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Speciation and gene flow across an elevational gradient in New Guinea kingfishers

RUNNING HEAD: SPECIATION IN NEW GUINEA KINGFISHERS

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AUTHOR CONTRIBUTIONS: All authors designed the study, collected data, and drafted the manuscript. E.B.L. analyzed data and produced figures.

DATA AVAILABILITY: All code used in this study can be found at `https://github.com/elinck/syma_speciation/`. Processed data are available from Dryad [doi: pending], and sequence data are available from the NCBI SRA [accession: pending].

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Speciation and gene flow across an elevational gradient in New Guinea kingfishers

Abstract

Theory suggests speciation without geographic isolation is plausible if divergent natural selection is strong enough to counteract gene flow. Tropical mountains provide strong and temporally stable environmental gradients that can promote local adaptation and population genetic structure. Pairs of closely related species with adjacent but divergent elevational ranges are common in these environments, a pattern that suggests parapatric speciation (i.e., speciation with moderate gene flow), but evidence for this process is scarce. Here we use genomic data from modern and historical museum specimens to investigate speciation in a pair of New Guinea kingfishers that segregate ranges by elevation. We find that the lowland species *Syma torotoro* and montane species *S. megarhyncha* form discrete genotypic clusters with bimodal variance in phenotypic traits. Phylogenetic relationships among lineages are discordant between mitochondrial and nuclear genomes, which *D*-tests and demographic inference indicate is partly driven by interspecific gene flow over long time periods. Summary statistics reveal differentiation is concentrated in a handful of small regions of the genome. These data are consistent with ecological speciation driven by adaptation to abiotic or biotic factors varying with elevation. More broadly, they suggest selection across elevational gradients can maintain species boundaries without intrinsic reproductive isolation, a mechanism contributing to high tropical biodiversity.

Key words: parapatric speciation, speciation genomics, ecological speciation, *Syma*

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Introduction

Adaptation across environmental gradients is ubiquitous in nature (Turesson, 1925; McCormack and Smith, 2008; Cheviron and Brumfield, 2009), but its role in promoting speciation remains contentious (Coyne and Orr, 2004; Mallet, 2005; Fitzpatrick et al., 2008; Mallet, 2008). Disruptive natural selection can lead to local adaptation that restricts gene flow between populations in different environments if it becomes paired with a mechanism to promote nonrandom mating such as a pleiotropic "magic trait," linkage disequilibrium between separate loci involved with local adaptation and assortative mating, or habitat preference leading to ecogeographic isolation (Rundle and Nosil, 2005; Via, 2009; Nosil, 2012). A robust body of theory suggests this process is possible under a range of circumstances (Smith, 1966; Endler, 1977; Kirkpatrick and Ravigné, 2002; Doebeli and Dieckmann, 2003; Doebeli et al., 2005; Hua Xia, 2016). However, many evolutionary biologists dismiss its relevance in natural systems as it is unlikely compared to models of speciation without gene flow and because there are few obvious empirical examples (Coyne and Orr, 2004).

Both the traditional emphasis on geography in speciation research and skepticism towards speciation with gene flow were deeply seeded by the writings of German-American zoologist Ernst Mayr (Mayr, 1942, 1963; Coyne, 1994), a prominent architect of the Modern Synthesis. Mayr's theory of "geographic" (or allopatric) speciation was rooted in his detailed study of the birds of the island of New Guinea and nearby islands in Northern Melanesia (Mayr, 1942, 1963). Mayr observed that putative sister species or geographically differentiated races nearly always had non-overlapping geographic ranges on separate islands, isolated mountains, or in lowland basins separated by mountain ranges. Consequently, he suggested geographic differentiation in isolation was nearly always a better explanation for speciation than divergence in sympatry, in part because it allowed Bateson-Dobzhansky-Muller Incompatibilities (Dobzhansky, 1937) or other isolating factors to accumulate in the absence of the homogenizing influence of gene flow. Initially

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implicating drift as the primary evolutionary force driving speciation, his views later shifted to emphasize founder effects (Provine, 2004) while conceding a role for natural selection (Nosil, 2008).

Recent theory and empirical work has undermined the generality of Mayr's intuitive central assumption, which held that contemporary distribution of species can be used to infer their arrangement at the time of divergence. Models of speciation that invoke biotic interactions between diverging populations suggest that reproductive isolation may evolve more readily in the presence of competition and an environmental gradient than in strict allopatry (Doebeli and Dieckmann, 2003). Phylogenetic comparative approaches have been applied to evaluate the frequency of alternate modes of speciation, but are hampered by the lability of species ranges over evolutionary time scales and their limited information on underlying mechanisms (Losos and Glor, 2003). Demographic modeling using genomic data from multiple young species pairs has similarly found that the arrangement of current species ranges fails to predict inferred rates of ancestral gene flow (Penalba et al., 2017). Moreover, genome-wide DNA sequence data has profoundly altered our understanding of the speciation process. We now understand speciation is far more dynamic than previously assumed: reticulation of lineages is common across the tree of life and may be a generative force for adaptive variation or even speciation itself, while species limits can be maintained by selection in the face of significant gene flow (Brelsford et al., 2011; Kumar et al., 2017; Schumer et al., 2018; Edelman et al., 2018).

Ironically, Melanesia, the very region that was so important to Mayr's theory and ecology and evolution more broadly (Wilson, 1959; Diamond, 1972b, 1973), has seen comparatively little modern research in the emerging discipline of speciation genomics (but see Stryjewski and Sorenson (2017)), in part due to logistical difficulties facing fieldworkers. While allopatric divergence following dispersal or vicariance is doubtless an important factor in the origin of species in tropical areas like New Guinea (Smith et al., 2014), the importance of abiotic and biotic ecological variables in driving evolutionary

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processes agnostic to geography is increasingly recognized (Polato et al., 2018). Ecologists have long known that reduced seasonal variation in temperature drives strong thermal stratification across tropical mountainsides (Polato et al., 2018; Janzen, 1967; Sheldon et al., 2018), a pattern correlated with high beta diversity (Jankowski et al., 2009; Cadena et al., 2012), especially among closely related species (Terborgh and Weske, 1975; Freeman and Freeman, 2014). Because many tropical taxa are residents that appear to show low rates of dispersal and have a "slow pace of life" (Wiersma et al., 2007; Harvey et al., 2017; Smith et al., 2017), strong disruptive selection could plausibly counteract migration to drive adaptive divergence.

Local adaptation across tropical elevational gradients has been documented by clines in functional genes and intraspecific population genetic structure (Cheviron and Brumfield, 2009; DuBay and Witt, 2014; Funk et al., 2016; Gadek et al., 2018). This has been shown theoretically to "scale up" to speciation under a scenario of niche expansion (Hua Xia, 2016), but empirical evidence remains mixed. Phylogenetic and phylogeographic comparative studies suggest this process has occurred in *Ithioma* butterflies (Elias et al., 2009) and Andean amphibians and reptiles (Arteaga et al., 2016). In Andean birds, a broad consensus holds that elevational replacements primarily form through divergence in allopatry followed by secondary contact and displacement (Cadena et al., 2012; DuBay and Witt, 2014; Caro et al., 2013; Cadena and Céspedes, 2019), despite equivocal results in some tests. Yet we are aware of only one study that has used population genomics to evaluate speciation across elevational gradients, which found strong evidence that adaptation to altitude drives speciation in *Senecio* ragwort plants in temperate Italy (Chapman et al., 2013; Osborne et al., 2013). The question of the relative contribution of adaptation in the presence of gene flow across elevational gradients to speciation and broader patterns of tropical biodiversity remains open.

