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Quaternary land bridges have not been universal conduits of gene flow

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Abstract

Quaternary climate oscillations are a well-known driver of animal diversification, but their effects are most well studied in areas where glaciations lead to habitat fragmentation. In large areas of the planet, however, glaciations have had the opposite effect, but here their impacts are much less well understood. This is especially true in Southeast Asia, where cyclical changes in land distribution have generated enormous land expansions during glacial periods. In this study, we selected a panel of five songbird species complexes covering a range of ecological specificities to investigate the effects Quaternary land bridges have had on the connectivity of Southeast Asian forest biota. Specifically, we combined morphological and bioacoustic analysis with an arsenal of population genomic and modelling approaches applied to thousands of genome-wide DNA markers across a total of more than 100 individuals. Our analyses show that species dependent on forest understorey exhibit deep differentiation between Borneo and western Sundaland, with no evidence of gene flow during the land bridges accompanying the last 1–2 ice ages. In contrast, dispersive canopy species and habitat generalists have experienced more recent gene flow. Our results argue that there remains much cryptic species-level diversity to be discovered in Southeast Asia even in well-known animal groups such as birds, especially in nondispersive forest understorey inhabitants. We also demonstrate that Quaternary land bridges have not been equally suitable conduits of gene flow for all species complexes and that life history is a major factor in predicting relative population divergence time across Quaternary climate fluctuations.

KEYWORDS

climate change, connectivity, cross island gene flow, Sundaland

1 | INTRODUCTION

The Quaternary started about 2.8 million years ago and has been characterized by a succession of 20–30 glacial cycles (Berger, Loutre, & Laskar, 1992; Gregory et al., 2009; Woodruff, 2010). These climate fluctuations have played an important role in generating the intraspecific variation observed in many species today (Avise & Walker, 1998; Fouquet et al., 2012; Leonard et al., 2015; Schneider, Cunningham, & Moritz, 1998). Studies on the role of Quaternary glacial cycles in promoting diversification have mainly focused on the northern hemisphere, where the expansion of ice sheets has resulted in species range contraction and fragmentation (Hewitt, 2000, 2004; Provan & Bennett, 2008). In some regions, however, glacial maxima have had an opposite effect, as the accumulation of ice

has resulted in global sea level drops of up to 120m, leading to exposed shelf and the appearance of land bridges between otherwise isolated landmasses. The mechanisms of Quaternary climate variation in shaping patterns of diversification in such regions remain poorly understood (Brown et al., 2013; Ericson et al., 2019; Garg, Chattopadhyay, Wilton, Prawiradilaga, & Rheindt, 2018; Heaney, Walsh, & Peterson, 2005; Hosner, Sánchez-González, Peterson, & Moyle, 2014; Irestedt et al., 2013; Leonard et al., 2015; Ng, Wilton, et al., 2017; Peterson et al., 2015).

The largest area of shallow shelf globally is the Southeast Asian Sunda shelf (Hall, 1998; Hewitt, 2000; Inger & Voris, 2001), which comprises the western part of the Indonesian archipelago (Figure 1), making this unique region particularly interesting for studying the role of Quaternary climate change on biotic diversification. Despite cyclically recurring connections between landmasses throughout the Quaternary, including some very recent ones (Sarr et al., 2019; Voris, 2000), certain Sundaic species show much deeper interisland divergences than others (Buckley-Beason et al., 2006; Campbell, Schneider, Adnan, Zubaid, & Kunz, 2004; Janečka et al., 2008; Karin, Das, Jackman, & Bauer, 2017; Leonard et al., 2015; Lim, Rahman, Lim, Moyle, & Sheldon, 2011; Moyle, Schilthuizen, Rahman, & Sheldon, 2005; Veera Singham, Othman, & Lee, 2017; Warren et al., 2001). The exposed Sunda shelf may not have provided suitable habitat for all species during glacial periods based on specific niche requirements (Cannon, Morley, & Bush, 2009; Heaney et al., 2005). In order to gain a more detailed understanding of the impact of sea level changes, it is therefore essential to explore how ecological characteristics may affect a species' response to climate change. Furthermore, as previous research focusing on more than one species is scarce (Leonard et al., 2015; Peterson et al., 2015), more comparative studies are needed.

Research on the impact of Quaternary climate fluctuations on the evolutionary history of Sundaic fauna has thus far been hampered by limitations in genetic sample size (Peterson et al., 2015). New molecular methodologies, such as restriction enzyme-associated DNA sequencing (RADseq) in combination with high-throughput sequencing platforms, offer improved resolution in the investigation of diversification in this region (Angeloni, Wagemaker, Vergeer, & Ouborg, 2012; Lim et al., 2017; McCormack, Hird, Zellmer, Carstens, & Brumfield, 2013; Peterson, Weber, Kay, Fisher, & Hoekstra, 2012). The concomitant development of a wide variety of analytical methods (e.g. coalescent-based approaches [Beaumont, Zhang, & Balding, 2002; Excoffier & Foll, 2011]) offers new options to investigate species demographic history.

To understand and characterize the contribution of Quaternary land bridges to gene flow across different terrestrial guilds, we used genome-wide markers in combination with modelling approaches to investigate the timing of the cessation of gene flow between western (Singapore/Sumatra) and eastern (Borneo) Sundaic landmasses that have repeatedly been connected throughout the Quaternary (Sarr et al., 2019) and between which limited interisland gene flow has been demonstrated for a number of Southeast Asian songbird species (Lim et al., 2011). We additionally contrasted observed genomic patterns with plumage and bioacoustic information. To explore whether ecological characteristics have influenced observed responses, we studied five songbird species of the superfamily Sylvioidea: two babblers (*Cyanoderma erythropterum* [family Timaliidae sensu Moyle, Andersen, Oliveros, Steinheimer, & Reddy, 2012] and *Trichastoma rostratum* [family Pellorneidae]) and three bulbuls (*Pycnonotus plumosus*, *Pycnonotus brunneus* and *Pycnonotus simplex* [family Pycnonotidae]) with varying ecological characteristics, spanning different habitat requirements, levels of edge tolerance and use of forest strata. Both babbler species are found in the understorey, whereas the three bulbul species occupy the canopy (Wells, 2007). While the babbler *C. erythropterum* is highly forest-dependent, *T. rostratum* is found in a wider range of woodland habitats (Eaton, van Balen, Brickle, & Rheindt, 2016; Wells, 2007). Similarly, while the bulbul *P. plumosus* is a woodland habitat generalist and edge-tolerant, *P. simplex* and *P. brunneus* are forest-dependent canopy specialists (Wells, 2007). We hypothesized that populations of dispersive, generalist and edge-tolerant species would have experienced punctuated gene flow whenever west and east-Sundaland landmasses have been connected throughout the Quaternary, such that potential signs of interisland differentiation would be more recent than those observed for less dispersive forest interior-adapted species. In this study, we show that Quaternary land bridges have not been equally suitable conduits of gene flow across Sundaic species of differing ecological requirements.

