SPECIES LIMITS IN THE RUSTY-BREASTED ANTPITTA (*GRALLARICULA FERRUGINEIPECTUS*) COMPLEX

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ABSTRACT.---The Rusty-breasted Antpitta (*Grallaricula ferrugineipectus*) is widely distributed within the tropical Andes of South America. We analyzed 73 study specimens, 25 vouchered tissue samples and 123 audio recordings to assess geographic genetic, vocal, and morphological variation and evaluate species limits. We found that *Grallaricula ferrugineipectus* as currently defined is polyphyletic because populations from Colombia and Venezuela form a clade that is closely related to Andean populations of *G. nana*, whereas populations from Peru and Bolivia are recovered as sister to *G. lineifrons*. Birds in Colombia and Venezuela (the northern group) last shared a common ancestor with birds from Peru and Bolivia (the southern group) more than 10 million years ago. Northern and southern groups additionally differ in song, suggesting that they may have evolved substantial premating reproductive isolation. Discriminant function analysis reliably distinguished songs from northern and southern groups in multivariate acoustic space, but univariate analyses found non-overlapping acoustic variation between northern and southern groups in only one trait, mean note maximum frequency (and other correlated measures of song pitch). This suggests that the "three-trait" threshold for using vocalizations to inform species limits, which was developed for another suboscine group, the antbirds (Thamnophilidae), may be conservative when applied to antpittas (Grallariidae). In addition, we document apparent clinal variation in song pace within the southern group, a rare example of a suboscine with geographic clinal variation in a vocal trait. Finally, we show that northern and southern groups differ markedly in morphology. In summary, northern and southern groups of Rusty-breasted Antpittas are divergent in genetics, vocalizations and morphology, demonstrating that these taxa are best classified as two monophyletic, biological species with allopatric distributions.

Keywords: Andes, clinal variation, songs, suboscine, South America, taxonomy, vocalizations

The Rusty-breasted Antpitta (*Grallaricula ferrugineipectus*) is a small antpitta in the family Grallariidae distributed from Venezuela to Bolivia, where it inhabits the understory of humid forests in the Andes and other montane systems, such as the Sierra Nevada de Santa Marta and the coastal Venezuela mountains (Hilty and Brown 1986, Krabbe and Schulenberg 2003, Greeney 2013). Like other *Grallaricula* species, *G. ferrugineipectus* is a plump bird with long legs and a very short tail, and it is shy and seldom seen (Greeney 2013). Three subspecies are currently recognized, each of which inhabits a distinct montane region: *G. f. ferrugineipectus* is found in northern and western Venezuela and the Sierra Nevada de Santa Marta in adjacent northern Colombia; *G. f. rara* in the Eastern Andes of Colombia and the Sierra de Perijá, which straddles the Colombia-Venezuela border; and *G. f. leymebambae* in the Andean foothills from extreme southern Ecuador to western Bolivia (Greeney 2013).

Current knowledge of the distribution of the species has been improved by recent discoveries of populations outside its traditionally known range. Although its presence in Peru north of the Marañón River had been documented since the mid 1950's based on two specimens taken independently by M. Koepcke and T. A. Parker in Cancheque, Piura (Schulenberg and Parker 1981, Parker et al. 1985), there are now recent records in the departments of Piura (Vellinga et al. 2004) and Lambeyeque (Angulo Pratolongo et al. 2012), as well as in the Ecuadorian provinces of Loja and Pichincha (Athanas and Greenfield 2016; P. Coopmans unpublished data). Likewise, only in the early 1980's was the species first recorded in Bolivia (Schulenberg and Remsen 1982). More recently, MAR and collaborators discovered a population in the Cauca Valley of the Central Andes in the department of Caldas, Colombia; this population appears to be geographically isolated from other conspecific populations. Taxonomic affinities of

these populations have never been formally assessed, and their taxonomic treatment has been assumed to correspond to that of the geographically closest populations.

Populations differ somewhat in elevational distribution and habitat: subspecies *rara* and *ferrugineipectus* inhabit forested foothills from ~250 m to 2200 m (Krabbe and Schulenberg 2003), and the Cauca Valley antpittas are currently known only from one locality at 1000-1100 m. Hereafter, these three populations will be referred to as the northern group. In contrast, birds belonging to the southern group (populations from Ecuador, Peru, and Bolivia) range substantially higher and inhabit montane forest from 1750-3350 m (Ridgely and Tudor 2009). Southern group birds appear closely tied to bamboo in the genus *Chusquea* (Fjeldså and Krabbe 1990, Athanas and Greenfield 2016). This habitat association that has not been documented for the northern group, which can tolerate some degree of habitat degradation (Hilty and Brown 1986, Niklison et al. 2008, N. Athanas pers. comm.).

The three subspecies of *G. ferrugineipectus* were described based on differences in plumage and morphology. The *rara* subspecies is the most divergent with respect to plumage, with a rich rufous-brown underside and clear rufous tones on the crown and face, in contrast to the dark brown to slate-brown upperside and rufous underside of other subspecies (*ferrugineipectus* and *leymebambae*). These latter two subspecies both lack rufous on the head, have duller underparts, and show an obvious white throat crescent; *G. f. leymebambae* differs from *G. f. ferrugineipectus* in larger size and darker overall coloration (Greeney 2013). Songs of the species also vary across its geographic range. Most noticeably, *G. f. leymebambae* gives slower and higher pitched songs than *G. f. ferrugineipectus* (Krabbe and Schulenberg 2003). The song of *G. f. rara* is poorly known and not described in recent reference volumes (Krabbe and Schulenberg 2003, Greeney 2013). Finally, recordings from northwest Ecuador demonstrate that

this population's songs appear to be slower than those of other populations (e.g., see **XC35333** on xeno-canto.org).

Because populations of *G. ferrugineipectus* are distributed allopatrically throughout the tropical mountains of northern South America, reproductive isolation between subspecies cannot be directly assessed. Early on, systematists proposed arrangements of species level taxonomy based on plumage differentiation within this complex. For example, *G. f. rara* was originally described as a species due to its distinctive plumage (Hellmayr and Madarász 1914), while *G. f. leymebambae* was first described as a subspecies (Carriker 1933). More recently, differences in distribution, morphology, and vocalizations between *G. f. leymebambae* and the northern group have led some authors (e.g., Ridgely and Tudor 2009, BirdLife International 2017) to classify it as a distinct species. However, no formal comparative analysis has systematically examined genetic, vocal, and morphological variation within this complex. These data types are complementary: whereas genetic data reveal the evolutionary histories of taxa, vocalizations provide information directly related to the likelihood of premating reproductive isolation (e.g., Isler et al. 1998, Zimmer 2002, Seddon and Tobias 2007, Donegan 2008, Zimmer 2008).

Here, we present an analysis of genetic, vocal, and morphological variation across the range of *G. ferrugineipectus*. We construct a phylogenetic hypothesis including all named taxa therein and their closest relatives to test the monophyly of the species. We then examine intraspecific vocal and morphological variation and assess the diagnosability of vocal traits among populations as a proxy for the level of behavioral premating isolation. With these data, we ask whether populations in the northern and southern groups indeed represent different species and evaluate variation in their constituent subpopulations.

METHODS

Molecular Data.---An ongoing study aiming at elucidating the species-level relationships within the Grallariidae (Bravo, G. A., Cuervo, A. M., Aristizábal, N., Rice. N., Carneiro, L., Aleixo, A., Pérez-Emán, J., Brumfield, R. T. & Bates, J. M., unpubl. data) has generated mitochondrial and nuclear sequences for multiple individuals for 52 of the 53 species currently recognized in the family (sensu Remsen et al. 2017). Based on preliminary phylogenetic analyses of this dataset, we selected 25 grallariid individuals of the following species, which include all genera in the family: *Grallaria guatimalensis* (1 sample), *Hylopezus berlepschi* (1), *Myrmothera campanisona* (1), *Grallaricula flavirostri*s (2), *G. lineifrons* (2), *G. nana* (7), and *G. ferrugineipectus* (11) (Supplemental Material, Appendix 1). Five of these samples were derived from toepads of museum specimens (three samples of *G. ferrugineipectus rara,* one sample of *G. flavirostris* and one sample of *G. lineifrons*). This sampling encompasses all currently recognized subspecies within *G. ferrugineipectus* and also includes birds from the Cauca Valley in Colombia. However, it does not yet include samples north of the Marañón River in Peru and Ecuador. For outgroups, we included sequences from the genera *Scytalopus and Thamnophilus* (Supplemental Material, Appendix 1).

