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A rare case of interspecific hybridization in the tracheophone suboscines: Chestnut-naped Antpitta *Grallaria nuchalis* × Chestnut-crowned Antpitta *G. ruficapilla* in a fragmented Andean landscape

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Based on analyses of variation in plumage, morphometrics, vocalizations, and mitochondrial and nuclear DNA sequences, we document the occurrence of interspecific hybridization between a Chestnut-naped Antpitta *Grallaria nuchalis* and a Chestnut-crowned Antpitta *G. ruficapilla* in a high-elevation forest fragment in the Cordillera Central of the Andes of Colombia. One hybrid individual was collected and at least two were recorded singing. The hybrid specimen exhibits a combination of phenotypic traits that exclude other species of antpittas as potential parents, and its vocalizations combine elements of songs of both of its parental species. Genetic analyses demonstrate that the hybrid has *G. nuchalis* mitochondrial DNA and mixed nuclear DNA with copies corresponding to *G. nuchalis* and *G. ruficapilla* alleles, demonstrating that the female parent was *G. nuchalis*. Hybridization is considered extremely rare in most groups of suboscine passerines, but it may be facilitated when populations are decimated as a consequence of deforestation and habitat fragmentation.

On 5 June 1999, while conducting fieldwork in a 25-ha forest fragment at 2750 m elevation in Finca San Miguel, 3 km west of the town of Roncesvalles, Departamento de Tolima, Cordillera Central of Colombia (04°053′N, 75°030′W), B.L.L. obtained a tape recording of what at that time seemed to be the song of an antpitta in the White-bellied Antpitta *Grallaria hypoleuca* complex (*G. hypoleuca* or Yellow-breasted Antpitta *G. flavotincta*). Following an imitated whistle of the song, however, a bird that closely resembled the Chestnut-crowned Antpitta *Grallaria ruficapilla* came into view. Three days later, at least two individuals were found calling simultaneously in the same forest patch. These encounters suggested the possibility that an undescribed species of *Grallaria* resembling *G. ruficapilla* in plumage and members of the *G. hypoleuca* complex in voice occurred in the area. This prompted further fieldwork directed at obtaining specimens and additional tape recordings.

On 14 July 1999, one individual was sound-recorded, lured into a mist-net with playback, and collected at 2830 m elevation. F.G.S. prepared the specimen, an adult male in fairly fresh plumage with larger (left) testis (10 × 6 mm), fully ossified skull, no bursa, traces of fat in the furcular area and feather tracts, and remains of a small weevil and a few other

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insect fragments in the stomach: this specimen is deposited in the ornithological collection of the Instituto de Ciencias Naturales, Universidad Nacional de Colombia (catalogue number ICN 33401). Upon close examination with other specimens, this bird was noted to differ substantially from *G. ruficapilla*, especially in terms of its much larger body size and noticeably more massive bill (Fig. 1). Initially, based on its vocalizations and distinctive morphology, the Roncesvalles bird appeared to represent a novel species of *Grallaria*. However, while preparing the specimen, F.G.S. noted a decided resemblance to Chestnut-naped Antpitta *G. nuchalis* in the shape of the head and bill and large size, as well as the greyish tones of the underparts, and began to suspect that it might represent a hybrid between this species and *G. ruficapilla*. Therefore, it was decided to postpone any formal description in the hope of obtaining additional specimens and recordings and, if possible, placement in a molecular phylogenetic analysis.

On 12 January 2000, B.L.L. recorded another bird vocalizing in the same forest fragment, confirming that at least two individuals of this enigmatic *Grallaria* had been present in the area. However, multiple attempts at obtaining new specimens proved futile, and seemingly identical habitat patches in other sectors of the study area were searched for singing birds to no avail. Faced with the lack of additional records, we began to pursue the possibility that the Roncesvalles specimen might indeed represent an aberrant individual resulting from hybridization. The obvious similarity in plumage to *G. ruficapilla* and the close resemblance to *G. nuchalis* in the flat crown and large body and bill size pointed to these two species, both common in the study region, as potential parents. We also noted that the song of the Roncesvalles *Grallaria* seemed to combine features of those of *G. ruficapilla* and *G. nuchalis*, with its similarity to that of members of the *G. hypoleuca* complex being only superficial. Thus, we reasoned that describing this *Grallaria* as a new species would need to await formal rejection of the hypothesis that it was a hybrid. Here we address this hypothesis on the basis of morphological, vocal, and mitochondrial and nuclear DNA sequence data. Our analyses demonstrate conclusively that the Roncesvalles

Figure 1. Ventral (a), dorsal (b) and lateral (c) view of Colombian specimens of *Grallaria ruficapilla ruficapilla* (left or top), *G. nuchalis ruficeps* (right or bottom), and a hybrid between them (middle).

Grallaria is indeed a hybrid between *G. nuchalis* and *G. ruficapilla*, and we are able to identify *G. nuchalis* as the female parent. This represents one of the very few documented instances of interspecific hybridization in the tracheophone suboscines (Furnarioidea, *sensu* Wetmore 1960), a large Neotropical radiation of passerine birds comprising some 600 species.

