	LSUMZ B427	*	*	*	*	*	*	*	*	*	*	*	*	*	Absent	Absent	HQ342160
A. t. tacarcunae	LSUMZ B28362	*	*	*	*	C	*	*	*	*	*	*	*	*	Absent	Absent	HQ342161
A. b. brunneinucha	FMNH 393770	A	*	*	G	*	*	*	*	*	G	*	*	*	Present	Absent	HQ342150
A. b. alleni	MBM DAB 1751	A	*	*	G	*	*	*	*	*	G	*	A	*	Present	Absent	HQ342151
A. b. apertus	FMNH 393763	A	*	*	G	*	*	*	T	*	G	*	*	*	Absent	Absent	HQ342163
A. b. allinornatus	COP IC 965	A	*	*	G	*	*	*	*	*	G	*	*	*	Absent	Absent	HQ342158

extensive phylogeographical study (Cadena *et al.*, 2007), supplemented with the sequence of *A. t. phaeopleurus* mentioned earlier and additional sequences for *A. t. larensis* (two individuals) and *A. t. perijanus* (one individual). Additionally, *Arremon virenticeps* was included in the analysis of *A. brunneinucha*, because phylogenetic analyses showed that this species is nested within *A. brunneinucha* (Cadena *et al.*, 2007). In sum, analyses included sequence data collected for a total of 82 and 145 individuals for *A. torquatus* and *A. brunneinucha*, respectively. These samples provide thorough coverage of the distributional range of both groups (see maps in Cadena *et al.*, 2007).

Larger *s* values indicate greater levels of incomplete lineage sorting (suggesting more recent population divergence), and smaller *s* values indicate lower levels of incomplete lineage sorting (suggesting older population divergence). For *A. torquatus*, *s* was 17, and for *A. brunneinucha*, *s* was 11.

Second, coalescent simulations (using Mesquite) were used to estimate the upper 95% confidence limit (CL) for the number of generations since subspecies divergence (scaled by effective population size, N_e) that would be expected to give the observed *s* value for the mtDNA tree. We used 10 000 simulations and $N_e = 500$, but choice of N_e does not affect results because all calculations are scaled by N_e. Using the upper 95% CL for time since divergence rather than the mean reduces the chance of a significant result, because a greater time since divergence increases the probability of fixation of phenotypic traits under neutrality. Following Masta & Maddison (2002), we ran simulations using two different scenarios for population divergence: (i) simultaneous divergence in which all subspecies diverge at the same time, and (ii) bifurcating divergence in which subspecies branch off one at a time. For *A. torquatus*, the upper 95% CL for time since subspecies divergence was 2.06 N_e generations assuming simultaneous divergence and 5.40 N_e generations assuming bifurcating divergence. For *A. brunneinucha*, the upper 95% CLs for time since divergence were 2.93 N_e generations and 8.26 N_e generations assuming simultaneous and bifurcating divergence, respectively.

Lastly, estimates of generations since subspecies divergence were used in coalescent simulations of nuclear genes to estimate the probability of complete fixation of plumage traits within populations ($s = 13$ for 14 taxa in *A. torquatus* and $s = 9$ for 10 taxa in *A. brunneinucha*) by genetic drift, assuming that such traits are encoded by nuclear genes. These simulations used $N_e = 2000$, because N_e of nuclear genes is four times that of mtDNA.

