The interplay of color and bioacoustic traits in the speciation of a Southeast Asian songbird complex

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Abstract

Morphological traits have served generations of biologists as a taxonomic indicator, and have been the main basis to delineate species for museum taxonomists for centuries. Widespread awareness of the importance of behavioural characters, such as vocalizations, has arisen much more recently, and the relative importance of these different traits in the speciation process remains poorly understood. To shed more light on the interplay between morphological and behavioral traits in the speciation process, we generated a draft genome of a cryptic Southeast Asian songbird, the Limestone Wren-babbler *Napothera crispifrons*, and re-sequenced whole genomes of multiple individuals of all three traditional subspecies and a distinct leucistic population that had previously been misinterpreted as a plumage polymorphism. We demonstrate strong genomic and mitochondrial divergence among all three taxa, pointing to the existence of three species-level lineages. Despite its great phenotypic distinctness, the leucistic population was characterized by shallow genomic differentiation from its neighbor, with only a few localized regions emerging as highly-diverged. Quantitative bioacoustic analysis across multiple traits revealed deep differences especially between the two taxa characterized by limited plumage differentiation. Our study demonstrates that speciation in these furtive songbirds is not governed by the evolution of marked color differences, but is regulated by an interplay between color and bioacoustic traits. Extreme color differences can be anchored in few genomic loci and may therefore arise and subside rapidly.

Keywords

Cryptic plumage, vocalization, genomic landscape, limestone wren-babbler, passerine

Introduction

Speciation occurs when a population evolves traits that enable it to distinguish itself from another closely related lineage. Evolutionary biologists have sought to quantify these characteristics through various methods, but chief among them in earlier centuries has been morphological inquiry (Mayr, 1942). Coloration and morphometric traits have served as an important species delimitation tool for generations of biologists because phenotypically distinct populations are unlikely to mate with each other (Mayr, 1942; Zapata & Jiménez, 2012). This approach has assisted museum taxonomists in inferring species relationships, and is relatively reliable for higher level taxa and/or when morphological differences are discrete and obvious. However, morphological traits may become unreliable when phenotypic distinctions are diffuse or subtle, including in exceptionally well-known animals such as birds, which boast a high degree of cryptic species di-

versity characterized by nearly identically-colored sister species (Töpfer, 2018). In fact, a growing number of studies have revealed the pitfalls of relying on morphology alone to discern phenotypically conserved species (Bickford et al., 2007; Lohman et al., 2010; Satler et al., 2013).

In view of this limitation, taxonomists have seen a need to expand their arsenal of species delimitation tools to reproductively important behavioral traits. In particular, the application of bioacoustic tools has gained popularity in recent decades for animal groups which use vocalizations for maintaining reproductive isolation, including birds, mammals and amphibians (Baptista & Kroodsma, 2001; Alström & Ranft, 2003; Philippe et al., 2017; López-Baucells et al., 2018a). Most notably, bioacoustic comparisons have helped uncover high levels of cryptic avian diversity, sometimes with valuable implications for conservation (Baptista & Kroodsma, 2001; Rheindt et al., 2008; Gwee et al., 2017; Gwee et al., 2019b). At the same time, bioacoustic species delimitation is not a panacea as vocal divergence may be subtle in genetically differentiated cryptic species (Garg et al., 2016; Dufresnes et al., 2018). The relative importance and interplay of biological traits, such as morphology and bioacoustics, in the speciation process thus remain poorly understood.

To test this interplay between morphological and bioacoustic traits, one ideally requires a species complex in which vocalizations are known to be important for reproduction, but which equally provides a striking morphological contrast. The Limestone Wren-babbler, *Napothera crispifrons* , is exactly such a model. It is an example of a highly non-dispersive, morphologically conserved songbird species complex closely associated with limestone karst formations across Southeast Asia. Members of this species complex are characterized by an inconspicuous brown plumage, a distinctly streaked throat common to the genus, and either a grey or dark rufous belly. The species complex comprises three taxa: nominate *crispifrons* from Myanmar and western Thailand*, annamensis* centered in Vietnam and Laos, and*calcicola* from lower northeast Thailand (Figure 1a).Although Deignan (1945, 1963) assigned birds from Phrae Province – the only far northern Thai population then known – to nominate*crispifrons* , the more recent discovery of an adjacent population in Nan Province abutting the Lao border (PDR, pers. obs.) points to the taxonomic identity of these far northern Thai birds as *annamensis*(grey area in Figure 1a). The Limestone Wren-babbler has traditionally been treated as a single species due to its members' conserved morphology and a general lack of biological understanding of this group of birds (Robson & Allen, 2002; Cai et al., 2019). However, a recent evaluation based on plumage differences of museum specimens split the rufous-bellied taxon as a distinct species, *N. calcicola* , from the remaining two taxa, *N. crispifrons* (Collar et al., 2018).

