Short Communication

Genome-wide data help identify an avian species-level lineage that is morphologically and vocally cryptic

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Abstract

Species identification has traditionally relied on morphology. However, morphological conservatism can lead to a high incidence of cryptic species, as characters other than morphological ones can be biologically important. In birds, the combined application of bioacoustic and molecular criteria has led to an avalanche of cryptic species discoveries over the last two decades in which findings of deep vocal differentiation have usually been corroborated by molecular data or vice versa. In this study, we use genome-wide DNA data to uncover an unusual case of cryptic speciation in two species within the South-east Asian Streak-eared Bulbul Pycnonotus blanfordi complex, in which both morphology and vocalizations have remained extremely similar. Despite a considerable pre-Pleistocene divergence of these two bulbul species, bioacoustic analysis failed to uncover differences in their main vocalization, but examination of live birds revealed important differences in eye color that had been overlooked in museum material. Our study demonstrates that genome-wide DNA data can be helpful in the detection of cryptic speciation, especially in species that have evolved limited morphological and behavioral differences.

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1. Introduction

Cryptic species provide a challenge to taxonomists because they lack features differentiating them from other, better-known species, leading to serious underestimates of global animal diversity (Bickford et al., 2007). While knowledge about the planet’s invertebrate fauna is relatively fragmentary, certain vertebrate groups such as birds were thought to be understood in great detail (Mayr, 1946) until the application of DNA barcoding techniques demonstrated an unexpected incidence of cryptic bird species diversity even in extremely well-explored regions such as North America and Europe (Hebert et al., 2004; Kerr et al., 2007). However, the barcoding approach has its own limitations, especially in cases where cryptic species are unusually young (Wagner et al., 2013), or where genetic introgression has resulted in a reduction of raw sequence divergence in the barcoding gene, COI (cytochrome oxidase I), leading species to be overlooked by barcoders (Rheindt and Edwards, 2011). In these instances, the use of genome-wide markers is called for to disentangle patterns of potential gene flow (McCormack et al., 2013).

In birds, plumage coloration and external morphology have been the main taxonomic guiding lights for the last few centuries. However, many bird lineages use vocal cues as their main agents of mate selection, and bioacoustic characters have been shown to be of greater taxonomic importance than plumage characters in some lineages (e.g. Irwin et al., 2001; Isler et al., 1998; Rheindt et al., 2008). Bird vocalizations were only occasionally recruited for species diagnosis before the 1980s (Stein, 1963; Thie Uecke, 1970); their routine application for this purpose is a relatively new phenomenon (Alström and Ranft, 2003) that has led to the discovery of high levels of cryptic species especially across tropical bird genera from less well-explored regions.

Coincidentally, many of the cryptic species-level lineages first exposed by mtDNA sequencing have been quickly confirmed by field ornithologists familiar with their vocalizations. In some cases, mtDNA has alerted ornithologists to bird forms that are highly likely to exhibit distinct vocalizations (Rheindt et al., 2013). In
other cases, vocal distinctions were pointed out first but then quickly corroborated by mtDNA studies (Isler et al., 2007; Tobias et al., 2008). It is extremely rare to find cases in which bird lineages deeply diverged in mtDNA fail to exhibit some sort of pronounced differentiation either in plumage or vocalizations upon closer inspection.

In this study, we used genome-wide DNA markers along with mtDNA, vocal and observational data to examine subspecific differentiation within the Streak-eared Bulbul Pycnonotus blanfordi from South-east Asia. The species is divided into two subspecies: nominate blanfordi (mostly confined to Myanmar) and conradi (from Thailand and Indochina) (Fig. 1A). The two taxa are reported to be poorly differentiated in coloration, with conradi having slightly yellower underparts than blanfordi (Fishpool and Tobias, 2005; Robson, 2008), and nothing is published about potential vocal differences. We present data showing conradi to be a rare example of a genetically deeply-diverged avian lineage with limited differentiation in plumage and vocalizations. However, we expose a diagnostic morphological trait (eye color) that has previously been overlooked by museum taxonomists (probably because eyes are not preserved in dry museum skins). Differences in eye color may serve as a reproductive barrier in this complex and may partly account for the deep genomic differentiation.