The Yellow-billed and Mountain Kingfishers *Syma torotoro* and *S. megarhyncha* (Aves: Alcedinidae) are putative sister taxa that segregate by elevation and vary only

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subtly in phenotype (Mayr, 1942; Pratt and Beehler, 2015; Diamond, 1972a). The lowland species *S. torotoro* is reportedly smaller with a higher-pitched call, and primarily found below 700 m, above which it is replaced by the slightly larger, deeper-voiced *S. megarhyncha* (Pratt and Beehler, 2015) in at least two discontinuous mountain ranges. Among New Guinea's many elevational series, the distribution and morphological conservatism of *Syma* has led systematists to suggest speciation driven by colonization of montane forest (Rand, 1936), though Mayr discounted this possibility in New Guinea mountain birds generally (Mayr, 1942). However, species limits and range-wide variation have never been quantitatively assessed, and observed differences might instead reflect phenotypic plasticity or clinal variation of a single widespread lineage (Caro et al., 2013).

Here we use genome-wide DNA sequences, bioacoustic data, and morphometric analyses to investigate processes of speciation in *Syma* kingfishers. We assessed species limits and phylogenetic relationships, tested for introgression and inferred demographic history, and described the landscape of genomic divergence. Our results are consistent with parapatric speciation across an elevational gradient followed by range expansion into currently discontinuous montane forest habitat. More concretely, they highlight how selection across elevational gradients can maintain species boundaries in the face of incomplete reproductive isolation, a mechanism for generating high tropical biodiversity.

Methods

Study system

Yellow-billed Kingfisher *Syma torotoro* and Mountain Kingfisher *Syma megarhyncha* (Aves: Alcedinidae) are the sole members of their genus. Members of the tree kingfisher subfamily Halcyoninae, they are endemic to New Guinea, its satellite islands, and the Cape York Peninsula of Australia. *S. torotoro* is found in tropical lowland forest and savannah from sea level to ∼500 m elevation, or less commonly to ∼1100 m (Pratt and Beehler, 2015). *S. megarhyncha* is found from ∼600 m to 2700 m or higher (Pratt and

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Beehler, 2015). Though cited as a classic example of congeneric elevational replacements occurring in parapatry (Diamond, 1972a), their elevational ranges have also been reported to either overlap (Gregory, 2017) or be separated by a substantial gap (Freeman and Freeman, 2014; Sam et al., 2014). Both species are omnivorous territorial interior forest residents and differ only in *S. megarhyncha's* larger body size, deeper call, and the extent of black on the top of its bill in one subspecies (Pratt and Beehler, 2015). Insular *S. torotoro* subspecies *S. t. ochracea* differs substantially from its conspecifics and *S. megarhyncha* in call, and is intermediate in size, leading some authors to propose it represents a distinct species (Beehler and Pratt, 2016).

Morphological and bioacoustic data

We measured bill length, bill depth, tarsus, wing chord, and tail length from 72 museum specimens of *Syma torotoro* (n=40) and *S. megarhyncha* (n=32) at the American Museum of Natural History, collected from 1894-1965 and including only individuals of known sex as originally identified by the preparator. Using these data, we performed principal component analyses in R (R Core Team, 2018) with normalized variables, and used PC1 to build mixture models using the R package mclust v. 5.4.1, which we evaluated with a maximum likelihood classification approach (Scrucca et al., 2016). We downloaded all available vocalizations from *S. torotoro* ($n = 34$) and *S. megarhyncha* ($n = 14$) from xeno-canto and Cornell's Macaulay Library. We filtered these data for quality and quantified 36 standard bioacoustic variables using the warbleR package v. 1.1.14 in R (Araya-Salas and Smith-Vidaurre, 2017), analyzing 278 distinct vocalizations from *S. torotoro* and 106 from *S. megarhyncha* in total. (A full list of measured variables can be found in warbleR's documentation.) We ran PCA with normalized variables on the output, and used these data to alternate species delimitation models using the same approach as with our morphological data.

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Sampling, library preparation, and DNA sequencing

We extracted DNA from fresh tissues (n=6) and toepad samples from a historical museum specimens (n=34) from 30 individuals of *S. torotoro* (n=30) and 10 individuals of *S. megarhyncha* (n=10). Though only partially overlapping with samples used in morphometric analyses, these individuals represented the full extent of both species' ranges in New Guinea and Australia (Table 1), and included all described subspecies. We extracted DNA from fresh tissues using a Qiagen DNAeasy kit and the manufacturer's recommended protocol. For historical toepad samples (collected 1877-1973), we extracted DNA using either a using a phenol-chloroform and centrifugal dialysis method (Dumbacher and Fleischer, 2001) (for reduced representation sequencing) or a standard salt extraction protocol (for whole genome sequencing). Due to constraints of cost and time, we employed two complementary sequencing approaches. On a subset of samples (n=20), we performed reduced representation genome sequencing using a hybridization capture with RAD probes (hyRAD) approach, described in detail elsewhere (Linck et al., 2017). We sent the remaining samples to the UC Berkeley's Vincent J. Coates Genomic Sequencing Laboratory, where laboratory staff prepared genomic libraries for low coverage whole genome sequencing (WGS) using Illumina TruSeq Nano kits and a modified protocol that skipped sonication and enzymatically repaired fragments with RNAse. They then pooled (n=20) and sequenced these samples with 150 base pair paired-end reads on a single lane of an Illumina HiSeq 4000.

Sequence assembly and variant calling

We processed demultiplexed reads from both sequencing strategies together with a custom bioinformatic pipeline optimized for handling degraded DNA data and available at `https://github.com/elinck/syma_speciation/`. Briefly, we trimmed raw reads for adapters and low quality bases using bbduk from BBTools version 38.06 suite of bioinformatics tools. We aligned these reads to an unpublished draft genome of Woodland

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Kingfisher *Halcyon senegalensis* from the Bird 10K Genome Project using bbmap with a *k*-mer value of 12, a maximum indel length of 200 bp, and a minimum sequence identity of 0.65. These highly sensitive alignment parameters were necessary given the clade containing *Syma* diverged from the clade containing *Halcyon* approximately 15 mya (Andersen et al., 2018). We used PicardTools v. 2.17.8 and GATK v. 3.6.0 (McKenna et al., 2010) to append read groups and perform local realignment on .bam files. We then used mapDamage 2.0.9 to account for postmortem damage to DNA from historical museum specimens by rescaling quality scores (Jónsson et al., 2013). We performed multisample variant calling using the UnifiedGenotyper tool in GATK v. 3.6.0, and filtered our variant calls for missing data, coverage, and quality with VCFtools 0.1.16 (Danecek et al., 2011). We generated multiple SNP datasets by implementing different filters to suit the requirements and goals of different analyses; these settings and their underlying rationale are described in detail below. To complement our nuclear DNA sequences, we assembled near-complete and fully annotated mitochondrial genomes from a majority of individuals using mitofinder v. 1.0.2 and a complete mtDNA genome from close relative *Todiramphus sanctus* as a reference (Andersen et al., 2015). We then extracted NADH dehydrogenase 2 (ND2) from these genomes and performed multiple sequence alignment using MAFFT v7.407 under its "–auto" parameter setting package (Katoh and Standley, 2013).