Adding to these taxonomic complexities, a conspicuously white-faced population of *N.* [*c.*] *crispifrons* has been known historically from the far west of the distribution in Myanmar (Figure 1a), but has variously been regarded as a leucistic form or white "morph" (Collar et al., 2018). The type specimen of the entire complex is a Myanmar individual collected in 1855 from Mulayit Taung, a mountain east of the Salween River (in Burmese: Thanlwin River) close to the Thai border (Deignan, 1963). Based on the original description by Blyth (1855), the type specimen has a brown face. Subsequent museum inspections of *N. crispifrons* specimens from a wider range across Myanmar revealed that white-faced individuals can be commonly found at localities west of the Salween River or directly along it, such as Wimpong (16°49'N, 97°28'E) and Moulmein (16°30'N, 97°38'E) (Baker, 1922; Deignan, 1945). Our own fieldwork around Hpa-An, along the Salween River, has established a mutually exclusive distribution of white-faced and brown-faced individuals on either side of the Salween, with white-faced individuals to the west and brown-faced individuals to the east of the river. The discovery of such a conspicuous plumage-based cross-river divergence, at least at Hpa-An, in an otherwise cryptically colored songbird complex in which vocalizations are thought to be the most important defining character provides an ideal model to test the relative importance of coloration and vocal parameters in the differentiation process.

Previous tests of the relative importance of morphological and behavioural traits in speciation have largely relied on mitochondrial DNA (Rheindt et al., 2008; Wang et al., 2014; López-Baucells et al., 2018b), which is susceptible to genetic introgression and frequent selective sweeps (Ballard & Whitlock, 2004; Bazin et al., 2006; Rheindt et al., 2009; Rheindt & Edwards, 2011). Here, we have generated a new draft genome of the Limestone Wren-babbler based on a white-faced individual while employing an integrative approach

involving bioacoustic and whole genome resequencing methods across members of all described taxa to shed light on the drivers of differentiation and speciation in this complex. We tested for congruence of results from the different approaches to assess the importance of morphological and bioacoustic traits in the speciation process. We evaluated the levels of genomic differentiation in pairwise comparisons across chromosomes, including between white-faced and brown-faced populations of *N.*[*c.*] *crispifrons* , to investigate the presence of specific genomic regions that may be unique to a particular population.

Material and Methods

Bioacoustic analysis

A total of 25 sound recordings across the three taxa were collected from our field work and various online sound recording archives, such as xeno-canto (https://www.xeno-canto.org), Macaulay Library (https://www.macaulaylibrary.org) and Avian Vocalization Center (AVoCet, https://avocet.zoology.msu.edu) (Supporting information Table S1). Limestone Wren-babbler vocalizations constitute complex song motifs consisting of multiple vocal elements, i.e. traces on a sonogram. We used Raven Pro Version 1.5 (Bioacoustics Research Program & Program, 2014) to measure ten vocal parameters: 1) average maximum frequency of a motif, 2) average minimum frequency of a motif, 3) average bandwidth, 4) average center frequency of a motif (i.e., the darkest pixel on a sonogram), 5) average peak frequency of a motif (i.e., the frequency tranche with the highest amplitude), 6) average number of elements per motif, 7) largest element bandwidth, 8) longest break-length within a motif (i.e., between any two subsequent elements), 9) average pace (i.e., number of elements per second) and 10) average duration of a motif. We conducted principal component analysis (PCA) to examine the vocal variation among different populations. Additionally, we applied the criterion established by Isler et al. (1998), henceforth Isler criterion, to assess the significance of each vocal parameter across pairwise comparisons. The Isler criterion is based on two conditions: 1) the ranges of measurements between two populations must not overlap, and 2) the means (x) and standard deviations (SD) of the population with lower measurements (a) and the population with higher measurements (b) have to fulfil the following equation:$x_a + t_a\mathrm{SD}_a \ \leq \ x_b - t_b\mathrm{SD}_b$, where t_i refers to the t- score at the 95^{th} percentile of the distribution for n – 1 degrees of freedom. The Isler criterion was initially devised for New World antbirds (Isler et al., 1998), but has been applied across various songbirds (Cros & Rheindt, 2017; Prawiradilaga et al., 2018; Gwee et al., 2019b) and non-oscines (Sangster & Rozendaal, 2004; Rheindt et al., 2011; Ng et al., 2016; Ng & Rheindt, 2016; Gwee et al., 2017; Gwee et al., 2019a).

Whole genome resequencing library preparation

We collected blood, tissue or feather material from a total of 15 individuals: three *N.* [*c.*] *crispifrons* samples from Myanmar, including one white-faced individual; five *N.*[*c.*] *calcicola* and four *N.* [*c.*]*crispifrons* samples from Thailand; and three *N.*[*c.*] *annamensis* samples from Vietnam (Supporting information Table S2). We extracted DNA of each sample using the DNeasy Blood & Tissue Kit (Qiagen, Germany) following the manufacturers' instructions. For dried blood on filter paper, an additional incubation step after the addition of ATL buffer at 90@C for 15 minutes was made. For feather samples, 200 uL instead of 180 uL of ATL, and 20 uL of 1M DTT were added before the addition of proteinase K for keratin digestion.