This method assumes that variation in ND2 is neutral, which we verified for *A. brunneinucha* previously (Cadena *et al.*, 2007) and for *A. torquatus* here by calculating Tajima's *D* for each subspecies using Arlequin (Excoffier *et al.*, 2005). Tajima's *D* values for *A. torquatus* subspecies were low (mean = −0.263; range = 0 to −1.249) and insignificant (mean *P* value = 0.65; range = 0.105–1.000), strongly supporting the assumption of neutrality. A second assumption is that lack of reciprocal monophyly of subspecies in mtDNA is primarily because of incomplete lineage sorting, rather than gene flow. This assumption is likely valid too, because evidence indicates low migration across barriers separating subspecies (Cadena *et al.*, 2007; C. D. Cadena, unpublished). A third assumption is that fixation of alleles at one or more nuclear genes underlies the fixation of plumage traits (i.e. no assumptions are necessary concerning the number of genes that underlie plumage variation). The genetic basis of plumage variation in *Arremon* is unknown, but if phenotypes (presence or absence of a black pectoral band and a white superciliary) are dominant, then the alleles underlying them may not be fixed (i.e. some individuals may be heterozygotes). However, if this were the case, alternative phenotypes would occur within populations under random mating, yet we have never observed this in a large sample of specimens or in the field (Cadena & Cuervo, 2010), although a single exception was noted by Chapman (1923). Therefore, alleles underlying plumage traits are likely fixed, or nearly so.

Results

Phylogeny reconstruction and evolution of plumage traits

Arremon torquatus consists of several distinct lineages that seem to have differentiated rapidly; accordingly, relationships among several of them could not be confidently resolved using several mtDNA genes and multiple individuals per lineage in an earlier study (Cadena *et al.*, 2007). Adding *A. t. phaeopleurus* to the ND2 data set did not improve phylogenetic resolution; this taxon appears to be yet another divergent branch of uncertain affinities within the South American clade of *A. torquatus*. It appeared to be most closely allied to *A. t. phygas*, but this was not strongly supported (posterior probability 0.61; Fig. 2). However, we emphasize that uncertainty in phylogeny reconstruction does not affect our interpretation of patterns of character evolution because our inferences are not based on a single tree, but rather on analyses conducted across a large sample of plausible trees sampled from the posterior distribution in our Bayesian analysis.

Analyses of character evolution indicate that the phenotypic similarity of geographically distant populations can be attributed in many cases to convergent evolution or to retention of ancestral character states, but not to close phylogenetic affinities. Because of high lability in the pectoral band character and because two *A. torquatus* taxa connected by a short branch in the phylogeny differ in character states for this trait (present in *fimbriatus*, lacking in *borelli*), its estimated rate of evolution was so high that maximum-likelihood