2 | MATERIAL AND METHODS

2.1 | Sample collection and extraction

To investigate the role of Quaternary land bridges in facilitating gene flow, we focused on gene flow dynamics between the two main Sundaic land portions, the western Sundaic region, represented by samples from Singapore and/or Sumatra, and the eastern Sundaic region with samples from Borneo. Between 2014 and 2016, we collected blood samples of uniquely tagged babblers (*Cyanoderma erythropterum* and *Trichastoma rostratum*) and bulbuls (*Pycnonotus plumosus*, *P. brunneus* and *P. simplex*) from Singapore and north-east Borneo (Sabah, Malaysia) (Table S1). Additionally, tissue samples from Sarawak and Sabah (Malaysia), Sumatra (Indonesia) and Brunei were provided by the Burke Museum of Natural History and Culture (Seattle, USA) and the Yale University Peabody Museum (New Haven, USA) (Table S1). Genomic DNA of 59 samples was extracted using a DNeasy Blood & Tissue Kit (QIAGEN, Germany). We also included sequence data for 61 samples, including 18 *C. erythropterum*, 31 *P. simplex*, 10 *P. plumosus*, 1 *Mixornis gularis* and 1 *Pellorneum malaccense* used as out-groups, which were obtained from a recently published study (Cros, Ng, Oh, et al., 2020). In total, we had DNA material and sequences for 38 *Cyanoderma erythropterum* (12 from Singapore, 7 from Sumatra, 5 from Sarawak and 14 from Sabah), 17 *Trichastoma rostratum* (6 from Sumatra, 3 from Sarawak and 8 from Sabah), 34 *Pycnonotus simplex* (17 from Singapore, 15 from Sarawak

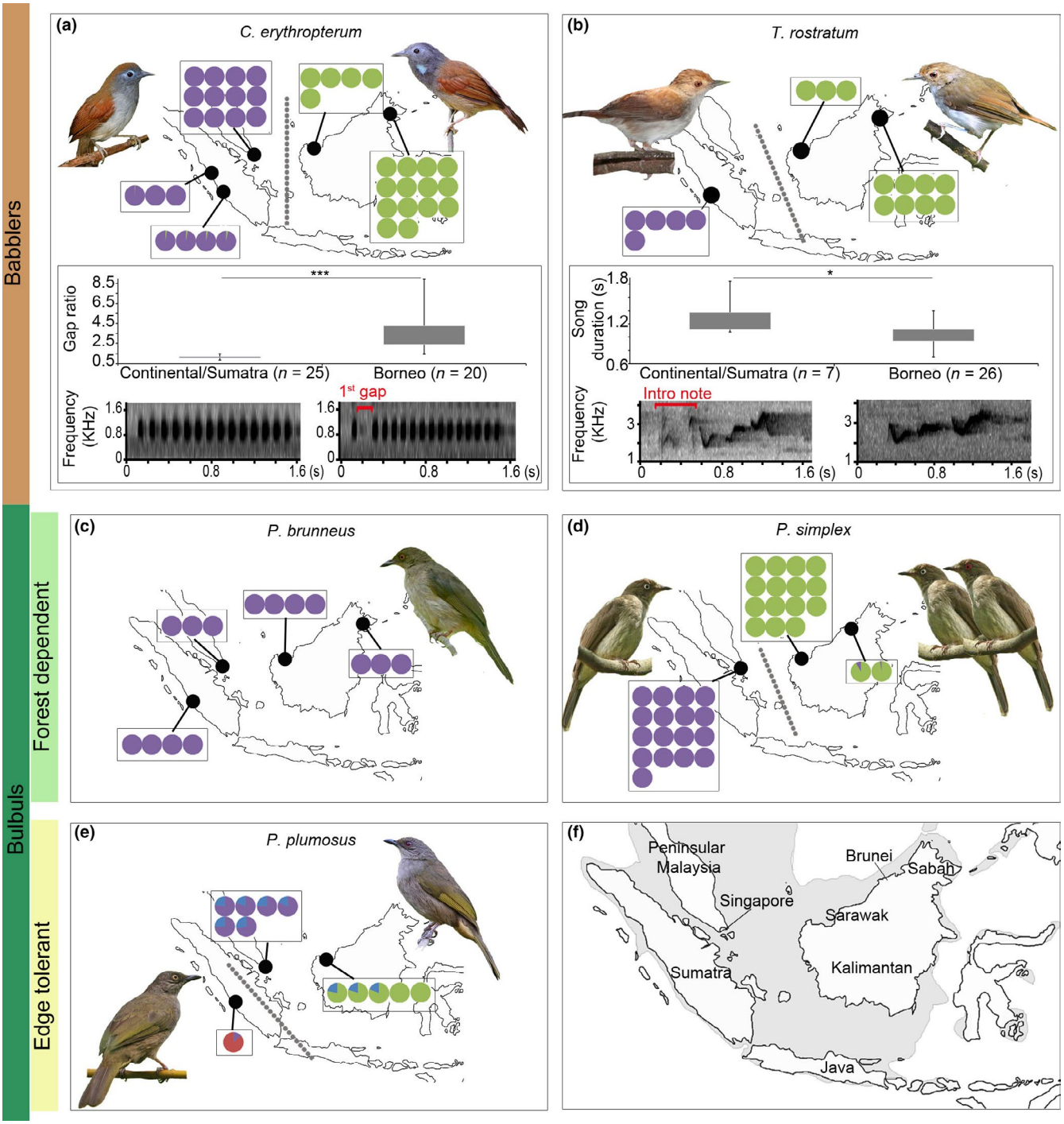

FIGURE 1 Population structure across all study species, with STRUCTURE results mapped to collection localities of samples (black dots). (a) *Cyanoderma erythropterum*: Pie chart circles depict STRUCTURE results at optimal $K = 2$ following Evanno et al.'s (2005) method, each circle corresponding to one individual. Boxplots depict results of bioacoustic analysis of first gap/second gap ratio with sonograms illustrating both populations' songs (***$p < .001$); (b) *Trichastoma rostratum*: STRUCTURE results at optimal $K = 2$. Bioacoustic analysis of song duration with sonograms illustrating both populations' songs (*$p < .05$). (c) *Pycnonotus brunneus*: STRUCTURE results at optimal $K = 1$; (d) *Pycnonotus simplex*: STRUCTURE results at optimal $K = 2$; (e) *Pycnonotus plumosus*: STRUCTURE results at optimal $K = 4$. (f) Map of the Sundaic region; grey indicates exposed land at a sea level of 120 m below the present (based on Voris, 2000). See Tables S1 and S3 for sample ID and information. The grey dashed line indicates major population separation based on plumage. Bird illustrations: *C. erythropterum* from Singapore (© M. Chua), from Borneo (© M. Wong/P. Wong), *T. rostratum* from Singapore (© J. W. K. Cheah), from Borneo (© D. O'Neill), *P. plumosus* from Sumatra (© J.A. Eaton), all other illustrations © D. Koh

and 2 from Brunei), 15 *Pycnonotus brunneus* (3 from Singapore, 4 from Sumatra, 5 from Sarawak and 3 from Sabah), 12 *Pycnonotus plumosus* (6 from Singapore, 1 from Sumatra and 5 from Sarawak), 1 *Mixornis gularis*, 1 *Pellorneum malaccense*,1 *Pycnonotus erythropthalmos* and 1 *Pycnonotus pseudosimplex*. We used mitochondrial barcodes (see below) to exclude misidentified samples in the case of cryptic species that are morphologically almost identical to our target species complexes (e.g. one Sabah sample that turned out to belong to the newly described *Pycnonotus pseudosimplex* rather than *P. simplex*; Shakya et al., 2019; one immature *Pycnonotus erythropthalmos* misidentified as *P. simplex* in the field).