We used standard methods described elsewhere (Brumfield and Edwards 2007, Brumfield et al. 2007, Kimball et al. 2009) to extract total DNA and to amplify and obtain sequences for two mitochondrial (NADH dehydrogenase subunit 2 – ND2, 1,041 bp; and NADH dehydrogenase subunit 3 – ND3, 351 bp) and three autosomal nuclear introns (transforming growth factor-β2 intron 5 – TGFβ2, 629 bp; muscle-specific kinase receptor intron 3 – MUSK, 651 bp; β-fibrinogen intron 5 – βF5, 568 bp). Because toepad samples came from recent museum

specimens collected in the 1990's, DNA extraction from these samples followed the same DNA extraction protocols with the addition of an extended lysis time in dithiothreitol (DTT).

We edited sequences and checked that protein-coding sequences did not include stop codons or anomalous residues using Geneious v. 8.1 (www.geneious.com, Kearse et al. 2012). We aligned sequences for each marker using the MAFFT v.7 multiple alignment plugin (Katoh and Standley 2013) implemented in Geneious and obtained a concatenated dataset using Geneious Pro v8.1. In the end, alignments for ND2 and ND3 included sequences for all individuals (27), TGFβ2 (26 individuals), MUSK (25), and βF5 (20). The final alignment included data for 3,223 base pairs. Newly obtained sequences were deposited in GenBank (Supplemental Material, Appendix 1).

Partition and Substitution Models.---We selected substitution models and the optimal partitioning regime using the greedy algorithm (Lanfear et al. 2012) and PhyML v. 3.0 (Guindon et al. 2010) implemented in PartitionFinder2 (Lanfear et al. 2017). We evaluated those models of molecular evolution available in BEAST (Drummond et al. 2012, Bouckaert et al. 2014) and the maximum number of partitions was set to be five (each marker treated separately). Using the corrected Akaike Information Criterion (AICc) (Hurvich and Tsai 1989) as a model selection parameter, we were able to partition data in three different subsets: ND2, ND3, and the three nuclear introns.

Phylogenetic inference.---Using the selected partitioned scheme, we conducted a maximum likelihood phylogenetic analysis with the GTR+Γ model of nucleotide substitution and 999 bootstrap replicates implemented in RAxML 8.2.9 (Stamatakis 2014) on the CIPRES Science Gateway V 3.3 (Miller et al. 2010). We estimated a time-calibrated species tree in a Bayesian framework using the multispecies coalescent model implemented in the program

169 *BEAST2 v2.4.4 (Drummond et al. 2012, Bouckaert et al. 2014) on the Cipres Science Gateway
170 V 3.3. We used unlinked substitution models across partitions and clock models linked by locus
171 (i.e. mtDNA and each intron separately). Gene trees were estimated independently for each of
172 the four loci (both mtDNA markers were treated as a single locus). Substitution parameters were
173 based on results previously obtained from PartitionFinder2. Based on the avian mtDNA
174 substitution rate of 2.1%/My (Weir and Schluter 2008), we used a lognormal relaxed molecular
175 clock with a mean rate of 0.0105 for mtDNA. Clock rates for nuclear introns were estimated
176 relative to the rate of mtDNA. We used a Yule prior with no restrictions on tree shape and a
177 randomly generated tree as a starting tree. We ran analyses for a total of 200 million generations
178 with a sampling frequency of 20,000. We determined that replicate analyses converged (effective
179 sample size values > 400) using Tracer v1.6 (Rambaut et al. 2014). Using TreeAnnotator v2.4.4
180 (Drummond et al. 2012, Bouckaert et al. 2014) and a burn-in of 20%, we estimated a posterior
181 distribution of topologies and the maximum clade credibility (MCC) tree.
182 *Morphometric data.*---We measured six morphological variables (wing length, tail length,
183 tarsus length, bill length from nostril to tip, bill gape, bill depth at nostrils) from 73 study
184 specimens of all subspecies of *Grallaricula ferrugineipectus* (Supplemental Material, Appendix
185 2) following Baldwin et al. (1931). We measured nine specimens of *G. f. ferrugineipectus*
186 (including six from the Sierra Nevada de Santa Marta), six from the Cauca Valley of Colombia,
187 10 from *G. f. rara*, and 48 of *G. f. leymebambae*, including one from north of the Marañon
188 Valley. All measurements were taken to the nearest 0.01 mm with a Mitutoyo Digimatic Point
189 Caliper by GAB.
190 *Audio recordings.*---We compiled recordings of *Grallaricula ferrugineipectus* from the
191 Macaulay Library at the Cornell Lab of Ornithology (macaulaylibrary.org), xeno-canto (xeno-

192 canto.org), the natural sound collection of Instituto de Investigación de Recursos Biológicos
193 Alexander von Humboldt (humboldt.org.co)**,** and private collections of recordists (Supplemental
194 Material, Appendix 3). We retained recordings that contained high-quality examples of the
195 species' song. Variation in the song of an individual is present in suboscines but is relatively
196 small (e.g., Bard et al. 2002, Kirschel et al. 2011); we confirmed that individual variation was
197 minor (at least within a single recording session) by manually inspecting vocalizations of the
198 same individual. We selected one song per individual for further analysis, typically the best-
199 recorded song in a series or the one with the least background noise. From these recordings, we
200 obtained 110 songs from as many individuals (Fig. 1): 49 of *G. f. ferrugineipectus* (including 13
201 from the Sierra Nevada de Santa Marta), four of *G. f. rara* from the Eastern Andes of Colombia,
202 five from the Cauca Valley in the Colombian Central Andes, 12 from northwest Ecuador, four
203 from the Andes between the Marañón River and central Ecuador, and 36 of *G. f. leymebambae*
204 south of the Marañón River. To compare vocal variation within the *G. ferrugineipectus* complex
205 to closely related species, we included 7 songs from *G. nana* and 6 from *G. lineifrons* in our
206 analysis, but we did not include *G. flavirostris* because the song of this species seems to consist
207 of only one note and thus cannot be meaningfully compared to the other species, which all have
208 multi-note songs.

209 *Acoustic data processing.*---We used Raven Pro 1.5 (Bioacoustics Research Program
210 2014) to make measurements of the acoustic properties of vocalizations. *Grallaricula* songs are
211 typically composed of a series of repeated notes that are structurally simple (Greeney 2013). It is
212 therefore straightforward to capture acoustic variation of *Grallaricula* vocalizations with a series
213 of acoustic measurements. For each song, we manually identified its constituent notes by
214 drawing selection boxes. We then used the Raven software to record the following measurements

for each note: the frequency of peak power, the minimum and maximum frequencies of the peak frequency contour, the average slope of the peak frequency contour, the duration containing 90% of the energy of the note, and the bandwidth containing 90% of the energy of the note. We summarized these note measurements using the following 19 statistics for each song: 1) mean note slope (Hz/ms), 2) mean note peak frequency (Hz), 3) mean note bandwidth (Hz), 4) mean note peak frequency bandwidth (Hz), 5-6) mean note maximum and minimum frequencies (Hz), 7) number of notes per song (note count), 8) the duration of the song (s), 9) the mean duration of each note (s), 10) the rate of note delivery (note rate or song pace; notes per second), 11) the frequency slope of the song (Hz per note), 12) the song peak frequency bandwidth (Hz), 13-14) the position of the frequency minima and maxima within each note of the song (proportion of note from 0-1), 15) the change in note pacing through the song (s per note), 16) the maximum frequency of the song (Hz), 17) the minimum frequency of the song (Hz), 18) the difference between the two (song bandwidth; Hz), and 19) and the position of the maximum frequency note in the song (proportion of song from 0-1). We subsequently examined measurements for collinearity and found that measurements 2, 5, 6, 16, and 17 were closely correlated. Of these, we retained only mean note maximum frequency (5) because it showed the highest correlations with the other frequency variables (all $r > 0.95$).