METHODS

Phenotypic analyses

The first step in the process of hybrid diagnosis is to identify potential parental species (Graves 1990). Four species of *Grallaria* are known to occur in the forest remnant where the Roncesvalles specimen was collected: Undulated Antpitta *G. squamigera*, Brown-banded Antpitta *G. milleri*, *G. nuchalis* and *G. ruficapilla*. This assemblage is typical of forests located at comparable elevations in other nearby sites in the Cordillera Central (Kattan & Beltrán 1999). Bicolored Antpitta *G. rufocinerea* and Moustached Antpitta *G. alleni* have not been recorded at the site, but may occur there. To compare them with the Roncesvalles specimen based on external morphology, we examined specimens of these antpitta species available in the ornithological collections of the Instituto de Ciencias Naturales, the Academy of Natural Sciences of Philadelphia (ANSP) and Instituto Alexander von Humboldt (IAvH).

Based on preliminary inspections of specimens, it became clear that the most likely parental species of the Roncesvalles *Grallaria* were *G. nuchalis* (subspecies *ruficeps*, the form that occurs in the Cordillera Central of Colombia) and *G. ruficapilla* (see below). Thus, we conducted more in-depth examinations of these two taxa, including detailed descriptions of plumage coloration and pattern and morphological measurements on specimens. Colours were described with reference to Smithe (1975, 1981). Morphological measurements taken (to the nearest 0.1 mm) were exposed culmen, total culmen, commissure width (i.e. the distance from one corner of the mouth to the other), height of bill at nares, tarsus length, chord of folded wing and tail length. We compared these univariate measurements with those of the Roncesvalles bird, and also summarized the morphological variation by conducting a principal components analysis with varimax rotation in SPSS version 14.0. This analysis was conducted after standardizing the data by transforming the original measurements of each variable to *Z* scores (Gotelli & Ellison 2004).

Vocalizations

Recordings of the song of the Roncesvalles specimen have been archived with the Macaulay Library, Cornell Laboratory of Ornithology. To compare the song of the specimen with those of other *Grallaria* species, we prepared spectrograms of selected cuts and took measurements on note duration and frequency on them using the program CoolEditPro (Syntrillium Software).

Molecular genetics

As part of ongoing studies on antpitta phylogeny (Krabbe *et al.* 1999, Rice 2005, N.H. Rice & J.M. Bates unpubl. data), a fragment of the second subunit of the mitochondrial NADH dehydrogenase gene (ND2) and the fifth intron of the nuclear β-fibrinogen gene (β-fib5) have been sequenced for multiple species of *Grallaria* (see Appendix). Therefore, we focused on these two markers to determine whether the Roncesvalles *Grallaria* could be a hybrid, and if so, to ascertain which species served as the female parent and which as the male parent. Because mitochondrial DNA is inherited maternally and nuclear DNA biparentally, if the bird were a hybrid it would have the mitochondrial DNA of its maternal parent and mixed nuclear DNA, with alleles of both parental species.

We extracted genomic DNA from a sample of skin taken from a foot of the Roncesvalles specimen using the DNeasy Tissue Kit (Qiagen) following the manufacturer's protocol. In order to avoid contamination, DNA was extracted in a laboratory where no samples of *Grallaria* antpittas had ever been housed, and negative controls were used in DNA extraction and PCR amplifications. Standard PCR protocols (available from C.D.C.) were used to amplify approximately half of the ND2 gene using primers L5758 and H6313 (Sorenson *et al.* 1999), and the complete β-fib5 with primers FIB5 and FIB6 (Marini & Hackett 2002). PCR products were purified using a QIAQuick PCR kit (Qiagen), and in the case of the mitochondrial product, used as template for direct sequencing using the same primers employed for amplification. Nuclear PCR fragments were cloned using a TOPO-TA cloning kit (Invitrogen) following the manufacturer's protocol. Bacterial colonies containing a vector with gDNA (i.e. white colonies) were used as a template for subsequent PCR. Both strands of each purified product were cycle-sequenced using an ABI Dye Terminator Kit. Products were treated with ethanol and sodium acetate to remove unincorporated dyes, and run on an ABI 3730XL DNA sequencer.

Sequences were assembled and proofread using the program SeqMan (DNAstar), and then aligned manually using a text editor. Insertions and deletions were lacking in ND2 and were sufficiently rare in β-fib5 that alignment was straightforward. We used the program PAUP*4b10 (Swofford 2002) to calculate uncorrected pairwise distances between sequences and to conduct maximum-likelihood phylogenetic analyses. We ran phylogenetic analyses including sequences for all the samples listed in the Appendix, but we present results of analyses only including taxa that were found to be closely allied to sequences from the Roncesvalles specimen, and were thus relevant to establish its status. The analyses reported here employed the TrN+I+G and HKY models of nucleotide substitution, which were selected as the best fit to the mitochondrial and nuclear datasets, respectively, according to the Akaike Information Criterion in ModelTest version 3.7 (Posada & Crandall 1998). Tree searches were heuristic, each consisting of ten replicates with random taxon addition and tree-bisection reconnection branch swapping. We assessed support for nodes via bootstrap resampling (100 pseudoreplicates). Sequences of the Roncesvalles *Grallaria* were submitted to GenBank (accession numbers EF370121–EF370123).

RESULTS

External morphology

Assuming that plumage and other morphological traits are polygenic, hybrid individuals should exhibit a combination of features present in their parental species (Graves 1990). Given the strong overall resemblance in plumage colour and pattern between it and the Roncesvalles specimen, *G. ruficapilla* seemed to be an obvious choice for one parent. On the basis of plumage colour and pattern, tarsal scutellation, and overall proportions (tail/wing and tarsus/wing ratios, see Lowery & O'Neill 1969), we can exclude *G. squamigera* and *G. alleni* as potential parents. The much smaller size of *G. rufocinerea* (especially its much smaller bill) would also seem to exclude it, whereas *G. milleri* is also considerably smaller than *G. ruficapilla* and aside from a broad, indistinct brown breast-band and whitish belly, this species is virtually unpatterned and totally lacking in greyish tones, which are present in the Roncesvalles bird (see below). The large size, head and bill shape, and greyish underparts combine to make *G. nuchalis* (subspecies *ruficeps*) the most likely second parent on morphological grounds. Detailed descriptions of plumage, which confirm this interpretation, follow.