2.2 | Mitochondrial DNA sequencing

We downloaded 59 NADH dehydrogenase subunit 2 (ND2) sequences available on GenBank for our five target species complexes (Table S2). Additionally, ND2 was sequenced for 27 samples (Table S2) following previously published protocols (Sorenson, Ast, Dimcheff, Yuri, & Mindell, 1999) using the primers L5219Met (5'-CCCATACCCCGAAAATGATG-3′) and H6313Trp (5'-CTCTTATTTAAGGCTTTGAAGGC-3′). ExoSAP-IT (Affymetrix, USA) was used for clean-up steps, and a BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA) was used to cycle sequence the samples. An Applied Biosystems 3730xl DNA Analyzer was used to generate the DNA sequences.

2.3 | Mitochondrial divergence time analysis

We assembled and trimmed the ND2 sequences with CodonCode Aligner 7.0.1 (CodonCode Corporation, Dedham, Massachusetts). We tested for the best-fitting model and for data compatibility with a molecular clock in MEGA 7.0.21 (Kumar, Stecher, & Tamura, 2016). We then estimated the divergence time with two independent runs of 10 million iterations with one tree stored every 1000th iteration, using BEAST (Bouckaert et al., 2014). In the absence of an appropriate fossil record, we used a rate of 2% divergence per million years to estimate divergence time between Borneo and the western Sundaic landmasses based on a previously published mitochondrial clock rate widely used in birds (Weir & Schluter, 2008). We also used MEGA to calculate raw pairwise distances using 1,000 bootstrap replicates.

2.4 | ddRADSeq library preparation

Four double-digest RADseq (ddRADseq) libraries were prepared for all samples (Table S1) using a modified version of Peterson et al.'s (2012) protocol (Ng, Garg, et al., 2017). Sera-Mag magnetic beads were used to select fragments of 250–650 bp length and to perform the clean-up step (Thermo Scientific, USA). The last clean-up step was replaced by a second size selection step to ascertain that final products are clean and of the desired fragment size. The final libraries were spiked with 5% PhiX and sequenced on an Illumina HiSeq 2,500 lane (150 bp paired-end run) at the Singapore Centre of Environmental Life Sciences and Engineering.

2.5 | Single nucleotide polymorphism calling

We used "process_radtags" in STACKS 1.42 (Catchen, Hohenlohe, Bassham, Amores, & Cresko, 2013) to demultiplex ddRADseq reads. Based on quality FastQC control checks (Andrews, 2010) indicating that the per base quality dropped towards the end, reads were trimmed to 145bp and any read with an uncalled base was removed. We used BBMAP v35.85 (Bushnell, 2016) to rename files and bwa-0.7.13 (Li, 2013) to align reads to the genome of *Mixornis gularis* (Tan et al., 2018). The resulting.sam files were sorted and converted to.bam files with a minimum required MAPQ score of 20 using samtools-1.3.1 (Li et al., 2009). For each species complex separately, the programs "ref_map.pl" and "populations" in STACKS 1.42 were used to call and filter single nucleotide polymorphisms (SNPs) (Catchen et al., 2013). The stack depth was set to 10, and SNPs had to be present in at least 90% of individuals in each species complex to be called. Only one random SNP was called per locus to avoid analysing linked SNPs. We then used BAYESCAN2.1 (Foll & Gaggiotti, 2008) to scan for and filter SNPs under selection. We checked for and removed individuals with excessive missing data and SNPs in linkage disequilibrium using PLINK 1.9 (Chang et al., 2015, https://www.cog-genomics.org/plink2). We investigated the impact of different levels of missing data on the population genomic results and discarded individuals with an excessive number of missing data as necessary. We created an additional data set for each species complex in which we removed all loci with missing data.

In order to check the distribution of SNPs throughout the genome, we additionally mapped the *Mixornis gularis* reference genome assembly against the chromosome-level reference genome of *Parus major* (Laine et al., 2016) using SATSUMA version 3.1.0 (Grabherr et al., 2010). We then calculated the number of SNPs per base pair per chromosome.

2.6 | Population genomic analysis

To investigate population structure within each of the five species complexes, we used Bayesian model-based clustering algorithms in STRUCTURE v. 2.3.4 (Pritchard, Stephens, & Donnelly, 2000). We ran ten replicates of $K = 1$ to 10 with 100,000 burn-in steps and 500,000 Markov Chain Monte Carlo samples each. To determine the optimal number of genetic clusters for each species complex, we used Evanno, Regnaut, and Goudet's (2005) delta K method, but we additionally inspected results across all utilized K values (Pritchard et al., 2000). We also performed principal component analysis (PCA) and employed a network-based cluster approach using the R packages SNPRelate and NetView, respectively (Neuditschko, Khatkar, & Raadsma, 2012; Steinig, Neuditschko, Khatkar, Raadsma, & Zenger, 2016; Zheng et al., 2012).

TABLE 1 Summary statistics for genomic reads and ND2 (=mitochondrial) sequences across *C. erythropterum*, *T. rostratum*, *P. brunneus*, *P. simplex* and *P. plumosus*

Species	Average number of genomic reads per individual	Stacks pipeline					
		Number of samples				Total	
		Total	West Sundaland	East Sundaland	Number of SNPs[a]	H_o[b]	H_e[c]
Cyanoderma erythropterum	2,925,947	38	19	19	19,364	0.09	0.13
Trichastoma rostratum	2,507,288	16	5	11	16,301	0.18	0.22
Pycnonotus simplex	3,106,226	34	17	17	12,897	0.11	0.13
Pycnonotus brunneus	2,885,603	14	7	7	14,025	0.17	0.21
Pycnonotus plumosus	3,054,977	12	7	5	18,072	0.21	0.25

[a]Single nucleotide polymorphism.
[b]Average observed heterozygosity.
[c]Average expected heterozygosity.
[d]Base pair.

