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TITLE: Speciation with gene flow across an elevational gradient in New Guinea kingfishers

Ethan Linck1\*, Benjamin G. Freeman2, John P. Dumbacher3

*1Department of Biology and Burke Museum of Natural History & Culture, University of Washington, Seattle WA, USA 98195*

*2Biodiversity Research Center and Department of Zoology, University of British Columbia, Vancouver BC, CA V6T1Z4*

*3Ornithology & Mammalogy, California Academy of Sciences, San Francisco CA, USA 94118*

*\*Corresponding Author: elinck@uw.edu; UW Biology, Box 351800, Seattle, WA 98195; (802) 777-6662*

**Abstract:** The role of environmental gradients in speciation remains contentious. While geographic isolation is assumed to be a requirement for the majority of speciation events, theory suggests speciation with gene flow is widely plausible and caution against inferring modes of divergence from current geography. Tropical mountains are strong and temporally stable environmental gradients that can promote local adaptation and population genetic structure. Pairs of closely related species with divergent elevational ranges are a ubiquitous feature of these environments and may arise from parapatric speciation, but have rarely been evaluated in a speciation genomic framework. Here we use genomic data from historical museum specimens to provide evidence for speciation with gene flow across an elevational gradient in a pair of New Guinea kingfishers. We find lowland species *Syma torotoro* and montane species *S. megarhyncha* form discrete genotypic clusters with subtle bimodal variance in phenotypic traits. Despite this, demographic inference, *D*-statistics, and phylogenetic networks indicate extensive range-wide gene flow over long time periods, with divergence concentrated in small regions of the genome shaped by positive selection. We propose these data are consistent with a “magic trait” model of ecological speciation, where selection on body size affects mate choice. Our results provide a rare validation of theoretical models of adaptive speciation and the influence of tropical thermal stability on diversification. We suggest the primary role of selection across elevational gradients is the maintenance of species boundaries in the face of incomplete reproductive isolation, a mechanism for generating high tropical biodiversity.

*Keywords*: *Syma,* speciation genomics, natural selection, tropical biodiversity, Ernst Mayr

**Significance:** Speciation without geographic isolation can occur across environmental gradients when disruptive natural selection reduces migration between locally adapted populations. Theory suggests this process is widely plausible in a range of circumstances, but empirical examples remain rare. Strong and temporally stable thermal stratification in tropical mountains can select for narrow thermal tolerances and reduce dispersal across elevations, potentially promoting this process and contributing to globally high levels of biodiversity in these environments. We provide multiple lines of evidence for speciation across an elevational gradient in a pair of New Guinea kingfisher species, which segregate by elevation, maintain species limits in the face of high levels of long-term gene flow across their range, and differ primarily in small regions of the genome shaped by positive selection. These results indicate selection across elevational gradients can maintenance of species boundaries in the face of incomplete reproductive isolation, a mechanism generating high tropical biodiversity.

**Introduction:** Adaptation across environmental gradients is ubiquitous in nature (1–9), but its role in promoting speciation remains contentious (10–13). 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 (14–16). Though a robust body of theory suggests this process is possible under a range of circumstances (17–24), its relative difficulty to achieve compared to models of speciation without gene flow combined with a lack of obvious empirical examples has led many evolutionary biologists to dismiss its relevance in natural systems (10). The sometimes-confusing language of speciation has likely exacerbated this debate (25). Despite a recent shift from the traditional emphasis on the geography of diverging populations to a focus on measuring relative rates of gene flow (10, 12, 26, 13, 27), intermediate values of migration only imprecisely map on to verbal models. Perhaps as a result, the most compelling studies of speciation without isolation have focused on host shifts, allochrony, or divergent resource specialization within a single, relatively compact deme (28–33). Yet if speciation with some degree of gene flow is common, as Nosil has argued (34), it is likely parapatric divergence that predominates, given its less stringent requirements for the strength of selection. Additional tests for its occurrence in a diverse range taxa will help clarify its plausible parameter space and spur the development of more accurate models.

