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The interplay of colour and bioacoustic traits in the differentiation 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 for defining and classifying species diversity for centuries. A quantitative integration of behavioural characters, such as vocalizations, in studies on biotic differentiation has arisen more recently, and the relative importance of these different traits in the diversification process remains poorly understood. To provide a framework within which to interpret the evolutionary interplay between morphological and behavioural traits, we generated a draft genome of a cryptic Southeast Asian songbird, the limestone wren-babbler *Napothera crispifrons*. We resequenced whole genomes of multiple individuals of all three traditional subspecies and of a distinct leucistic population. 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 neighbour, 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 differentiation in these furtive songbirds has resulted in a complex mosaic of colour-based and bioacoustic differences among populations. Extreme colour differences can be anchored in few genomic loci and may therefore arise and subside rapidly.

KEYWORDS

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

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1 | INTRODUCTION

Biotic differentiation is often accompanied by the evolution of traits that enable a lineage to distinguish itself from another closely related lineage. Such traits accumulate over time due to processes directly or indirectly associated with selection pressure, or through random genetic drift (Barton et al., 2007; Ridley, 2003). Evolutionary biologists have sought to quantify these characteristics through various methods, but chief among them in earlier centuries has been morphological inquiry (Mayr, 1948). In particular, coloration and morphometric traits have served—with varying levels of success—as an important species delimitation tool for generations of biologists because phenotypically distinct populations are less likely to mate with each other. 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 (Zapata & Jiménez, 2012). However, morphological traits may become unreliable and misleading when phenotypic distinctions are diffuse or subtle, especially in animals such as birds, which boast a high degree of cryptic species diversity characterized by nearly identically coloured sister species (Mayr, 2000; 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 behavioural traits. Bioacoustic analysis within a rigorous quantitative framework has only begun to be applied to taxonomy in recent decades, even if there has been an awareness of the importance of vocal traits much earlier (Lanyon, 1960, 1961, 1965, 1978). Bioacoustic characters are useful to consider in systematic research across various animal groups, including birds, insects, mammals and amphibians (Alström & Ranft, 2003; Baptista & Kroodsma, 2001; Desutter-Grandcolas & Robillard, 2003; López-Baucells et al., 2018b; Philippe et al., 2017; Tishechkin & Vedenina, 2016). Even in the most well-known animal class on Earth, namely birds, bioacoustic comparisons have helped uncover high levels of cryptic diversity, sometimes with valuable implications for conservation (Ford & Parker, 1973; Ford, 1983; Baptista & Kroodsma, 2001; Gwee et al., 2017, 2019a; Rheindt et al., 2008), and have been one of the driving forces behind a minor renaissance in genuine new species discovery in the 21st century (Alström et al., 2010; Krabbe et al., 2020; Prawiradilaga et al., 2018; Rheindt et al., 2020). At the same time, bioacoustic species delimitation is not a panacea as vocal divergence may be subtle or ambiguous in genetically differentiated cryptic species (Dufresnes et al., 2018; Garg et al., 2016; Harris et al., 2014). The relative importance and interplay of biological traits, such as morphology and bioacoustics, in biotic differentiation 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 nondispersive, 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 streaky plumage common to the genus. The species complex comprises three allopatric taxa: nominate *crispifrons*, *annamensis* and *calcicola* (Figure 1a). The limestone wren-babbler has traditionally been treated as a single species (Cai et al., 2019; Robson, 2014). However, a recent evaluation based on plumage differences of museum specimens split the sole rufous-bellied taxon as a distinct species, *N. calcicola*, leaving the remaining two grey-bellied taxa within *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). Subsequent museum inspection 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 (Baker, 1922; Deignan, 1945). Our own fieldwork around Hpa-An, along both sides of the Salween River, has established a mutually exclusive distribution of white-faced individuals to the west and brown-faced individuals to the east of the river in this otherwise cryptically coloured songbird complex. This differentiation pattern provides an ideal model to test the relative importance of coloration and vocal parameters as outcomes of the differentiation process.

Previous tests of the relative importance of morphological and behavioural traits in biotic differentiation have largely relied on mitochondrial DNA (López-Baucells et al., 2018a; Rheindt et al., 2008; Wang et al., 2014). This marker choice is mainly due to the status of mitochondrial DNA as the workhorse of phylogenetic research before the advent of next-generation sequencing technology (Avise et al., 1987). However, a large body of literature attests to mitochondrial DNA's high susceptibility to genetic introgression and to 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 one white-faced individual while employing an integrative approach involving bioacoustic and whole genome resequencing methods across members of all currently recognized taxa to shed light on the interplay between vocal and other phenotypic traits. We tested for congruence of results from the different approaches to assess the importance of morphological and bioacoustic traits in the differentiation 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 any particular population.