2.7 | Phylogenomic analysis

Demultiplexed reads obtained from STACKS were additionally clustered and aligned using PYRAD 3.0.66 (see tutorial of Eaton, 2014a, b) to reconstruct a maximum likelihood tree with the concatenated data using the GTR + gamma model and a rapid bootstrap algorithm with 1,000 replicates in RAXML v8.2.9 (Stamatakis, 2014). The maximum likelihood tree was visualized with FIGTREE 1.4 (http://tree.bio.ed.ac.uk/software/figtree/). Although our study is primarily of a population genomic nature, this phylogenomic maximum likelihood approach was helpful in assuring that our gene flow modelling is based on correct evolutionary premises regarding a split between Borneo and western Sundaland.

2.8 | Approximate Bayesian Computation modelling approach

The history of periodic Quaternary connectivity among Sundaic land masses across successive glacial periods at increasing intervals and intensity is extremely complex (Sarr et al., 2019). As no model would appropriately be able to account for multiple such ice ages individually, we implemented a no-gene flow model that searches for the most recent divergence across these five species complexes as a proxy for the most recent significant bout of connectivity. To test at what time gene flow last ceased between populations from western Sundaland versus Borneo, we used an Approximate Bayesian Computation (ABC) approach as implemented in DIYABC v2.1.0 (Cornuet et al., 2014) and compared evolutionary models of divergence. For ABC analysis, we used the SNP data sets with no missing data, always comparing two populations: one comprising all Bornean individuals of a species complex and the other comprising the individuals from Sumatra and peninsular Malaysia (including Singapore). We did not specifically test for a nested set of hypotheses that populations from one part of Sundaland (e.g. west) might have newly invaded the other part (e.g. east) during one of these most recent glacial periods as this is merely a special scenario of "most recent gene flow." However, we strongly doubt that such a new colonization occurred in any of our species complexes, given that fluctuating extents of lowland rainforest would have been present on both sides of Sundaland for much of the Quaternary (Cannon et al., 2009) and given that our estimates of effective population size of eastern and western Sundaic populations are always within the same order of magnitude (see Results).

We referred to sea level estimates (Bintanja, van de Wal, & Oerlemans, 2005) to identify periods of connectivity between western Sundaland and Borneo during the Quaternary and to define four windows of potential population subdivision: (1) 10–12 kya (around the time when the last land bridge between these landmasses severed); (2) 80–130 kya (around the time when the penultimate land bridge severed; (3) 190–420 kya (a period encompassing the three interglacials before the last interglacial, including two glacial land bridges each lasting a few tens of thousands of years); and (4) 500–1,000 kya (a longer, more ancient period encompassing approximately 5–6 ice ages during which Sundaland is thought to have been continuously emerged; Sarr et al., 2019). We combined multiple ice ages in the fourth model because it spans a period when the present regime of land submergence in Sundaland during glacial peaks had not yet commenced, so that the entire region would have been one large terrestrial subcontinent across periods of cooling and warming (Sarr et al., 2019). Based on these time windows, four possible evolutionary scenarios were constructed (Figure S1). A generation time of one year, commonly used for small passerines, was chosen (Sibley & Ahlquist, 1990).

Uniform prior distributions were used for all parameters, and four million simulations (one million simulations per scenario) were performed for each species complex (Table S6). We assessed whether the observed data fell within prior space and refined the models by removing summary statistics which were not close to the data (Cornuet et al., 2014). We performed model selection using

					Concatenated sequences		ND2	
West Sundaland		East Sundaland						
H_o[b]	H_e[c]	H_o[b]	H_e[c]	Number of SNPs with no missing data	Number of SNPs	Alignment length (bp[d])	Number of samples	Alignment length (bp[d])
0.8	0.9	0.11	0.12	6,178	12,047	1,758,408	30	757
0.17	0.21	0.18	0.19	9,274	11,560	1,723,477	8	893
0.11	0.13	0.11	0.12	4,527	7,095	1,037,661	8	781
0.18	0.20	0.17	0.18	8,420	12,135	1,791,864	10	681
0.20	0.23	0.21	0.22	12,876	15,579	2,368,693	30	831

the logistic regression approach (Beaumont, Cornuet, Marin, & Robert, 2009; Fagundes et al., 2007) and tested the power of the data to differentiate among models by calculating the posterior predictive error rates of these models. For the best scenario, a model check was then performed to assess whether the model posterior parameter combination fell close to the observed data set. As the main purpose of these analyses was to obtain a temporal window which maximizes the signal of divergence between populations, analyses were restricted to model selection.

2.9 | Site Frequency Spectrum-based parameter estimation

Based on the best model obtained from ABC analysis, we estimated the time of the most recent significant divergence and effective population size for each species complex using the Site Frequency Spectrum (SFS) approach as implemented in FASTSIMCOAL2 version 2.5.2.21 (Excoffier, Dupanloup, Huerta-Sánchez, Sousa, & Foll, 2013). This SFS-based approach allows for the evaluation of more complex demographic models and is not biased by summary statistics (Excoffier et al., 2013; Sovic, Fries, & Gibbs, 2016). Each parameter was allowed to follow a uniform prior distribution with a range similar to that used in the best diversification model obtained from DIYABC for each species complex (Table S7). For instance, where two models had similar posterior probabilities, we used the information from both models to define prior space in fastsimcoal2 simulations. We obtained a folded SFS based on minor allele frequency (as the ancestral state is unknown) in ARLEQUIN 3.5.2.2 (Excoffier & Lischer, 2010). We did not include information about monomorphic sites and hence fixed the ancestral effective population size (effective population size of the ancestor of Bornean and western Sundaic populations) for all analyses. Ancestral effective population size was estimated based on nucleotide diversity ($\theta_\pi = 2N_e\mu$) for haploid populations, where N_e is the effective population size and μ is the mutation rate per generation. This is a commonly used estimate for ancestral N_e based on RADseq data (Lanier, Massatti, He, Olson, & Knowles, 2015; Papadopoulou & Knowles, 2015, 2017). We calculated nucleotide diversity for each population in STACKS based on the whole sequence data associated with the filtered SNPs present in the Bornean and western Sundaic populations, then generated an average estimate from these two values. We assumed μ as $2.3 \times 10E{-}9$ per generation (Smeds, Qvarnström, & Ellegren, 2016). For all analyses, we assumed a generation time of one year.

We performed 50 independent runs to estimate parameters in fastsimcoal2 for each species complex. In each run, we performed 100,000 simulations to estimate the expected SFS and likelihood of the given set of demographic parameters based on the prior distribution. To avoid issues of local maxima, we performed 40 cycles of a conditional maximization algorithm (Expectation Conditional Maximization) for parameter estimation. From the 50 independent runs, we chose the run in which the maximum estimated likelihood was closest to the maximum observed likelihood to obtain point estimates of divergence time, following the fastsimcoal2 manual's instruction that the maximum observed likelihood should be obtained by using the observed SFS as the expected SFS when computing the likelihood. We further performed parametric bootstraps to estimate confidence intervals for our point estimates. We simulated 100 SFS based on the point estimates to assess uncertainty among point estimates.