We included recordings made following the use of conspecific playback in our dataset. Although most recordings did not have associated metadata describing whether they followed playback or not, the vast majority of recordings with such metadata were made using playback (see Supplemental Material, Appendix 3). We therefore have too few recordings made without playback for each taxon to investigate a possible effect of its use at this time. However, we believe that the use of playback is unlikely to affect our conclusions regarding acoustic

238 differentiation for three reasons. First, the use of playback was relatively consistent among
239 subspecies. Second, we have no *a priori* reason to believe that playback differentially influences
240 different subspecies. Third, even if playback does induce vocal differences, including recordings
241 made both with and without playback provides a fuller range of vocal variation of each
242 population (i.e., calmer and agitated individuals), which is a statistically conservative approach
243 to assessing diagnosability.

244 *Statistical Analyses.*---We conducted several analyses to quantify levels of differentiation
245 and diagnosability in acoustic and morphological traits in our sample. We began by examining
246 differentiation between the northern and southern groups, which have been proposed to represent
247 different species (e.g., Ridgely and Tudor 2009). We first tested for differences in means of
248 vocal and morphological traits, using the Tukey Test to correct for multiple comparisons. We
249 applied a log transformation when it reduced the skewness of the given trait distribution. We
250 then calculated 95% prediction intervals for all traits, which estimate the spread of observations
251 in a population; non-overlapping prediction intervals indicate that trait distributions are unlikely
252 to overlap even as a larger sample is gathered (Isler et al. 1998). Species delimitation criteria
253 derived for another suboscine group, the antbirds, suggest that allopatric populations should be
254 classified as distinct biological species when they have diagnosable (non-overlapping)
255 differences in at least three independent vocal traits (Isler et al. 1998, Isler et al. 2007a, Isler et
256 al. 2007b, Isler et al. 2008). This yardstick approach has also been used in antpittas (Donegan
257 2008), but its efficacy has not been widely evaluated in the Grallariidae (e.g. by systematically
258 analyzing existing sympatric species pairs). As acknowledged by its proponents, this yardstick
259 approach as a point of reference (Isler et al. 1998) can be relaxed in certain cases, especially

when taxa are parapatric, or show distinct morphological differentiation (Isler et al. 2007b, Isler et al. 2012).

As an additional measure of diagnosability, we conducted discriminant function analyses on the above groupings. We scaled and centered our data before analysis by subtracting the mean and dividing by the standard deviation. We quantified diagnosability with a cross-validation approach: we withheld one datum, trained the model with the remaining data, and then asked the model to classify the withheld datum. We repeated this procedure 1,000 times and then calculated a misclassification rate for each taxonomic grouping. We analyzed vocal and morphological data separately because our vocal and morphological measurements were made on different individuals.

In addition, we tested for geographic variation within northern and southern groups in vocal and morphological traits. Within the northern group, we examined four subpopulations: 1) *G. f. ferrugineipectus* from Venezuela, 2) *G. f. ferrugineipectus* from the Sierra Nevada de Santa Marta, 3) *G. f. rara* from the Eastern Andes of Colombia, and 4) the population from the Cauca Valley of the Colombian Central Andes. We treated the population from the Sierra Nevada de Santa Marta as a distinct group to test for differentiation due to their isolated distribution. Within the southern group, we considered two subgroups: 1) antpittas from Ecuador and northwestern Peru, which are vocally similar (P. Coopmans, unpubl. data), hereafter "northern *leymebambae*; and 2) antpittas from northeastern Peru south (hereafter "southern *leymebambae*"). These groups are divided by the Marañon River, an important isolating barrier for Andean avifauna (Winger and Bates 2015). We primarily used discriminant function analyses as described above, but we also conducted tests of the "75% rule," which is satisfied if 75% of the trait values for a population lie outside 99% of the trait values of the other population (Patten et al. 2002). This

283 test has previously been used as a criterion for subspecies classification in the genus *Grallaricula*
284 (Donegan 2008).

285 RESULTS

286 *Phylogenetic analyses.*---Maximum-likelihood and Bayesian analyses produced identical
287 topologies supporting the non-monophyly of *Grallaricula ferrugineipectus*. Northern
288 populations (i.e., *G. f. ferrugineipectus*, *G. f. rara*, Cauca Valley, and Sierra Nevada de Santa
289 Marta) form a strongly supported clade that is sister to Andean populations of *G. nana* (albeit
290 with low support in the Bayesian species tree), whereas populations from Peru and Bolivia (i.e.,
291 *G. f. leymebambae*) are recovered as sister to *G. lineifrons* (Figs. 2 and 3). The time-calibrated
292 species tree estimated that the most recent common ancestor between northern and southern
293 groups split between 10.8 and 16.8 mya (Fig. 3). Additionally, northern populations of *G.*
294 *ferrugineipectus* exhibit some degree of geographic structure and differentiation that is not
295 entirely consistent with current subspecific boundaries. Further insights regarding taxonomic
296 limits of northern populations and their close relationship with *G. nana* will be published
297 elsewhere.

298 *Overall vocal variation.*---Northern and southern groups show strong divergence in song
299 (Fig. 4). To visualize these differences, we plotted the first two factors of a DFA run on all
300 populations (Fig. 5). The first factor, which explained 86.7% of between-group variance, loaded
301 strongly for mean note maximum frequency and song pace (Fig. 6). The second factor, which
302 explained 9.3% of the variance, was primarily composed of the number of notes per song and
303 additional variation in song pace (Supplemental Material, Table S1).

304 *Overall morphological variation.*---We visualized divergence in morphometrics in the
305 same way as for vocal variation (Fig. 7). The first factor explained 85.6% of the variance

306 between measured populations and loaded strongly for tarsus, tail length, and wing length. The
307 second factor, which explained 9.3% of the variance, was primarily composed of bill length and
308 gape (Supplemental Material, Table S2).

309 *Tests of species rank.*---Northern and southern groups significantly differed in the mean
310 values of 13 out of 15 vocal traits (Supplemental Material, Fig. S1). However, mean note
311 maximum frequency was the only vocal character for which 95% prediction intervals did not
312 overlap (Table 1). To place this result into context, we considered the number of vocal characters
313 for which 95% prediction intervals did not overlap between currently recognized *Grallaricula*
314 species. The southern group differed from *G. nana* in only one vocal character, song pace,
315 whereas populations in the northern group differed from *G. nana* in three characters: mean note
316 maximum frequency, the frequency slope of the song, and the position of the maximum
317 frequency in the song. Both northern and southern groups differed from *G. lineifrons* by several
318 vocal traits (seven and four, respectively), and *G. nana* differed from *G. lineifrons* by eight vocal
319 traits (Table 1). Although northern and southern groups differed significantly from one another
320 in the mean value of all six morphological characters (Supplemental Material, Fig. S2), none was
321 diagnosable at the 95% prediction level.

322 Discriminant function analysis performed well at separating northern and southern groups
323 based on both vocal and morphological traits. For vocal traits, the cross-validated correct
324 classification rate was 100% for the northern group and 97.6% for the southern group. The single
325 discriminant factor loaded most heavily for mean note maximum frequency (Supplemental
326 Material, Table S3). For morphological traits, the cross-validated correct classification rate was
327 95.5% for the northern group and 100% for the southern group; the discriminant factor was
328 primarily composed of tarsus and tail length (Supplemental Information, Table S4).