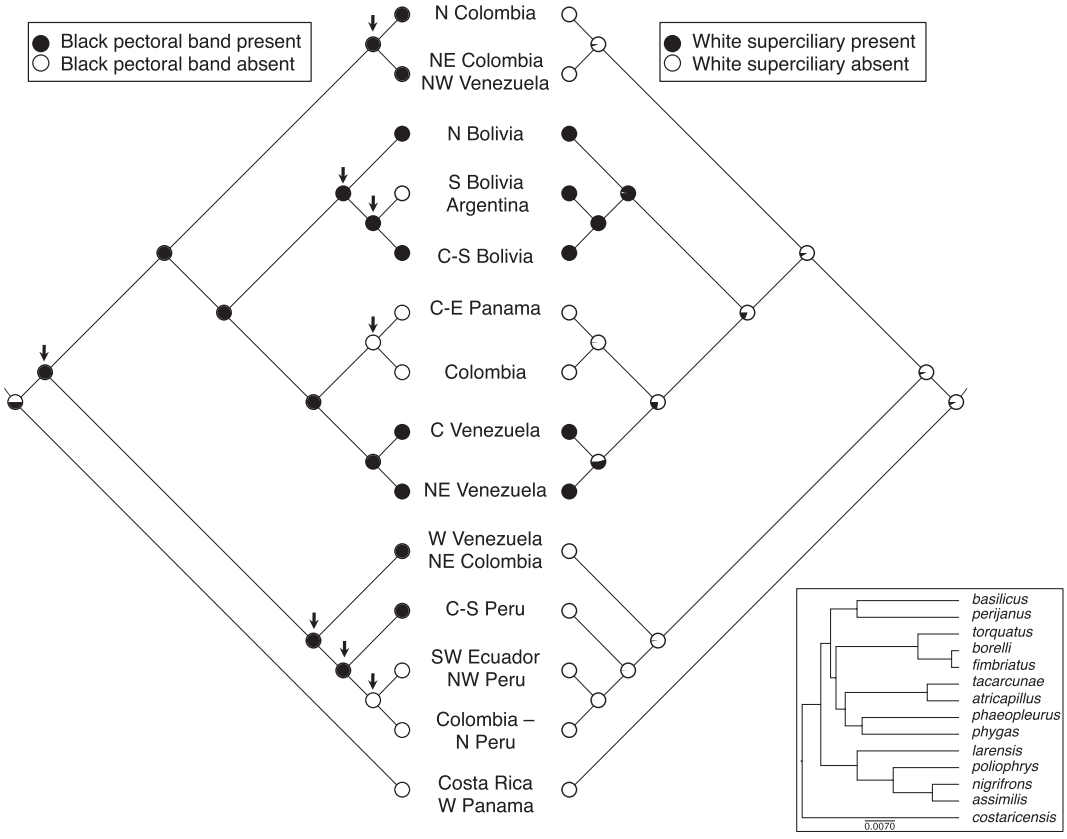

Fig. 2 Phylogeny and evolution of two plumage characters in *A. torquatus* (pectoral band on the left, superciliary on the right) showing that phenotypic similarity of geographically distant populations results from homoplasy or retention of ancestral character states. The topology is the maximum clade credibility tree recovered in the Bayesian analysis of ND2 sequences. Nodes with posterior probabilities = 1 are indicated with arrows on the left; other nodes had posterior probabilities < 0.80. Circles indicate character states for extant taxa and ancestral state reconstructions for nodes based on parsimony for the pectoral band and maximum-likelihood for the superciliary, with area proportional to the likelihood of alternative states in the latter. The inset shows the phylogram replacing geographical distributions with subspecies names.

ancestral state reconstructions were completely ambiguous (proportional likelihoods of 0.5 were assigned for both states at all nodes). A parsimony-based reconstruction on the maximum clade credibility tree revealed four switches between alternative character states (presence or absence of a pectoral band; Fig. 2). Analyses across the set of 15 000 credible trees considered to account for phylogenetic uncertainty indicate that the pectoral band has likely been lost (mean 3.36 times, range 0–5 times), and also possibly gained (mean 0.61 times, range 0–5 times) repeatedly. In most of the trees (98.8%), multiple (2–5) losses of this character were recorded; one or more gains were inferred in 54.1% of the trees and no gains in the remaining 45.9%. Tests for phylogenetic signal further confirmed that the pectoral band character is highly labile; closely related lineages of *A. torquatus* differ considerably more in this trait than expected based on the branch lengths separating them in the phylogeny (K = 0.645 and 0.284 for the data sets with 82 individuals and with a single individual of each of the 14 subspecies, respectively). Likewise, forms of *A. brunneinucha* lacking pectoral bands and occurring in distant locations (Mexico, Ecuador, Venezuela) are not each other's closest relatives (Cadena *et al.*, 2007), implying that each has evolved its phenotype independently.

The evolution of head colour pattern in *A. torquatus* appears more conserved, with two gains of white superciliaries (i.e. losses of pigmentation) and no reversals to the ancestral state, which most likely lacked this trait (Fig. 2). However, analyses accounting for phylogenetic uncertainty do not allow rejection of the hypothesis that the presence of a white superciliary in the two lineages possessing it is homologous because these lineages form a sister group in multiple trees in the posterior sample: 62% of trees indicated two independent gains of the white superciliary and a single gain was the most parsimonious reconstruction in 25% of trees. Across trees, the average number of gains of the white superciliary was 1.85 (range 0–3) and the average number of losses of this trait was 0.35 (range 0–3). The

test for phylogenetic signal indicated that this character tends to be phylogenetically conserved (K = 7.771 and 1.738).