2.10 | Bioacoustic analysis

We analysed 45 and 33 homologous song recordings of the two babblers *C. erythropterum* (25 for western Sundaland and 20 for Borneo) and *T. rostratum* (7 for western Sundaland and 26 for Borneo), respectively (Table S3). One recording was made on Natuna using an Olympus LS-12 recorder. All other recordings were compiled from colleagues, the British Library Sound Archive and three online sound

libraries: www.xeno-canto.org, www.avocet.zoology.msu.edu and www.macaulaylibrary.org (see Acknowledgements). Based on preliminary analysis of the song recordings, we focused on measuring the first gap/second gap ratio for *C. erythropterum* and total song duration for *T. rostratum* as these were perceived to be diagnostic song parameters for population differentiation. For each individual recording, two to three song bouts were analysed using Raven Pro 1.5 Beta version (Cornell Lab of Ornithology, Ithaca, NY, USA). The three *Pycnonotus* bulbuls are not known for their diagnostic songs and tend to have a comparatively muted vocal behaviour compared to other songbird groups (Wells, 2007). Consequently, we did not have access to sufficient homologous recordings for the bulbul species to conduct similar analyses. Permutation tests were performed using STATXACT 8 (Citel Software Corporation, Cambridge, MA, USA).

2.11 | Plumage analysis

We inspected museum specimens from the historic Raffles Collection in the Lee Kong Chian Natural History Museum (Singapore) of 26 *C. erythropterum*, 10 T. *rostratum*, 12 *P. brunneus*, 14 *P. simplex* (35 for eye colour comparison) and 69 *P. plumosus* (Table S4) in order to identify potential plumage and other morphological differences which could reflect genomic patterns. We compiled original subspecies comparisons and descriptions and used the information therein to investigate and score coloration in different body parts for each individual. We also recorded eye colour as and when indicated on specimen labels for *P. plumosus* and *P. simplex*.

3 | RESULTS

We obtained an average of 2,896,008 genomic reads per individual across 114 individuals (Table 1). Using the STACKS pipeline (Catchen et al., 2013), we created two SNP data sets, one allowing for some level of missing data for population genomic analyses and the other with no missing data for ABC analysis, with an average of 16,131 and 8,255 SNPs per species complex, respectively (Table 1). None of the loci exhibited a signature of selection. Average expected heterozygosity across species complexes ranged from 0.09 to 0.25 (Table 1). Using the pyRAD pipeline (see tutorial of Eaton, 2014a, b), we generated concatenated genomic sequences of about 1,736,020 bp length, with an average of 11,683 SNPs across all species for phylogenomic analysis (Table 1). Two samples were discarded, one *T. rostratum* with 69% missing data and one *P. brunneus* with 32% missing data. Finally, we amplified mitochondrial ND2 sequences for 27 individuals and combined them with additional ones from Genbank for a total of 86 individuals to obtain five sequence alignments (one per species complex) ranging between 681–893bp (Table 1).

We successfully mapped 60.1% of scaffolds of the *Mixornis gularis* reference genome to the chromosome-level reference assembly of *Parus major*, allowing us to map an average of 99.2% of SNPs across all five target species complexes to specific songbird chromosomes. To investigate whether some chromosomes are unusually enriched for our SNPs, we compared the number of SNPs per base pair across all chromosomes. These analyses suggested that our SNPs are roughly equally distributed throughout the genome for each species complex, perhaps allowing for a slight tendency of denser SNP distributions on smaller chromosomes, with the exception of the sex chromosome Z, which exhibited a distinct scarcity of SNPs (Figure S14), consistent with interpretations of the avian Z chromosome as a hotspot of reproductively important genes with strong negative selection counteracting genetic drift (Backström et al., 2006; Ellegren et al., 2012; Handley, Ceplitis, & Ellegren, 2004).

3.1 | Population genomic analysis

Preliminary data exploration indicated an unusually deep divergence between a single sample from Sabah initially identified as *P. simplex* versus the remaining populations (including from adjacent Sarawak). This sample was then identified as belonging to the newly described cryptic species *P. pseudosimplex* (Shakya et al., 2019) and was removed from further population genomic analyses.

Across the five species complexes, there was a general division into separate Bornean versus western Sundaic lineages (Figure 1, Figures S2–S6). Network plots corresponding to intermediate *k* (maximum number of nearest neighbours) values, STRUCTURE plots at $K = 2$, as well as first principal components in PCA clearly separated populations into Bornean versus western Sundaic clusters (Figures S2-S6). At increasing k values in Netview analysis, individuals from the same location clustered together first, followed by those found on the same landmass. While STRUCTURE and PCA results indicated three relatively deep clusters for *P. plumosus*, a division between Borneo and western Sundaic populations did apply (Figure S6). For *P. brunneus*, the STRUCTURE results based on Evanno et al.'s (2005) Delta K method suggested no population structure across the sampling area (Figure 1, Figure S5).

3.2 | Phylogenomic analysis

The results of maximum likelihood analysis based on genome-wide data for the two babblers *C. erythropterum* and *T. rostratum* were congruent with population genomic results, indicating that Bornean and western Sundaic populations form two separate and well-supported clades (Figures S7–S8). Similarly, in the bulbul *P. simplex*, Sarawak and western Sundaic populations appeared as two separate and well-supported clades (Figure S9). In *P. plumosus*, however, maximum likelihood analysis indicated a basal position of the sole western Sumatran sample (Figure S10). The results for *P. brunneus* were inconclusive, with an unresolved tree topology even when up to 50% missing data were allowed (Figure S11), supporting the results of population genomic analysis that indicated a general lack of genetic subdivision in this species.

The analysis of the mitochondrial ND2 sequences for the babblers *C. erythropterum* and *T. rostratum* was in agreement with the

results of all other analyses, indicating deep mitochondrial differentiation between Borneo and western Sundaland (Figures S7–S8). Results for *P. plumosus* indicated a deep divergence between western Sumatra and the other populations (Figure S10). We therefore excluded western Sumatran populations from the pairwise comparisons to estimate mitochondrial divergence time between the Bornean and western Sundaic populations. In *P. simplex*, *P. brunneus* and *P. plumosus*, average p-distances between Borneo and western Sundaland were lower than for babbler populations (Figures S7–S11).

3.3 | ABC modelling approach

For all five species, PCA showed that the observed data fell within the prior distribution for all four scenarios of population division tested (Figure S12). Posterior predictive checks indicated a very low predictive error rate. Comparison of the four scenarios revealed a single best-fitting scenario for *P. simplex* (scenario 2 [80–130 kya]), *P. plumosus* (scenario 1 [10 y–12 kya]) and *P. brunneus* (scenario 2 [80–130 kya]) each (Table 2, Figure S1 and S12). For *C. erythropterum* and *T. rostratum*, we identified two scenarios with high probabilities (Table 2). As the confidence intervals did not overlap (Table S5), the scenario (scenario 3 [190–420 kya]) with the highest probability was selected. The results indicated that the three canopy bulbuls experienced gene flow more recently than the two understorey babblers (Table 2) and that within both groups, effective diversification took place at an earlier time for strictly forest-dependent species.