2 | MATERIAL AND METHODS

2.1 | Bioacoustic analysis

A total of 25 sound recordings across the three taxa were collected from our field work and various online sound recording archives,

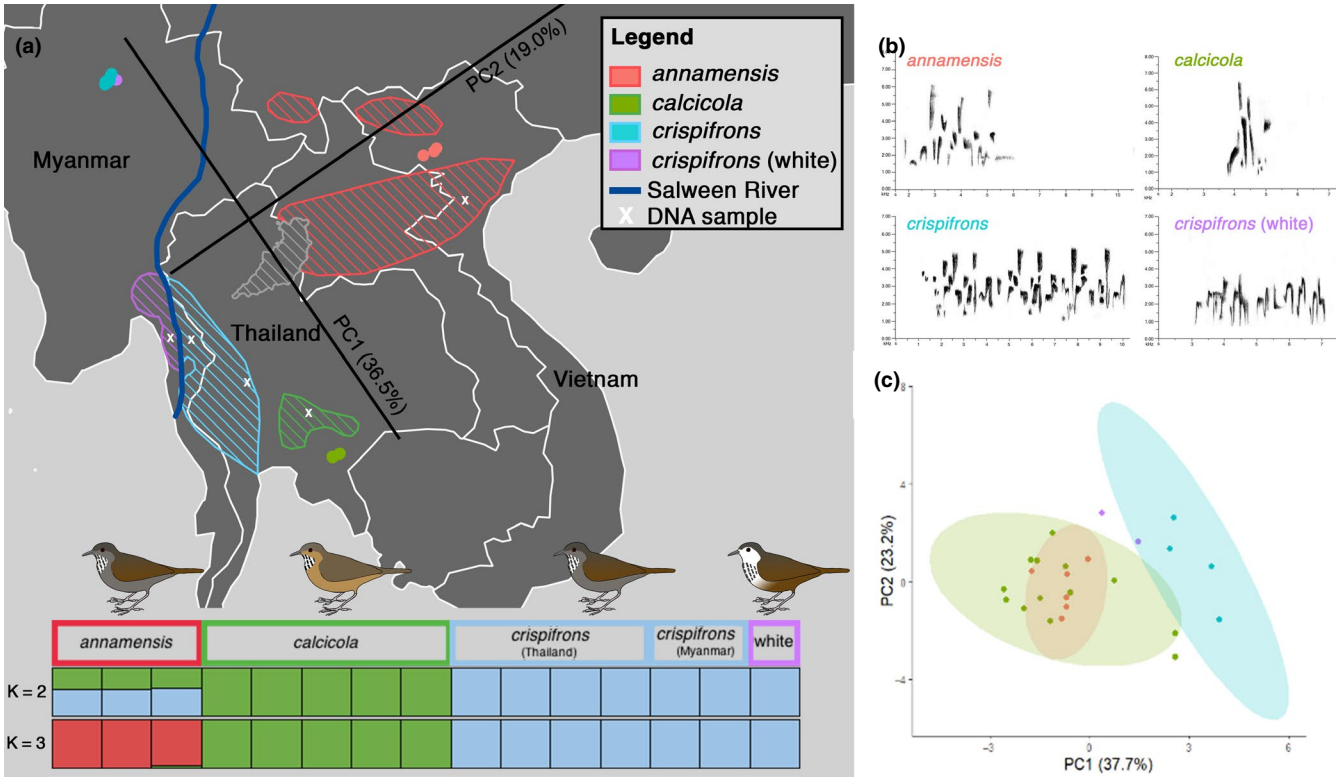

FIGURE 1 (a) DNA sampling localities and geographical distribution of the limestone wren-babbler *Napothera crispifrons* complex, including the three described taxa and the white-faced population, shaded respectively on the map. The taxonomic identity of the northern Thai population from Phrae and Nan provinces (cross-shaded in light grey) is controversial. Principal component analysis (PCA) of the genome-wide data is superimposed onto the distribution map, with each individual represented by a dot coloured according to taxon affinity. STRUCTURE results are depicted at the bottom for $K = 2$ and $K = 3$. (b) Sample spectrograms of the vocalizations of each population examined. One full motif is depicted in each spectrogram. (c) PCA of bioacoustic measurements with ellipses indicating 95% confidence intervals of each group. Colours of dots correspond to panel (a)