Geographic variation within the northern group.---Populations in the northern group exhibited significant variation in mean vocal trait values, with 12 of 15 traits showing significant differences among populations (Supplemental Material, Fig. S3). However, discriminant analysis showed a relatively poor ability to distinguish northern populations based on vocal traits. The most distinctive populations were *G. f. ferrugineipectus* from Venezuela and the population from the Cauca Valley of Colombia, which were classified with 85.1 and 82.6% accuracy, respectively. Songs from the Sierra Nevada de Santa Marta were classified with 78.0% accuracy and from the subspecies *rara* with 76.9% accuracy. The first discriminant factor (72.4% of variance) loaded very strongly for song pace and number of notes, and the second (19.1%) for additional variation in song pace (Supplemental Material, Table S5). By the 75% rule, these four populations were generally not diagnosable; *G. f. rara* from the Eastern Andes of Colombia differed from *G. f. ferrugineipectus* from Venezuela in song pace, but this was the only difference among northern populations.

Populations from the northern group showed significant differences in mean values of four morphological traits (Supplemental Material, Fig. S4), but there were no differences at the level of the 75% rule. The discriminant analysis of morphological traits performed poorly at distinguishing G. *f. ferrugineipectus* from Venezuela (20.9% correct), *G. f. rara* (51.1% correct), and individuals from the Cauca Valley (65% correct) (Supplemental Material, Table S6), whereas those from the Sierra Nevada de Santa Marta achieved a correct classification rate of 76.8%. Note, however, that the sample sizes for these groups were generally small (N = 3-8).

Geographic variation within the southern group.---Southern Rusty-breasted Antpitta populations also varied in mean vocal trait values, with significant differences between northern and southern *leymebambae* in 8 of 15 traits (Supplemental Material, Fig. S5). Discriminant

analysis performed well in distinguishing the two groups, with cross-validated correct classification rates of 95.5% and 97.2% for northern and southern *leymebambae*, respectively. The single discriminant factor (100% of variance) loaded strongly for the number of notes per song, song duration, and song pace (Supplemental Material, Table S7). Considering the 75% test, however, the groups showed no diagnosable vocal differences. Morphological tests were not conducted because there was only one measured individual from the "northern *leymebambae*" group.

DISCUSSION

We find that *Grallaricula ferrugineipectus*, as currently recognized, is polyphyletic. The southern subspecies *G. f. leymebambae* is more closely related to *G. lineifrons* and *G. flavirostris* than it is to the northern subspecies *G. f. ferrugineipectus* and *G. f. rara*; in turn, these northern populations are more closely related to *G. nana* than to *G. f. leymebambae*. In fact, the split between the northern and southern clades represents the earliest divergence within the genus *Grallaricula* (Fig. 2, Bravo et al. unpubl. data), and the age of this split is close to the start of diversification of the Hylopezus-Myrmothera-Gallaricula clade, estimated at approximately 13-21 mya (Ohlson et al. 2013). Hence, *G. ferrugineipectus,* as currently defined, is polyphyletic and comprises populations that belong to highly divergent and distinctive clades. We therefore propose to elevate *G. f. leymebambae* to species rank. We recommend the complex be considered to consist of two species and, provisionally, two subspecies.

Grallaricula ferrugineipectus (Sclater 1857)

Grallaricula f. ferrugineipectus (Sclater 1857)

Grallaricula f. rara (Hellmayr and Madarász 1914)

Grallaricula leymebambae (Carriker 1933)

Hereafter, we refer to these taxa by the above naming scheme.

Taxonomic status and nomenclature of the populations in the Cauca Valley of Colombia and north of the Marañón River in Peru and Ecuador will be assessed elsewhere. Here, they are maintained as part of *G. ferrugineipectus* and *G. leymebambae*, respectively, with no defined subspecific ascription.

Even though the split between *G. ferrugineipectus* and *G. leymebambae* was one of the earliest divergence events in *Grallaricula*, these allopatric taxa were diagnosable at the 95% prediction level in only one measured vocal trait, the mean note maximum frequency, and in no measured morphological traits. In addition, *G. leymebambae* differed from *G. nana* by only one vocal character, song pace. In contrast, *G. ferrugineipectus* differs from *G. nana* by three vocal characters. A point of reference of diagnosability in at least three vocal traits for species status has been frequently used in suboscine taxonomy, traditionally with antbirds (Isler et al. 1998, Isler et al. 2007a, Isler et al. 2007b, Isler et al. 2008) but also in antpittas (Donegan 2008). The results of the present study suggest that this framework, which does not consider how multiple traits covary among populations, may be conservative when applied to allopatric antpitta taxa. For example, the northern and southern groups did not differ in song pace at the 95% prediction level, but the combination of song pace and note frequency yielded fully distinguishable groups (Figs. 5 and 6). Further assessment of the "three-trait" rule is still required for the Grallariidae.

We emphasize that, though diagnosably different in only a single measured vocal trait, songs from northern and southern groups were fully distinguishable in our multivariate discriminant function analyses. Other differences may also be present in calls, which we did not examine here.

Divergence in vocal traits can in part be a by-product of changes in body size, with larger birds emphasizing lower-pitched sounds (Ryan and Brenowitz 1985, Martin et al. 2011). The primary vocal difference between *G. ferrugineipectus* and *G. leymebambae* was in frequency, with *ferrugineipectus* singing lower-pitched songs. However, morphological measurements (tarsus, tail, and wing) showed that *ferrugineipectus* were generally smaller overall, which is a difference in the opposite direction than that expected by body size alone. This suggests that the primary vocal difference between these groups may be due to selection (e.g. linked to habitat or sexual selection).

We conducted additional analyses to investigate variation in vocalizations and morphology within *G. ferrugineipectus* and *G. leymebambae*. As expected, levels of differentiation were generally lower within than between these groups. We found that northern populations could be vocally distinguished with moderately high accuracy (>75%), but none of them could be reliably classified with morphological measurements. By the 75% rule, none of these groups showed consistent differences in vocal or measured morphological traits. The small sample sizes of *G. f. rara* and Cauca Valley songs make it difficult to reliably evaluate vocal differences for these groups. Overall, moderate vocal differentiation exists across northern populations, but measured morphological variation is less evident. We note that, though some of these populations (e.g., *G. f. rara*) show divergent plumage, we did not analyze plumage variation. It is interesting to note that *rara* did not show particularly distinct vocalizations, despite distinctive plumage.

We documented moderate vocal differentiation in *Grallaricula leymebambae*, with northern and southern *leymebambae* distinguishable with 95-97% accuracy. Song pace and number of notes were the most important distinguishing variables; southernmost birds sang almost twice as fast as did the northernmost birds. This trait appears to vary clinally, with no evidence for distinct jumps in trait value with latitude (Fig. 8). Although previous work has documented clinal variation in morphology in tropical Andean birds (e.g., Graves 1991), examples of clines in suboscine song traits has seldom been noted (but see Isler et al. (2005), who demonstrated clinal variation in song pace in the Variable Antshrike *Thamnophilus caurelescens*). Given that vocal traits are presumed to be innate in suboscines, observed vocal clines should reflect underlying genetic clines in the gene(s) and/or regulatory regions that influence the clinal vocal trait (see Isler et al. 2005), and they potentially offer a case example to investigate the genetic basis of suboscine song. However, there are additional potential barriers to dispersal through the species' range (e.g., the Huallaga and Apurímac valleys; Winger et al. 2015), and further research is necessary to rigorously evaluate whether latitudinal patterns in song pace in the southern population are truly clinal or instead show a step-wise pattern of variation.

Our results support the well-documented role of Andean geographic features (i.e., valleys and ridges) in driving and maintaining population structure of Andean birds (e.g., Gutiérrez-Pinto et al. 2012, Benham et al. 2015, Winger and Bates 2015). Specifically, features such as the Maranon valley in northern Peru and the Cauca and Magdalena valleys in the Northern Andes have been widely supported as primary barriers to dispersal in the Andes (Vuilleumier 1969, Parker et al. 1985, Fjeldså and Krabbe 1990, Weir 2009, Cuervo 2013, Winger and Bates 2015). These valleys act as pronounced barriers for antpittas and have likely shaped observed patterns

444 of phylogeographic structure within both *G. ferrugineipectus* and *G. leymebambae*. It is also
445 important to note that the apparently disjunct range of *G. ferrugineipectus sensu lato*—with a
446 large gap in distribution between northern Ecuador and central Colombia—is an artifact of
447 taxonomic bias. As this study shows, the populations to the north and south of this "gap" (i.e. *G.*
448 *ferrugineipectus* and *G. leymebambae*) are not closely related. In fact, the "gap" is largely
449 occupied by their respective sister taxa, *G. nana* and *G. lineifrons*. Truly disjunct ranges in
450 Neotropical mountain birds are uncommon, and such distributions may indicate a need for
451 taxonomic revision.