Population structure inference

We evaluated population genetic structure within and between species using a nonparametric clustering approach. We performed principal component analysis of genotypes (PCA) and identified putative genetic clusters for K=2 through K=4 using adegenet v. 2.1.1 and a 95% complete dataset with 66,917 SNPs from all individuals in both datasets with a minimum depth of coverage of 3x per individual, a maximum depth of coverage of 120x per individual, a minimum quality score of 30, and a minimum minor allele frequency of 0.05. These relatively lenient filtering parameters were chosen to permit

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the inclusion of samples from both sequencing strategies in our initial assessment of probable species limits. We evaluated the best-fit clustering model using the Bayesian Information Criterion, again implemented in adegenet.

Phylogenetic inference

We evaluated phylogenetic relationships among lineages with three complementary approaches. Using our mitochondrial DNA (ND2) alignment, we inferred a time-calibrated phylogeny in BEAST 2.6.0 including all samples and Sacred Kingfisher *Todiramphus sanctus* as an outgroup (Andersen et al., 2015). We used a strict molecular clock with a rate of $2.9x10^{-8}$ following Lerner et al. (2011), a GTR+GAMMA model of nucleotide evolution, and a Yule species tree prior. We ran BEAST for 50 million generations, storing trees every 1000 generations, and assessed mixing, likelihood stationarity and adequate effective sample sizes (ESS) above 200 for all estimated parameters using Tracer v.1.7.1. We then generated a maximum clade credibility tree using TreeAnotator v.2.6.0, discarding the first 10% of trees as burnin. We next inferred a nuclear DNA phylogeny using SVDquartets (as implemented in PAUP v.4.a165) without *a priori* species assignments. To reduce the influence of sequencing errors, we used a stringently filtered SNP dataset, including only whole genome sequencing data, sites with a minimum minor allele count of 3, no more than 10% missing data, a minimum depth of 5x and a maximum depth of 120x per individual, and a minimum quality score of 30. We converted this diploid alignment to a single consensus IUPAC ambiguity code per individual using vcf2phylip v2.0 (Ortiz, 2019), and included the reference sequence from *H. senegalensis* as an outgroup. We ran SVDquartets with 2000 quartets and 100 bootstrap replicates. Lastly, we infered a species tree under a multispecies coalescent model with SVDquartets and the same SNP dataset as above, using taxon paritions concordant with k-means clustering results and ND2 lineages and the speciesTree=Yes parameter setting.

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Demographic inference

We inferred the demographic history of diverging lineages using moments v.1.0.0, which uses ordinary differential equations to model the evolution of allele frequencies (Jouganous et al., 2017). We used moments to calculate a folded joint site frequency spectrum (JSFS) using a SNP dataset that included only whole genome sequencing data, a minimum minor allele count of 1, no more than 10% missing data per site, a minimum depth of 6x per individual, and a minimum quality score of 30. We chose these filtering parameters to reduce the influence of sequencing error without strongly biasing the JSFS. This dataset was further thinned to 1 site for every 50 kbp to reduce the influence of linkage disequilibrium, and did not include *S. t. ochracea*. To account for the remaining missing data, we projected the JSFS down to an effective sample size of 16 x 12 chromosomes for *S. megarhyncha* and *S. torotoro* respectively. To infer changes in population size and migration rate over time, we defined an isolation-with-migration model that included two time periods following an initial split. We allowed both population size for each lineage and a symmetrical migration rate parameter between lineages to vary in each. After initially optimizing parameters using the "optimize_log" method, we ran 10 additional optimizations, randomly sampling starting parameters from a uniform distribution within the bounds of $1x10^{-5}$ and 10. We converted parameter values to real units using a genome-wide mutation rate of $2.3x10^{-9}$ (Smeds et al., 2016), an effective sequence length scaled to reflect our LD-thinned SNP dataset, and a generation time estimate of two years, and generated parameter uncertainty estimates by fitting the IM model to 200 bootstrapped site frequency spectra generated using the fs.sample() function on our data.

Introgression

We explicitly tested for introgression between lineages using the four taxon D-test (Green et al., 2010), which relies on asymmetries in discordant site patterns to distinguish

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incomplete lineage sorting from introgression. We randomly selected one representative sequence for *S. torotoro*, *S. megarhyncha*, and *S. t. ochracea* from the nuclear DNA SNP alignment used in our phylogenetic analyses, and again used the reference sequence for *H. senegalensis* as an outgroup. We then calculated D using a custom python script (available at `https://github.com/elinck/syma_speciation/`), estimating uncertainty using a block jacknife with 100 replicates.

Genome scans

We assessed levels of divergence across the genome by calculating Wright's F_{ST} and D_{XY} for each species in 50 kbp windows with a 10 kbp step size using scripts written by Simon Martin (`https://github.com/simonhmartin/genomics_general`), using a VCF file generated from our whole genome sequencing data that included invariant sites and was filtered for a minimum Q score of 30 and a minimum depth of 3x per individual, for a total of 1,858,764 SNPs and 102,606,487 total sites. We permitted a lower depth of coverage threshhold in this analysis as our goal was to describe the overall landscape of differentiation as completely as possible given the constraints of our data. To assign chromosome identity to scaffolds and windows, we aligned the *H. senegalensis* draft genome to the chromosome-level genome assembly of *Taenopygia guttata* (Warren et al., 2010) using NUCmer in MUMmer v. 3.1 (Delcher et al., 1999), allowing a maximum gap of 1000 bp and using a minimum sequence identity threshold of 10 kbp per contig. We evaluated correlations among summary statistics using simple linear models implemented in R (R Core Team, 2018).

Results

DNA sequencing from historical specimens

We extracted DNA and generated genome-wide sequence data from all historic toepad samples across both species' relatively inaccessible distributions, with collection

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dates ranging from 1896 to 1973 and including 3 individuals collected by Ernst Mayr himself in 1929. We present a detailed description of reduced representation hyRAD data elsewhere (Linck et al., 2017). On average we were able to align 83.3% of reads to the draft *Halcyon senegalensis* genome, ranging from 35.5% to 92.37% across individuals; this broad range likely reflects high DNA degradation in a handful of samples. Whole genome sequencing data had an average depth of coverage of 5.38x per individual, ranging from 1.92x to 12.12x. Following variant calling and filtering for depth of coverage and quality, a 95% complete data matrix including 37 individuals and both WGS and hyRAD data had 66,917 SNPs, which was further reduced to 10,351 SNPs after thinning to 1 site for every 50 kbp to reduce the influence of linkage disequilibrium. A second matrix of whole genome sequencing data alone had 78,882,912 SNPs, which was reduced to 1,858,764 SNPs after filtering for a minimum depth of coverage of 3x and a minimum quality score of 30.

Species limits and phylogeny

Analysis of genome-wide DNA sequence data, morphometrics, and calls supported *Syma torotoro* and *S. megarhyncha* as distinct, assortatively mating lineages (Figure 1). Principal component analysis of genotypes separated *S. torotoro* and *S. megarhyncha* into discrete clusters (Figure 1A; Figure 1C) with little evidence of hybrid genotypes along the primary axis of genetic differentiation, with PC1 explaining 17.02% of total variance. PC2 explained 6.31% of total variance and separated insular subspecies *S. t. ochracea* into a third discrete cluster. The best-fit model from a *k*-means clustering analysis (as identified by BIC scores) perfectly recovered these groups (Figure 1C).