Both the emphasis on the geography of speciation and early skepticism towards models of speciation with gene flow were deeply seeded by the writings German-American zoologist Ernst Mayr (35–39), 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 Northern Melanesia (35, 36). Observing that putative sister species or geographically differentiated races nearly always had non-overlapping geographic ranges, frequently on separate islands or in lowland basins separated by mountain ranges, Mayr suggested geographic differentiation in isolation was nearly always a better explanation for speciation history than divergence in sympatry, in part because it allowed Bateson-Dobzhansky-Muller Incompatibilities (40, 41) or other isolating factors to accumulate in the absence of the homogenizing influence of gene flow. Initially implicating drift as the primary evolutionary force driving speciation, his views later shifted to emphasize founder effects (42) while conceding a role for natural selection (39).

Regardless of mechanism, Mayr’s central assumption–that contemporary distribution of species can be used to infer their arrangement at the time of divergence–is intuitive, but recent theory and empirical work has somewhat undermined its generality. Models of speciation that invoke biotic interactions between diverging populations suggest it is often easier to achieve reproductive isolation in the presence of competition and an environmental gradient than in strict allopatry (21). Demographic modeling of codistributed population pairs using genomic data has similarly found that the arrangement of current species ranges fails to predict inferred rates of ancestral gene flow, a conclusion that underscores the lability of geographic ranges over evolutionary time scales (43, 44). Moreover, DNA sequence data (first from a handful of loci and now from across the entirety of the genome) 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, and species limits can be maintained by selection in the face of significant gene flow (45–48).

Ironically, the very region that was so important to Mayr’s theory and ecology and evolution more broadly (49–51) has seen comparatively little modern research in the emerging discipline of speciation genomics (but see 52), 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 (53), the importance of abiotic and biotic ecological variables in driving evolutionary processes agnostic to geography is increasingly recognized (54). Ecologists have long recognized that reduced seasonal variation in temperature drives strong thermal stratification across tropical mountainsides (54–56), a pattern reflected in high beta diversity (57–59), especially among closely related species (60, 61). Because many tropical taxa are residents appear to show low rates of dispersal and have a “slow pace of life” (62–64), strong disruptive selection could plausibly exceed migration to drive adaptive divergence.

Local adaptation across tropical elevational gradients has been documented by clines in functional genes and intraspecific population genetic structure (5, 65–68), and has been theoretically been shown to “scale up” to speciation under a scenario of niche expansion (24), but evidence remains mixed. Phylogenetic and phylogeographic comparative studies suggest this process has occurred in *Ithioma* butterflies (69, 70) and Andean amphibians and reptiles (71, 72), but in terrestrial vertebrates such as Andean birds, a broad consensus holds that elevational replacements primarily form through divergence in allopatry followed by secondary contact and displacement (59, 66, 73, 74), 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 (75, 76). The question of the relative contribution of adaptation 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: Halcyonidae) are putative sister taxa that segregate by elevation and vary only subtly in phenotype (35, 77, 78). 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 slightly larger, deeper-voiced *S. megarhyncha* (79)*.* Among New Guinea’s many elevational series, the distribution and strong morphological conservatism of *Syma* has led systematists to suggest speciation driven by colonization of montane forest (80), though Mayr discounted this possibility in New Guinea mountain birds generally (35). However, species limits and range-wide variation have never been quantitatively assessed, and observed differences, if valid, might instead reflect phenotypic plasticity or clinal variation of a single widespread lineage (73).

Here we use genome-wide DNA sequences, bioacoustic data, and morphometrics analyses to ask whether parapatric speciation across an elevational gradient occured in *Syma* kingfishers. Under this hypothesis (**Figure 1A**), we predicted 1) discrete genotypic and phenotypic clusters supporting a two-species model, rather than clinal variation; 2) evidence of extensive historical and contemporary gene flow resulting from long-term interbreeding at range margins; and 3) evidence of positive selection shaping highly divergent regions of the genome, independent of structural features that can inflate metrics of relative differentiation (81). If speciation occurred in allopatry by an alternate mechanism and current elevational distributions are the result of secondary contact and range displacement (**Figure 1B**), we predicted 1) discrete genotypic and phenotypic clusters; 2) limited gene flow restricted to recent timescales (as secondary contact prior to adaptive differentiation would collapse gene pools into a single population and intrinsic reproductive isolation would restrict the extent of introgression); and 3) no evidence of positive selection in highly divergent regions of the genome.