We used Bioruptor® to shear the extracted DNA to 250 base pair (bp) fragments with sonification conditions of 30 seconds on and off time each for 13 cycles. We used NEBNext Ultra II DNA Library Prep Kit (New England Biolabs, UK) for whole genome library preparation, following the manufacturer's protocol. All samples were run with five polymerase chain reaction (PCR) cycles, except samples with an input DNA amount of less than 100 ng which were run with eight PCR cycles. Each sample was prepared with a unique dual index barcode using NEBNext Multiplex Oligos for Illumina (New England Biolabs, UK). Each library was quantified with Qubit and an AATI Fragment Analyzer to check DNA concentration and fragment size respectively. The average peak fragment size was approximately 370 bp including adapters and barcodes. The libraries were sequenced using the Illumina Hiseq 4000 platform with 150 bp paired-end runs.

Draft genome assembly

We produced a mid-coverage (approximately 38.8X) whole genome of the white-faced individual of nominate *crispifrons* (KK05) by sequencing the prepared library with Illumina 150 bp paired-end runs on two Illumina Hiseq 4000 lanes. Adapter contamination was removed using cutadapt 1.18 (Martin, 2011). We mapped the reads to the complete mitogenome of *N. epilepidota* (accession no. KX831093) (Huang et al., 2019) using BWA-MEM 0.7.15 (Li, 2013) and used SAMtools 1.9 (Li et al., 2009) to remove mitochondrial reads and retain paired-end reads that did not map to the mitogenome. MaSuRCA 3.3.0 (Zimin et al., 2013) was utilized for the *de novo* assembly of the *Napothera*draft genome due to its superior performance on short-read assemblies. The MaSuRCA pipeline was run with a jellyfish size of 5 billion bp and Celera Assembler (Myers et al., 2000) was chosen for the assembly of contigs and scaffolds as it requires a minimum overlap of 40 bp and is thus more stringent. The initial assembly produced a genome with N50 = 48,862 bp, a total of 82,195 scaffolds and a size of 1.08 Gb. We further processed the scaffold output by removing short scaffolds of less than 3000 bp with a custom script and filtering repeat regions with RepeatMasker 4.0.9 (Smit et al., 2015), thus reducing the number of scaffolds to 36,669. We assessed the completeness of the draft genome using BUSCO 3.1 (Simão et al., 2015), which showed that the genome was 81.4% complete with the *Gallus gallus* genome as a reference. We inferred chromosomal position of the assembled draft genome by assuming conserved synteny with *Taeniopygia guttata* , and applied Satsuma 3.1.0 (Grabherr et al., 2010) to align the draft genome against the*T. guttata* genome (accession no. GCF_003957565.1).

Raw data processing and SNP calling

We used cutadapt 1.18 (Martin, 2011) to remove adapter contamination and reads with a quality score lower than 20. Each sample was aligned to the*Napothera* draft genome using BWA-MEM 0.7.15 (Li, 2013). The generated sam files were then converted to bam files with SAMtools 1.9 (Li et al., 2009). Read groups were attached to the sequenced reads using Picard 1.8 (http://broadinstitute.github.io/picard/) AddReadGroups, followed by Picard 1.8 MarkDuplicates to filter out PCR duplicates for subsequent downstream analysis. Finally, GATK 3.8.1.0 IndelRealigner (McKenna et al., 2010) was applied to perform local realignment around insertions and deletions to minimise mismatches. The mean coverage of each sample was approximately 15.7X (5.9X – 38.8X; Supporting information Table S2).

We conducted SNP calling using ANGSD 0.923 (Korneliussen et al., 2014) with the following parameters: doMaf 2, GL 2, doMajorMinor 1, SNP_pval 1e-6, minMapQ 20, minMaf 0.05, minInd 15, minIndDepth 5, and geno_mindepth 5. A total of 1,244,043 SNPs were called and recorded in two different ways to be processed separately: the genotype likelihoods were saved in the BEAGLE (Browning & Yu, 2009) format and the called genotypes were saved in the transposed (tped) format. First, we conducted PCA on the genotype likelihoods of all individuals using PCAngsd 0.981 (Meisner & Albrechtsen, 2018) to account for statistical uncertainty in the genotypes of low coverage samples. Then, we used the tped file as an input file for PLINK 1.90 (Chang et al., 2015) to prune SNPs in high linkage disequilibrium. We set the independent pairwise filter at a correlation threshold of 0.1 for a window size of 25 kbp and a step size of 10 kbp, resulting in a final set of 104,092 unlinked SNPs for PCA and STRUCTURE (Pritchard et al., 2000) analyses. Additionally, PCA was run on datasets with more relaxed linkage disequilibrium filters applied: a correlation threshold of 0.3 giving 285,724 SNPs, and a correlation threshold of 0.5 giving 490,054 SNPs. We ran STRUCTURE (Pritchard et al., 2000) using the multithreading program Structure_threader (Pina-Martins et al., 2017) to assess the population admixture of *Napothera* at K=1 to 5 with three replicates each.