2. Methods

2.1. Field observations and morphological comparisons

We carried out detailed observations of the less well-known nominate taxon on the occasion of two field expeditions to Myanmar, one from 25–31 May 2014 around Bago (17.3333° N, 96.4833° E) and the second from 21–28 February 2015 around Bagan (21.1667° N, 94.8667° E). We also examined online photo collections of Asian birds (e.g. www.orientalbirdimages.org) to compare images of live individuals with our own observational data from Myanmar. As detailed plumage comparisons have previously failed to reveal pronounced differentiation in coloration, we restricted ourselves to morphological comparisons of live birds (as opposed to museum specimens).

2.2. Bioacoustic sample collection and analysis

We collected seven recordings of the species’ main vocalization from six individual streak-eared bulbuls from our own fieldwork as well as from other sound recordists (Table 1). Streak-eared bulbuls, like many other Pycnonotus bulbuls, have inconspicuous and non-stereotypical call notes (Fishpool and Tobias, 2005; Robson, 2008) that are challenging to use for field identification and taxonomic purposes. While the streak-eared bulbul’s vocalizations have not been studied in great detail, the call type we are comparing is the main vocalization known in this species. It may serve as a general contact call, while additional vocalizations are rarely heard. We are confident that all recordings belong to the same homologous vocalization. Of the six individuals, three were from nominate blanfordi (Myanmar) and three were from conradi (Thailand: 1, Vietnam: 1, Cambodia: 1; Fig. 1, Table 1).

We used the program RavenPro 1.5 (Cornell Lab of Ornithology, Ithaca, NY, USA) under default settings to measure vocal parameters in these recordings. Each streak-eared bulbull call bout comprises of a series of short, serially repeated vocal elements (Fig. 1D). We measured four vocal characters: (1) element duration, (2) inter-element duration, (3) pace, and (4) dominant frequency (Table 1). For all recordings containing multiple call

Fig. 1. (A) Streak-eared bulbull distribution and vocal collection localities. Distribution of the nominate blanfordi from Myanmar is denoted in red and conradi in blue. Diagonal lines denote montane areas largely uninhabited by either form, but may encompass possible areas of overlap; (B and C) streak-eared bulbull blanfordi from Myanmar (© James Eaton) and conradi from Thailand (© Simon van der Meulen), respectively, with lines pointing to the approximate locality where photos were taken; (D) example sonogram of a main vocalization given by nominate blanfordi showing the succession of nine and four song elements, respectively, making up two successive song bouts; (E) principal component analysis of four bioacoustic parameters on six individuals (conradi blue, blanfordi green), also showing the amount of variation explained by each principal component (PC). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Table 1

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Locality (coordinates)</th>
<th>Date</th>
<th>Recordist</th>
<th>Source/Accession number</th>
<th>Element duration (s)</th>
<th>Pace (elements/s)</th>
<th>Inter-element duration (s)</th>
<th>Dominant frequency (Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nominate blanfordi</td>
<td>Myanmar; Bago (17.33° N, 96.48° E)</td>
<td>May-14</td>
<td>Frank</td>
<td>Private recording</td>
<td>0.12</td>
<td>4.81</td>
<td>0.10</td>
<td>3273.05</td>
</tr>
<tr>
<td>Nominate blanfordi</td>
<td>Myanmar; Bagan (21.17° N, 94.87° E)</td>
<td>27-May-15</td>
<td>Frank</td>
<td>Private recording</td>
<td>0.13</td>
<td>4.43</td>
<td>0.11</td>
<td>3445.30</td>
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<tr>
<td>Nominate blanfordi</td>
<td>Myanmar; Kazakhstan (21.0° N, 94.24° E)</td>
<td>13-Apr-00</td>
<td>Per</td>
<td>Alström</td>
<td>0.16</td>
<td>3.50</td>
<td>0.15</td>
<td>3358.65</td>
</tr>
<tr>
<td>Conradi</td>
<td>Vietnam; Cat Tien National Park (11.34° N, 107.15° E)</td>
<td>Mar-08</td>
<td>David</td>
<td>Xeno-canto/XC26276</td>
<td>0.17</td>
<td>3.88</td>
<td>0.10</td>
<td>3100.77</td>
</tr>
<tr>
<td>Conradi</td>
<td>Cambodia; Banteay Stei, Siem Reap (13.60° N, 103.96° E)</td>
<td>06-May-13</td>
<td>Edwards</td>
<td>Xeno-canto/XC133782</td>
<td>0.14</td>
<td>3.56</td>
<td>0.16</td>
<td>1894.90</td>
</tr>
<tr>
<td>Conradi</td>
<td>Thailand; Phetchaburi (12.84° N, 99.59° E)</td>
<td>24-Feb-12</td>
<td>Timothy</td>
<td>Macaulay/</td>
<td>0.07</td>
<td>4.80</td>
<td>0.18</td>
<td>3100.73</td>
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</tbody>
</table>