**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 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 (82). On average, we were able to align 83.3% of reads to the draft *Halcyon senegalensis* genome, ranging from 35.5% to 92.37%. Whole genome resequencing data had an average depth of coverage of 5.38x per sample, 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 samples and both WGS and hyRAD data had 66,917 SNPs, which was further reduced to 10,351 SNPs after trimming for linkage disequilibrium. A second matrix of whole genome resequencing 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 2**). Principal component analysis of genotypes identified elevation (and by extension current species limits) as the primary axis of genetic differentiation, with PC1 explaining 17.02% of total variance and separating *S. torotoro* and *S. megarhyncha* into discrete clusters (**Figure 2A; Figure 2B**) with little evidence of hybrid genotypes. PC1 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 2B**). Using assignments from this best-fit model as population priors, supervised inference of population structure in ADMIXTURE (83) recovered identical clusters (log-likelihood=-104066.81; cross validation error: 0.5974) with no evidence of recent hybridization (**Figure S1**). Unsupervised inference of population structure for *K=*2 through *K=*5 assigned clusters that were discordant with both species identity and geography, with high cross-validation error (0.6822-1.113), likely due to uneven sampling (84) or small total sample size.

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 (*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 2D**). 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 24 bioacoustic variables quantified in warbleR (85), 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 2E**).

A maximum likelihood phylogeny (86) from near-complete mtDNA genomes further supported the distinctiveness of *S. megarhyncha* and *S. torotoro,* but unexpectedly recovered mainland *S. torotoro* assister with a clade containing *S. megarhyncha* and insular endemic subspecies *S. t. ochracea* (**Figure 2F**). Within *S. torotoro*, a fully supported clade of four individuals from the southeast peninsula of Papua New Guinea was separated from the remaining individuals, but with little other apparent geographic structure across the tree. Because we expected the assumption of strict bifurcation underpinning many methods of phylogenetic inference to be explicitly violated by recent gene flow between species, we evaluated evolutionary relationships using resampled neighbor joining trees. Neighbor-joining trees are an empirical description of the distance matrix among samples, and their behavior under admixture is known exactly (87). We recovered the same four major clades as in our mtDNA phylogeny, with resampled neighbor joining trees revealed substantial discordance within species. Bipartitions separating *S. torotoro* from *S. megarhyncha* and *S. t. ochracea* and *S. megarhyncha* from *S. t. ochracea* were present in all 500 resampled trees. The bipartition separating four *S. torotoro* individuals from the southeast peninsula from the remained of the clade was present in 469/500 trees.

*Demographic history and introgression.*A formal test for introgression, a phylogenetic network analysis, and demographic inference indicate a long history of gene flow between *S. torotoro* and *S. megarhyncha.* Replicate ABBA-BABA tests (88) to distinguish gene flow from incomplete lineage sorting in discordant genealogies indicated widespread introgression between *S. torotoro* and each of three allopatric *S. megarhyncha* populations, with all tested pairwise comparisons showing significant median *Z* scores after correction for multiple comparisons (**Figure 3A**). Of these, the proportion of individual tests that were significant ranged from 0.74-1.0. Intriguingly, there was also evidence of introgression between *S. t. ochracea* and mainland *S. torotoro,* and *S. t. ochracea* and two out of three *S. megarhyncha* populations. There was no evidence of gene flow between the far western population of *S. megarhyncha* and far eastern *S. t. ochracea.* These results were mirrored by a distance-based phylogenetic network analysis (**Figure 3B**), which showed clear distinctions among the three lineages shaped by large reticulations, a pattern consistent with introgression over long time periods (89).

We formally evaluated alternate demographic hypotheses by fitting the empirical joint site frequency spectrum (JSFS) to the its expected distribution using *moments* v. 1.0.0, which uses ordinary differential equations to model the evolution of allele frequencies (90)**.** We tested models that shared growth in the most recent of two time periods following an initial divergence but differed in which of the two time periods migration was allowed in, if any (**Figure S2**). Across all parameter optimizations, a model of isolation with migration (IM; e.g. parapatric speciation with incomplete reproductive isolation) had the highest log-likelihood (-538.72), followed by a model of initial isolation with migration (IIM; parapatric speciation leading to strong reproductive isolation), with a maximum log-likelihood of -552.46, and then a model of secondary contact, with a maximum log-likelihood of -565.69 (**Figure 3C**). An allopatric speciation (“strict isolation”; SI) model was poorly supported (maximum log-likelihood -723.79). Both the wide range of likelihoods across runs and large standard deviations associated with parameter values for all models (**Table S1**) indicated difficulty reaching a global optima and / or poor fits for a more complex scenario involving selection.