Detection of secondary gene flow with ABBA-BABA tests

The ABBA-BABA test, also known as the D-statistic, detects introgression by assessing if there is an excess of shared derived alleles between populations (Green et al., 2010; Patterson et al., 2012). We computed ABBA-BABA statistics using ANGSD 0.923 (Korneliussen et al., 2014) with the same parameters as applied for SNP calling for a block size of 50,000 bp, and with Rufous-capped Babbler*, Cyanoderma ruficeps* , assigned as an outgroup (H4) population. We obtained the raw reads sequenced for the whole genome assembly of *C. ruficeps* (Yang et al., 2019), from the European Nucleotide Archive (accession no. PRJNA573563). The raw reads were processed using the same pipeline as above and a consensus alignment was obtained with the doFasta 2 option in ANGSD 0.923 (Korneliussen et al., 2014). The jackknife.R script in ANGSD 0.923

(Korneliussen et al., 2014) was applied to test for significance of ABBA-BABA statistics. A positive critical value of at least three (Z > 3) suggests a significant excess of ABBA-like alleles as compared to BABA-like alleles (Patterson et al., 2012).

Genome scan for candidate genes

To compute the absolute genetic differentiation for each population pair, *C. ruficep* was used an outgroup to polarize the allele frequencies of segregating variants. We recalled SNPs using ANGSD 0.923 (Korneliussen et al., 2014) with *C. ruficeps* as an ancestral sequence, applying the same parameters as used in the previous SNP calling procedures, but additionally implementing a filter to discard tri-allelic sites. We then used the Python script popgenWindows.py (https://github.com/simonhmartin/genomics_general) to compute d_{XY} values for each population pair with the following parameters: 50,000 bp sliding window, 10,000 bp step size and at least three sites must be present per window size. Outlier windows above the 99.9^{th} percentile of each population pair comparison were identified.

Additionally, we computed net d_{XY} differences for pairwise comparisons of the white-faced and brown-faced populations using the d_{XY} comparison of brown *N.*[*c.*] *crispifrons* between Thailand and Myanmar as a baseline. We expect a high net d_{XY} value in regions which are highly differentiated between white-faced and brown individuals but are highly-conserved between the two brown *N.*[*c.*] *crispifrons* populations. The peak regions found in the net d_{XY} comparisons between white-faced and both brown-faced populations (Myanmar and Thailand) were identified. These peak regions were aligned against the Zebra Finch genome (assembly bTaeGut_v1.p) and associated genes were inferred using the Ensembl database (Yates et al., 2019).

Genome-wide SNP tree

We conducted SNP calling using ANGSD 0.923 (Korneliussen et al., 2014) under the same parameters as outlined above with the inclusion of a*C. ruficeps* individual as an outgroup. The variants were filtered using PLINK 1.9 (Chang et al., 2015) to remove SNPs under linkage disequilibrium by applying the same parameters as previously described, and a final set of 306,874 variants were retained for the construction of a SNP tree. We applied RAxML 8.2.12 (Stamatakis, 2014) to construct a Maximum Likelihood (ML) tree using a GTR + GAMMA model with 1000 rapid bootstraps.

Mitochondrial tree

We extracted mitochondrial reads from each sample by mapping the reads to the complete mitogenome of *N. epilepidota* (accession no. KX831093) (Huang et al., 2019) using BWA-MEM 0.7.15, followed by the application of Picard MarkDuplicates to remove all duplicates. CLC Workbench 7 was used to re-align the extracted mitogenomic reads to the ND2 gene of *N. epilepidota* (accession no. JN826601.1) (Moyle et al., 2012), perform local realignment and produce a consensus alignment in FASTA format. All reads with a coverage lower than 100X were discarded. For blood samples which had a low coverage of mitogenomic regions, we conducted Sanger sequencing separately to retrieve the ND2 sequence following Lim et al.'s (2019) protocol. All the ND2 sequences were exported to MEGA7 (Kumar et al., 2016) and aligned with ClustalW. MEGA7 was also used to construct a neighbour joining (NJ), maximum likelihood (ML) and maximum parsimony (MP) tree with 10,000 bootstraps each.

Results

Bioacoustic comparison of all three taxa

PCA results of vocal measurements revealed that *N.*[*c.*] *crispifrons* was vocally distinct from *N.*[*c.*] *annamensis* and *N.* [*c.*]*calcicola* , while the latter two emerged as vocally undifferentiated from each other on the basis of the bioacoustic parameters investigated (Figure 1b). Using the Isler criterion, we detected one to two diagnostic vocal parameters between *N* . [*c.*] *crispifrons* and each of the other two taxa, but none of the vocal parameters emerged as diagnostic between *N.*[*c.*] *annamensis* and *N.* [*c.*]*calcicola* (Supporting information Table S3). Despite a large penalty imposed on the pairwise comparisons involving

the white-faced*N. [c.] crispifrons* due to low sample size (n = 2), the white-faced *N . [c .] crispifrons* emerged as vocally distinct from all other populations except from the brown form of *N . [c .] crispifrons* .