Analyses of phenotypic data formed discrete clusters concordant with genomic results and consistent with morphological differentiation by elevation. Bill width and depth, tarsus, wing chord, and tail length were significantly larger in *Syma megarhyncha* after correcting for multiple comparisons in Welch's two sample T-tests (all comparisons $p < 1x10^{-7}$), and species was a significant predictor of PC1 in a linear model

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($p < 1x10^{-14}$). Following principal component analysis, PC1 explained 81.47% of variance across all five traits. Bayesian Information Criterion (BIC) selected two distinct normal distributions out of normal mixture models (NMMs) fit to PC1 (log-likelihood$= -378.0914$) (Figure 1D). Calls of *S. torotoro* had a significantly higher frequency ($p < 1x10^{-5}$) but did not differ in duration. Species was a significant predictor $p < 1x10^{-5}$) of PC1 in a principal component analysis of the 36 bioacoustic variables quantified in warbleR (Araya-Salas and Smith-Vidaurre, 2017), and explained 35.72% of total variance. Because NMMs assume independence of observations, we did not perform formal model fitting for bioacoustic data, but a frequency distribution strongly suggests values are bimodally distributed by species identity (Figure 1E).

A time-calibrated phylogeny of the mitochondrial gene ND2 supported the reciprocal monophyly of *S. megarhyncha* and *S. torotoro*, but unexpectedly recovered mainland *S. torotoro* as sister with a clade containing *S. megarhyncha* and insular endemic subspecies *S. t. ochracea* (Figure 1F). Within *S. torotoro*, a clade of four individuals with full bootstrap support from the southeast peninsula of Papua New Guinea was separated from the remaining individuals, but with little other apparent geographic structure across the tree. Based on a strict molecular clock with a rate of $2.9x10^{-8}$, *S. megarhyncha* and *S. t. ochracea* initially diverged from *S. torotoro* approximately 790,000 years ago (95% CI: 607,421.8-972,618.8 years); a subsequent split between *S. megarhyncha* and *S. t. ochracea* occured approximately 377,997 years ago (95% CI: 243,261.9-512,732.0 years), and a split between *S. torotoro* populations in the SE peninsula of Papua New Guinea and the rest of the species occurred approximately 428,580 years ago (95% CI: 304,804.8-552,355.0 years). In contrast, our nuclear DNA lineage phylogeny was more poorly resolved and conflicted with both the ND2 tree and current taxonomy (Figure 1G). An initial divergence event with full bootstrap support separated a clade containing *S. megarhyncha* individuals from far western New Guinea and *S. t. ochracea*; a second fully supported split separated *S. torotoro* populations in SE Papua New Guinea from an ingroup containing poorly resolved

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clades containing the remainder of *S. torotoro* and *S. megarhyncha* individuals. Lastly (and again conflicting with both other phylogenies), our nuclear DNA species tree from SVDquartets grouped *S. torotoro* and *S. megarhyncha* together in a clade that was sister to *S. t. ochracea*, with full bootstrap support at all internal nodes (Figure 1H)

Demographic history and introgression

D-tests indicated asymmetric genealogies among lineages that were the product of introgression or ancestral population structure rather than incomplete lineage sorting alone. For two of three possible species tree topologies, *D* differed signficantly from 0 (one sample t-tests: $p < 2x10^{-16}$ and $t = 877.23$ given (ochracea,megarhyncha),torororo); $p < 2x10^{-16}$ and $t = 837.36$ given (ochracea,torotoro),megarhyncha)), with signs indicating introgression between *S. torotoro* and *S. megarhyncha*, rather than either of these species and *S. t. ochracea* (Figure 2A). Bootstrapped *D*-test values for the topology (megarhyncha,torororo),ochracea) also different significantly from zero (one sample t-test: $p < 2x10^{-16}$ and $t = -19.416$), but with a range which encompassed it (-0.023 to 0.003).

Parameter estimates from demographic inference under an isolation-with-migration model provided additional evidence for introgression, and over long time periods (Figure 2B). During the time period immediately following divergence, which was estimated to last approximately 463,311 years (SD: 22,301.72 years), the symmetrical migration rate parameter was equivalent to approximately 3.41 migrants per generation (SD: 0.001). During the second time period, which was estimated to be approximately 280,852 years (SD: 13,686.37 years), symmetrical migration rate parameter increased slightly to 3.69 migrants per generation (SD: 0.01589379). Summing the first and second periods indicates an initial divergence event approximately 744,163 years ago—a close match to the estimate from the mitochondrial DNA molecular clock analysis. Population size changed in both species over time: *S. megarhyncha* was inferred to decline from approximately 780,894 individuals (SD: 37,243 individuals) to approximately 346,054 individuals (SD: 16,517

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individuals), while *S. torotoro* was inferred to grow from 256,736 individuals (SD: 12,254 individuals) to 596,653 individuals (SD: 28,464 individuals).

Genome scans

Analysis of whole genome sequencing data suggests *S. torotoro* and *S. megarhyncha* differ primarily in small regions of the genome (Figure 3). Genome scans of divergence in 50 kb sliding windows with a step size of 10 kb revealed globally low to moderate genetic divergence (mean F_{ST}=0.0947) defined by 21 autosomal F_{ST} peaks (Figure 3A; Figure 3B). The Z chromosome showed low interspecific divergence, suggesting a limited role in speciation (Irwin, 2018), though this may represent an artifact of high intraspecific diversity due to misincorporation of reads from the W chromosome Correlations between genome-wide summary statistics suggested a role for both structural reductions in recombination and positive selection in shaping F_{ST} peaks. F_{ST} was negatively but weakly correlated with D_{XY} ($p < 1x10^{-15}$, $R^2 = 0.1271$) (Figure 3A). Considering a subset of chromosomes with prominent F_{ST} peaks (Figure 3B), this correlation remained significant but was slightly reduced ($p < 1x10^{-15}$, $R^2 = 0.0.1044$) (Figure 3C).

DISCUSSION

Speciation across environmental gradients is commonly invoked in explanations of high tropical species richness and the latitudinal biodiversity gradient (Caro et al., 2013; Schneider et al., 1999; Portik et al., 2017; Beheregaray et al., 2015). Tropical elevational gradients have high levels of temporally stable thermal stratification (Janzen, 1967) that has been linked to selection for narrow thermal physiologies (Sheldon et al., 2018), reduced upslope or downslope dispersal (Polato et al., 2018), and high beta diversity (Jankowski et al., 2009). As a result, they have frequently been studied as a stage for this process (Mayr, 1942; Cadena et al., 2012; Funk et al., 2016; Arteaga et al., 2016; Caro et al., 2013; Moyle et al., 2017). Yet previous comparative phylogenetic studies have overwhelmingly

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supported models of divergence in allopatry followed by secondary contact and range displacement, particularly in vertebrates (Arteaga et al., 2016; Caro et al., 2013; Moyle et al., 2017). Our finding of well-defined genotypic and phenotypic clusters (Figure 1) in the face of extensive historical and contemporary introgression (Figure 2) suggests that selection across elevational gradients is sufficient to maintain species boundaries with little evidence of strong postzygotic isolation. This result provides rare validation of an intuitive and widely cited but poorly buttressed theory: that selection across environmental gradients can serve as a motor for tropical diversification, either as the primary driver of speciation (e.g., in ecological speciation models) or as a force for reinforcement and disruptive selection after the build-up of partial reproductive isolation in allopatry.