Molecular basis of phenotypic variation

We found no clear associations between sequence variation at *MC1R* and the presence or absence of a melanic pectoral band or white superciliary. This finding implies that the repeated evolution of these traits cannot be attributed to parallel genetic changes at this locus. There were 20 variable sites within *A. torquatus*, eight of which were nonsynonymous substitutions (Table 1). None of the substitutions were fixed across taxa with the same phenotype for either trait. Instead, seven of the eight nonsynonymous substitutions were singletons, occurring in only one taxon, and at the remaining position (site 346) both alleles (Ser^{115} & Gly^{115}) are present in taxa with alternative phenotypes (Table 1). There were eight variable sites within *A. brunneinucha*, only two of which were nonsynonymous, and in both cases they were singletons (Table 1).

The role of drift and selection

We used coalescent simulations of plumage traits and mtDNA sequence data (ND2) to differentiate the roles of selection and genetic drift in plumage evolution. If variation in plumage traits (presence or absence of a black pectoral band in both species and of a white superciliary in *A. torquatus*), assumed to be encoded by nuclear genes, has sorted (become fixed) within subspecies significantly faster than mtDNA haplotypes, then the hypothesis of selection rather than drift is supported. Coalescent simulations gave very low probabilities that nuclear genes would be fixed in different subspecies, as observed for plumage traits, under neutrality ($P < 0.0001$ and $P = 0.0005$ for *A. brunneinucha* assuming simultaneous or bifurcating branching, respectively; $P < 0.0001$ for *A. torquatus* regardless of branching pattern). This suggests that plumage variation is better explained by some form of selection causing divergence.

Discussion

Phylogenetic reconstructions of ancestral character states reveal that regardless of uncertainties in phylogeny reconstruction, phenotypic similarity of geographically distant populations separated by phenotypically distinct populations in *A. torquatus* and *A. brunneinucha* (particularly in relation to the presence or absence of a pectoral band) is best explained by parallel evolution or retention of ancestral traits in distant populations with differentiation of central populations, and not by close phylogenetic affinity. The same conclusion was reached by other studies examining leapfrog patterns in a phylogenetic context (García-Moreno & Fjeldså, 1999; Dumbacher & Fleischer, 2001; Norman *et al.*, 2002; Pavlova *et al.*, 2005). This raises the questions of (i) what are the genetic and developmental mechanisms leading to the parallel origin of the same phenotype in different populations, and (ii) what is the relative importance of different microevolutionary processes (i.e. genetic drift and different forms of selection) in the independent fixation of alternative phenotypes in different populations.

Chapman (1923) proposed that geographical variation in *Arremon* was likely caused by recurrent mutations (see also Parkes, 1954), a hypothesis later dismissed because it was put forward at a time when mutations 'had attracted the fancy of biologists' (Paynter, 1978). However, mounting recent evidence suggests that the repeated evolution of plumage traits might be a result of substitutions (i.e. mutations in the modern sense) in genes with large phenotypic effects, which sets the stage for testing Chapman's hypothesis. Our results demonstrate that the expression of melanic plumage traits (pectoral bands and a lack of a white supercilliary) in distantly related *Arremon* taxa is not because of shared mutations within the coding region of *MC1R*. The only possible case is a nonsynonymous substitution at site 346 shared by *torquatus, fimbriatus* and *borelli*, three closely related taxa exhibiting a white (i.e. unmelanized) supercilliary. Although to our knowledge, this site has not been shown to be associated with phenotypic variation in any species of vertebrate, it is located near the third transmembrane domain of *MC1R* (a part of the protein important in agonist binding), which contains mutations associated with melanic plumage in *Monarcha* flycatchers (Uy *et al.*, 2009) and melanic coat colour in mammals (Kijas *et al.*, 1998; Våge *et al.*, 1999). However, a causal association between genetic variation in *MC1R* and the absence of a melanized superciliary in these *Arremon* taxa seems unlikely, because associations between *MC1R* variation and expression of derived non-melanic traits in highly localized plumage patches remain to be demonstrated (Badyaev, 2006). We note, however, that our data are not sufficient to investigate the functional consequences of singleton nonsynonymous substitutions within taxa with melanic traits. Likewise, our data do not enable us to reject the hypothesis that regulatory variation influencing the expression of *MC1R* accounts for phenotypic variation.