Parameter estimates varied widely across models, but broadly agreed in indicating greater ancestral than contemporary population sizes, larger populations in lowland species *S. torotoro,* and greater gene flow from *S. torotoro* to *S. megarhyncha* than the reverse(approximately 2-17 migrants per generation versus 0-2, respectively). Historical effective population size trajectories inferred in SMC++ (91) were nearly identical in both species (**Figure 3D**), likely due to the confounding effects of gene flow, but agreed with results from *moments* in indicating large ancestral effective population sizes and a pronounced bottleneck at the last glacial maximum assuming a two-year generation time (92).

*Natural selection and genomic divergence.*Analysis of WGS data suggests *S. torotoro* and *S. megarhyncha* diverge primarily in small regions of the genome shaped by positive selection. Genome scans of divergence in nonoverlapping 50 kb windows were broadly consistent with expectations of speciation with gene flow driven by disruptive natural selection, revealing globally low genetic divergence (mean *FST*=0.0445) punctuated by *FST* peaks on chromosomes 2, 5, 11, and a handful of scattered outlier regions elsewhere across the genome (**Figure 4A; Figure S3A**). The Z chromosome showed low interspecific divergence, suggesting a limited role in speciation (93), though we are unable to rule out the possibility this represents an artifact of high intraspecific diversity due to misincorporation of reads from the W chromosome in unsexed females. Correlations between summary statistics suggested a role for both structural reductions in recombination and positive selection in shaping *FST* peaks. *FST*was negatively but weakly correlated with *DXY* (*P<*1e-15, *R2=*0.0113), *πtorotoro* (*P<*1e-15, *R2=*0.1571), and *πmegarhyncha* (*P<*1e-15, *R2=*0.1208) (**Figure S3B**). *DXY* was positively and strongly correlated with *πtorotoro* (*P<*1e-15, *R2=*0.7663) and *πmegarhyncha* (*P<*1e-15, *R2=*0.7331) (**Figure S3B**). *FST* was approximately exponentially distributed, while *DXY, πtorotoro,* and *πmegarhyncha* showed higher median values (**Figure S3C**). However, a method for detecting selective sweeps based on multiple signatures (94) indicated a strong role for positive selection in generating *FST* outliers. First, the value of the composite selective sweep summary statistic *μ* was significantly greater within peaks than without(Wilcox-Mann–Whitney U test; *P<*2e-15) (**Figure 4B**). Second, the total number of candidate selective sweeps within *FST* outlier regions was enriched relative to a random subset of nonoutlier windows, given a false positive rate of 0.05 (**Figure 4C**). Lastly, empirical values for *πmegarhyncha* on chromosome 5 were significantly lower than expected under purifying selection alone (Wilcox-Mann–Whitney U test; *P<*2e-15), based on values generated by forward-time simulations (95) (**Figure 4D**).

**Discussion:** Speciation across environmental gradients is commonly invoked in explanations of high tropical species richness and the latitudinal biodiversity gradient (73, 96–98). Tropical elevational gradients have high levels of temporally stable thermal stratification (55) that has been linked to selection for narrow thermal physiologies (56), reduced upslope or downslope dispersal (54), and high beta diversity (58). As a result, they have frequently been studied as a stage for this process (35, 59, 73, 66, 67, 72, 99). Yet previous comparative phylogenetic studies have overwhelmingly supported models of divergence in allopatry followed by secondary contact and range displacement, particularly in vertebrates (73, 74, 99). Our finding of well-defined species limits (**Figure 2**) in the face of extensive historical and contemporary introgression (**Figure 3**) 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.

We believe *Syma* provides a rare example of speciation with gene flow driven by niche expansion and disruptive selection across an elevational gradient (24), rather than allopatric speciation by another mechanism. (This could occur either within New Guinea or on a neighboring island, as potentially suggested by the unexpected sister relationship of insular *S. t. ochracea* and *S. megarhyncha.*)Multiple lines of evidence difficult to reconcile with 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 2D, 2E**), is largely thought to reflect ecological adaptation in allopatric populations (100, 101) but may arise as a result of character displacement from competition in populations experience secondary contact (102). However, this presumes sufficient reproductive isolation has developed to permit coexistence without genetic homogenization and the fusion of lineages. In *Syma,* *D*-statistics in replicated pairwise comparisons across *Syma’s* full range, (**Figure 3A**), demographic inference (**Figure 3C**), a phylogenetic network analysis (**Figure 2B**), and genome scans (**Figure 4A**) reveal extensive shared genetic variation from introgression that suggests few intrinsic incompatibilities and weak postzygotic isolation when lineages come in to contact. Further, several lines of evidence suggest gene flow has occurred for long periods: extensive reticulation in a phylogenetic network paired with an apparent absence of individuals with recent hybrid ancestry in our dataset (89) (**Figure 3B**), provisional support for an isolation-with-migration model (**Figure 3C**), and significant introgression between mainland *S. torotoro* and insular endemic *S. t. ochracea,* most easily explained by the sorting of introgressed loci following its split with *S. megarhyncha*.