452 In sum, we demonstrate that northern and southern populations of *Grallaricula*
453 *ferrugineipectus sensu lato* are 1) deeply divergent genetically, and not even sister lineages, 2)
454 differ markedly in song (particularly in song pace), and 3) are morphologically divergent. As
455 such, these two groups merit classification as distinct biological species. We further document
456 variation within each group, laying the groundwork for future taxonomic assessments. In
457 particular, further work could incorporate both genetic data and playback experiments to "ask the
458 birds themselves" if the differences in note shape and pacing between southern populations are
459 sufficient to generate premating reproductive isolation based on voice (e.g., Seddon and Tobias
460 2007, Zimmer 2008, Areta and Pearman 2009, Pegan et al. 2015).

461

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645

646 TABLES

647 Table 1. Vocal traits that differ between northern and southern Rusty-breasted Antpittas and two

648 congeners following the 95% prediction interval test.

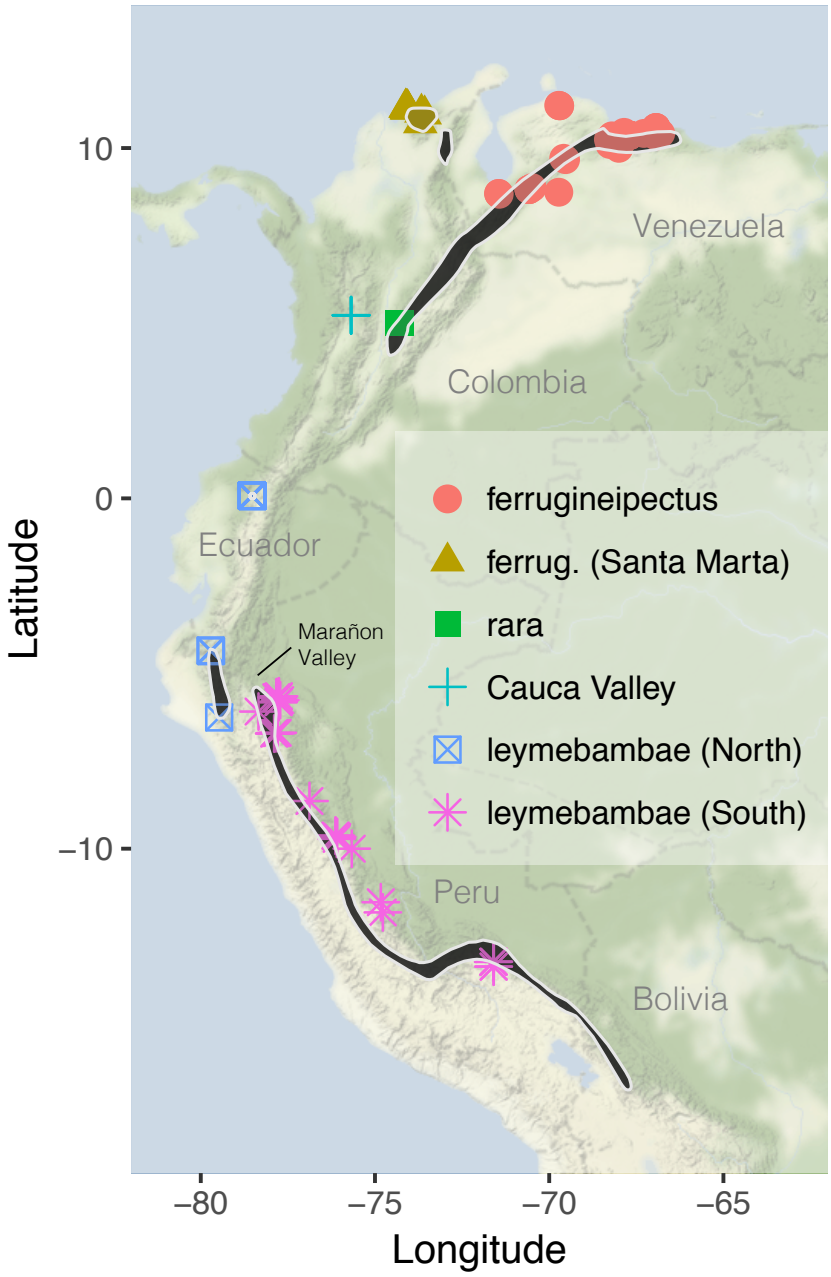
	north	**nana**	**lineifrons**
south	MNMX	RND	MNMX FSS SPFB SB
north		MNMX FSS PHFN	MNMX DS MDN RND FSS SPFB SB
nana			MNPFB MNB DS MDN RND FSS SPFB PHFN

Mean note slope (Hz/ms)	MNS
Mean note peak frequency (Hz)	MNPF
Mean note peak frequency bandwidth (Hz)	MNPFB
Mean note bandwidth (Hz)	MNB
Mean note maximum frequency (Hz)	MNMX
Mean note minimum frequency (Hz)	MNMN
Number of notes per song	NNS
Duration of song (s)	DS
Mean duration of each note (s)	MDN
Rate of note delivery (notes per s)	RND
Frequency slope of the song (Hz per note)	FSS
Song peak frequency bandwidth (Hz)	SPFB
Position of the frequency min within each note	PFMN
Position of the frequency max within each note	PFMX
Change in pace (s per note)	CP
Maximum frequency of the song (Hz)	MXFS
Minimum frequency of the song (Hz)	MNFX
Song bandwidth (Hz)	SB
Position of the highest frequency note	PHFN