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) (Table S1). Limestone wren-babbler vocalizations constitute complex song motifs consisting of multiple vocal elements (i.e., traces on a sonogram). We identified duetting behaviour in multiple recordings, including almost all recordings of *Napothera c. annamensis*, but we did not observe any vocal differences between presumed males and females in duets. For such recordings, we measured the longest nonoverlapping stretch of a song which could be confidently identified as belonging to one of the two individuals. We used RAVEN PRO version 1.5 (https://ravensoundsoftware.com/software/raven-pro) to measure 10 vocal parameters: (a) average maximum frequency of a motif, (b) average minimum frequency of a motif, (c) average bandwidth, (d) average centre frequency of a motif (i.e., the darkest pixel on a sonogram), (e) average peak frequency of a motif (i.e., the frequency tranche with the highest amplitude), (f) average number of elements per motif, (g) largest element bandwidth, (h) longest break-length within a motif (i.e., between any two subsequent elements), (i) average pace (i.e., number of elements per second) and (j) average duration of a motif (Figure S1, Table S2). 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), hereafter Isler criterion, to assess the significance of each vocal parameter across pairwise comparisons. The Isler criterion is based on two conditions: (i) the ranges of measurements between two populations must not overlap, and (ii) 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: $-x_a + t_a SD_a \leq -x_b - t_b SD_b$, where t_i refers to the t-score at the 95th 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 since been applied across various songbirds (Cros & Rheindt, 2017; Gwee et al., 2019a; Prawiradilaga et al., 2018) and nonoscines (Gwee et al., 2017, 2019b; Ng et al., 2016; Ng & Rheindt, 2016; Rheindt et al., 2011; Sangster & Rozendaal, 2004).

2.2 | Whole genome resequencing library preparation

We collected blood, tissue or feather material from 15 individuals: three *Napothera c. crispifrons* samples from Myanmar, including one white-faced individual; five *Napothera c. calcicola* and four *N. c. crispifrons* samples from Thailand; and three *N. c. annamensis*

samples from Vietnam (Table S3). We extracted DNA of each sample using the DNeasy Blood & Tissue Kit (Qiagen) following the manufacturer's instructions. For dried blood on filter paper, an additional incubation step after the addition of ATL buffer at 90°C for 15 min was made. For feather samples, 200 μl instead of 180 μl of ATL, and 20 μl of 1 m dithiothreitol was added before the addition of proteinase K for keratin digestion.

We used Bioruptor to shear the extracted DNA to 250-bp fragments with sonification conditions of 30 s on and off time each for 13 cycles. We used NEBNext Ultra II DNA Library Prep Kit (New England Biolabs) 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 <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). 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 ~370 bp including adapters and barcodes. The libraries were sequenced using the Illumina Hiseq 4000 platform with 150-bp paired-end runs.

2.3 | Draft genome assembly

We produced a midcoverage (~38.8×) whole genome of one of the 15 individuals (i.e., the sole white-faced individual of nominate *crispifrons* [KK05; Table S3]), 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 the eye-browed wren-babbler *Napothera 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 <3,000 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).

2.4 | 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 (Table S3). 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 minimize mismatches. The mean coverage of each sample was ~15.7× (5.9×–38.8×; Table S3).

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 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 at high linkage disequilibrium as detected across all individuals. 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 data sets 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 population admixture of *Napothera* at $K = 1$ to 5, for 1,100,000 MCMC (Monte Carlo Markov chain) iterations and 100,000 burn-in iterations, with three replicates for each K value. A maximum of $K = 5$ was chosen as it reflects the highest number of biological entities possible in this data set. The standard deviation of log likelihood values across each K value remained zero even when the number of replicates was increased from three to 10, precluding a computation of *delta K* with the present data set. Therefore, we did not run Evanno et al.'s (2005) method to obtain an estimate of the best K. Instead, we assessed the distribution of mean likelihood values, also known as $L(K)$, generated by STRUCTURE HARVESTER (Earl & vonHoldt, 2012) to infer the most appropriate K value for the data set (Porras-Hurtado et al., 2013; Pritchard et al., 2000).