Population genomics of N. crispifrons

Both PCA and STRUCTURE results support three distinct genomic clusters:*annamensis* , *calcicola* and *crispifrons* , in agreement with the mitochondrial results which revealed pairwise divergences in excess of 5% among all three populations (Figure 1; Supporting information Figures S1, S2; Supporting information Table S4). Among the three populations, *calcicola* and *crispifrons* were most differentiated from each other, while *annamensis* was almost genomically equidistant to both taxa (Figure 1; Supporting information Figure S1). PCA analyses were congruent across the four different datasets, including the ones based on genotype likelihoods of all SNPs and those based on genotypes of two sets of unlinked SNPs (Supporting information Figure S1).

Although only limited genomic differentiation was found between the Myanmar and Thai populations of *N . [c.]crispifrons* , a relatively deep mitochondrial divergence of 2.7% was detected between the two populations (Figure 1; Supporting information Table S4). Both genomic and mitochondrial results support a lack of differentiation between the white-faced and brown-faced*N . [c.] crispifrons* populations from Myanmar (Figure 1; Supporting information Figure S1; Supporting information Table S4).

A phylogeny of the Limestone Wren-babbler complex constructed with over 300,000 genome-wide SNPs revealed a sister relationship between*annamensis* and *calcicola* , with *crispifrons* basal to them. In contrast, the phylogeny of the three taxa generated by mitochondrial ND2 gene was unresolved as the key node was poorly supported by both maximum parsimony and maximum likelihood methods (Figure 1c).

Absence of secondary gene flow

We tested for the presence of secondary gene flow among the three taxa and among the intra-taxon populations of nominate *crispifrons*using the genome-wide SNP tree as the backbone topology. We found no evidence of secondary gene flow in both inter and intra-taxon comparisons (Table 1).

Genomic landscape of differentiation

The overall absolute genetic pairwise divergence (d_{XY}) across chromosomes was greater between taxa (i.e., *annamensis* vs.*crispifrons* , *annamensis* vs. *calcicola* , and*calcicola* vs. *crispifrons*) than within taxa (i.e., Thai vs. Myanmar populations of *crispifrons* , and white-faced vs. brown*crispifrons* from Myanmar) (Figure 2). In pairwise divergences between taxa, but not within taxa, the great majority of d_{XY} values across the entire genome was generally above 0.2 and multiple regions emerged as highly differentiated (Figure 2; Supporting information Figure S3).

Although pairwise genetic differentiation across the chromosome was relatively low in intra-taxon comparisons, the differentiation landscape exhibited several outlier peaks (Figure 2c, d). These peaks were almost exactly congruent between the pairwise comparisons that contrast the white-faced population against the adjacent brown population in Myanmar as opposed to the more distant brown population in Thailand (Figure 2c, d). Similarly, net d_{XY} values computed between white-faced and brown *N. crispifrons* individuals and corrected for differentiation between the two brown populations exhibited divergence peaks in several localized regions, each spanning about 150 kb (Figure 2c, d). Two outlier peaks, one identified in chromosome 1A and the other in chromosome 18, were found to be associated with genes implicated in pigmentation pathways (Table 2).

Discussion

The speciation process is based on disruptions in gene flow often thought to arise from phenotypic differentiation. However, the suite of characters that are relevant to the maintenance of gene flow differs among animal groups. In songbirds such as wren-babblers, vocalizations are thought to be the most consequential defining character in the speciation process (e.g. Rheindt et al., 2008; van Balen et al., 2013), but color traits may also be important (Tobias et al., 2010; Topfer, 2018), and little is known about their interplay

in generating taxon diversity. The Limestone Wren-babbler has long been treated as a single species due to the overall conservatism of its plumage and a previous lack of attention to existing bioacoustic information. A recent taxonomic classification split the Limestone Wren-babbler into two distinct species, *N. crispifrons* and *N. calcicola* , based on their grey and rufous bellies, respectively (Collar et al., 2020). However, this treatment erroneously interpreted the white-faced phenotype of birds west of the Salween River as a plumage polymorphism rather than geographic variation, and included no more than a rough assessment of qualitative vocal differences among taxa (Collar et al., 2018).

Three deeply diverged lineages in the complex

Our genome-wide approach revealed strong support for three deeply diverged lineages at the species level: *N. crispifrons* from Myanmar and western Thailand, *N. annamensis* from Vietnam, and*N. calcicola* from north-eastern Thailand (Figure 1). All three emerged in well-separated clusters in analyses incorporating thousands of genome-wide markers, without any signs of intermediacy or secondary gene flow (Figure 1; Table 1). Mitochondrial divergences of ˜5 – 6.4% provide a convenient yardstick (Supporting information Table S4), and are over twice as deep as those between typical sister species pairs in bird phylogenetics (Hebert et al., 2003; Kerr et al., 2009; Campbell et al., 2016). Our results attest to the dangers of primarily relying on plumage characters in modern taxonomic overhauls: Collar et al.'s (2018) color-based separation of *N. calcicola* , but not *N. annamensis* , as a monotypic species is not supported by the genomic results, which indicates that *N. annamensis* shares a closer affinity with *N. calcicola* than with the similarly grey-bellied *N. crispifrons* (Figure 1).