3.4 | SFS-based parameter estimation

Based on the best-fitting scenario identified through ABC, we estimated effective population size and the time of divergence for each species complex using fastsimcoal2 (Excoffier et al., 2013) (Figure 2, Table 3). Analyses for demographic parameter estimation using the site frequency spectrum in fastsimcoal2 revealed that for all species complexes, observed point estimates were within the parametric bootstrap minimum and maximum values (Table 3). The results were broadly congruent with ABC analysis, indicating that time of divergence between western Sundaic and Bornean populations was more recent for bulbuls than for babblers when comparing species with similar habitat requirements (Figure 2, Table 3). Additionally, within both groups, habitat generalists experienced more recent gene flow than strictly forest-dependent species. Our results also revealed that for some species, the contemporary effective population size (N_e) in Borneo was higher than that in western Sundaland (Table 3).

3.5 | Bioacoustic analysis

The bioacoustic differences detected were congruent with genomic analysis. We found consistent bioacoustic differences between western Sundaic and Bornean populations in both babbler complexes. The song of Bornean *C. erythropterum* has a pronounced gap between the first note and the rest of the song motif, whereas the song of the western Sundaic population has a regularly spaced gap at this position (first gap/second gap ratio: m (Borneo) = 3.70 s ± 1.81; m (western Sundaland) = 1.14 s ± 0.16; $p < .001$) (Figure 1). Similarly, the song of *T. rostratum* from western Sundaland exhibits an introductory note extending the total duration of the song which is absent from the Bornean population (song duration: m (Borneo) = 1.03 s ± 0.15; m (western Sundaland) = 1.30 s ± 0.27; $p = .003$) (Figure 1). We did not analyse bulbul songs as they do not lend themselves well to bioacoustic analysis (see Methods).

3.6 | Plumage analysis

Plumage differences congruent with the genomic Bornean versus western Sundaic split were detected in both babblers, *T. rostratum* and *C. erythropterum* (Figure 1). Specifically, side-by-side comparison of 10 specimens of *T. rostratum* showed that subspecies *macropterum* from Borneo was separable from nominate *rostratum* from western Sundaland by a more olive, less rufescent back, as documented in the field literature (Collar & Robson, 2018; Eaton et al., 2016). Similarly, comparison of 26 specimens of *C. erythropterum* revealed that Bornean birds had a slightly more intense reddish brown back as compared to western Sundaic specimens. Bornean specimens also had a dark grey cap and brownish flanks, whereas western Sundaic (including Natuna) specimens had a chestnut cap and cream flanks, becoming cream-brown towards the vent. These differences – again – reflect a traditional separation into Bornean and western Sundaic subspecies groups (e.g. Eaton et al., 2016).

In contrast, no western Sundaic versus Bornean plumage differences were observed in *P. brunneus* and *P. simplex* after comparison across 12 and 14 specimens, respectively. While this lack of plumage

TABLE 2 Posterior probabilities (DIYABC) of the best and second best scenarios for *C. erythropterum*, *T. rostratum*, *P. simplex*, *P. brunneus* and *P. plumosus*

Species	Best scenario	Posterior probability	2nd best scenario	Posterior probability
Cyanoderma erythropterum	3	.592	4	.4
Trichastoma rostratum	3	.523	2	.477
Pycnonotus simplex	2	.79		
Pycnonotus brunneus	2	.882		
Pycnonotus plumosus	1	.987		