Table 2
List of samples including museum and taxon name, locality, source tissue, Genbank accession number for COI (if applicable) and sample code. Museum abbreviations: LKCNHM = Lee Kong Chian Natural History Museum, Singapore; PSUZC = Princess Maha Chakri Sirindhorn Natural History Museum Zoological Collections, Hatyai, Thailand and MTD-C = Senckenberg Natural History Collections Dresden, Germany.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Museum ID</th>
<th>Taxon</th>
<th>Locality</th>
<th>Source of DNA</th>
<th>Genbank accession number</th>
<th>NGS data generated</th>
</tr>
</thead>
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<tr>
<td>HL1</td>
<td>LKCNHM-</td>
<td>P. blanfordi</td>
<td>Hlawaga National Park, Yangon</td>
<td>Blood</td>
<td>NA</td>
<td>Yes</td>
</tr>
<tr>
<td>HG61</td>
<td>LKCNHM-</td>
<td>P. blanfordi</td>
<td>Hlawaga National Park, Yangon</td>
<td>Blood</td>
<td>KT307974</td>
<td>Yes</td>
</tr>
<tr>
<td>TC997</td>
<td>PSUZC-AV</td>
<td>P. blanfordi</td>
<td>Ban Phu Toei, Sai Yok,</td>
<td>Muscle</td>
<td>KT307972</td>
<td>No</td>
</tr>
<tr>
<td>TC992</td>
<td>PSUZC-AV</td>
<td>P. blanfordi</td>
<td>Ban Phu Toei, Sai Yok,</td>
<td>Muscle</td>
<td>KT307972</td>
<td>No</td>
</tr>
<tr>
<td>KAN20</td>
<td>MTD-C-KAN20</td>
<td>P. blanfordi</td>
<td>Ban Tha Lor, Kanchanaburi</td>
<td>Feather</td>
<td>KT307973</td>
<td>No</td>
</tr>
<tr>
<td>KAN24</td>
<td>MTD-C-KAN24</td>
<td>P. blanfordi</td>
<td>Ban Tha Lor, Kanchanaburi</td>
<td>Feather</td>
<td>KT307970</td>
<td>No</td>
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<tr>
<td>605.1</td>
<td>PSUZC-AV</td>
<td>P. finlaysoni</td>
<td>Phu Luang Wildlife Research</td>
<td>Muscle</td>
<td>KT307975</td>
<td>Yes</td>
</tr>
<tr>
<td>M0830</td>
<td>LKCNHM-M</td>
<td>P. plumosus</td>
<td>Singapore</td>
<td>Blood</td>
<td>KT321535</td>
<td>Yes</td>
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<tr>
<td>M1108</td>
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<td>P. plumosus</td>
<td>Singapore</td>
<td>Blood</td>
<td>KT321565</td>
<td>Yes</td>
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<tr>
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<td>P. plumosus</td>
<td>Singapore</td>
<td>Blood</td>
<td>KT321566</td>
<td>Yes</td>
</tr>
</tbody>
</table>
were filtered using the 'populations' program. We allowed for a maximum of 10% missing data per locus and called only a single SNP per locus.