However, we emphasize that parapatric speciation and (and its vaguer, spatially agnostic variant “speciation with gene flow”, which is used here given its current popularity) 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 (103, 104). More likely than a “pure” model of parapatric speciation and equally consistent with our data are 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 (105). 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* (61, 106), in contrast to earlier studies (79) and suggesting effective allopatry at short horizontal distances, likely exacerbated by the high geographic complexity of New Guinea’s mountains. We suspect cyclical gene flow is difficult to distinguish using data from site frequency spectra alone, but may leave a unique signature in the distribution of haplotype lengths, possibly reflected in extremely high gene tree discordance and the pronounced heterogenous divergence across the genome in our data (**Figure 3, Figure 4**).

Though we did not identify the targets of selection, our data suggest disruptive positive selection has lead 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 has been shown in wide range of nonmodel organisms (16, 107). Early interpretations of the pattern as a clear signature of speciation with gene flow--where *FST* peaks correlate to genes for traits under disruptive selection while low *FST*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 (81, 107–113). Using multiple signatures of selective sweeps paired with simulations to establish a false positive threshold, we found *FST* peaks were enriched for candidate sweeps in both species (**Figure 4B, Figure 4C**), with values of *π* on *S. megarhyncha* chromosome 5 (the site of one major peak) reduced below expectations under purifying selection alone. Evidence from multiple summary statistics across the same genomic windows can also help tease apart underlying mechanisms in some cases (113): for instance, low absolute differentiation (*DXY*) in genomic islands can indicate *FST* values are inflated by low intraspecific diversity due to reduced recombination, casting doubt on models of divergence with gene flow (111). Here, our data defy easy description, highlighting the limits of this approach. A handful major *FST* peaks stand out against a background of low *FST* windows, with a global average of 0.0445; several other minor peaks are interspersed across the genome (**Figure 4A**). However, *DXY* shows only a weak correlation with *FST* values (**Figure S3**): low in some *FST* peaks and high in others, consistent with 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.*

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) we suggest *Syma* represents a case of ecological speciation *sensu* Nosil and Schluter (14, 16, 114). In the absence of strong evidence for the target of disruptive selection and the mechanism that links it to reproductive isolation, several hypotheses provide plausible fits for our genomic and phenotypic data. First, morphometric data from *Syma* is consistent with Bergmann’s rule and its prediction of larger body size in cooler climate (high elevation) species *S. megarhyncha* (but see 115)*.* As one of only three kingfisher species found above 2000 m in New Guinea, and the only regional high elevation specialist, thermal physiologies across the clade may be 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 correlate with lower frequency calls, and assortative mating might act on either trait individually or in tandem (116-118). This scenario implicates a “magic trait” model of ecological speciation, where environmentally-mediated selection acts directly on a trait responsible for reproductive isolation (119). Magic trait models are among the easiest ways to achieve speciation with gene flow (16), and perhaps especially apply to birds, where intrinsic incompatibilities are rare and prezygotic isolation is thought to play a major role in speciation (120, 121).