Though evidence from genotypic and phenotypic clustering analyses suggests *S. torotoro* and *S. megarhyncha* are assortatively mating lineages that are largely match current taxonomy, alternate approaches to phylogenetic inference provide conflicting data on species limits and evolutionary relationships. A well-sampled phylogeny of the mitochondrial gene ND2 shows an unexpected sister relationship between *S. megarhyncha* and insular *S. torotoro* subspecies *ochracea*, previously hypothesized to represent a species-level lineage (Pratt and Beehler, 2015), but also revealed the expected pattern of monophyly for *S. torotoro* and *S. megarhyncha*. In contrast, a phylogeny from nuclear DNA is strongly discordant with both mitochondrial relationships and the results of clustering analyses (Figure 1E). We propose this conflict can be primarily explained by introgression between *S. torotoro* and *S. megarhyncha* in eastern New Guinea, as supported by evidence from *D*-tests and demographic inference (Figure 2). Increased structure in mitochondrial DNA relative to the nuclear genome may also be generated by with reduced hybrid fitness in heterogametic females (in accordance with Haldane's rule), or male biased dispersal (Toews and Brelsford, 2012)—both of which are plausibly contributing to patterns in *Syma*. However, we note that the unavoidable autocorrelation between collecting date and geography among the toepad samples in our dataset is likely contributing to noise in our

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data to some degree, as drift and selection will have contributed to allele frequency differences over the nearly 50 generations between 1877 and 1973.

In spite of this phylogenetic uncertainty, we believe our data from *Syma* are more consistent with speciation with gene flow driven by niche expansion and disruptive selection across an elevational gradient (Hua Xia, 2016) rather than allopatric speciation during an extensive period of isolation followed by secondary contact and niche displacement. Divergence in morphometric traits, the most visible phenotypic difference between *S. torotoro* and *S. megarhyncha* (Figures 1D, 1E), is largely thought to reflect ecological adaptation in allopatric populations (Ricklefs and Bermingham, 2007; Winger and Bates, 2015). While this may arise as a result of character displacement from competition in populations experiencing secondary contact (Diamond et al., 1989), it presumes sufficient reproductive isolation has developed to permit coexistence without genetic homogenization and the fusion of lineages. In *Syma*, tests for introgression and demographic inference indicate gene flow over long time periods, rendering the presence of strong genetic incompatabilities unlikely. Though we cannot rule out a role for ancestral population structure in generating significant D-test results, parameter estimates from the isolation-with-migration model and reports of occasional hybridization in the field (Beehler and Pratt, 2016) provide additional evidence for introgression. Additionally, the failure of *S. torotoro* to expand its elevational range upslope in absence of *S. megarhyncha* in the mountains of the Vogelkop Peninsula in West Papua supports a hypothesis of adaptation to divergent elevational niches, rather than competitive segregation of a shared, broader realized niche.

However, we emphasize that for most cases, parapatric speciation (and its vaguer, spatially agnostic variant "speciation with gene flow") are overly simplified models of what is likely a highly dynamic process over evolutionary time scales. Nor are genomic data ever likely to completely eliminate the possibility of a period of allopatry in the history of diverging lineages (Strasburg and Rieseberg, 2011; Martin et al., 2013), especially given

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the challenges of optimizing multiple parameters while attempting to represent complex biological processes with simple models. While parameter estimates from demographic inference suggest significant gene flow occurred between *S. megarhyncha* and *S. torotoro* occurred early on in the history of their divergence, a recent increase in migration (Figure 2B) indicates a model intermediate between isolation-with-migration and secondary contact may be more appropriate. A scenario in this vein might consist of numerous interdigitated periods of gene flow and isolation, driven by fluctuating local population sizes or by Pleistocene glacial cycling and its compression of elevational zones throughout New Guinea (Hope, 2014). Indeed, recent surveys on Mt. Wilhelm and Mt. Karimui in Papua New Guinea found a large gaps in the elevational ranges of *S. torotoro* and *S. megarhyncha* (Freeman and Freeman, 2014; Sam et al., 2014), in contrast to earlier studies (Diamond, 1972a), suggesting that populations are effectively allopatric at short horizontal distances. However, we again note that the large time span covered by our toepad samples provides an additional source of noise to our data and may bias our parameter value estimates, with few clear predictions.

Though we did not identify the targets of selection, our data are consistent with disruptive positive selection leading to high divergence in small regions of the genome against a background of long-term introgression. These genomic "islands" have become a hallmark of the high throughput sequencing era and have been shown in wide range of nonmodel organisms (Nosil, 2012; Bay and Ruegg, 2017). Early interpretations of the pattern as a clear signature of speciation with gene flow—where F_{ST} peaks correlate to genes for traits under disruptive selection while low F_{ST} regions remain susceptible to introgression—have been complicated by evidence numerous processes can produce similar distributions, including selective sweeps in allopatry or recombination rate variation (Cruickshank and Hahn, 2014; Bay and Ruegg, 2017; Irwin et al., 2018; Via, 2012; Burri, 2017). Evidence from multiple summary statistics across the same genomic windows can also help tease apart underlying mechanisms in some cases (Irwin et al., 2018): for

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instance, low absolute differentiation (D_{XY}) in genomic islands can indicate F_{ST} values are inflated by low intraspecific diversity due to reduced recombination, casting doubt on models of divergence with gene flow (Cruickshank and Hahn, 2014).

Here, our data defy easy description, highlighting the limits of this approach. A handful of major F_{ST} peaks stand out against a background of low F_{ST} windows, with a global average of 0.0947 (Figure 3A; Figure 3B). However, D_{XY} shows only a weak correlation with F_{ST} values (Figure 3C), which is further reduced in outlier windows. Low in some F_{ST} peaks and high in others, this relationship is supports the likely hypothesis that recombination rate, selection against hybrid ancestry, disruptive selection, and gene flow have interacted to form a complex mosaic in the genomes of *S. torotoro* and *S. megarhyncha*. Given the limitations of short-read sequencing data, however, we concede these patterns may be influenced by either alignment artifacts or undetected structural rearrangements. In particular, we point to large peaks on chromosomes 5 and 11 that deserve further scrutiny as possible inversions contributing to reproductive isolation (Hooper and Price, 2017) (Figure 3A and B).

As adaptive differentiation in morphology appears to have occurred during the initial stages of lineage divergence (either in the presence of gene flow or accompanied by it shortly afterwards), *Syma* is plausibly a case of ecological speciation *sensu* Nosil and Schluter (Rundle and Nosil, 2005; Nosil, 2012; Schluter, 2009). If this is the case, we propose that it may have been facilitated by a so-called “magic trait”, or trait under environmentally-mediated selection that also influences reproductive isolation (Servedio et al., 2011). Magic trait models are among the easiest ways to explain speciation with gene flow (Nosil, 2012), and perhaps especially apply to birds, where intrinsic incompatibilities are rare and prezygotic isolation is thought to play a major role in speciation (Edwards et al., 2005; Price, 2008). While we lack strong evidence for a target of disruptive selection or a specific mechanism to link it to reproductive isolation, we speculate selection on body size may also affect mate choice. Morphometric data from

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Syma are consistent with Bergmann's rule and its prediction of larger body size in the higher elevation cooler climate species, *S. megarhyncha* (but see Freeman (2017)). As one of only three kingfisher species found above 2000 m in New Guinea and the only regional kingfisher that is a high elevation specialist, *Syma megarhyncha* is likely to have a thermal physiology under stronger selective constraint for warm environments, requiring greater adaptive divergence in body size to colonize montane environments. An increase in body size might directly lower frequency calls as a biproduct of morphological divergence, and assortative mating might act on either trait individually or in tandem (Derryberry et al., 2018; Slabbekoorn and Smith, 2002; Zhen et al., 2017).