649

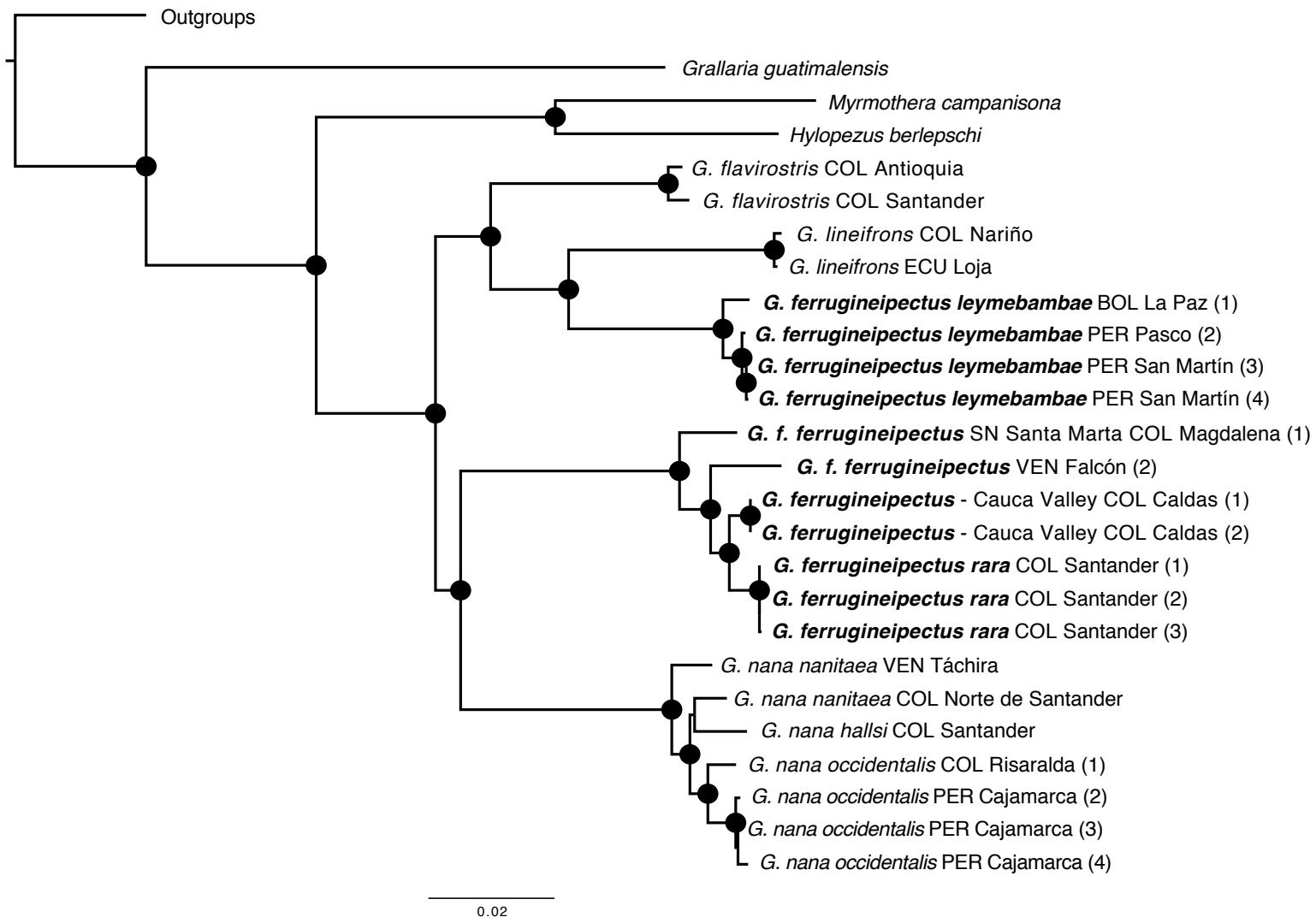
650

651 FIGURES



652

653 FIG. 1. Locations of audio recordings included in this study, colored by population. The range
654 map of this species from BirdLife International (BirdLife International and NatureServe 2015) is
655 indicated in black. Note that this map is not fully accurate (e.g., there are recordings from sites
656 outside the range indicated in black).