The repeated evolution of plumage traits in *Arremon* and other taxa exhibiting leapfrog variation may be partly explained by changes in developmental processes and restrictions imposed by development (Badyaev, 2006). For example, once a particular plumage pattern has evolved in a lineage, slight alterations in development, such as changes in threshold mechanisms that alter melanocyte sensitivity to stimulating or inhibitory factors, can allow for the repeated gain and loss of melanic elements (Price & Pavelka, 1996). Another developmental mechanism potentially involved in the

evolution of leapfrog patterns in *Arremon* is heterochrony, broadly defined as a shift in the relative timing of developmental events (Raff & Wray, 1989). Because juveniles of *A. torquatus* and *A. brunneinucha* lack distinct pectoral bands, losses of this trait (Fig. 2) essentially reflect retention of a juvenile trait in adults (Fig. S1). Retention of juvenile plumage in adults also occurs in *Sporophila,* another confusing emberizid genus (Areta, 2009). The varying width and sharpness of pectoral bands across *A. torquatus* taxa (Chapman, 1923; Paynter, 1978) might also reflect that the development of this trait is arrested at different stages in different populations; however, it may also have resulted from the fixation of variants that may have existed among adults within populations (Chapman, 1923; Fig. S1).

Regardless of the molecular and developmental mechanisms involved, another fundamental question about leapfrogging in *Arremon* and other taxa is what microevolutionary forces underly this pattern? In particular, what is the relative role of different forms of selection versus genetic drift? Reflecting the pre-Modern Synthesis thinking of his time, Chapman (1923) hypothesized that the presence or absence of plumage traits in brushfinches '…does not materially affect a species' chance of success or failure. I also believe that natural selection has played no part in their development'. Because Remsen (1984) did not observe concordance in the geographical distribution of taxa showing leapfrog variation in the Andes, he concurred with Chapman in attributing such patterns of variation to chance events, and further suggested that '…if this hypothesis is correct, much of the phenotypic differentiation in the speciation process may be due to stochastic factors, absence of gene flow, and transilience, rather than to more predictable, environmentally induced factors.'. Similarly, it has been suggested that speciation in high-elevation Andean birds is largely driven by persistence of relict populations in climatically stable areas, followed by divergence as a consequence of genetic drift (García-Moreno & Fjeldså, 2000). In contrast with these views, our coalescent analyses indicate that fixation of plumage traits in *Arremon* has occurred faster than expected under drift alone, implying that some form of selection has driven phenotypic divergence. More generally, because phenotypic differentiation in plumage among populations can be considered a precursor to species-level diversification in birds (Phillimore *et al.*, 2007; Martin & Tewksbury, 2008), our findings are consistent with reviews highlighting a prominent role for selection and a negligible role for genetic drift in speciation (Coyne & Orr, 2004; Price, 2008). However, we note that although our analyses reveal that genetic drift is not the only factor driving population differentiation, this mechanism may facilitate speciation when acting in concert with selection (Templeton, 2008; Uyeda *et al.*, 2009). In any event, an outstanding question is whether leapfrog patterns of geographical variation in *Arremon* result from the action of natural selection or social (including sexual) selection.

It seems hard to imagine how the presence or absence of ornaments like melanized pectoral bands or superciliaries might directly affect the performance or survival of individuals differentially in different populations/environments (e.g. via effects on thermoregulation or camouflage), so the hypothesis that plumage divergence has been driven by natural selection would, at first glance, appear unlikely. However, because the production of melanic plumage ornaments might be costly and influenced by environmental and physiological factors such as nutrition and hormonal profiles (McGraw, 2008), differences among populations in the resource base or in factors influencing hormone levels might lead to variation in the adaptive value of different phenotypes across populations as a result of tradeoffs, and thereby result in phenotypic differentiation via natural selection.