Unexpectedly, the Myanmar and western Thai populations of *N. crispifrons* (*sensu stricto* , excluding *annamensis* and*calcicola*) displayed a relatively high mitochondrial divergence of approximately 2.7% (Supporting information Table S4). While borderline for purposes of species delimitation, this depth of mitochondrial divergence is sometimes found between distinct sister species in other songbird groups (Hebert et al., 2003; Kerr et al., 2009; Campbell et al., 2016). Yet genomically these two populations exhibited limited differentiation, occurring in the same tight PCA clusters (Figure 1, Supporting information Figure S1) and remaining undivided even at higher levels of K in Structure analysis (Supporting information Figure S2). This mito-nuclear discordance may have a variety of causes, such as 'ghost introgression' of mitochondrial haplotypes of extinct or unsampled populations (Rheindt & Edwards, 2011; Zhang et al., 2019), but ultimately demonstrates the shortcomings of relying on mitochondrial data alone.

Are bioacoustic and morphological traits congruent?

Despite sharing a similar grey-bellied plumage, *N. crispifrons*was found to be vocally distinct from *N. annamensis* (Figure 1b). This strong vocal divergence between two morphologically similar but genomically distinct species suggests that bioacoustic traits may be especially important in maintaining species integrity when visual cues are limited. On the one hand, the grey-bellied *N. annamensis*emerged as vocally indistinguishable from the rufous-bellied *N. calcicola* on the basis of the ten vocal parameters investigated in this study (Figure 1b): their different belly colors may render the need for vocal differentiation less important. On the other hand, the lack of perceived bioacoustic divergence may be attributed to their proclivity for duetting between partners, which is usual in these two taxa of wren-babblers, resulting in difficulties for vocal analysis and a large variation in measurements of some temporal parameters (Hall, 2009). In summary, the incongruence detected between plumage and bioacoustic patterns highlights the limitation in relying on a single approach and advocates the importance of integrative taxonomy.

Future sampling will be able to shed further light on the affinity of populations not included herein. Citing morphological differences, but providing limited details, Deignan (1945, 1963) assigned populations from northernmost Thailand in Phrae Province to *N. crispifrons*instead of *N. annamensis* . However, the recent discovery of an adjacent population in Nan Province, abutting the Lao border, fills a geographic gap (PDR, pers. obs.) and suggests that northern Thai populations may form a continuum with the Lao population and may ultimately belong to *N. annamensis* (grey area in Figure 1a).

White faces are likely a recent occurrence driven by mutations in a few localized regions

Among the different populations of Limestone Wren-babbler, the white-faced population from Myanmar arguably has the most distinct plumage, yet it has always been regarded as a leucistic morph of*N. crispifrons* instead of a geographic race deserving of subspecies recognition. The results of our genomic work confirmed that the white-faced population is genomically cohesive with the brown-plumaged *N. crispifrons* from Myanmar and Thailand (Figure 1), supporting a sizeable body of research that has shown that limited genomic regions and sometimes even single loci can be responsible for stark plumage differences in pigmentation (Theron et al., 2001; Kerje et al., 2004; Mundy et al., 2004; Uy et al., 2009; Cibois et al., 2012; Toews et al., 2016; Knief et al., 2019). While the involvement of the melanocortin-1 receptor (*MC1R*) gene in melanic polymorphisms is extremely well documented across various animal species (Theron et al., 2001; Mundy et al., 2004; Baiao et al., 2007; Uy et al., 2009; Cibois et al., 2012; Switonski et al., 2013), our understanding of other candidate loci that may be indirectly associated with the expression of pigmentation is still rudimentary (MacDougall-Shackleton et al., 2003; Cheviron et al., 2006; Bourgeois et al., 2016).

Comparing the genomes of the white-faced and brown populations of*N. crispifrons* , we identified several outlier regions which may be associated with this partial albinism. Specifically, we identified two genes from chromosomes 1A and 18 that may be involved in the pigmentation pathway, including *RAB3IP* and *SLC16A3* . The gene *RAB3IP* encodes for a *RAB3A* interacting protein, which is responsible for converting inactive GDP-bound Rab proteins into their active GTP-bound form. Various Rab proteins have been shown to play a significant role in melanosome transport (Araki et al., 2000; Chakraborty et al., 2003; Chabrillat et al., 2005), and mutations to these genes can lead to a loss of pigmentation in the coat, such as lightened skin colour in mice and silvery hair in humans (Wilson et al., 2000; Matesic et al., 2001; Ohbayashi & Fukuda, 2012). Therefore, mutations to the gene *RAB3IP* may indirectly affect the expression of pigmentation in white-faced *N. crispifrons* individuals. The gene *SLC16A3* , also known as *MCT4* , has been shown to play an important role in the pigmentation of chicken skin, muscle and bone (Yu et al., 2018). Given the shallow mitochondrial divergence of the white-faced population and the virtual lack of substantive genomic differentiation outside of those few divergence peaks (Figure 2), mutations in these genes leading to the evolution of white faces are likely to have occurred relatively recently, and perhaps only in one or a few individuals and driven by a founder effect amplified by the poor dispersal capability of these babblers. The Salween River may therefore be a formidable secondary barrier, preventing the easy spread of this plumage innovation across the river towards the east.