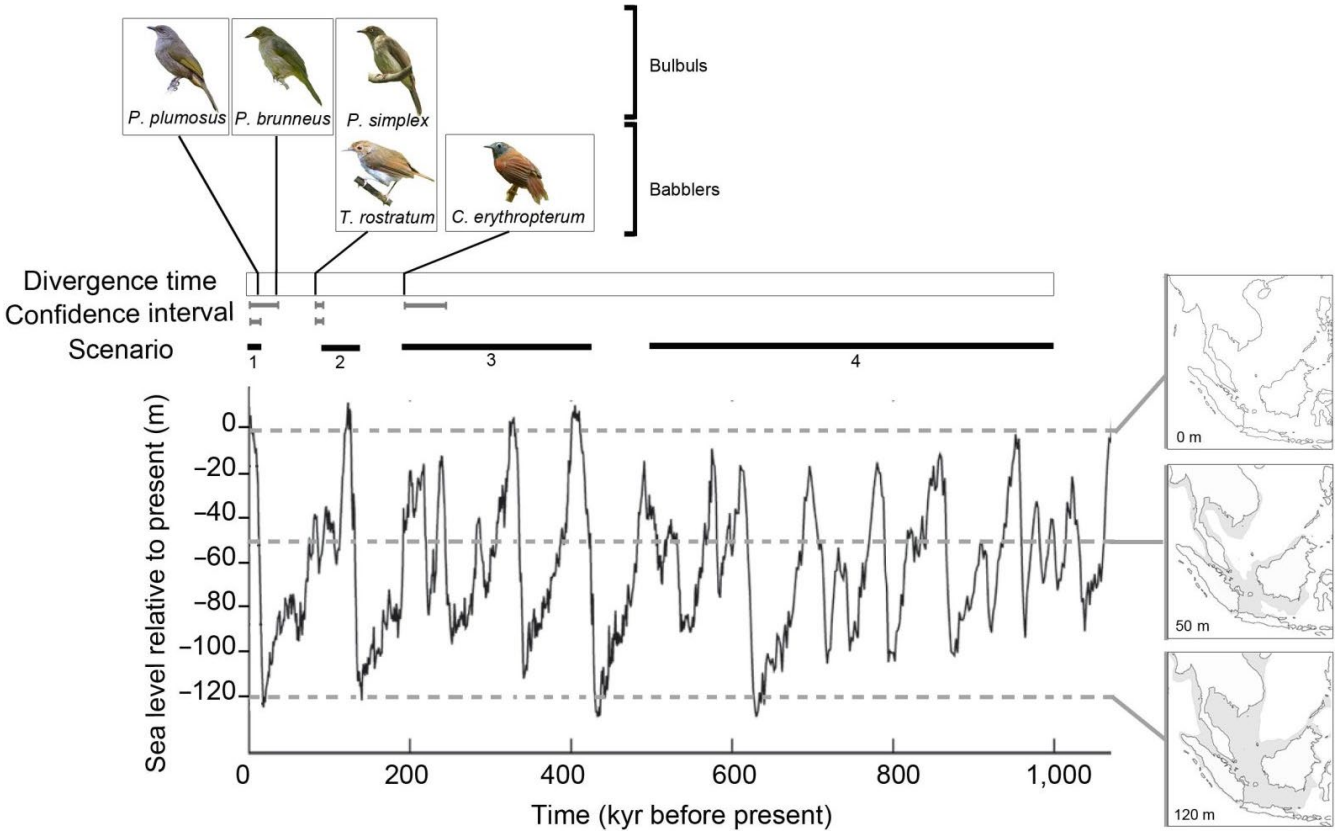

FIGURE 2 Estimated time of divergence for *Cyanoderma erythropterum*, *Trichastoma rostratum*, *Pycnonotus brunneus*, *Pycnonotus simplex*, and *Pycnonotus plumosus*, and sea level variation relative to present (modified from Bintanja et al., 2005). The maps show the Sundaic region at sea levels 0, 50, and 120 m below the present sea level (based on Voris, 2000). The exposed shelf is indicated in grey. The uppermost bar indicates the divergence time obtained from fastsimcoal2 analysis. The four thick black numbered lines correspond to the four tested scenarios. Bird pictures courtesy of D. Koh

differentiation is expected for *P. brunneus*, which is classified into a pan-Sundaic subspecies, it is surprising in *P. simplex*, which lacked discernible plumage differences even between specimens labelled as *P. simplex* from Sabah and the rest of Borneo. Similarly, in *P. plumosus*, only minor differences in belly colour hue were observed between nominate *plumosus* in western Sundaland (darker) and *hutzi* on Borneo (paler, more cream-coloured) across 69 specimens. According to label information, the eye colour differed between west Sumatran *P. porphyreus* (ochre/yellow/orange) and all other populations of *P. plumosus* (red/reddish brown/brown) (Figure 1, Table S4), supporting the separation of west Sumatran birds as an independent taxon that seems to be deeply diverged genomically and mitochondrially (Figure S10). Furthermore, when compared to specimens from the rest of western Sundaland (except Anamba Islands), specimens from West Sumatra had darker, more brownish underparts, especially on the belly.

4 | DISCUSSION

In this study, we used 4,527–19,364 SNPs to investigate how Quaternary climate change has affected population connectivity across five Sundaic songbird complexes with different ecological characteristics. We carefully selected a range of species complexes exhibiting different preferences for vegetative strata and forest dependence to understand how life-history traits affect dispersal across Quaternary land bridges. Using a model-based approach, we found that the Bornean and western Sundaic populations of more canopy-dependent bulbuls experienced gene flow more recently than babblers from lower strata of the forest (Figure 2). Within both groups, we found that gene flow between Sundaic landmasses ceased earlier in forest specialists as compared to habitat generalists (Figure 2).

4.1 | Cryptic diversity in Southeast Asia

Our analysis revealed deep genomic and mitochondrial divergence as well as important phenotypic differences within some Southeast Asian babbler and bulbul species complexes and adds to previous research documenting a high incidence of cryptic diversity in tropical Southeast Asian forest and woodland birds (Garg et al., 2016; Lim et al., 2011; Lohman et al., 2010; Rheindt & Eaton, 2010; Sadanandan & Rheindt, 2015; Van Balen, Eaton, & Rheindt, 2012).

We found consistent plumage and bioacoustic differences between populations from western Sundaland and Borneo in the two

TABLE 3 Estimates of divergence time and effective population size (N_e) for *C. erythropterum*, *T. rostratum*, *P. simplex*, *P. brunneus* and *P. plumosus* obtained from fastsimcoal2 analysis (generation time was set to 1 year and mutation rate per generation to 2.3E−09) and estimates of divergence time for ND2 (=mitochondrial)

	ddRADSeq											ND2	
	Ancestral N_e estimation			N_e point estimate					Time of divergence (years)			Time of divergence (years)	
				Western Sundaland			Borneo						
Species	θ	N_e (ancestral)	Rounded N_e	Observed	Minimum	Maximum	Observed	Minimum	Maximum	Observed	Minimum	Maximum	Mean	95% confidence interval
Cyanoderma erythropterum	0.0007	152,174	152,000	187,196	182,907	221,982	683,309	647,062	828,410	192,301	192,000	244,348	4,048,000	2,950,000–5,243,500
Trichastoma rostratum	0.0013	282,609	283,000	175,489	167,837	207,386	312,678	297,197	354,791	80,832	80,832	91,779	6,576,900	4,632,900–8,815,500
Pycnonotus simplex	0.0009	195,652	196,000	544,569	496,830	641,604	662,076	614,919	771,384	81,144	80,944	92,891	3,056,100	1,574,000–4,706,100
Pycnonotus brunneus	0.00135	293,478	293,000	329,612	9,782	375,521	306,091	8,081	356,115	32,102	698	36,325	2,046,300	1,390,200–2,783,900
Pycnonotus plumosus	0.0017	369,565	370,000	92,427	7,879	143,680	68,404	5,582	114,052	10,125	787	16,533	1,157,900	745,900–1,589,800

understorey babblers, *C. erythropterum* and *T. rostratum* (Figure 1). Corresponding mtDNA sequence divergences of 6.5%–9.4% are considerably higher than mitochondrial thresholds widely used for species delimitation in avian barcoding studies (Hebert, Stoeckle, Zemlak, & Francis, 2004; Kerr, Lijtmaer, Barreira, Hebert, & Tubaro, 2009) (Figures S7–S8). These differences closely mirrored genomic results indicating that – despite opportunity – eastern and western populations have not experienced gene flow during the land bridges accompanying the last 1–2 glacial maxima (Figure 2) and suggest the possibility of at least two biological species in each complex.

While our estimates based on genome-wide SNPs indicated that *C. erythropterum* populations diverged before those of *T. rostratum*, mtDNA-based results suggested that *T. rostratum* populations are the most deeply divergent (Table 3). As the methods used to calculate divergence time from mtDNA and nuclear DNA are vastly different and as the effective population size of haploid markers is one-fourth that of diploid markers (Nei & Tajima, 1981), results cannot be directly compared. Strong sex-specific differences in dispersal capability could potentially lead to an incongruence between the maternal and global population structure (Spottiswoode, Stryjewski, Quader, Colebrook-Robjent, & Sorenson, 2011), but no such strong differences are known to apply in these songbirds. On the other hand, such mito-nuclear discordance is extremely widespread in nature, particularly when secondary contact follows geographic isolation (Toews & Brelsford, 2012; Zhang et al., 2019), because mitochondrial divergence is based on a single marker that is particularly prone to introgression and selection (Bazin, Glémin, & Galtier, 2006), and therefore does not necessarily reflect the true taxon divergence (Fontenot, Makowsky, & Chippindale, 2011; Lim et al., 2011; Ng, Wilton, et al., 2017; Rheindt & Edwards, 2011; Ruane, Bryson, Pyron, & Burbrink, 2014).