Description of the Roncesvalles specimen (colour names and numbers follow Smithe 1975, 1981)

Dorsum immaculate dark rich brown (nearest 23, Raw Umber, tending toward 33, Cinnamon-Brown). Crown, sides of head and nape chestnut (near 32, Chestnut), brightest on postocular area (123A, Brick Red), auriculars darker (132, Burnt Sienna). Bases of loreal feathers buffy-whitish; faint paler eyering (38, Tawny). Throat and underparts white, tinged with grey; upper throat with fine, faint, sparse blackish streaking; sides and flanks with central shaft-streaks white, bordered with black; lateral portion of these feathers concolour with back; centre of lower breast and abdomen with indistinct mottling and faint suggestion of streaking dusky (between 79, Glaucous, and 30, Olive). Thighs rich brown (near 121C, Mikado Brown); wing linings rusty (near 39, Cinnamon, some feathers nearer 40, Cinnamon-Rufous). Secondaries broadly edged with reddish brown (near 38, Tawny). Iris brown, bill black, legs and feet pale bluish grey. Twelve rectrices, sternum two-notched (skeleton of carcass prepared).

Description of Colombian specimens of G. ruficapilla

Dorsum immaculate olive-brown (between 23, Raw Umber, and 223B, Verona Brown). Crown, nape and sides of head deep rufous (nearest 240, Kingfisher Rufous), darkest on crown (in some nearly 136, Raw Sienna) and brightest on facial area (approaching 40, Cinnamon-Rufous); shading posteriorly through 36, Amber, into the olive-brown of the back. Bases of loreal feathers more or less buffy-whitish. Throat immaculate white, slightly tinged with pale grey; sides of breast, sides and flanks with feathers broadly white along shafts, this bordered with black, the lateral portion of the vanes concolour with the back producing a heavily streaked pattern; feathers across the centre of breast and sides of the abdomen white with the lateral borders black, more or less suffused or bordered basally with bright rufous (132C, Orange-Rufous) producing a more lightly streaked effect of mixed black and rufous (rufous nearly lacking in some); centre of abdomen immaculate white. Thighs paler brown (near 139, True Cinnamon, or 223B, Verona Brown). Wing linings rufous (240, Kingfisher Rufous); secondaries edged with paler brown often tinged with vinaceous (varying between 223C, Sayal

Brown, and 121D, Pale Pinkish Buff). Iris brown to greyish-brown, maxilla black, mandible grey; legs and feet lead grey.

Description of G. nuchalis ruficeps *(from specimen IAvH 13255 taken at Berlín, Depto. Caldas, Colombia)*

Dorsum immaculate rich brown (between 33, Cinnamon-Brown, and 223A, Mars Brown), becoming brighter and more rufescent (approaching 136, Raw Sienna) on the tail and secondaries; the outer secondaries and primaries edged with dark rufous (near 340, Robin Rufous) to pale brown (139, True Cinnamon). Crown, forehead and sides of head near chestnut (32, Chestnut), the crown darker and the postocular area brighter, between Chestnut and 123b, Mahogany Red. Loreal feathers blackish with pale whitish bases. Malar area mixed olive-brown (near 23, Raw Umber) and dark grey. Chin mottled with blackish and dull white, passing into grey (between 84, Medium Neutral Gray, and 79, Glaucous) over the lower throat, breast, sides and flanks; abdomen slightly paler grey with an olive tinge (near 79, Glaucous); thighs olive-brown (near 23, Raw Umber). Iris 'reddish grey', bill black, legs and feet grey.

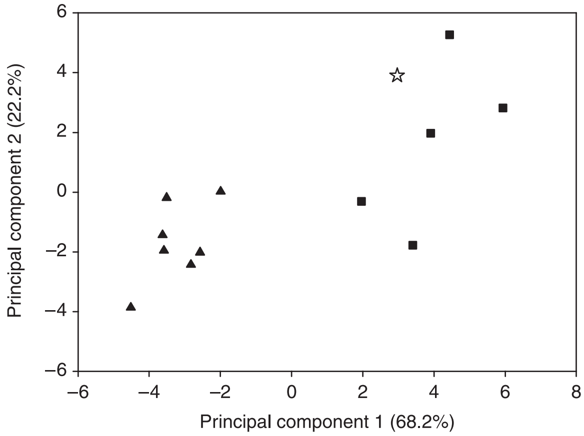
In summary, the Roncesvalles specimen resembles *G. nuchalis* most in the colours of the head and upperparts, but is more like *G. ruficapilla* on the underparts. Indeed, the effect of its ventral plumage is as though one had tried to overpaint the underparts of *G. nuchalis* with a *G. ruficapilla* colour and pattern, but with a thin white paint that let the grey 'show through'.