What does speciation in *Syma* say about the relative importance of the process in generating the elevational series of congeners and by extension their significant contribution to tropical montane biodiversity (61)? First, as emphasized above, our results suggest disruptive selection across elevational gradients can effectively maintain species limits in the absence of strong intrinsic reproductive isolation, a finding with broad relevance to speciation in tropical mountains regardless of the geographic mode of divergence or levels of gene flow. Second, though indicating blanket skepticism of the role of environmental gradients in tropical diversification is unwarranted, we concur with previous authors that at least for tropical birds, divergence and allopatry followed by secondary contact likely predominates (73, 74). Still, several aspects of *Syma’s* natural history serve as a guide to where to expect similar findings: intrinsic vocalizations, a putatively conserved thermal niche, and both a small realized range and total area of suitable habitat. In particular, though New Guinea is large, geologically complex, and the second smallest island to have obvious *in situ* avian speciation (120), it is dwarfed by other tropical montane regions, which potentially increasing the odds for speciation in allopatry. In the tropical Andes, for instance, the width of the central cordillera is a sufficient barrier to gene flow to have generated unique east slope and west slope sister taxa, a phenomena largely absent (or at least yet to be described) in the New Guinea avifauna (77, 122). Furthermore, the horizontal extent of species ranges of many elevational specialists in the Andean tropical avifauna exceeds that of similarly distributed New Guinean birds, increasing the odds of allopatric divergence over major barriers such as the Marañon Valley (101) or from stochastic extinction due to low population density (79). Yet even if it remains a rare exception among birds, the conditions that make speciation across elevational gradients possible in *Syma* are more common in other taxa, and preliminary studies suggest it may be a much more common mechanism in amphibians, insects, and plants (67, 70, 72, 75). We highlight the 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 (37, 38). Though famously a skeptic of sympatric speciation and its relatives (36), he retained an appreciation of ecological factors in population divergence (39)--both 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 (52), 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 (88), 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 complicating previously simple narratives of the role of selection and gene flow in the origin of species.

**Materials and Methods:** *Reproducibility and data availability****.*** All code written for this study and all processed data matrices can be found at https://github.com/elinck/syma\_speciation/. Large data matrices are available via Dryad (pending), and raw sequence data is available via the NCBI Sequence Read Archive (pending).

*Study system***.** The Yellow-billed Kingfisher *Syma torotoro* and Mountain Kingfisher *Syma megarhyncha* (Aves: Alcedinidae), sole members of their genus, are tree kingfishers (subfamily Halcyoninae) 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 ~500m, or less commonly ~1100m (77, 79). *S. megarhyncha* is found from 600m to 2700m or higher (98). Though cited as classic example of congeneric elevational replacements occurring in parapatry (79), their elevational ranges have also been reported to either overlap (123) or be separated by a substantial gap (61, 106). Both species are omnivorous, territorial interior forest residents, and differ only in *S. megarhyncha’s* larger body size and deeper call, and the extent of black on the top of its bill in one subspecies (77, 78). Insular *S. torotoro* subspecies *S. t. ochracea* differs substantially from its conspecifics and *S. megaryncha* in call, and is intermediate in size.

*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, representing all described subspecies (**Table S1**). Using these data, we performed principal component analysis in R (124) 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 (125). We downloaded all available vocalizations from both species from xeno-canto and Cornell’s Macaulay library. We filtered these data for quality and quantified a suite of bioacoustic variables using the warbleR package v. 1.1.14 in R (85), and ran PCA with normalized variables on the output. We then fit these data to alternate species delimitation models using the same approach as with our morphological data.

*Sampling, library preparation, and DNA sequencing***.** We extracted DNA from fresh tissues (n=6) and toepad samples from historical museum specimens (n=34) from 30 individuals of *S. torotoro* (n=30) and 10 individuals of *S. megarhyncha* (n=10). These samples represented all described subspecies and the full extent of both species’ ranges in New Guinea and Australia (**Table S2**). We extracted DNA from fresh tissues using a Qiagen DNAeasy kit and the manufacturer’s recommended protocol. For historical toepad samples (collected 1877-1977), we extracted DNA using either a using a phenol–chloroform and centrifugal dialysis method (126) (for reduced representation sequencing) or a standard salt extraction protocol (for whole genome resequencing). 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 (82). 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 with a custom bioinformatic pipeline optimized for handling degraded DNA data and available at https://github.com/elinck/syma\_speciation/blob/master/pipeline.md. Briefly, we trimmed raw reads for adapters and low quality bases using bbduk from BBTools version 38.06 suite of bioinformatics tools (https://jgi.doe.gov/data-and-tools/bbtools/). We aligned these reads to an unpublished draft genome of Woodland Kingfisher *Halcyon senegalensis* from the Bird 10K Genome Project (https://b10k.genomics.cn/) using bbmap with a *k*-mer value of 12, a maximum indel length of 200 bp, and a minimum sequence identity of 0.65. We used PicardTools v. 2.17.8 (127) and GATK v. 3.6.0 (128) 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 (129). 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 (130). To complement our nuclear DNA sequences, we assembled near-complete mitochondrial genomes from all samples using a baiting and iterative mapping approach implemented MITObim v 1.9.1. (131), which we for 10 cycles using a complete mtDNA genome from close relative *Todiramphus sanctus* as a reference (132).