What does *Syma* reveal about the origin of elevational series of congeners and by extension their significant contribution to tropical montane biodiversity (Freeman and Freeman, 2014)? First, our results suggest disruptive selection across elevational gradients can effectively maintain species limits in the absence of strong postzygotic reproductive isolation, a finding with broad relevance to speciation in tropical mountains regardless of the geographic mode of divergence or levels of gene flow. In a scenario of secondary contact without adaptation to divergent elevational niches, *Syma* lineages would likely have fused. This suggests allopatric divergence in isolation may not be sufficient to drive many speciation events in tropical mountains. Second, aspects of *Syma's* natural history may serve as a guide to where to expect speciation with gene flow in birds: species that are vocal nonlearners, have a conserved climatic niche, and have a small realized range and total area of suitable habitat. While New Guinea is large and geologically complex, it is dwarfed by other tropical montane regions, which potentially have increased odds for speciation in allopatry (Pratt and Beehler, 2015; Price, 2008). Yet even if speciation across elevational gradients remain a rare exception among birds, the conditions that make it possible in *Syma* are more common in other taxa. Indeed, preliminary studies suggest it may be a much more common mechanism in amphibians, insects, and plants (Funk et al., 2016; Elias et al., 2009; Arteaga et al., 2016; Chapman et al., 2013). We highlight the

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importance of natural history studies of poorly known tropical organisms in establishing candidates for further investigation with genomic and experimental approaches.

Ernst Mayr's emphasis on geographic isolation profoundly shaped the study of speciation and diversification, establishing the primacy of divergence in allopatry and highlighting the significance of coexistence in sympatry to species concepts and speciation theory (Coyne, 1994; Nosil, 2008). Though famously a skeptic of sympatric speciation and its relatives (Mayr, 1963), he retained an appreciation of ecological factors in population divergence (Nosil, 2008)—contrasting perspectives shaped by his foundational experience as an ornithologist, natural historian and systematist in New Guinea. Yet contemporary speciation research integrating genomic data with traditional analyses of phenotype and distributional data has only recently been applied to the New Guinea birds Mayr knew so well (Stryjewski and Sorenson, 2017), and continues to be difficult to implement due to massive logistical hurdles. Much as the study of ancient DNA has revolutionized our understanding of human prehistory (Green et al., 2010), widespread use of whole genome sequences from historic museum specimens has the potential to reshape our understanding of the speciation process in understudied tropical regions with few contemporary data and pressing conservation challenges. As this occurs, we expect much of Mayr's seminal work to gain new significance, as case studies like *Syma* highlight the continued relevance of the spatial organization of populations while enriching previously simple narratives of the role of selection and gene flow in the origin of species.

Data availability

All code used in this study can be found at `https://github.com/elinck/syma_speciation/`. Processed data are available from Dryad [doi: pending], and sequence data are available from the NCBI SRA [accession: pending].

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Figures & Tables

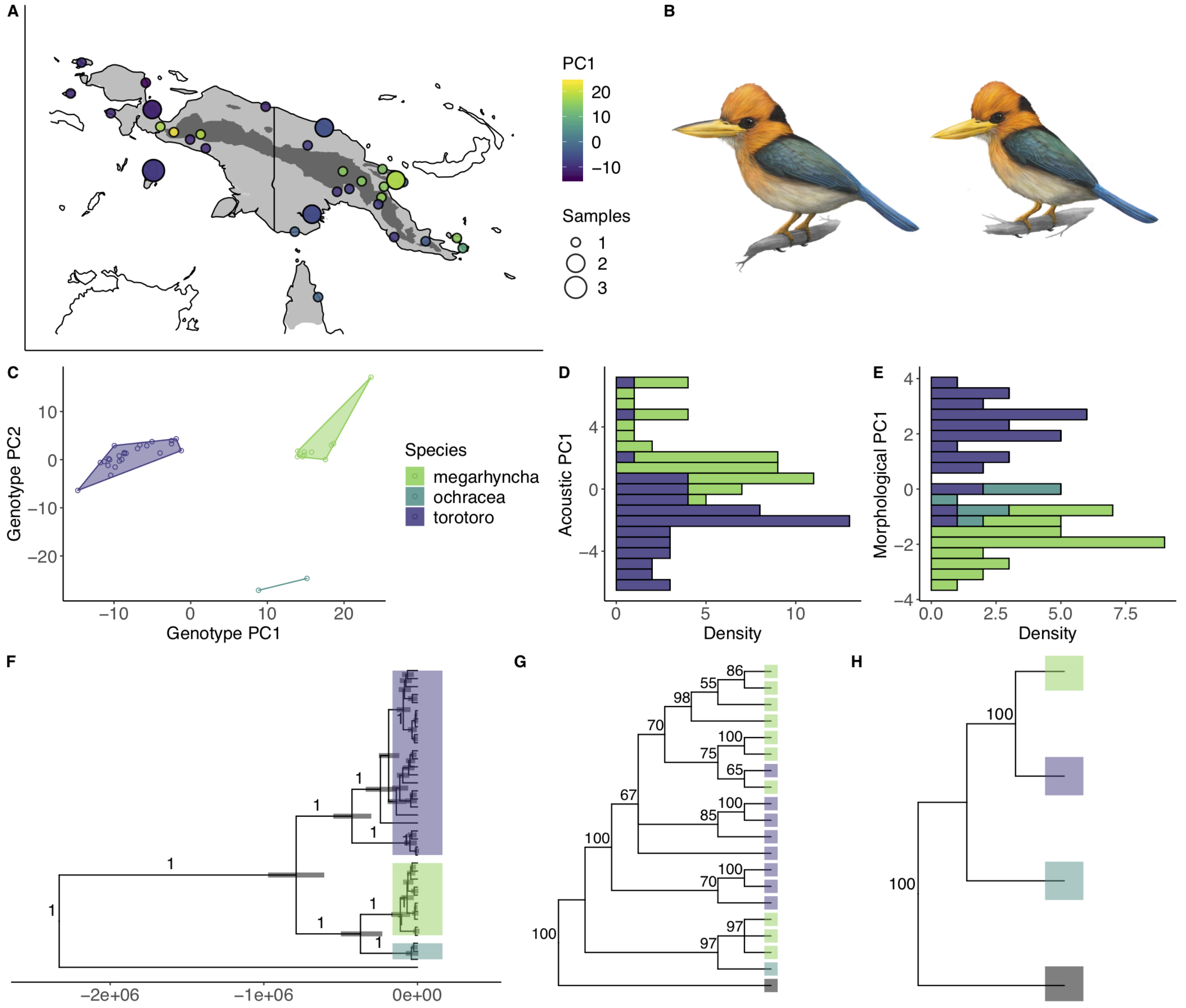

Fig. 1. **Genomic and phenotypic data provide evidence of assortative mating.** A) Sampling localities for *Syma* kingfishers across New Guinea and Australia, color-coded by genotype PC1 and scaled by number of individuals. B) Illustration of *S. megarhyncha* (top) and *S. torotoro* (bottom), by Kevin Epperly. C) Principal component analysis of genotypes, clustered by the best fit *k*-means result (K=3) and color-coded by the mean PC1 value for all individuals in a given cluster. D) The first principal component of bioacoustic parameters measured from vocalizations is bimodally distributed by species. E) The first principal component of morphological data is bimodally distributed by species. F) A time-calibrated ND2 phylogeny supports reciprocal monophyly of *S. torotoro* and a clade with *S. megarhyncha* and *S. t. ochracea*. G) SVDquartets nuclear DNA phylogeny. H) SVDquartets species tree, using *k*-means clustering results and mitochondrial DNA clades as taxa.