2.5 | 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 a significant 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) for a block size of 50,000 bp using the same parameters as applied for SNP calling. For outgroup (H4) rooting, we obtained the raw reads sequenced for the whole genome assembly of the rufous-capped babbler *Cyanoderma ruficeps* (Yang et al., 2019) from the European Nucleotide Archive (accession no. PRJNA573563). Within the superfamily Sylvioidea, *C. ruficeps* is currently the closest-available genome to limestone wren-babblers (Moyle et al., 2012). 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 above three ($Z > 3$), corresponding to a *p*-value below 0.0013, suggests a significant excess of ABBA-like alleles as compared to BABA-like alleles (Green et al., 2010; Patterson et al., 2012). This critical value is widely applied as a threshold for detecting introgression and reduces the likelihood of false positives (Zheng & Janke, 2018).

2.6 | Differentiation across the genome and scans for candidate genes

Because of sample size limitations, we quantified differentiation across the genome using absolute distance (d_{XY}) instead of the more commonly applied relative measure of differentiation (F_{ST}), which is highly dependent on intrapopulation genetic diversity and tends to overestimate differentiation between populations of small sample size. To compute d_{XY} for each population pair, *C. ruficeps* was used as 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} 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 99.9th percentile of the d_{XY} values 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 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).

2.7 | Genomic trees

To obtain a rooted 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 with an outgroup. We applied RAXML 8.2.12 (Stamatakis, 2014) to construct maximum likelihood (ML) trees using a General Time Reversible model with a Gamma distribution (GTR + G) and 1,000 rapid bootstraps for the SNP data sets with and without the outgroup.

To address SNP bias, we also generated trees using sequence alignments from the 33 largest chromosomes by calling consensus sequences using mpileup in BCFTOOLS (Li et al., 2009) and converting them into FASTA files using the perl script vcfutil.pl. We aligned sequences across all 15 individuals with MAFFT 7.0 (Katoh & Standley, 2013) under default settings for each of the 33 chromosomes, including chromosome Z. For the largest chromosomes, which exceeded alignment limits, we extracted the first 1 million base pairs of each individual and reran MAFFT 7.0 (Katoh & Standley, 2013) for alignment. ML trees were constructed for each chromosomal alignment with RAXML 8.2.12 using a GTR + G model and 100 rapid bootstraps. All generated trees were viewed with FIGTREE version 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree).

2.8 | Mitochondrial tree

We extracted mitochondrial reads from each sample by mapping its read harvest 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 a FASTA file. Loci with a coverage lower than 100× were discarded. Avian blood is characterized by an extremely low mitochondrial content as red blood cells are nucleated (Sorenson & Quinn, 1998). Therefore, we additionally conducted Sanger sequencing for those blood samples with a low coverage of mitogenomic regions in order to retrieve the ND2 sequence following Lim et al.'s (2019) protocol. All ND2 sequences were exported to MEGA7 (Kumar et al., 2016) and aligned with CLUSTALW. MEGA7 was also used to construct a neighbour joining (NJ) and maximum parsimony (MP) tree with 10,000 bootstraps each. Following the results of a model scan with JMODELTEST 2 (Darriba et al., 2012), a GTR + G model was applied for the construction of an ML tree with RAXML 8.2.12 (Stamatakis, 2014) using 10,000 bootstraps. *N. epilepidota* was incorporated as an outgroup in the construction of NJ, MP and ML trees. To detect the presence of

nuclear paralogues of mitochondrial genes (numts), which are a possible artefact of mitochondrial DNA sequencing of avian blood samples, resultant trees were assessed regarding whether blood and tissue samples from the same population emerged in identical clades (Sorenson & Quinn, 1998). All blood samples formed cohesive clades across mitochondrial trees, ruling out artefacts of numt contamination.

3 | RESULTS

3.1 | Bioacoustic comparison

PCA results of vocal measurements revealed that *Napothera c. crispifrons* was vocally distinct from *Napothera c. annamensis* and *Napothera c. calcicola*, while the latter two did not exhibit any deep vocal differentiation on the basis of the bioacoustic parameters investigated (Figure 1). 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* (Table S4). 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*.

3.2 | Population genomics

PCAs were congruent across the four different data sets, including those based on genotype likelihoods of all SNPs and those based on genotypes of two sets of unlinked SNPs (Figure 1; Figure S2), separating our samples into three clusters along taxonomic lines. Similarly, STRUCTURE analysis at $K = 3$, which produced the highest mean likelihood values, divided samples into the same three clusters (Figure 1), with no additional population differentiation apparent at higher K values (Figure S3). Mitochondrial ND2 tree inference revealed the same three clusters separated by pairwise divergences in excess of 5% (Figure 2a; Table S5), although there was poor branch support with respect to which two of these three lineages are most closely related. The rooted and unrooted SNP trees depicted *annamensis* as more closely related to *calcicola* than to *crispifrons* (Figure 2a, b). All 33 independent chromosomal alignments, except chromosome 4A, revealed a similar topology that divides *annamensis*, *calcicola* and *crispifrons* into distinct clusters each (Figure 2c; Supporting File).