*Population structure inference***.** We evaluated population genetic structure within and between species using both nonparametric and model-based clustering approaches. 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 samples passing quality filters and a minimum minor allele frequency of 0.05. We plotted axes PC1 and PC2 using ggplot v. 3.1.0. After first determining small sample sizes made unsupervised inference inappropriate for our data and resulted in extremely high cross validation error (84), we implemented model-based clustering using ADMIXTURE v. 1.3.0 (83) and population priors from the *k=*3 *k-*means clustering result.

*Phylogenetic inference***.** We first constructed a consensus tree in the R package ape v. 5.1 (133), implementing Sanderson's nonparametric rate smoothing with λ = 1 using a 22,226 SNP dataset of all individuals passing quality filters with no more than 5% missing data per site, a minimum minor allele frequency of 0.05, and a minimum depth of coverage of 3x. To evaluate and visualize gene tree variance, we generated 500 additional neighbor joining trees by resampling 10,000 SNPs from the matrix with replacement, and plotted them on the consensus tree using the densiTree() function in ape. We used the same underlying distance matrix to compute a phylogenetic network using the Neighbor-Net approach (134) implemented in SplitsTree (135) with default parameters. Using near-complete mtDNA genomes from all samples, we inferred a maximum likelihood tree using RAxML through the CIPRES portal (136) using a GTR + I + G model of sequence evolution.

*Demographic inference.*We calculated the JSFS using a SNP dataset with a minimum per site depth of 3x and a minimum quality of Q=30, thinned to 1 site for every 50000 bp to reduce the influence of linkage disequilibrium. We then defined four nested models which differed by level and timing of gene flow (**Figure S1**): isolation with migration (IM), isolation with initial migration (IIM), secondary contact (SC), and strict isolation (SI). All models featured a split of an ancestral population into two daughter lineages of arbitrary size, followed by a second time period allowing population growth. After initially optimizing parameters using the ‘optimize\_log’ method, we ran 25 additional optimizations, allowing a 1-fold random perturbation of parameter values each time. We selected the model with the maximum log-likelihood and estimated parameter uncertainty using 100 bootstrapped frequency spectra and the Godambe Information Matrix. We converted parameter values to real units using a genome-wide mutation rate of 2.3 x 10-9 (137), 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 using a Godambe Information Matrix. To evaluate changes in historical effective population size through time in greater detail, we used SMC++ (91) to implement a simple demographic model accounting for distribution of variation across the genome rather than working a single genome-wide estimate of the SFS. We masked continuous stretches of homozygosity greater than 30kb, included all contigs longer than 1 x 106 bp, assumed a generation time of 2 years, and again assumed mutation rate of 2.3 x 10-9. We visualized uncertainty by refitting models to 10 bootstrap replicates per population, resampling both samples and contigs with replacement.

*Introgression.*We explicitly tested for introgression between lineages using a four taxon test implemented in ANGSD with the “doAbbababa” function, described in detail elsewhere (88, 138). Briefly, given three ingroup lineages and an outgroup with a tree topology of ((P1, P2), P3), O) and a randomly chosen haploid genome sequence, sites where P2 and P3 share a derived allele (“B”) are denoted “ABBA”, with P1 carry ancestral copy “A”. Similarly, sites where P1 and P3 share a derived allele are denoted “BABA.” As these patterns are discordant with the underlying species tree, they may reflect either incomplete lineage sorting (ILS) or introgression. Under incomplete lineage sorting, we expect approximately equal proportions of ABBA and BABA sites. Alternatively, if introgression has occurred, we expect a significant excess of either ABBA or BABA sites. We quantify deviations in the balance of ABBA/BABA using the statistic *D*, which reflects the difference in the sum of *ABBA* and *BABA* patterns across all surveyed sites divided by their total sum. A negative value of *D* reflects introgression between P1 and P3, while a positivie value of *D* reflects introgression between P2 and P3. We calculated *D* for each combination of individuals conforming to the topology (mega,ochr),toro), and assessed significance by block jackknife over 1 mbp windows. We summarized results by collapsing individual tests representing the same geographic comparisons and calculating median *Z* scores and the proportion of individual tests that were significant given a cutoff of *Z*=3.59, which is equivalent to *P*=0.005 after performing a Bonferroni correction for multiple comparisons (*n*=31). These comparisons included *S. torotoro,* insular *S. t. ochracea* and three *S. megarhyncha* populations: *S. megarhyncha* in the mountains of the Huon Peninsula, *S. megarhyncha* in the eastern Central Ranges, and *S. megarhyncha* in the far western Central Ranges.