To prepare a dataset for phylogenomic analysis we used the pyRAD pipeline. We used the filtered reads obtained from STACKS as input data for this pipeline. For each individual we kept the minimum locus coverage at 10 reads and allowed for a maximum of four bases in a read with a quality score below 20 (Phred score). The clustering threshold for within- and between samples was set to 0.88, which is the default setting. We subsequently generated a concatenated data matrix for phylogenomic analysis without allowing for any missing data.

2.6. Mitochondrial DNA barcoding

For all individuals used in mtDNA analysis we amplified the COI gene using the BirdF1 and Bird R2 (Hebert et al., 2004) or AWCF1 and AWCR6 primers (Patel et al., 2010). We used published PCR protocols for amplification. We performed either gel extraction (QIAGEN) or used ExoSAP-IT (Affymetrix, USA) to clean the PCR products before sequencing from both ends. We aligned and edited the sequences manually in MEGA 6 (Tamura et al., 2013).

2.7. Population genetic analyses

We calculated average heterozygosity in CERVUS 3.03 (Kalinowski et al., 2007) and level of missing data in PLINK 1.0.7 (Purcell et al., 2007) for the unlinked SNPs generated using STACKS. We tested for neutrality of SNPs in Bayesian 2.1 at 5% false discovery rate (Foll and Gaggiotti, 2008). We used the default settings to test for loci under selection. For the neutral loci, we used two approaches to understand population subdivision. We first performed a PCoA (Principle coordinate analysis) using the unlinked SNPs in GenAlEx 6.5 (Peakall and Smouse, 2012) and plotted the results in R 3.1.2 (R core Team, 2014). We also used the Bayesian clustering approach in STRUCTURE 2.3.4 (Pritchard et al., 2000) to infer the number of genetic clusters (K') within the data. For each K (1–6), we performed ten iterations, each of which consisted of 100,000 generations of burnin and 500,000 generations of MCMC sampling.

2.8. Phylogenomic analyses

We reconstructed the phylogeny for our concatenated data within a maximum likelihood framework implemented in Raxml GUI 1.3 (Silvestro and Michalak, 2012). We used the GTR+ gamma model of sequence evolution as suggested in the manual for large datasets with few taxa and performed a single full maximum likelihood tree search. We used the rapid bootstrap algorithm with 1000 replicates. The final tree was viewed in Figtree 1.4 with midpoint rooting.

We also estimated divergence times by performing an additional analysis using COI sequences only in combination with BEAST 2.2.0 (Bouckaert et al., 2014). In the absence of suitable fossil or earth-historic calibration points for comparison between these two bulbul taxa, we used a clock rate of 2% sequence divergence/million years for dating because it has been shown to apply across a wide variety of bird lineages for mitochondrial coding genes (Weir and Schluter, 2008), with slight variations across different loci. We tested for a molecular clock using maximum likelihood in MEGA. We used the uncorrelated lognormal clock as the strict clock model was rejected (p value <0.001) and allowed for variation (0.1%) in the divergence rate. In addition to sequences generated in this study (KT307970–KT307975) we also used sequences from Genbank (P. finlaysoni, FJ473154.1; P. simplex, FJ473143; P. plumosus, KT321535, KT321565-66). We used the HKY+G model of evolution based on jModelTest 2.1.2 (Darriba et al., 2012), performing two independent runs for 10^7 iterations with a default burnin and sampling trees every 1000th iteration. For both runs we checked for parameter convergence as well as divergence time estimation using Tracer v1.6. We did not observe any significance difference either in parameter convergence or in divergence time estimation between the independent runs and hence report the results from the combined dataset within Tracer. We further concatenated data from both runs using logCombiner 2.2.0 and annotated the tree using TreeAnnotator 2.2.0. We also calculated average raw p-distances between groups in MEGA using the pairwise deletion option.

3. Results

3.1. Bare parts coloration

During our field trips in the distribution range of the rarely observed and less well-known nominate subspecies blanfordi, we paid special attention to bare parts coloration. Based on the observation of ~40 different individuals across different parts of Myanmar stretching from Yangon in the south to Bagan in the north, we recorded an invariably dark-red eye coloration in nominate individuals (subspecies blanfordi), clearly different from the off-white to dark-grey eye color known from the well-studied race conradi from Thailand and Indochina (Fishpool and Tobias, 2005; Robson, 2008). Inspection of internet photo collections (see Section 2) with locality data confirmed that all individual attributable to conradi have a greyish or off-white eye color, while nominate blanfordi has dark-red eyes.