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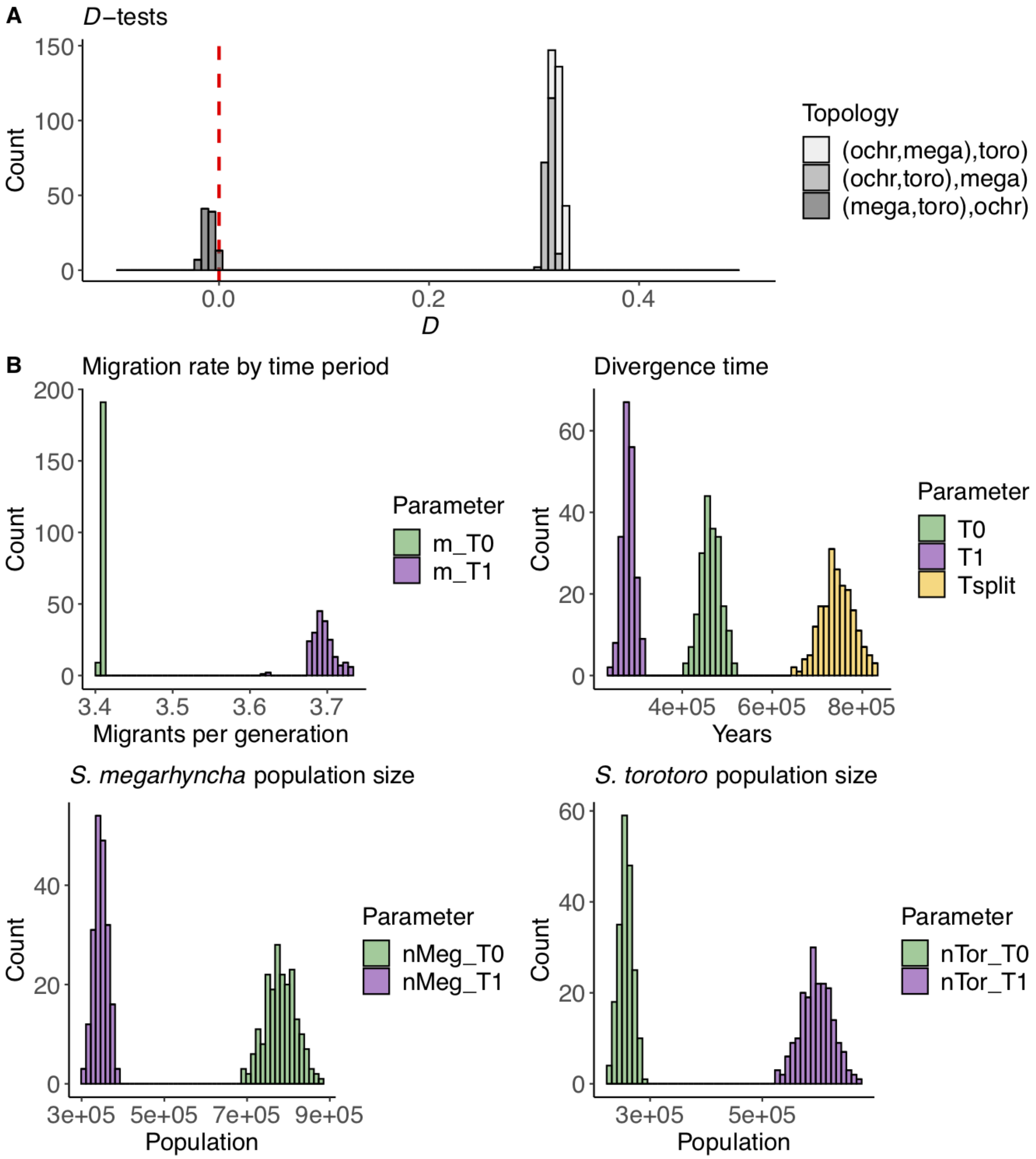

Fig. 2. **Evidence for long-term introgression between species.** A) Bootstrapped results for *D* tests for introgression across all three possible species tree topologies. The dashed red line indicates 0, the expectation under incomplete lineage sorting alone. B) Parameter estimates from an isolation-with-migration model allowing migration rate and population size to vary across two time periods.

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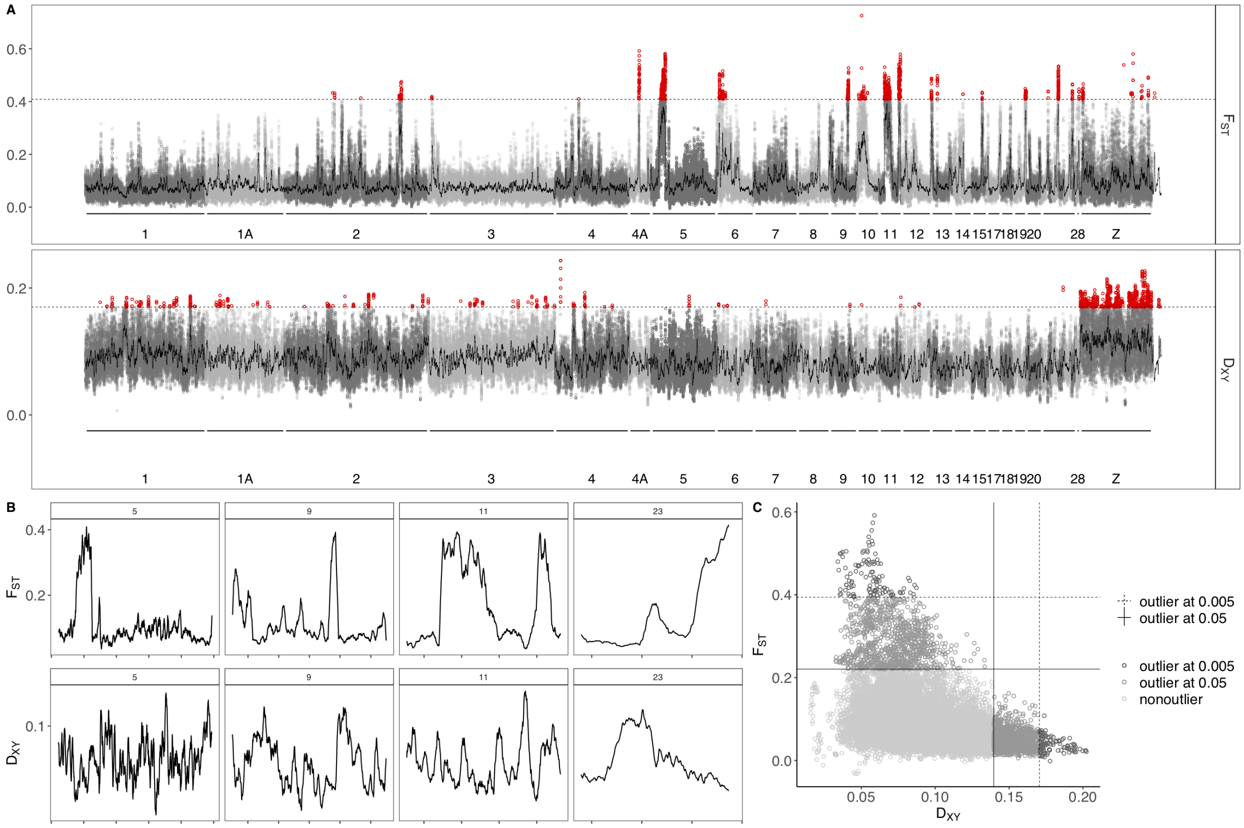

Fig. 3. **Heterogeneous genomic divergence.** A) Relative divergence (F_{ST}), and absolute divergence (D_{XY}) in 50 kb sliding windows across the genome. Outliers at an arbitrary 0.005 significance level are highlighted in red. B) The genomic landscape of divergence in four chromosomes with strong F_{ST} peaks. C) The correlation between F_{ST} and D_{XY} in the same four chromosomes.