Morphometrics

The Roncesvalles *Grallaria* exhibits greater similarity in body dimensions to *G. nuchalis* than to *G. ruficapilla*. In all measurements, the values obtained for the Roncesvalles specimen were close to those observed among specimens of *G. nuchalis ruficeps*, and appeared to be significantly different from those of *G. ruficapilla* (Table 1). The variable in which the Roncesvalles specimen most appeared to be intermediate was bill height, but even then, its value was closest to that of *G. nuchalis ruficeps.* The principal components analysis reduced morphological measurements to two main axes with eigenvalues greater than one, which explained most (90%) of the variation (Fig. 2). The first axis (eigenvalue 4.77) accounted for 68.2% of the variation. Variables loading heavily along the first axis were commissure width, bill height, bill width and tail length, whereas exposed culmen length, total culmen length and tarsus length loaded heavily

Table 1. Morphological measurements (in mm) of the Roncesvalles *Grallaria*, and of specimens of *G. nuchalis* and *G. ruficapilla* corresponding to subspecies occurring in the Cordillera Central of Colombia. Data for *G. nuchalis* and *G. ruficapilla* are means and standard deviations, with the range for each measurement shown in parentheses.

Measurement	Roncesvalles *Grallaria*	*G. nuchalis ruficeps*	*G. ruficapilla ruficapilla*
Exposed culmen	25.5	24.13 ± 1.46 (22.0–26.3) *n* = 7	22.96 ± 0.62 (22.3–24.0) *n* = 10
Total culmen	29.8	27.55 ± 1.47 (25.7–30.1) *n* = 7	27.61 ± 1.00 (26.3–29.2) *n* = 10
Commissure width	16.7	17.30 ± 0.84 (16.0–18.4) *n* = 8	14.41 ± 0.75 (12.9–15.5) *n* = 10
Bill-height	9.8	10.43 ± 0.52 (9.9–11.4) *n* = 6	8.86 ± 0.24 (8.6–9.2) *n* = 7
Wing length	111.7	113.25 ± 4.10 (107.5–120.3) *n* = 9	98.86 ± 2.42 (95.3–102.7) *n* = 10
Tarsus length	60.8	59.35 ± 2.96 (55.0–62.6) *n* = 8	54.00 ± 1.86 (50.9–56.0) *n* = 10
Tail length	60.5	63.26 ± 3.93 (58.1–69.8) *n* = 9	53.02 ± 1.36 (51.7–56.2) *n* = 10



	PC1	PC2
Exposed Culmen	0.361	0.731
Total Culmen	0.007	0.983
Commissure	0.634	0.046
Bill Height	0.917	0.285
Bill Width	0.885	0.231
Tarsus	0.486	0.822
Tail	0.933	0.104

Figure 2. Results of principal components analysis based on seven morphological measurements (see Table 1) taken on specimens of the Roncesvalles *Grallaria* (star), *G. nuchalis ruficeps* (squares) and *G. ruficapilla ruficapilla* (triangles). Data are shown only for the first two components, which accounted for a combined total of 90% of the variation. The values on the right correspond to the loadings of different variables (rotated factor matrix) along the first two components. Specimens included in the analysis were only those having data for the seven measurements. Note the close resemblance between the Roncesvalles *Grallaria* and specimens of *G. nuchalis ruficeps.*

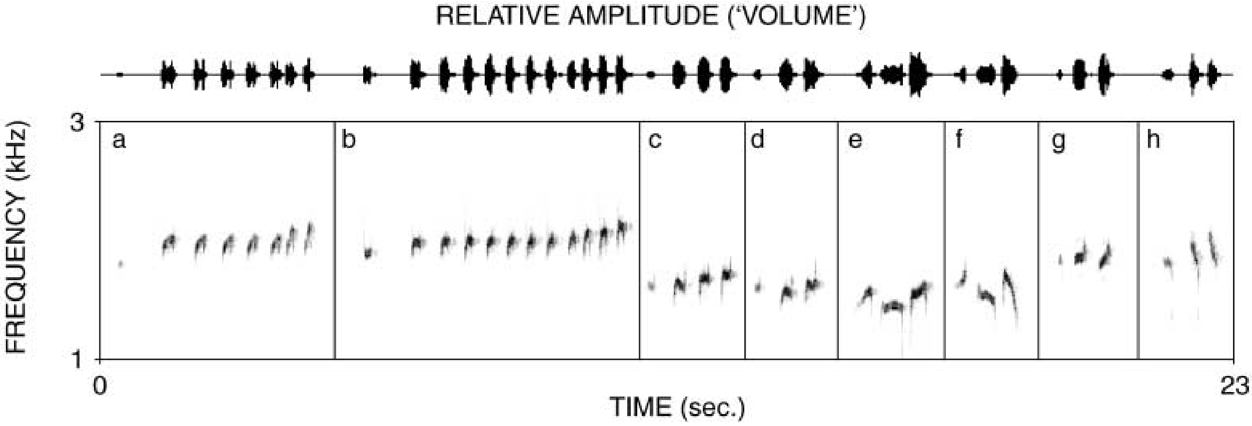


Figure 3. Spectrograms showing natural songs of species of *Grallaria* occurring in the Colombian Andes and of the Roncesvalles *Grallaria*. (a) *G. nuchalis ruficeps*, Alto del Escobero (6°06′N 75°32′W, 2400–2800 m), Envigado, Antioquia, Colombia. A. Cuervo. 28 October 2001. (b) *G. nuchalis ruficeps*, Finca La Estrella (05°13′N 75°24′W, 3100 m), La Cristalina, Neira, Caldas, Colombia. M. Álvarez-Rebolledo (BSA 7666). (c,d) Roncesvalles *Grallaria*, Finca San Miguel, Roncesvalles (04°02′N 75°38′W, 2830 m), Tolima, Colombia. B. López-Lanús. 14 July 1999. (e) *G. ruficapilla ruficapilla*, Finca Termópilas (05°13′N 75°29′W, 2250 m), El Laurel, Aranzazu, Caldas, Colombia. M. Álvarez-Rebolledo (BSA 7873). (f) *G. ruficapilla ruficapilla*, Tandayapa ridge (00°01′N 78°41′W, 2250 m), Pichincha, Ecuador. N. Krabbe. 11 July 1998 (CLXXIVB 205–219). (g) *G. flavotincta*, Mesenia (05°29′N 75°54′W, 2250 m), Jardín, Antioquia, Colombia. N. Krabbe. 23 August 2004 (A 165–212). (h) *G. hypoleuca*, La Forzosa Reserve (06°59′N, 75°08′W, 1935 m), Anorí, Antioquia, Colombia. A. Cuervo 1999.