Although only limited autosomal differentiation was found between the Myanmar and Thai populations of *N. c. crispifrons* (Figures 1 and 2), a relatively deep mitochondrial divergence of 2.7% was detected between the two populations (Figure 2a; Table S5). Both genomic and mitochondrial results support a lack of differentiation between the white-faced and brown-faced *N. c. crispifrons* populations from Myanmar (Figures 1 and 2; Table S5).

3.3 | Absence of secondary gene flow

We tested for the presence of secondary gene flow among the three taxa and among 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 intrataxon comparisons (Table 1).

3.4 | Genomic landscape of differentiation

The overall absolute genetic pairwise divergence (d_{XY}) across chromosomes was greater between taxa (i.e., *annamensis* versus *crispifrons*, *annamensis* versus *calcicola*, and *calcicola* versus *crispifrons*) than within taxa (i.e., Thai versus Myanmar populations of *crispifrons*, and white-faced vs. brown *crispifrons* from Myanmar) (Figure 3; Table S6). 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 3; Figure S4).

Although pairwise genetic differentiation across the chromosome was relatively low in intrataxon comparisons, the differentiation landscape exhibited several outlier peaks (Figure 3c, d). Comparing the white-faced population of *crispifrons* from Myanmar with brown populations from each Thailand and Myanmar produced almost exactly congruent genomic peaks of differentiation (Figure 3c, d). Similarly, net d_{XY} values computed between white-faced and brown populations of *crispifrons* and corrected for differentiation between the two brown populations exhibited divergence peaks in several localized regions, each spanning about 150 kb (Figure 3c, d). Two outlier peaks, one identified in chromosome 1A and the other in chromosome 18 (Figure 3c, d), were found to be possibly associated with genes implicated in pigmentation pathways (Table 2).

4 | DISCUSSION

Biotic diversification is promoted by disruptions in gene flow that may eventually lead to phenotypic differentiation. However, the suite of characters affected by these processes differs among animal groups. In songbirds such as wren-babblers, vocalizations are thought to be an essential driver of diversification (Alström & Ranft, 2003). Colour traits may also be important (Tobias et al., 2010; Töpfer, 2018), but little is known about the interplay of these different traits during the differentiation process. 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 species, *Napothera crispifrons* and *Napothera calcicola*, based on their grey versus rufous bellies respectively (Collar et al., 2020). However, this treatment interpreted the white-faced phenotype of birds west of the Salween River as a plumage polymorphism within one population rather than geographical variation, and