*Genome scans and tests for positive selection***.** We assessed levels of divergence across the genome by calculating Wright’s *FST*, *DXY*, and *π*for each species in 50,000 bp nonoverlapping windows using scripts written by Simon Martin (https://github.com/simonhmartin/genomics\_general), using a VCF file filtered for a minimum Q score of 30 and a minimum depth of 3x with a total of 1,858,764 SNPs and 102,606,487 total sites. To assign chromosome identity to scaffolds and windows, we aligned the *H. senegalensis* draft genome to the chromosome-level genome assembly of *Taenopygia guttata* (139) using NUCmer in MUMmer v. 3.1 (140), allowing a maximum gap of 1000 bp and using a minimum sequence identity threshold of 10,000 bp per contig. We evaluated correlations among summary statistics using simple linear models implemented in R (124). To formally test for the signature of positive selection in the genomes of both species, we used the program RAiSD (94). We first identified a set of scaffolds hosting *FST*  outlier windows at the *P=*0.05 threshold, and then created an identically sized set non-*FST*  outlier windows using random sampling without replacement. We then created species-specific .vcf files including only these scaffolds and ran RAiSD on each. To establish a value of composite selective sweep summary statistic *μ* to use as a threshold for a false positive rate of 0.05, we used the program SFS\_CODE (95) to simulate data similar to our own but under the influence of purifying selection, outputting a .vcf file to analyze with RAiSD using the “-k 0.05” option.

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**Figures:**

**Figure 1. Simplified hypotheses for the origin of elevational elevational series.** A) Range expansion across an elevational gradient exposes a population to disruptive natural selection, leading to local adaptation that restricts migration across the gradient until reproductive isolation develops as a byproduct. B)A single population splits following a dispersal event or vicariance and develops reproductive isolation in allopatry. Following range expansion and secondary contact, niche displacement leads to separate elevational ranges.

**Figure 2. 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) Principal component analysis of genotypes, color-coded by the best fit *k-*means clustering result (*k=*3). C) Illustration of *S. megarhyncha* (top) and *S. torotoro* (bottom), by Kevin Epperly. 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 maximum likelihood phylogeny from near-complete mitochondrial genomes supports reciprocal monophyly of *S. torotoro* and a clade with *S. megarhyncha* and *S. t. ochracea.* Highlighted clades have full bootstrap support. G) A consensus neighbor-joining tree overlaid with trees bootstrapped from 10,000 loci is concordant with relationships revealed by mitochondrial data.

**Figure 3. Evidence for long-term introgression between species.** A) Median values for *Z-*scores from *D-*statistics (“ABBA-BABA tests”) in pairwise comparisons between *S. torotoro* and allopatric *S. megarhyncha* and *S. t. ochracea* populations. Comparisons are color-coded by the proportion of individual combinations in each test that were significant, ranging from 0 to 1.0 and from black to off-white. B) A phylogenetic network supports previously inferred species limits but reveals extensive reticulation. C) Demographic inference from the joint site frequency spectrum supports a model of isolation with migration, but with broad uncertainty across replicate parameter optimizations. D) Trajectories of ancestral population sizes for indicate a bottleneck at the last glacial maximum, and are likely biased by gene flow.

**Figure 4. Heterogeneous genomic divergence shaped by positive selection.** A) Relative divergence (*FST*), absolute divergence (DXY), and intraspecific diversity (*πtorotoro, and πmegarhyncha*) calculated in 50 kb nonoverlapping windows across the genome. B) Significantly higher values of selective sweep summary statistic *μ* in *FST* peaks relative to a random sample of windows. C) *FST* peaks are enriched for the total number of candidate sweeps relative to non-outlier windows. D) Values of *π* on *S. megarhyncha* chromosome 5 are reduced below expectations from purifying selection alone.