along the second axis (eigenvalue 1.55), which accounted for 22.2% of the variation (Fig. 2). Consistent with patterns shown by the univariate data, the Roncesvalles *Grallaria* is far more similar to *G. nuchalis ruficeps* in multivariate space than it is to *G. ruficapilla* (Fig. 2).

Vocalizations

Vocalizations offer additional evidence that the most likely combination of parents for the Roncesvalles specimen are *G. nuchalis* and *G. ruficapilla*. The song of *G. nuchalis ruficeps* (Fig. 3a,b) is fairly high-pitched, around 2 kHz. It is composed of 8–12 notes, all fairly short (0.13–0.26 s). The first note is weak, whereas the rest are all equally loud and similar to one another, but the terminating 2–5 notes are of increasingly higher pitch and are delivered at slightly shorter intervals. Except for the first note, individual notes always fall in pitch at their ends. The song of *G. ruficapilla* (Fig. 3e,f) is lower-pitched, around 1.5 kHz. It is three-noted, with the second note distinctly lower-pitched than the first and third, and the third note always louder than the preceding two. All three notes, but especially the last two, are fairly long (0.30–0.45 s), and the last two fall in frequency at their ends. The song of the Roncesvalles *Grallaria* was usually four-noted (Fig. 3c), and occasionally three-noted (Fig. 3d). It resembles the song of *G. ruficapilla* in brevity (1.3–1.7 s) and pitch (1.6–1.7 kHz), and the song of *G. nuchalis* in the notes being short and of successively higher pitch. Thus, it sounds like an abbreviated version of the song of *G. nuchalis* given at or slightly above the pitch of the song of *G. ruficapilla*. However, it differs from songs of both of these species in that all the notes rise in pitch at their ends.

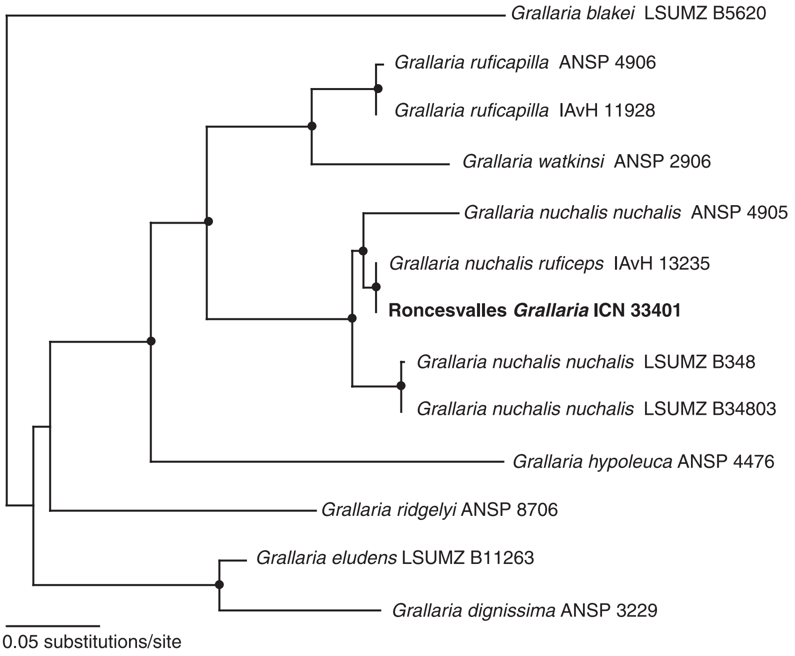
In the three-noted songs of *G. flavotincta* (Fig. 3g) and *G. hypoleuca* (Fig. 3h) the first note is weaker and lower-pitched than the following two notes. The songs of both are higher-pitched (1.8–2.3 kHz) than the song of the Roncesvalles *Grallaria* (1.6–1.7 kHz). The last two notes in the song of *G. hypoleuca* are alike, short (0.18–0.25 s), and given in rapid succession (interval 0.14–0.20 s), and are distinctive in quality by falling in frequency at their ends. In the song of *G. flavotincta*, the middle note is decidedly longer (0.27–0.37 s) and higher-pitched, and usually also louder, than the third note; the last two notes rise in pitch at their ends, in this respect resembling the notes of the song of the Roncesvalles bird. Despite this resemblance, spectrograms clearly indicate that the similarity noted in the field between the song of the Roncesvalles *Grallaria* and that of members of the *G. hypoleuca* complex was only superficial.