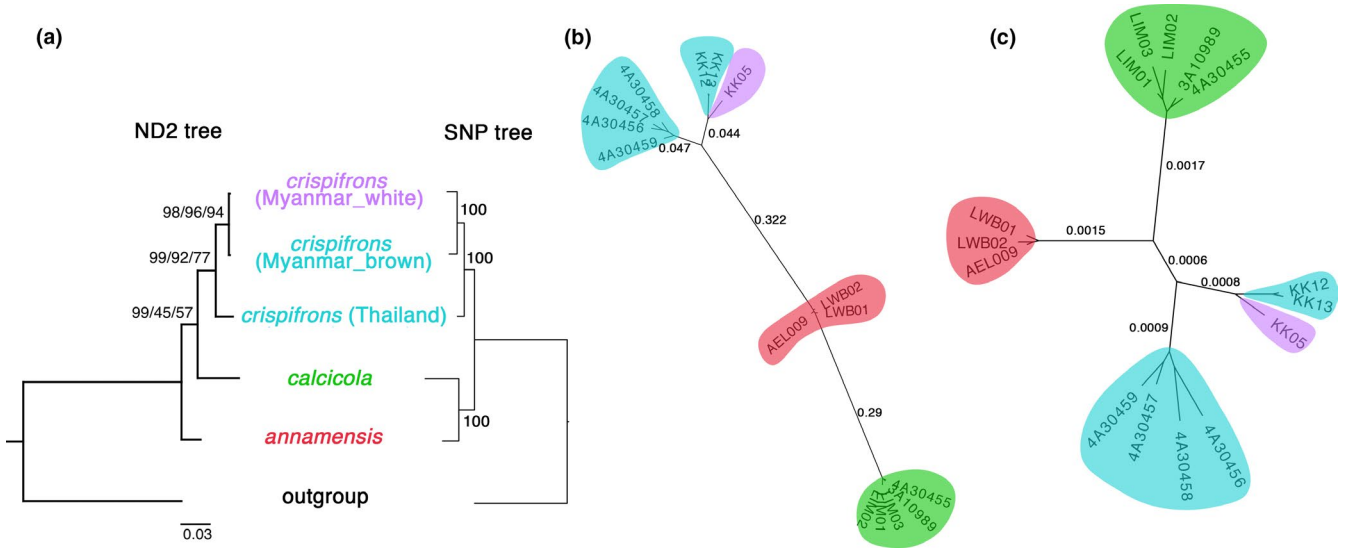

FIGURE 2 (a) Maximum likelihood (ML) trees of limestone wren-babblers using 887 bp of the mitochondrial ND2 gene (left) and 306,874 genome-wide SNPs (right). Bootstrap support generated from neighbour-joining, maximum parsimony and ML methods, respectively, is shown beside each node for the ND2 tree, while ML bootstrap is shown for the SNP tree. Scale of branch lengths, representing the number of substitutions per site, is depicted at the bottom of the ND2 tree. (b) Unrooted SNP tree based on ML. (c) ML tree based on sequence alignment of 1 million base pairs of chromosome Z

TABLE 1 *D*-statistics of selected population combinations to assess presence of secondary gene flow

D-stat	*Z*	*p*-value	ABBA	BABA	Blocks	H1	H2	H3
0.012	2.75	.006	16,016	15,647	7,770	*annamensis*	*calcicola*	*crispifrons* (Thailand)
0.002	0.53	.596	15,803	15,725	7,229	*annamensis*	*calcicola*	*crispifrons* (Myanmar_brown)
−0.007	−1.38	.168	15,367	15,579	6,736	*annamensis*	*calcicola*	*crispifrons* (white)
0.001	0.16	.874	13,582	13,562	7,468	*crispifrons* (Myanmar_brown)	*crispifrons* (white)	*crispifrons* (Thailand)

Note: A critical value of $Z > 3$, corresponding to a p-value $< .0013$, suggests a significant excess of allele sharing between recipient (H2) and donor (H3) populations.

only a rough assessment of qualitative vocal differences among taxa was conducted (Collar et al., 2018).

4.1 | Three deeply diverged lineages in the complex

We recovered 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 northeastern Thailand (Figures 1 and 2). 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 (Table S5), and are over twice as deep as those between typical sister species pairs in bird phylogenetics (Campbell et al., 2016; Hebert et al., 2003; Kerr et al., 2009). Our results attest to the dangers of primarily relying on plumage characters in modern taxonomic overhauls: our data support Collar et al.'s (2018) colour-based separation of *N. calcicola* as a monotypic species, but their retention of *N. annamensis* within *N. crispifrons* is contradicted by genomic results indicating that *N. annamensis* shares a closer affinity with *N. calcicola* than with the similarly grey-bellied *N. crispifrons* (Figure 2).

Unexpectedly, the Myanmar and western Thai populations of *N. crispifrons* (sensu *stricto*, excluding *annamensis* and *calcicola*) displayed a relatively high mitochondrial divergence of ~2.7% (Table S5). While borderline for the purposes of species delimitation, this depth of mitochondrial divergence is sometimes found between distinct sister species in other songbird groups (Campbell et al., 2016; Hebert et al., 2003; Kerr et al., 2009). Genomically, however, these two populations exhibited limited differentiation, occurring in the same tight PCA clusters (Figure 1; Figure S2), and remained undivided even at higher levels of *K* in STRUCTURE analysis (Figure S3). This mitonuclear 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.