3.2. Bioacoustic analysis

According to the four important temporal and frequency parameters herein investigated (Table 1), the vocalizations of streak-eared bulbuls from Myanmar (nominate blanfordi) are not noticeably differentiated from those found in Thailand and Vietnam (conradi), with all data points forming a single unstructured agglomeration on the PCA plot (Fig. 1E). The results from the Isler criterion test agree with PCA, none of the four measured parameters being diagnostically different between subspecies (Table 1, Supplementary materials).

3.3. Summary statistics

We successfully generated ddRAD-Seq data for seven out of ten samples (Table 2), obtaining an average of 2.03 ± 0.4 million reads per individual. We used different pipelines for population-genomic and phylogenomic analyses. For population-genomic analyses, we isolated 4643 unlinked SNPs using the STACKS pipeline (average heterozygosity of 0.08, polymorphic information content of 0.25). The overall level of missing data was less than 0.1%. Out of 4643 loci, one locus exhibited a signature of selection and was removed from subsequent analysis. For phylogenomic analyses we used the pyRAD pipeline and obtained a total of 8116 loci with no missing data. This generated a total of 619,572 bp of sequence data.

We amplified 613 bp of COI from ten individuals (Table 2). For one P. blanfordi sample from Myanmar (HL 1), the sequence was of poor quality and hence was discarded from further analysis.

3.4. Population subdivision

We observed three to four spatial clusters in PCoA analysis when including the two outgroup species P. plumosus and P. finlaysoni. All ingroup samples from the P. blanfordi group separated
out based on subspecific identity, although the two subspecies clustered more closely with each other than with the outgroups (Fig. 2A). We obtained the optimal number of clusters both from Evanno’s method (\(\Delta K\) criterion) (Evanno et al., 2005) as well as by comparing results across different \(K\) (Rosenberg et al., 2002). The \(\Delta K\) method suggests the presence of two major genetic clusters (\(P.\) blanfordi–\(P.\) finlaysoni and \(P.\) plumosus). A comparison across different \(K\) reveals presence of further subdivision. At \(K=3\), \(P.\) finlaysoni individual forms a separate cluster, whereas for \(K=4\), the individuals segregate into four distinct groups in agreement with the PCoA results (Fig. 2A and B).

3.5. Phylogeny

The phylogeny based on genome-wide markers fully reflects the population subdivision results (Fig. 2C, Fig. S1A). \(P.\) blanfordi formed two distinct lineages reflecting taxon identity. The COI tree was in agreement with the genome-wide tree (Fig. 2D, Fig. S1B). According to our molecular dating, the two lineages of \(P.\) blanfordi diverged during the late Pliocene or early Pleistocene (3.06 Mya; 95% confidence interval 1.36–4.78 Mya). The net \(p\)-distance between the two lineages was 8.7%.

4. Discussion

4.1. Morphological and vocal differentiation in streak-eared bulbuls

Although the division of streak-eared bulbuls into two forms has been recognized ever since Finsch (1873) described the taxon conradi, modern treatments have invariably thought of the species as being poorly differentiated (Fishpool and Tobias, 2005; Robson, 2008). The main difference given for conradi is the slightly more yellowish underparts coloration. Museum specimens are not always ideal for gauging morphological differentiation of closely related species as eyes are removed during sample preparation and other bare body parts undergo rapid post-mortem discoloration, leading to biases in assessment. Recourse to live material is therefore very important. Our own observations along with photographic material of live streak-eared bulbuls from dozens of naturalists demonstrate that previous treatments have overlooked an important character dividing the two taxa: eye color (dark-red in nominate blanfordi; off-white to dark-grey in conradi; Fig. 1B and C). Eye color may be an important trait in mate recognition and reproductive isolation of songbirds (Gill, 1995), including bulbuls.