Molecular genetics

Consistent with the hybridization hypothesis, the mitochondrial ND2 sequence obtained from the Roncesvalles specimen was identical to a sequence from a specimen of *G. nuchalis ruficeps* (IAvH 13235) from Berlín, Municipio Pensilvania, Departamento de Caldas, Colombia. This locality is some 130 km north of Roncesvalles. Uncorrected sequence divergence (*p*-distance) between the haplotype shared by these two specimens and a haplotype of nominate *G. nuchalis* from Ecuador (ANSP 185506, tissue 4905; Provincia de Napo, 8 km south of Cosanga, Cordillera Huacamayo) was 4.1%. In phylogenetic analyses, the haplotype shared by the Roncesvalles *Grallaria* and *G. nuchalis ruficeps* was nested within a clade formed by nominate *G. nuchalis* haplotypes: it formed a well-supported clade (> 70% bootstrap) with the Ecuadorean haplotype mentioned above, with a clade formed by two haplotypes from northern Peru (Depto. Cajamarca, Cerro Chinguela and Cordillera del Cóndor) as their sister group (Fig. 4a). The average mtDNA divergence between the Roncesvalles bird and *G. ruficapilla*, represented in our dataset by sequences from Colombia (Departamento de Caldas, Municipio Aranzazu, Vereda El Laurel) and from Ecuador (Provincia de Napo, 8 km south of Cosanga, Cordillera Huacamayo), was approximately 12%.

Sequences of the nuclear β-fib5 gene provide the final piece of evidence that demonstrates the hybrid status of the Roncesvalles *Grallaria*. Of the eight clones we sequenced, seven yielded identical sequences, but one clone was different. Phylogenetic analyses of the β-fib5 dataset (567 aligned base pairs for 11 species of *Grallaria* and multiple outgroup taxa; see Appendix) indicate that these two sequences are not each other's closest relatives (Fig. 4b). One of them was found to occupy an unresolved position in a well-supported clade formed by several sequences of *Grallaria* species, including *G. ruficapilla*, *G. watkinsi*, *G. capitalis*, *G. quitensis* and *G. przewalskii*. Although relationships within this clade are not clear owing to a limited number of informative characters, the sequence obtained from the Roncesvalles *Grallaria* exhibits indels that are only found in two other samples in our working β-fib5 dataset; both of these samples correspond to specimens of *G. ruficapilla*, one from Venezuela and one from Ecuador (Fig. 5). In turn, the other sequence we obtained was nearly identical to that of two specimens of *G. nuchalis* from Peru (Fig. 5); in phylogenetic analyses, the clade formed by these sequences was found to be sister to the large clade formed by the other sequence and its close relatives (Fig. 4b), which is consistent with relationships among species of *Grallaria* inferred based on mtDNA data. In summary, molecular data confirm that the Roncesvalles *Grallaria* has *G. nuchalis* mtDNA and mixed nuclear DNA of *G. nuchalis* and of *G. ruficapilla*, leaving no doubt that it is a hybrid, and that *G. nuchalis* served as the female parent.

(a) mtDNA (ND2)



(b) Nuclear DNA (β-fib5)

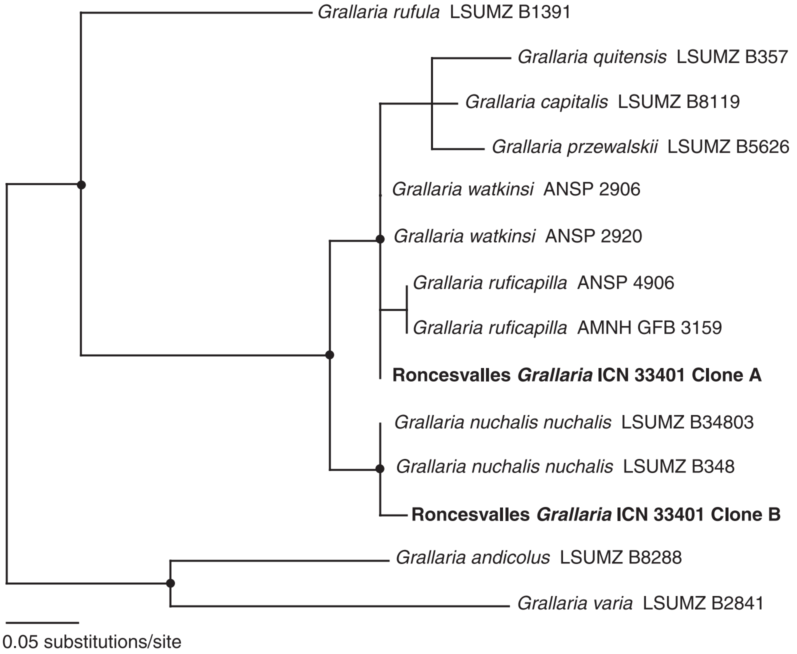


Figure 4. Phylogenetic relationships among selected *Grallaria* taxa and sequences obtained from the Roncesvalles *Grallaria* inferred by maximum-likelihood analyses of mitochondrial (a) and nuclear (b) DNA data. Black dots indicate nodes receiving maximum-likelihood bootstrap values exceeding 70%. See Appendix for information on specimens and museum acronyms.

	233	234	236	237	266	285	305	312	328	512	563
Grallaria nuchalis LSUMZ B34803	T	C	G	A	A	G	C	C	C	C	T
Grallaria nuchalis LSUMZ B308	.	.	.	.	.	.	.	.	.	.	-
Grallaria ruficapilla AMNH GFB 3159	-	-	-	-	G	.	T	A	T	T	.
Grallaria ruficapilla ANSP 4906	-	-	-	-	G	.	T	A	T	T	.
Roncesvalles *Grallaria* Clone A	-	-	-	-	G	.	T	A	T	.	.
Roncesvalles *Grallaria* Clone B	.	.	.	.	.	C	.	.	.	.	.