Long-term relevance of the plumage innovation in Limestone Wren-babblers

Our fieldwork for this study established that the white-faced phenotype of a peripheral population in Myanmar is not a polymorphism, but is geographically restricted to birds west of the Salween River, at least in the Hpa-An region. Although visually conspicuous, these far-western birds are virtually identical in their genomes to the conventional brown populations on the east side of the river. A few localized genomic areas of deep divergence are likely functionally tied to the difference in pigmentation and reflect a recent origin, possibly amplified by a founder effect and subsequent lack of regular gene flow across the river.

The rapid emergence of substantial melanin-based plumage differences has been attested to across many bird complexes (Theron et al., 2001; Doucet et al., 2004; Mundy et al., 2004; Baiao et al., 2007; Uy et al., 2009; Cibois et al., 2012). In the Limestone Wren-babbler, the long-term persistence of this pronounced difference in facial markings and its relevance for speciation remains to be determined. Phenotypic innovations involving the switching-off of pigmentation are characteristic of small-island populations (Doucet et al., 2004; Baiao et al., 2007; Cibois et al., 2012) and lineages otherwise isolated. Being found in a peripheral population with limited gene flow to the stronghold of the range, such a plumage innovation can probably disappear as quickly as it arises. While it is conceivable that we captured a population at the very beginning of the speciation process, when phenotypic differentiation is already evident in the absence of genomic divergence, it is equally conceivable that this population may be evolutionarily ephemeral, and succumb to climatic vagaries or be overrun by non-leucistic populations from the main range as soon as connectivity allows.

Conclusion

Our study reveals the Limestone Wren-babbler complex consists of three genomically diverged species, partially supported by plumage and vocal results individually. Although morphologically distinct, the elusive white-faced population of *N. crispifrons* is genomically cohesive with the conventional brown-faced population. We identified a few localized candidate genes apparently associated with the loss of pigmentation in the facial feathering of this population. In the presence of a secondary geographical barrier such as the Salween River, mutations in a few localized regions may have facilitated the establishment of a phenotypically distinct population within a short period of time.

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Data Accessibility

Draft genome of the *Napothera crispifrons* , raw reads and ND2 sequences generated by this study are available on Dryad Digital Repository: https://doi.org/XXXXX

Author Contributions

F.E.R. designed this research; F.E.R., S.P.M., H.L.M., R.T., P.D.R. conducted field research; Q.L.L. conducted molecular work; C.Y.G., Q.L.L., F.E.R. analyzed data; S.P.M. examined museum specimens; C.Y.G., Q.L.L., F.E.R. wrote the manuscript; and all authors reviewed and edited the manuscript.

Tables and Figures

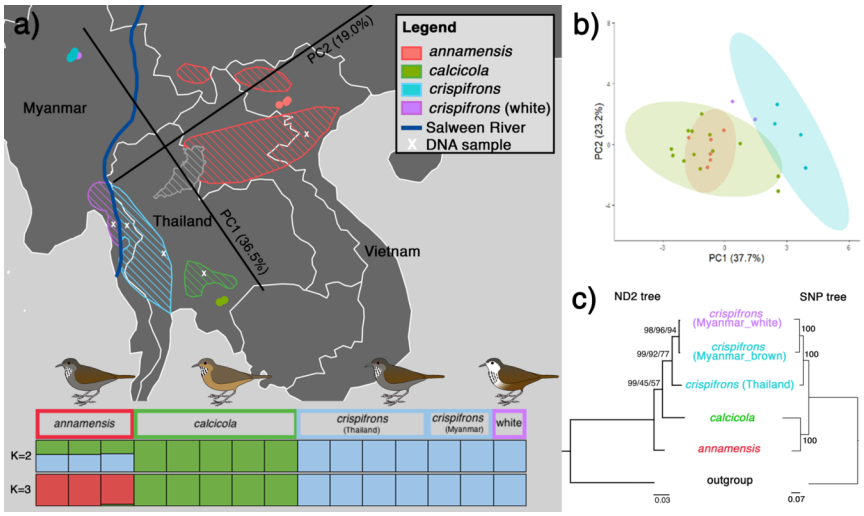

Figure 1 a) Geographical distribution of the Limestone Wren-babbler *Napothera crispifrons* complex, including the three described taxa and the white-faced population, shaded respectively on the map. The taxonomic identity of the northern Thai population from Phrae and Nan provinces (shaded in light grey) is controversial. Sampling localities of the DNA materials are represented by white crosses on the map. Principal component analysis (PCA) of the genome-wide data is projected onto the distribution map with each individual represented by a dot colored according to its respective taxon. STRUCTURE results are depicted at the bottom for K=2 and K=3. b) PCA of bioacoustic measurements with ellipses indicating 95% confidence intervals of each group. Colors of dots correspond to panel (a). c) Phylogeny of the Limestone Wren-babblers using 887 base pairs of the ND2 gene (left) and 306,874 genome-wide SNPs (right). Bootstrap support generated from neighbour-joining, maximum parsimony and maximum likelihood methods are shown beside each node in the corresponding sequence for the ND2 tree, while bootstrap support generated from maximum likelihood is shown for the SNP tree.