657

658 FIG. 2. Maximum-likelihood phylogeny of a subset of the Grallariidae. Note that *Grallaricula ferrugineipectus sensu lato* is

659 polyphyletic. Black circles at nodes indicate bootstrap support values > 70 based on 999 maximum-likelihood replicates.

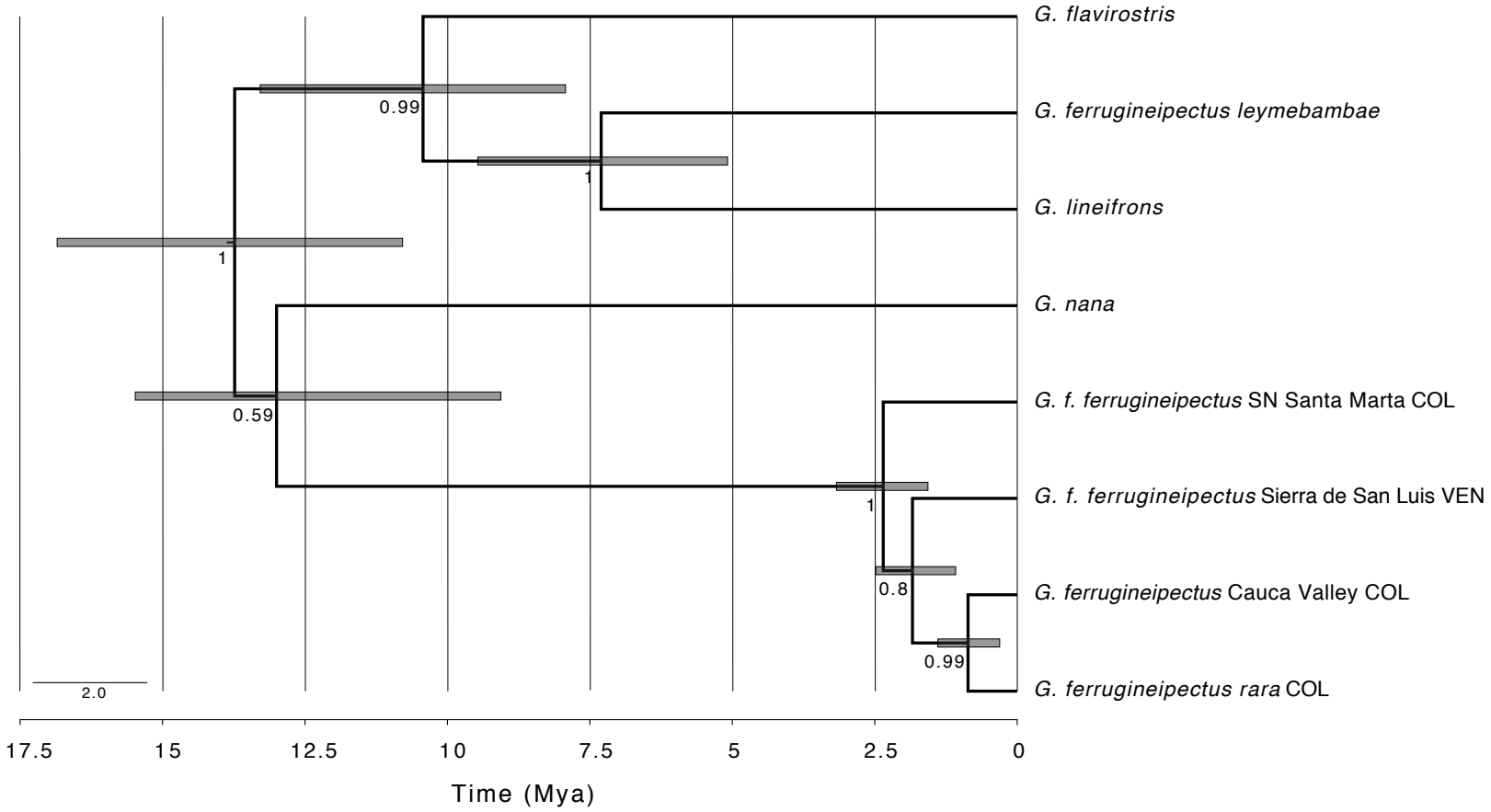
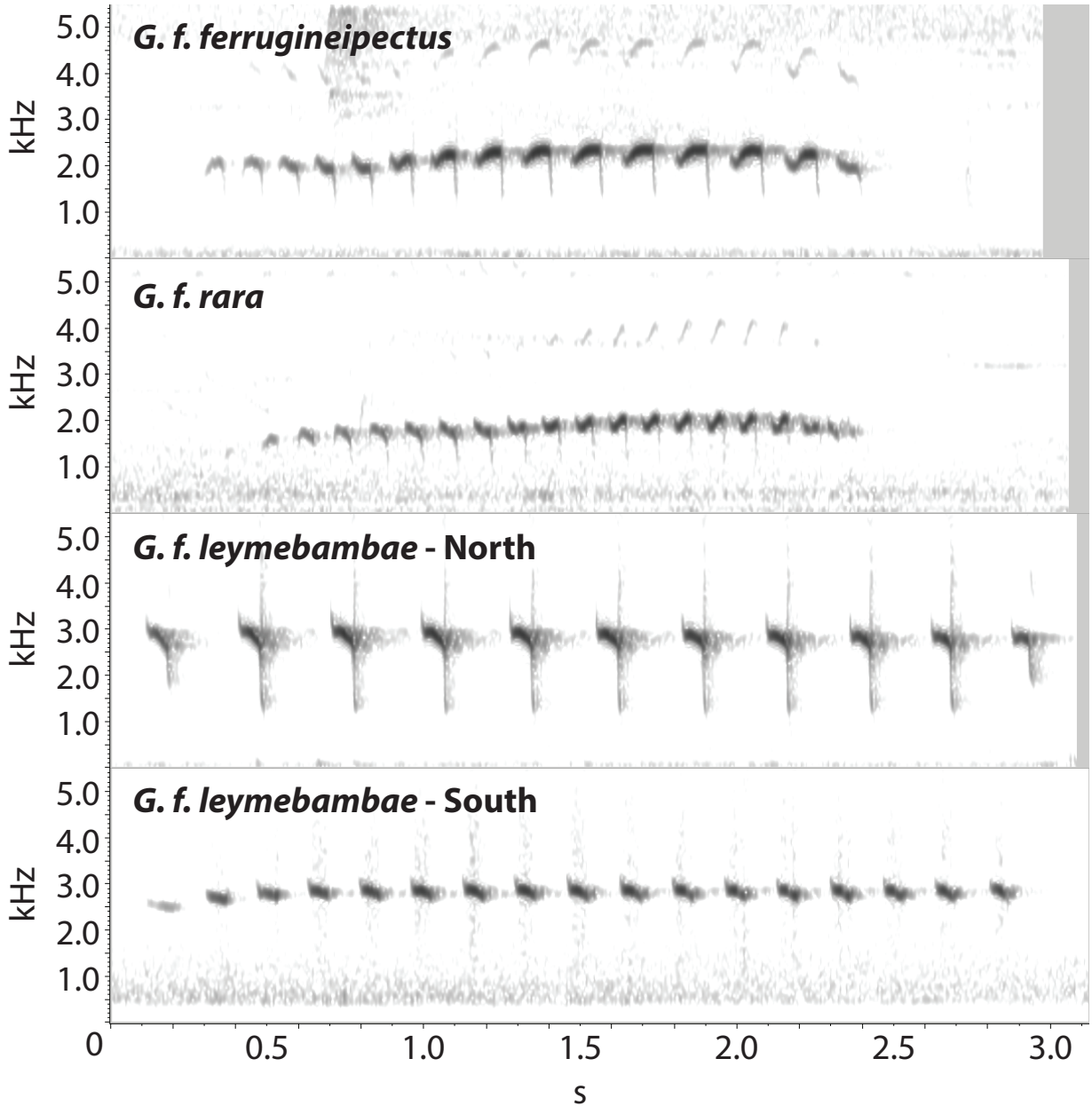
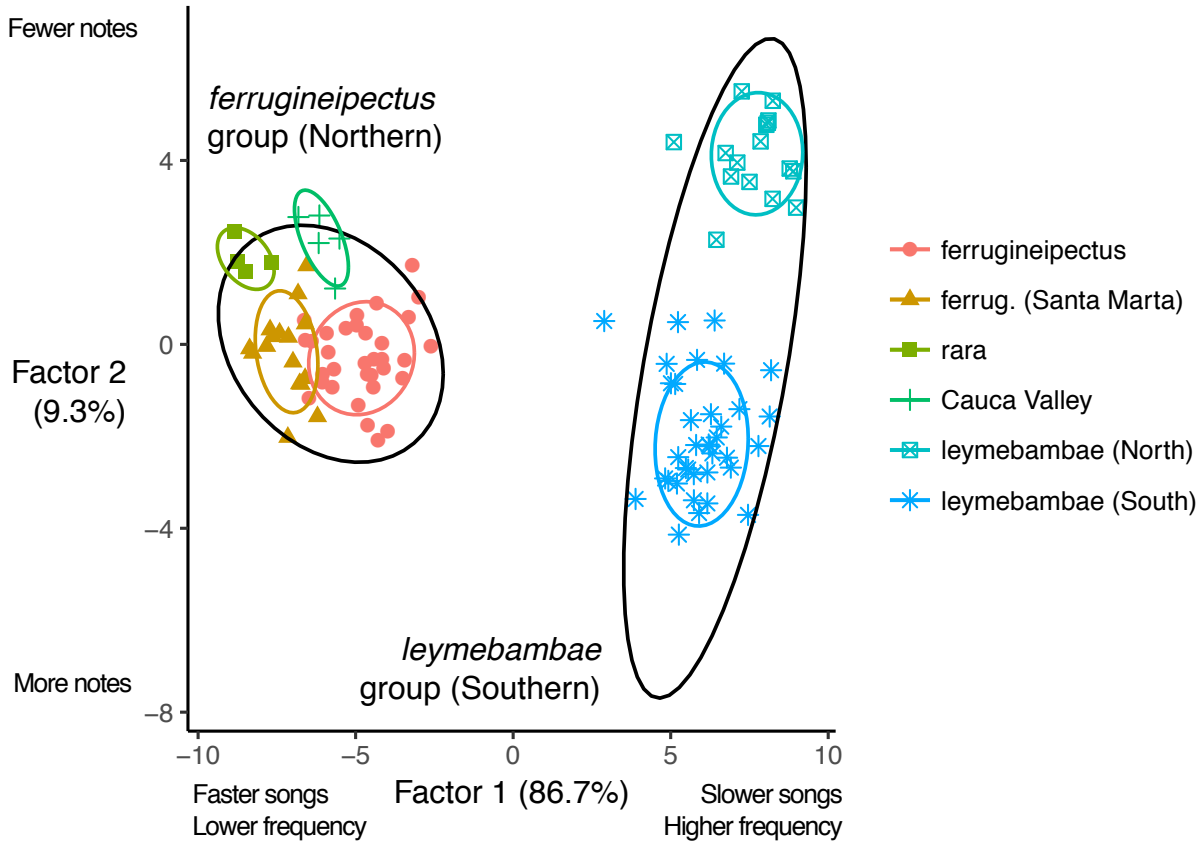