Figure 5. Nucleotide sites in the fifth intron of the β-fibrinogen gene exhibiting variation within or among *G. nuchalis*, *G. ruficapilla*, or the Roncesvalles *Grallaria*. Numbers indicate the position of nucleotides in the 567-bp alignment that included 11 species of *Grallaria* (see Appendix) and multiple outgroup taxa. Dots indicate sequences are identical to the first sequence, dashes indicate gaps resulting from insertions/deletions. Shaded nucleotide sites are those exhibiting variation that supports the hybrid status of the Roncesvalles *Grallaria.*

DISCUSSION

Our analyses of phenotypic, vocal and molecular data demonstrate that the Roncesvalles *Grallaria* is an F1 hybrid between a female *G. nuchalis* and a male *G. ruficapilla*. This represents one of the very few instances of hybridization documented between species of tracheophone suboscines (see below), a speciose and ecologically diverse clade encompassing the Furnariidae (including 'Dendrocolaptidae'), Thamnophilidae, Formicariidae, Grallariidae, Conopophagidae, Rhinocryptidae and Melanopareiidae (Graves 1992; family names follow Remsen *et al.* 2007). With the exception of a few species of woodcreepers (Skutch 1969, Willis & Oniki 1995), to the best of our knowledge all species of tracheophone suboscines are socially monogamous, form extended pair bonds, and males participate actively in all parts of the nesting cycle, such as incubation and nestling provisioning (Krabbe & Schulenberg 2003a, 2003b; Marantz *et al.* 2003, Remsen 2003, Whitney 2003). This social behaviour, in combination with an important role in mate choice for vocalizations that are presumably not learned, may explain why pre-mating species isolating mechanisms rarely break down under natural conditions in the group (Graves 1992).

The evolution of pre-mating isolating mechanisms such as differentiation in songs and behaviour often pre-dates the evolution of post-mating mechanisms such as hybrid inviability and infertility in avian speciation (Grant & Grant 1997). Uncorrected mtDNA sequence divergence between *G. nuchalis* and *G. ruficapilla* is 12%, which would imply these species last shared a common ancestor some 6 million years ago if one assumes an approximate rate of nucleotide substitution of 2% divergence per million years (reviewed by Lovette 2004; see also Weir 2006). Thus, this study demonstrates that although tracheophone suboscines very rarely hybridize in nature, sympatric species in this clade can retain the ability to produce hybrid offspring that can survive to adulthood even after millions of years of evolutionary isolation, pointing to the importance of pre-mating isolating mechanisms in maintaining species as distinct entities in the group.

Bird species that rarely hybridize under natural conditions may readily do so in non-natural settings (e.g. aviaries; Price & Bouvier 2002). Several studies have shown that understorey insectivores such as *Grallaria* antpittas are highly vulnerable to forest fragmentation (e.g. Kattan *et al.* 1994, Stouffer & Bierregaard 1995, Stouffer *et al.* 2006), and although *G. nuchalis* and *G. ruficapilla* are common species that are not threatened with extinction at national or regional scales, their population sizes can become severely decimated locally as forests become fragmented (Renjifo 2001). Additionally, the reluctance of antpittas to cross open areas in fragmented landscapes may cause them to become entrapped in small habitat patches, where population densities are reduced in comparison with continuous forests. We hypothesize that under such altered conditions, hybridization may be more likely than in undisturbed forests owing to an increased difficulty in finding conspecific mates. Consistent with this idea, the incidence of hybridization has been shown to be associated with scarcity of conspecifics across multiple species of birds (Randler 2002). Thus, a possible conservation implication of our finding is that even in tracheophone suboscines, a group noted for social monogamy and a very low incidence of natural hybrids, reduction of forest area might further threaten species of concern through hybridization facilitated by population declines.

We are certain that at least two hybrid individuals (i.e. individuals with vocalizations similar to those of the hybrid specimen) occurred in Finca San Miguel. Clutch size in G. *ruficapilla* is two (Martin & Greeney 2006), and although the nest and eggs of G. *nuchalis* have not been described, this species probably also lays two eggs, as observed in all species of *Grallaria* for which there is information (Krabbe & Schulenberg 2003a). If only two hybrid individuals occurred in the forest patch, it would be most parsimonious to assume they were siblings from a single clutch, but if a third individual occurred there, repeated instances of hybridization (presumably by the same pair) may have occurred. This would not be entirely unexpected considering that antpittas are thought to form extended pair bonds. Therefore, further fieldwork in the area would be of great interest to determine whether hybrid individuals have continued to be produced, resulting in a highly localized hybrid population. Determining the fate of hybrids (i.e. survival, pair formation, reproduction) would also be an important priority.

Many studies of vocalizations in suboscine passerines assume that songs of all species in this clade are not learned, an assumption that is often used to justify the use of vocal characters in species-level taxonomy (reviewed by Remsen 2005). However, that songs are not learned has been shown experimentally only in three species of flycatchers (Tyrannidae; Kroodsma 1984, 1985), which implies that extrapolating this finding to all suboscines may be inappropriate. Indeed, recent work on *Procnias* bellbirds (Cotingidae) demonstrates song learning (Kroodsma 2005), indicating that the hypothesis that songs are not subject to learning across suboscines needs re-evaluation. A prediction of the hypothesis that songs are transmitted genetically (i.e. not by learning) from parents to offspring is that songs should be intermediate in hybrid individuals. The stereotyped song of the Roncesvalles *Grallaria* is indeed intermediate between the songs of its two parental species in some aspects (Fig. 3), providing evidence that these vocalizations are probably under genetic control in *Grallaria*.