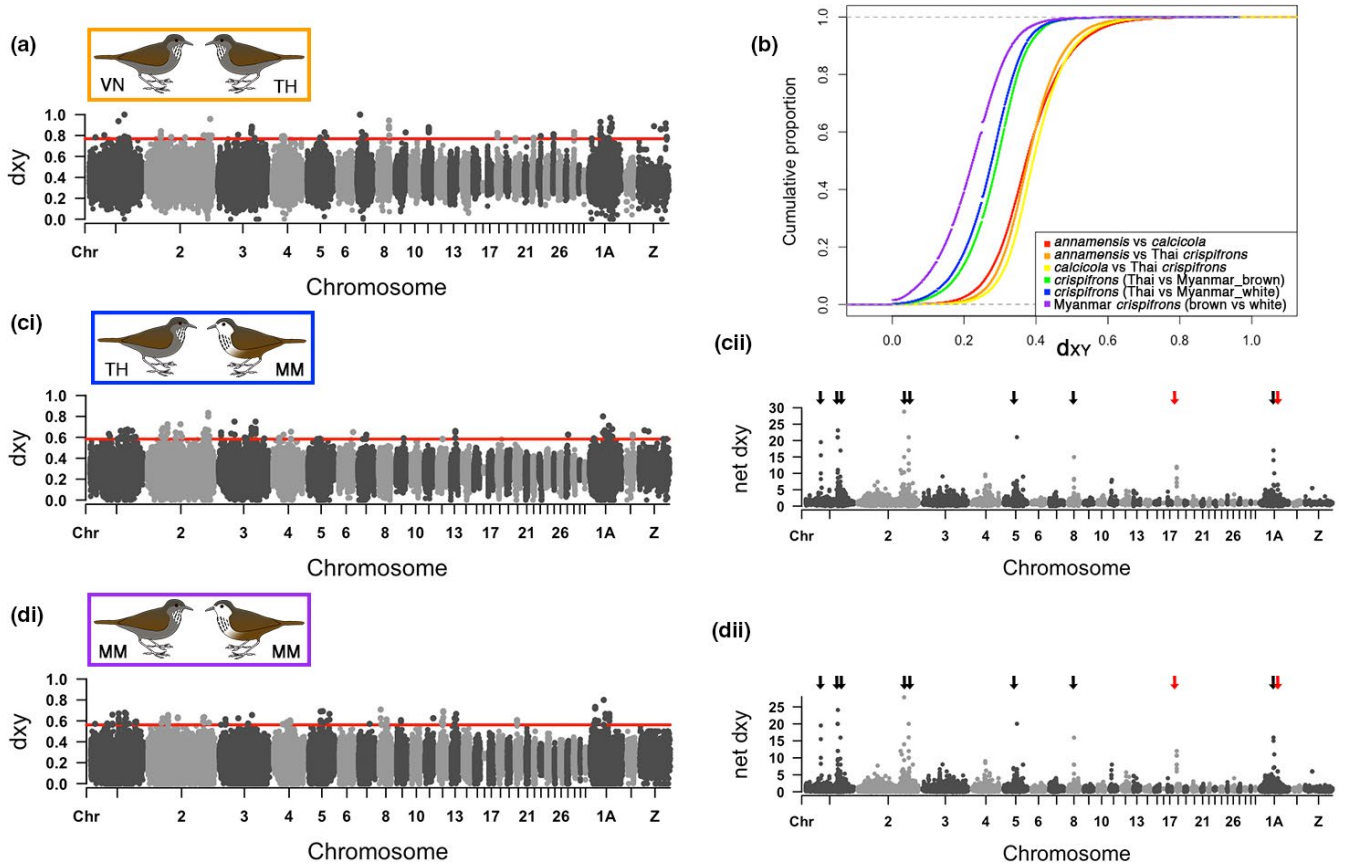

FIGURE 3 Absolute nucleotide divergence (d_{XY}) of selected pairs of populations. The red horizontal line in each d_{XY} plot reflects the 99.9th percentile threshold across pairwise comparisons. (a) d_{XY} between the Vietnam (VN) population of *Napothera c.annamensis* and Thai (TH) population of *Napothera c.crispifrons* across chromosomes (Chr). (b) Cumulative proportion of the d_{XY} values of selected pairwise population comparisons. Intertaxon comparisons (depicted by red, orange and yellow lines) had a higher proportion of high d_{XY} values, while intra-*crispifrons* 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 comparisons corresponding to (ci) and (di), respectively, across all chromosomes. Outlier regions are indicated by black or red arrows, the latter referring to regions containing pigment-related genes

There is no evidence in our data regarding potential secondary gene flow among the three main babbler lineages as ABBA-BABA tests did not indicate a significant excess of allele sharing among any taxa and populations investigated. Based on current genomic data availability across songbirds, our outgroup choice in ABBA-BABA analysis was limited to a fairly distantly related babbler, *Cyanoderma ruficeps*, that may have hampered the detection of ABBA- and BABA-like SNPs and impacted the sensitivity of our tests regarding recent gene flow, especially between the two *N. crispifrons* populations from Myanmar and Thailand. However, it is unlikely that strong signals of secondary gene flow would have been overlooked based on our outgroup choice.