FIG 3. Bayesian estimate of phylogenetic relationships and divergence times among a subset of the Grallariidae. *Grallaricula ferrugineipectus sensu lato* is polyphyletic; the northern and southern groups (*G. f. ferrugineipectus* and *G. f. leymebambae*) last shared a common ancestor around 13 million years ago. Bars at nodes indicate the 95% highest posterior density for the inferred divergence time estimates. Numbers at nodes indicate posterior probability support values.



665
666 FIG. 4. Representative spectrograms of Rusty-breasted Antpitta songs. Spectrograms were made
667 in Raven Pro 1.5 with the following parameters: Hann windows of size 553 samples; hop size
668 277 samples; DFT size 1024 samples.
669

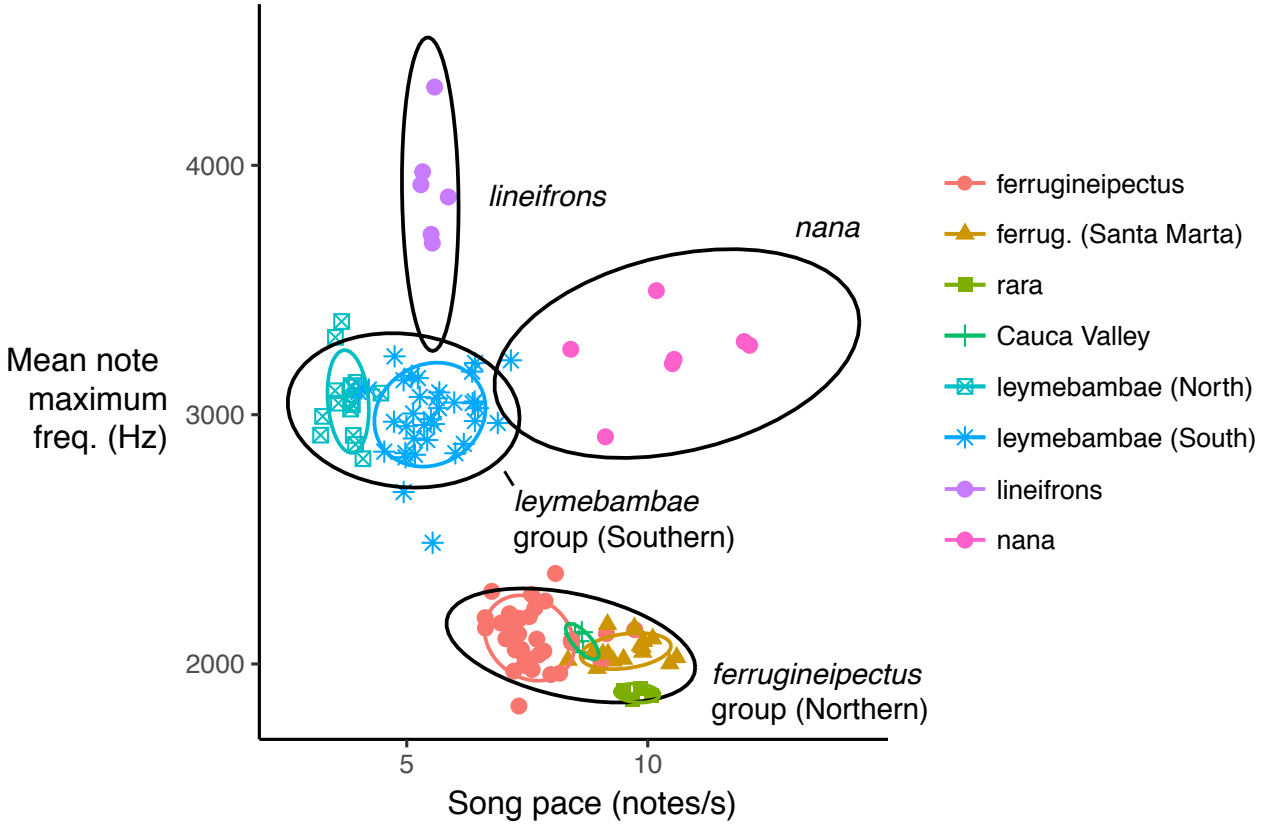


670

671 FIG. 5. First two factors from discriminant function analysis separating Rusty-breasted Antpitta

672 subpopulations by variation in 15 vocal traits. Black ellipses are 95% prediction ellipses for

673 northern and southern groups; colored ellipses are 75% prediction ellipses for subspecies.

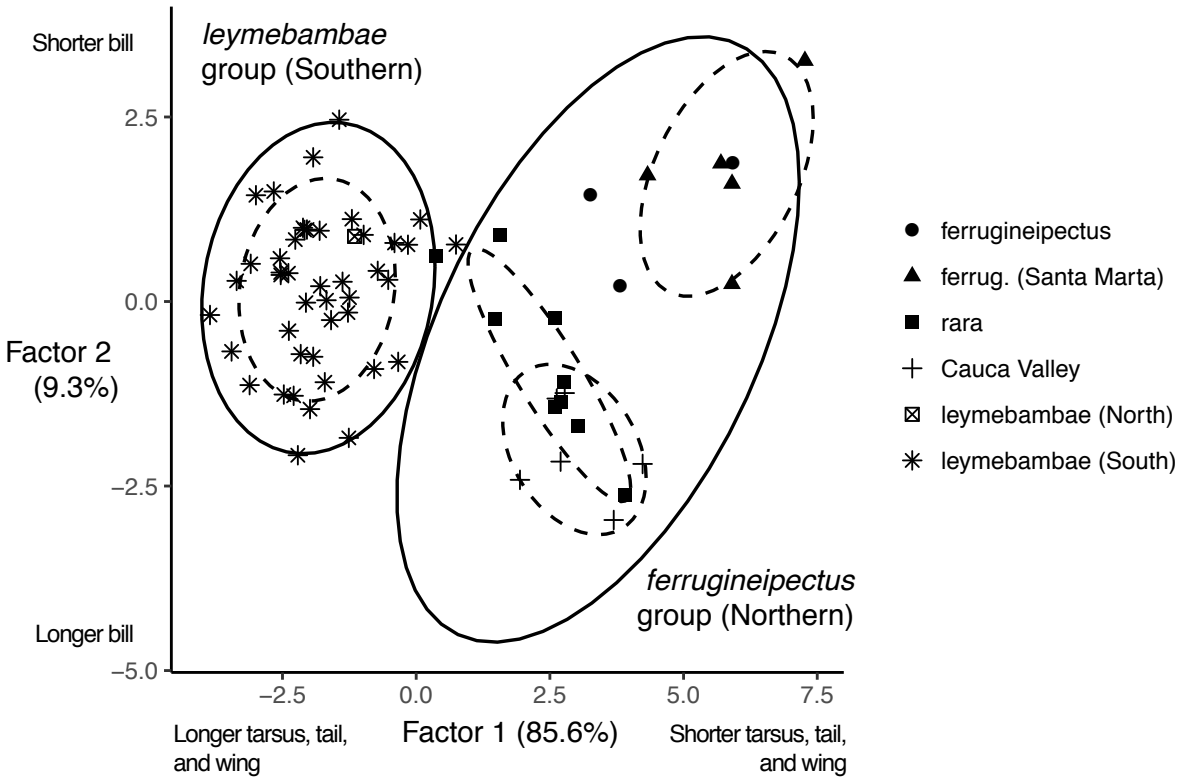


674

675 FIG. 6. Mean note maximum frequency versus note rate for all Rusty-breasted Antpitta

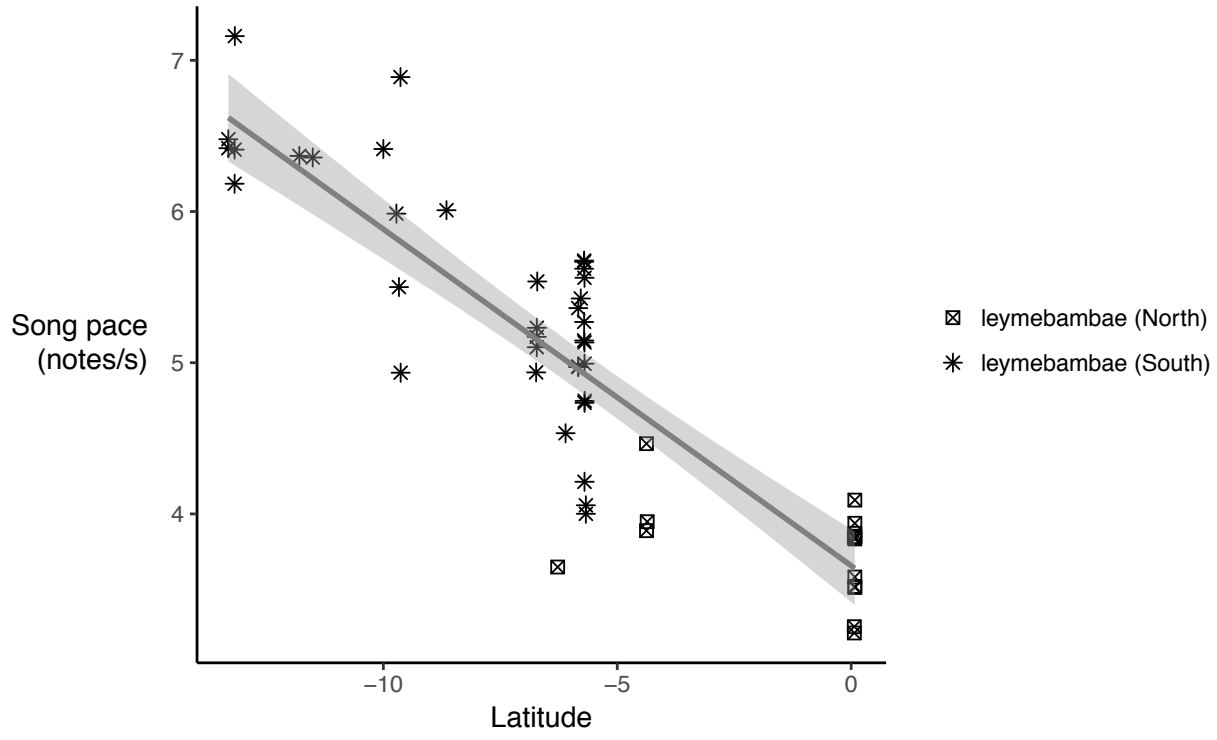
676 populations and two closely related species, *G. nana* and *G. lineifrons*. These two variables

677 generally showed the greatest differentiation across Rusty-breasted Antpittas.



678

679 FIG. 7. First two factors from discriminant function analysis separating Rusty-breasted Antpitta
680 subpopulations by variation in 6 morphological traits. Black ellipses are 95% prediction ellipses
681 for northern and southern groups; colored ellipses are 75% prediction ellipses for
682 subpopulations.



683

684 FIG. 8. Song pace versus latitude for southern Rusty-breasted Antpittas.