Despite the documentation of additional, hitherto unrecognized morphological differentiation between conradi and blanfordi, our bioacoustic analysis did not reveal any noticeable differences in the main vocalizations. Given the importance of vocalizations in songbirds, this may be surprising. However, Pycnonotus bulbuls are generally characterized by inconspicuous and not very stereotypical vocalizations (Fishpool and Tobias, 2005; Robson, 2008), and it is possible that the discovery of vocal differences must await a more complete bioacoustics inventory of the streak-eared bulbul.

4.2. Genetic differentiation in streak-eared bulbuls

Using both mtDNA and genome-wide markers amounting to \(~600\) kb (i.e. 8116 loci of 80 bp length scattered throughout the
may have been in contact at lower passes along the Thai-
mechanism during various Pleistocene epochs when their ranges
bulbuls, eye color could have emerged as an important isolating
brunneus
two species as these bulbuls avoid closed forest and higher eleva-
tions and Thailand may presently be an effective barrier between the
( Fishpool and Tobias, 2005 ). Other closely related

streak-eared bulbul calls and songs, to verify whether rare but
such as introgression ( Rheindt and Edwards, 2011 ) and young spe-
tial signaling importance ( Gill, 1995 ), indicates the presence of two
different species satisfying the criteria of the biological and many
other species concepts ( Meier, 2000 ).

Our results argue for the recognition of conradi as a cryptic
species-level lineage, leaving the form blanfordi as a monotypic
species endemic to the Myanmar dry central plain. This area com-
prises the Irrawaddy Plains Endemic Bird Area (EBA), with 3–4
other bird species restricted to its dry scrub and woodland
( Stattersfield et al., 1998 ). The border range between Myanmar
and Thailand may presently be an effective barrier between the
two species as these bulbuls avoid closed forest and higher eleva-
tions. This border range functions as a biogeographic divide for
many birds and other animal species ( Hughes et al., 2003 ). Addi-
tional vocal enquiries are needed, including an inventory of
streak-eared bulbul calls and songs, to verify whether rare but
diagnostic call types have been missed by the present study.

Eye color is an important isolating mechanism in bulbuls
( Fishpool and Tobias, 2005 ). Other closely related Pycnonotus bul-
bul species can only safely be identified using eye color in adult
birds, such as Cream-vented ( P. simplex ) and Red-eyed Bulbuls ( P.
brunneus ) in Sundaland, corroborating the importance of this trait
( Fishpool and Tobias, 2005 ; Robson, 2008 ). In the two streak-eared
bulbuls, eye color could have emerged as an important isolating
mechanism during various Pleistocene epochs when their ranges
may have been in contact at lower passes along the Thai-
Burme border range or along the coast of Tenasserim. In fact,
the latter area is ornithologically fairly unexplored to the present
day and may constitute a current zone of contact.

Our study confirms the importance of molecular tools, including
mtDNA barcoding ( Kerr et al., 2007 ), to identify cryptic diversity
even in well known animal classes such as birds. However, we have
additionally applied thousands of genome-wide markers given the
previous problems associated with species diagnosis based on COI,
such as introgression ( Rheindt and Edwards, 2011 ) and young spe-
cies ( Wagner et al., 2013 ). Our genome-wide markers corroborate
the mtDNA results of a deep genomic divergence between conradi and blanfordi.

4.3 Cryptic speciation in Pycnonotus bulbuls

Given the lack of diagnosable vocal differences and the close
plumage similarities of these two bulbuls, their deep genomic
differentiation is quite unexpected. The levels of genomic differentia-
tion suggest that the two lineages may not have engaged in regular
gene flow with each other for long periods of time, and the addi-
tional detection of differences in eye coloration, which is of poten-
tial signaling importance ( Gill, 1995 ), indicates the presence of two
different species satisfying the criteria of the biological and many
other species concepts ( Meier, 2000 ).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2016.05.028.

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(Thamnophilidae): the warbling antbird ( Hypocnemis cantator ) complex. Auk
program CERVUS accommodates genotyping error increases in
Notes 7 (4), 533–543.
University Press.

Data accessibility

SRA (genomic data): study accession number SRP061980.