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Table 1. Sampling, sequencing, and analysis information. WP=Indonesian New Guinea; PNG=Papua New Guinea.

Specimen	**Species**	**Subspecies**	**Locality**	**Source**	**Date**	**Sequencing**	**PCA**	**mtDNA phylogeny**	**nuDNA phylogeny**	**D-tests**	**Demographic inference**	**Genome scans**
AMNH:Birds:293714	*torotoro*	*torotoro*	Ifaar, WP	toepad	1928	hyRAD	yes	yes	no	no	no	no
AMNH:Birds:293715	*torotoro*	*torotoro*	Kepaur, WP	toepad	1897	hyRAD	yes	yes	no	no	no	no
AMNH:Birds:300723	*torotoro*	*torotoro*	Kepaur, WP	toepad	1897	hyRAD	yes	yes	no	no	no	no
AMNH:Birds:300723	*torotoro*	*torotoro*	Misol Island, WP	toepad	1900	hyRAD	yes	yes	no	no	no	no
AMNH:Birds:329542	*torotoro*	*torotoro*	Wasior, WP	toepad	1928	hyRAD	yes	yes	no	no	no	no
AMNH:Birds:437798	*torotoro*	*torotoro*	Amberbaki, WP	toepad	1877	hyRAD	yes	yes	no	no	no	no
AMNH:Birds:637429	*torotoro*	*torotoro*	Humbolt Bay, WP	toepad	1928	hyRAD	yes	yes	no	no	no	no
AMNH:Birds:637441	*torotoro*	*torotoro*	Mt. Mori, WP	toepad	1899	hyRAD	yes	yes	no	no	no	no
AMNH:Birds:637445	*torotoro*	*torotoro*	East Sepik Province, PNG	toepad	2003	hyRAD	yes	yes	no	no	no	no
AMNH:Birds:637446	*torotoro*	*torotoro*	Waigeu Island, WP	toepad	1900	hyRAD	yes	yes	no	no	no	no
AMNH:Birds:637450	*torotoro*	*tentelare*	Aru Islands, WP	toepad	1896	hyRAD	yes	yes	no	no	no	no
AMNH:Birds:637464	*torotoro*	*tentelare*	Aru Islands, WP	toepad	1900	hyRAD	yes	yes	no	no	no	no
AMNH:Birds:637464	*torotoro*	*ochracea*	Normanby Island, PNG	toepad	1934	hyRAD	yes	yes	no	no	no	no
CAS:Birds:7131	*torotoro*	*pseuestes*	Gulf Province, PNG	muscle	2002	hyRAD	yes	yes	no	no	no	no
KU:Birds:5215	*torotoro*	*pseuestes*	Gulf Province, PNG	muscle	2003	hyRAD	yes	yes	no	no	no	no
KU:Birds:5464	*torotoro*	*pseuestes*	Gulf Province, PNG	muscle	2003	hyRAD	yes	yes	no	no	no	no
KU:Birds:626	*torotoro*	*meeki*	Varirata National Park, PNG	muscle	2011	hyRAD	yes	yes	no	no	no	no
KU:Birds:6927	*torotoro*	*meeki*	Mt. Suckling, PNG	muscle	2011	hyRAD	yes	yes	no	no	no	no
NHMUK:Birds:1911.12.20.822	*torotoro*	*pseuestes*	Satakwa River, WP	toepad	1911	hyRAD	yes	yes	no	no	no	no
NHMUK:Birds:1911.12.20.823	*torotoro*	*pseuestes*	Mimika River, WP	toepad	1913	hyRAD	yes	yes	no	no	no	no
AMNH:Birds:301861	*megarhyncha*	*wellsi*	Weylendgep Kunupi, WP	toepad	1931	WGS	yes	yes	yes	yes	yes	yes
AMNH:Birds:302859	*megarhyncha*	*wellsi*	Mt. Derimapa, WP	toepad	1930	WGS	yes	yes	yes	yes	yes	yes
AMNH:Birds:339957	*megarhyncha*	*wellsi*	Bernhard Camp, WP	toepad	1939	WGS	yes	yes	yes	yes	yes	yes
AMNH:Birds:808986	*megarhyncha*	*megarhyncha*	Okapa, PNG	toepad	1965	WGS	yes	yes	yes	yes	yes	yes
ANWC:Birds:B02192	*megarhyncha*	*megrhyncha*	Western Highlands, PNG	toepad	1963	WGS	yes	yes	yes	yes	yes	yes
ANWC:Birds:B04293	*megarhyncha*	*megarhyncha*	Morobe, PNG	toepad	1966	WGS	yes	yes	yes	yes	yes	yes
ANWC:Birds:B25307	*megarhyncha*	*megarhyncha*	Wagau, PNG	toepad	1973	WGS	yes	yes	yes	yes	yes	yes
ANWC:Birds:B25652	*megarhyncha*	*sellamontis*	Huon Peninsula, PNG	toepad	1973	WGS	yes	yes	yes	yes	yes	yes
ANWC:Birds:B26140	*megarhyncha*	*sellamontis*	Huon Peninsula, PNG	toepad	1973	WGS	yes	yes	yes	yes	yes	yes
YPM:Birds:91444	*megarhyncha*	*sellamontis*	Huon Peninsula, PNG	toepad	1969	WGS	yes	yes	yes	yes	yes	yes
AMNH:Birds:329539	*torotoro*	*ochracea*	Fergusson Island, PNG	toepad	1928	WGS	yes	yes	yes	yes	yes	yes
AMNH:Birds:329540	*torotoro*	*ochracea*	Fergusson Island, PNG	toepad	1928	WGS	yes	yes	yes	yes	yes	yes
AMNH:Birds:426095	*torotoro*	*flavirostris*	Fly River, PNG	toepad	1936	WGS	yes	yes	yes	yes	yes	yes
AMNH:Birds:426096	*torotoro*	*flavirostris*	Fly River, PNG	toepad	1936	WGS	yes	yes	yes	yes	yes	yes
AMNH:Birds:426121	*torotoro*	*meeki*	Wassi Kussi River, PNG	toepad	1937	WGS	yes	yes	yes	yes	yes	yes
AMNH:Birds:637460	*torotoro*	*tentelare*	Aru Islands, WP	toepad	1900	WGS	yes	yes	yes	yes	yes	yes
AMNH:Birds:637471	*torotoro*	*meeki*	Simbang, PNG	toepad	1899	WGS	yes	yes	yes	yes	yes	yes
AMNH:Birds:637511	*torotoro*	*flavirostris*	Cape York Peninsula, AU	toepad	1913	WGS	yes	yes	yes	yes	yes	yes
ANWC:Birds:B07293	*torotoro*	*torotoro*	East Sepik Province, PNG	toepad	1966	WGS	yes	yes	yes	yes	yes	yes
ANWC:Birds:B07499	*torotoro*	*torotoro*	East Sepik Province, PNG	toepad	1966	WGS	yes	yes	yes	yes	yes	yes

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