Prior to this study, very few apparent instances of hybridization between species of tracheophone suboscines had been described, and most of the purported examples lack sufficient documentation to prove hybridization has actually occurred (McCarthy 2006). One of the cases with better documentation is that of the Argus Bare-Eye *Phlegopsis barringeri* (Thamnophilidae), known from a single specimen from southeast Colombia; Graves (1992) argued that the hypothesis that the specimen is a hybrid between Black-spotted Bare-eye *P. nigromaculata* and Reddish-winged Bare-eye *P. erythroptera* cannot be rejected. We note this is a different argument from demonstrating hybrid status as we have done for the Roncesvalles *Grallaria*, and some ornithologists regard the status of *P. barringeri* as unresolved (T.S. Schulenberg pers. comm.). In light of cases such as that of the White-masked Antbird *Pithys castaneus*, a species not recorded for many years and thus presumed by many to be a hybrid, until the proper habitats were surveyed (Lane *et al.* 2006), we suggest further work in the area where *P. barringeri* was collected is necessary to determine conclusively whether this bird is a valid species or a hybrid. Genetic analyses could also be illuminating.

To conclude, elements of this story can only make us wonder about the intriguing possibility that hybridization may play a role in species formation in antpittas and other suboscine birds. We found that: (1) two hybrid individuals (at least one of which was an adult) coexisted in a small forest patch where they were within range of one another; (2) song playback elicited strong responses by the hybrid, implying abilities for song recognition; and (3) songs are probably under strong genetic control, suggesting vocalizations could be transmitted from parents to offspring should hybrid individuals mate with each other. If mate discrimination abilities based on vocalizations are heritable or are acquired through imprinting on parental songs during development (Grant & Grant 1996, Payne *et al.* 2000), assortative mating between offspring of hybrid individuals is to be expected, which could potentially lead to speciation. Moreover, although these processes clearly occur over very different time scales, the reduction of population sizes brought about by anthropogenic forest fragmentation that we believe facilitated hybridization between G. *ruficapilla* and G. *nuchalis* might resemble the habitat contractions driven by climate change that have long thought to be important drivers of vicariant speciation in Neotropical birds (Haffer 1969, García-Moreno & Fjeldså 2000). Without information on whether hybrid individuals are able to mate and produce viable offspring, the hypothesis that hybrid speciation in suboscines might be possible is only conjectural at this point, but as more sequence data for genes in the mitochondrial and nuclear genomes become available, the possibility that some species may have hybrid origins (cf. Mavárez *et al.* 2006, Rieseberg 2006) will be amenable to evaluation.

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Appendix. Information on specimens of *Grallaria* antpittas included in comparisons of mitochondrial (ND2) and nuclear (β-fib5) DNA sequence data with the Roncesvalles *Grallaria*. GenBank accession numbers are given for sequences published in earlier studies. Those that are part of ongoing studies are indicated with an X, and are available upon request from J.M.B. and N.H.R. A dash indicates that sequence of the corresponding gene was not available for a particular specimen. AMNH = American Museum of Natural History; ANSP = Academy of Natural Sciences of Philadelphia; IAvH = Instituto Alexander von Humboldt; LSUMZ = Louisiana State University Museum of Natural Science; USNM = United States National Museum – Smithsonian Institution.

Taxon	Catalogue no.	Country	ND2	β-fib5
Grallaria squamigera	LSUMZ B215	Peru	–	X
Grallaria squamigera	LSUMZ B6254	Ecuador	AF127203	X
Grallaria varia	LSUMZ B7528	Venezuela	–	X
Grallaria varia	LSUMZ B2841	Peru	–	X
Grallaria varia	USNM CMM 786	Guyana	–	X
Grallaria guatimalensis	ANSP 5173	Ecuador	AF127205	–
Grallaria haplonota	LSUMZ B6106	Ecuador	–	X
Grallaria dignissima	ANSP 3229	Ecuador	AF127214	–
Grallaria eludens	LSUMZ B11263	Peru	X	–
Grallaria ruficapilla	IAvH 11928	Colombia	X	–
Grallaria ruficapilla	AMNH GFB 3159	Venezuela	–	X
Grallaria ruficapilla	ANSP 4906	Ecuador	X	X
Grallaria watkinsi	ANSP 2906	Ecuador	AF127209	X
Grallaria watkinsi	ANSP 2920	Ecuador	–	X
Grallaria andicolus	LSUMZ B8288	Peru	–	X
Grallaria ridgelyi	ANSP 8706	Ecuador	AF127210	–
Grallaria ridgelyi	ANSP 8731	Ecuador	AF127211	–
Grallaria nuchalis ruficeps	IAvH 13235	Colombia	X	–
Grallaria nuchalis nuchalis	ANSP 4905	Ecuador	AF127212	–
Grallaria nuchalis nuchalis	LSUMZ B308	Peru	X	X
Grallaria nuchalis nuchalis	LSUMZ B34803	Peru	X	X
Grallaria hypoleuca	ANSP 4476	Ecuador	AF127213	–
Grallaria przewalskii	LSUMZ B5626	Peru	–	X
Grallaria capitalis	LSUMZ B8119	Peru	–	X
Grallaria rufula	LSUMZ B1218	Bolivia	AF127206	X
Grallaria blakei	LSUMZ B5620	Peru	AF127207	–
Grallaria quitensis	LSUMZ B357	Peru	–	X