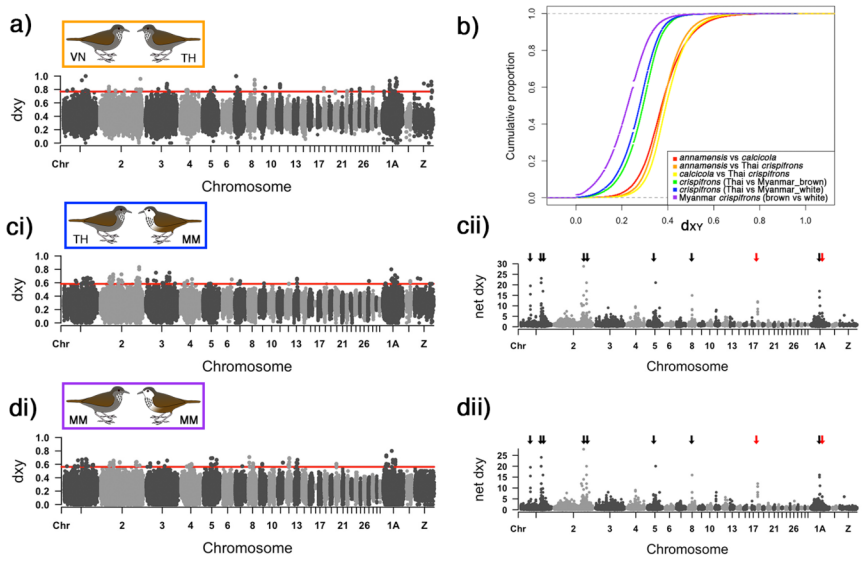

Figure 2 Absolute nucleotide divergence (d_{XY}) of selected pairs of populations. a) d_{XY} between the Vietnam (VN) population of *N.* [*c.*] *annamensis*and Thai (TH) population of *N.* [*c.*] *crispifrons*across chromosomes (chr). The red horizontal line in each d_{XY} plot reflects the 99.9^{th}percentile threshold across pairwise comparisons. b) Cumulative proportion of the d_{XY} values of selected pairs of populations. Inter-taxon comparisons (depicted by red, orange and yellow lines) had a higher proportion of high d_{XY} values, while intra-taxon comparisons (depicted by green, blue and purple lines) had a lower proportion of high d_{XY} values. ci) d_{XY} between *N. crispifrons* populations with white faces (Myanmar, MM) and brown faces (Thailand, TH) across chromosomes. di) d_{XY} between Myanmar *N. crispifrons*populations with white faces and brown faces across chromosomes. cii, dii) Net d_{XY} (see text) of the pairwise comparison corresponding to (ci) and (di), respectively, across all chromosomes. Outlier regions indicated by black or red arrows, the latter referring to regions containing pigment-related genes.

Table 1 D-statistics of selected populations to assess presence of secondary gene flow. A critical value (Z) above 3 suggests an excess of allele sharing between recipient (H2) and donor (H3) populations.

D-stat	Z	p-value	ABBA	BABA	Blocks	H1	H2	H3
0.012	2.75	0.006	16016	15647	7770	*annamensis*	*calcicola*	*crispifrons* (Th
0.002	0.53	0.596	15803	15725	7229	*annamensis*	*calcicola*	*crispifrons* (My
-0.007	-1.38	0.168	15367	15579	6736	*annamensis*	*calcicola*	*crispifrons* (wh
0.001	0.16	0.874	13582	13562	7468	*crispifrons* (Myanmar_brown)	*crispifrons* (white)	*crispifrons* (Th

Table 2 Outlier genomic regions identified between white-faced and brown-faced *N. crispifrons* populations from Thailand and/ or Myanmar. Genes found within each of the outlier regions are shown, and genes associated to pigmentation pathways are highlighted in bold.

Chromosome	Gene	Description
1: 38950001-39100000	Intronic region	NA
1: 82930001-83080000	NALCN (ENSTGUG00000010876)	Sodium leak channel, non-selective
	ITGBL1 (ENSTGUG00000010867)	Integrin subunit beta like 1
1: 89750001-89900000	ATP10A (ENSTGUG00000010310)	ATPase phospholipid transporting 10A (putative)
1A: 35730001-35880000	ENSTGUG00000023478	High mobility group AT-hook 2
1A: 37720001-37870000	**ENSTGUG00000006982**	**RAB3A interacting protein**
	ENSTGUG00000007004	
2: 119560001-119710000	FAM110B (ENSTGUG00000011117)	Family with sequence similarity 110 member B
2: 131400001-131550000	MMP16 (ENSTGUG00000011786)	Matrix metallopeptidase 16
5: 33910001-34060000	STXBP6 (ENSTGUG00000011795)	Syntaxin binding protein 6
8: 15490001-15640000	LMO4 (ENSTGUG00000006316)	LIM domain only 4
18: 2100001-2250000	CSNK1D (ENSTGUG00000003904)	Casein kinase 1 delta
	SLC16A3 (ENSTGUG00000003892)	**Solute carrier family 16 member 3**
	FASN (ENSTGUG00000003719)	Fatty acid synthase