In contrast to the babblers, plumage differences were generally slight to nondiscernible in canopy bulbuls, in which eye colour becomes a more important trait. Eye colour has been shown to be of central taxonomic importance and sometimes the only obvious phenotypic character differentiating closely related bulbul species (Fishpool & Tobias, 2018; Garg et al., 2016; Shakya et al., 2019). We observed deep genomic and mitochondrial divergence, arguably dating from the late Pliocene as per a previously validated molecular clock (Weir & Schluter, 2008), between pale orange-eyed *P. plumosus porphyreus* from western Sumatra and deeply dark red-eyed *P. plumosus* from Singapore (Figures S10 and S13 and Table S4). In the *P. simplex* complex, there was a relatively deep although less striking genomic division between western Sundaic and Bornean (i.e. Sarawak, Brunei) populations (Figure S9). We had no eye colour information for the Bornean samples, but a recent study suggested that all Bornean *P. simplex* are red-eyed and therefore starkly differ from the white-eyed populations in western Sundaland (Shakya et al., 2019). These cases of eye colour variation may underscore species-level status for the distinct lineages within the *P. simplex* and *P. plumosus* complexes.

4.2 | Quaternary land bridges as conduits of gene flow

Unlike in the northern hemisphere, differentiation in the fauna of many tropical areas, including Southeast Asia, has not yet been firmly placed in the context of Quaternary climate fluctuations. Our results based on genome-wide data indicate that gene flow between western Sundaland and Borneo generally ceased some time after 400kya, suggesting that up until then there had been some level of gene flow among populations during earlier glacial maxima (Figure 2). This timing is in precise agreement with recent insights that the cyclical pattern of Sundaic land submergence only commenced at 400kya, as the Sundaic subcontinent was far more emergent before then and started to subside more recently (Sarr et al., 2019).

The results of this comparative study suggest that the last glacial maximum (LGM) at 18,000–20,000 years before present was likely only one in a succession of roughly four glacial periods which have contributed to a gradual build-up of population differentiation in these Southeast Asian taxa. Indeed, three out of five species complexes showed signs of isolation which considerably predate the LGM, possibly indicating a completed speciation process between Bornean and western Sundaic populations. At the same time, *Pycnonotus plumosus*, the most dispersive species in our panel (Tang, Sadanandan, & Rheindt, 2015), showed a strong signature of continued gene flow between Borneo and western Sundaland through the LGM. This overall pattern is not surprising as glacial maxima of similar intensity have occurred before the LGM, and species for which Quaternary land bridges are not an efficient conduit for gene flow would have therefore been affected early on.

4.3 | Ecological parameters define potential for gene flow

This is the first comparative study for the Sunda shelf using genome-wide markers to identify Quaternary periods that may have affected some ecological guilds, but not others. It adds to previous research showing that Sundaland has been a complex biogeographic arena in which patterns of gene flow between landmasses would have differed among species (Lim & Sheldon, 2011; Peterson et al., 2015). Our results indicate that Pleistocene land bridges may not have been equally suitable conduits of gene flow for all members of the Sundaic avifauna.

We found that connectivity between western Sundaic and Bornean populations of the two undergrowth-inhabiting babblers, *C. erythropterum* and *T. rostratum*, has been more deeply affected than that of the canopy-dwelling bulbuls, *P. simplex*, *P. brunneus* and *P. plumosus*, underscoring the importance of forest stratum in determining a species' potential for gene flow. Additionally, *P. simplex*, which ranges lower in stratum than *P. brunneus* and *P. plumosus* (Wells, 2007), displays the least recent evidence of pan-Sundaic gene flow across all three bulbuls. Those results are consistent with previous findings showing forest stratum preferences to be a major predictor for the level of genetic differentiation in birds from the Neotropics (Burney & Brumfield, 2009).

Another important predictor of gene flow potential seems to be a species' habitat requirements. For instance, populations of *C. erythropterum* found on different landmasses have likely not experienced extensive gene flow for at least 200,000 years (Figure 2). Since *C. erythropterum* shows a strong forest dependence, this pattern may suggest that Quaternary land bridges have not continuously harboured stable forest habitats suitable for this species over long periods of time. At lower sea levels, the Sunda shelf is thought to have been temporarily occupied by open habitats such as swamp, woodland or savanna (Bird, Taylor, & Hunt, 2005; Cannon et al., 2009; Slik et al., 2011), which would have negatively impacted population connectivity for this babbler species. In contrast, *T. rostratum* is found in a wider range of habitats including mangrove, woodland, edge, mature and disturbed forest (Eaton et al., 2016; Wells, 2007) and has consequently experienced more recent gene flow between populations on different landmasses. A similar pattern emerged when comparing the habitat generalist and edge-tolerant bulbul, *P. plumosus*, with the two forest-dependent bulbuls, *P. simplex* and *P. brunneus*. Indeed, our least forest-dependent species *P. plumosus* displays the most recent evidence of gene flow between western Sundaland and Borneo. Habitat requirements seem to determine a species' response to Quaternary land formation across animal classes, as a high forest dependency has been shown to be similarly associated with deeper mitochondrial divergences between western Sundaland and Borneo in mammals (Mason, Helgen, & Murphy, 2019).

5 | CONCLUSION

The Sunda Shelf in Southeast Asia is the largest shelf area on Earth and has repeatedly emerged as dry land during Quaternary glacial cycles. Even so, its significance for biotic differentiation remains understudied. We show that population differentiation between Borneo (in the east of the Sunda Shelf) and the western Sundaic region across a panel of songbird species complexes predates the last glaciation. Only the most dispersive Sundaic species may be able to maintain gene flow during each glacial cycle of land connectivity, in our case *P. brunneus* and *P. plumosus*. On the other hand, species-level differentiation seems to have been attained between western Sundaland and Borneo in the remaining 3 species complexes (*C. erythropterum*, *T. rostratum* and *P. simplex*). This result has important implications for biodiversity assessments across the region, raising the possibility that in some Sundaic species complexes – even in highly mobile birds – the speciation process between Bornean and western Sundaic populations may be completed despite taxonomic conventions that generally treat these populations as members of single, pan-Sundaic species.

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AUTHOR CONTRIBUTIONS

E.C. and F.E.R. designed the research. E.C. performed laboratory work. E.C., N.S.R.N. and S.T. performed field work. S.B. and D.P.E. facilitated fieldwork. E.C., K.M.G. and B.C. analysed the data with input from N.S.R.N. and F.E.R. Finally, E.C. and F.E.R. wrote the paper with input from all co-authors.

DATA AVAILABILITY STATEMENT

The ddRADSeq data have been deposited in GenBank: BioProject Accession Numbers: PRJNA634759 (Cros et al., 2020a). Filtered data sets have been deposited on Dryad, Dataset, https://doi.org/10.5061/dryad.rfj6q577w (Cros et al., 2020b).

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SUPPORTING INFORMATION

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

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