4.2 | 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, c). 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 similar to the rufous-bellied *N. calcicola* on the basis of 10 vocal parameters (Figure 1c): their different belly colours 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). Future playback experiments may assist in gauging the level of vocal distinction, or lack thereof, between *N. annamensis* and *N. calcicola*. 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,

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

Chromosome	Gene	Description
1:38950001–39100000	Intronic region	NA
1:82930001–83080000	NALCN (ENSTGUG00000010876)	Sodium leak channel, nonselective
	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

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 geographical gap (P.D.R., pers. obs.) and suggests that northern Thai populations may form a continuum with the Lao population and may ultimately belong to *N. annamensis* (hatched grey area in Figure 1a).

4.3 | White faces are probably a recent occurrence driven by mutations in few localized genomic 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 geographical race. 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 (Figures 1 and 2), 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 (Cibois et al., 2012; Kerje et al., 2004; Knief et al., 2019; Mundy et al., 2004; Theron et al., 2001; Toews et al., 2016; Uy et al., 2009). While much is known about the involvement of the melanocortin-1 receptor (*MC1R*), Agouti and tyrosinase-related protein 1 (TYRP1) in melanic polymorphisms across various animal species (Baião et al., 2007; Cibois et al., 2012; Hiragaki et al., 2008; Jones et al., 2018; Mundy et al., 2004; Rieder et al., 2001; Switonski et al., 2013; Theron et al., 2001; Uy et al., 2009), colour variation can also be caused by mutations in other genes (Bourgeois et al., 2016; Cheviron et al., 2006; MacDougall-Shackleton et al., 2003; Poelstra et al., 2014). Our understanding of other candidate loci or processes associated with the expression of pigmentation remains rudimentary (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 leucism. None of these outlier regions falls within or near the MC1R, Agouti and TYRP1 genes. 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 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; Chabrillat et al., 2005; Chakraborty et al., 2003), and mutations in these genes can lead to a loss of pigmentation in the coat, such as lightened skin colour in mice and silvery hair in humans (Matesic et al., 2001; Ohbayashi & Fukuda, 2012; Wilson et al., 2000). Therefore, mutations to *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). However, we cannot rule out the possibility that these two regions may be highly differentiated due to factors unrelated to phenotypic mutations, such as through linked selection in regions of low recombination.

Given the shallow mitochondrial divergence of the white-faced population (0.1%–0.4%; Table S5) and the virtual lack of substantive genomic differentiation outside of those few divergence peaks (Figure 3), 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. Other processes, including ghost introgression from extinct populations or resistance to gene flow at a crucial locus, possibly aided by strong negative selection, may be alternative explanations for this pattern (see Zhang et al., 2019).

4.4 | Long-term relevance of the plumage innovation in western limestone wren-babblers

Our fieldwork for this study established that the white-faced phenotype of a peripheral population in Myanmar is geographically restricted to birds west of the Salween River, at least in the Hpa-An region. Therefore, it is not considered a polymorphism under the classical definition (see Ford, 1965, 1966). Although visually conspicuous, the 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 probably 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 (Baião et al., 2007; Cibois et al., 2012; Doucet et al., 2004; Mundy et al., 2004; Theron et al., 2001; Uy et al., 2009). 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 (Baião et al., 2007; Cibois et al., 2012; Doucet et al., 2004) 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 diversification process, when phenotypic differentiation is already evident in the absence of genomic divergence, it is equally plausible that this population may be evolutionarily ephemeral, and succumb to climatic vagaries or be overrun by nonleucistic populations from the main range as soon as connectivity allows.

5 | CONCLUSION

Our study reveals that the limestone wren-babbler complex consists of three mitochondrially and genomically diverged lineages, each supported by a combination of plumage and vocal characters that would allow them to be diagnosed as different species under many species concepts. Although morphologically distinct, the elusive white-faced population of *Napothera 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 genomic regions may have facilitated the establishment of a phenotypically distinct population within a short period of time.

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AUTHOR CONTRIBUTIONS

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

DATA AVAILABILITY STATEMENT

The draft genome of *Napothera crispifrons* and all raw reads are available on Dryad Digital Repository: https://doi.org/10.5061/dryad.q573n5th0. Raw FastQ files and ND2 sequences are also available on NCBI under accession nos. PRJNA669821 and MW148240–MW148254, respectively.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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