Alternatively, geographical variation in *Arremon* might have arisen as a consequence of social selection, i.e. selection arising out of competition for resources, including mates (West-Eberhard, 1983; Price, 2008). An obvious form of social selection that could account for the evolution of leapfrogging is sexual selection, particularly if female preferences for male traits vary geographically (Price, 1998). For example, studies in the Common Yellowthroat (*Geothlypis trichas*) indicate that features of male melanic plumage ornaments are involved in mate selection by females in some populations but not in others (Tarof *et al.*, 2005; Dunn *et al.*, 2008). Although the traits we examined are apparently not sexually dimorphic in *Arremon* (black plumage patches rarely show UV reflectance, but we cannot be certain there is no dimorphism in UV colour owing to the acute visual system of birds; Eaton & Lanyon, 2003; Eaton, 2005) and characters thought to evolve via sexual selection generally differ markedly between sexes (but see Lande, 1980; Badyaev & Hill, 2003), this mechanism might underly plumage evolution if sexual selection acts in both males and females or if female ornaments evolve as a consequence of constraints resulting from selection acting on males (Amundsen, 2000; Clutton-Brock, 2007; Cardoso & Mota, 2010). In addition, other forms of social selection merit consideration. For instance, social selection on sexually monomorphic plumage traits conferring information of relevance to territory defense and to dominance in agonistic contexts has been implicated as a driver of phenotypic differentiation in some passerine birds (Filardi & Smith, 2008; Tibbets & Safran, 2009). Conducting experiments to examine responses of females and males to mounted specimens with alternative phenotypes in different contexts (e.g. mate selection, territorial defense) across different *Arremon* populations would be of interest to understand how social selection might operate in this system.

Many Andean species exhibit strong population structure owing to their narrow, easily fragmented ranges

(Graves, 1988; Weir, 2009). This propensity for population isolation related to range geometry might explain why leapfrog patterns of geographical variation are especially pervasive and conspicuous in the Andes (Remsen, 1984). Additionally, convergent or parallel evolution resulting in leapfrog patterns is likely to arise if (i) the traits involved are adaptive and localities with similar selective environments are geographically separated (Wiens *et al*., 2006), or (ii) traits are subject to sexual selection and females exhibit variation in preexisting biases for particular traits or phenotypic traits and mating preferences are genetically linked (reviewed by Price, 2008).

Conclusions

Because of the central role that plumage coloration plays in mate selection and species recognition in birds, the origin of plumage diversity within species is likely a catalyst for avian speciation, and thus should be a central topic in the study of diversification in this group. Our study reveals that leapfrogging in *Arremon* arose as a consequence of convergent or parallel evolution in different populations and is consistent with macroevolutionary analyses indicating that plumage traits are highly labile, implying that such traits respond readily to selection and that they are often unreliable indicators of phylogenetic relationships (Omland & Hofmann, 2006). Although we could not identify the molecular basis of alternative phenotypes because they were not associated with nucleotide variation in the candidate locus we examined, traits involved in leapfrogging probably have a simple genetic basis and their recurrent appearance is likely an outcome of restrictions that developmental processes impose on phenotypic diversification. Most notably, our population genetic analyses indicate that, in contrast to prevailing views, leapfrog variation in *Arremon* is not entirely because of genetic drift, but rather reflects the action of deterministic selective processes. This calls for more studies assessing the adaptive significance of geographical variation and suggests that phenotypically differentiated populations, despite often not being unique based on neutral DNA markers (Zink, 2004), may be the result of adaptive processes (Leinonen *et al*., 2008; Pérez-Emán *et al*., 2010) and thus worthy of attention from an evolutionarily oriented conservation perspective (Moritz, 2002).

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Half-collared Sparrows (*Arremon semitorquatus*, a close relative of *A. torquatus*) from the same locality (Aripuanã, Mato Grosso, Brazil) in the ornithological collection of the Departamento de Zoologia at Universidade Federal de Minas Gerais showing variation in presence/absence and extent of a black pectoral band.

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