


A molecular genetic investigation into secondary contact and gene flow in island sister taxa


By

Robin M. Jones

Submitted to the graduate degree program in Ecology and Evolutionary Biology and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Master of Arts.



Chairperson Dr. Robert Moyle



Dr. Rafe Brown

Dr. Mark Holder

Date Defended: 8 July 2013

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The Thesis Committee for Robin Jones
certifies that this is the approved version of the following thesis:

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ABSTRACT

Investigations of colonization patterns have contributed to fundamental advances in evolutionary and ecological theory. Once populations have differentiated, secondary contact can lead either to the reinforcement of species boundaries or interbreeding. Its effects on the maintenance of biodiversity are not well known. Archipelagos like the Philippines are useful for studying the relationships of secondary contact because of their distinct geographic boundaries and simpler land vertebrate biota. A genus-level phylogeny of Philippine Tailorbirds (Passeriformes: Cisticolidae) inferred a deep split between two sister species on the island of Luzon. Some populations of *Orthotomus chloronotus* and *O. derbianus* co-occur parts of their range but they are phenotypically distinct. Because of that distinctiveness, these species were traditionally considered evolutionarily independent. Recently however, molecular and morphological evidence found evidence of gene flow between them (Sheldon et al. 2012). To investigate the extent of reproductive isolation between these species, molecular data was collected from populations across Luzon Island and three classes of DNA markers were sequenced: mitochondrial, autosomal, and sex-linked. The sex-linked chromosome was Green-backed *Orthotomus chloronotus* and gray-backed *O. derbianus* are paraphyletic at all loci; there is complete introgression in the sex chromosome, strong population structure in autosomal chromosomes, and strong phylogenetic structure in mitochondrial genes. These findings contradict taxonomic classification; allopatric populations of green- and gray-backed birds (respectively North and South Luzon) are genetically distinct from the sympatric population in Central Luzon. The southern population shows early signs of diverging from the rest of Luzon *Orthotomus* and coincides with the limits of the green-backed phenotype. The sympatric zone, Central Luzon, shows no signs of past or present genetic isolation, and this population is

exchanging significant amounts of alleles with North Luzon green-backed birds. The dataset finds no evidence of genes segregating by plumage in Central Luzon. This leaves unanswered questions about the emergence of the derived phenotype, whether plumage is stochastic or under selective pressure, and the amount of data necessary to statistically evaluate complicated evolutionary hypotheses in natural populations.

I. INTRODUCTION

Ecological and geographic isolating mechanisms play a vital role in speciation (Dobzhansky 1936). A traditional framework for studying avian speciation examines patterns of colonization and diversification across oceanic island archipelagos (Diamond, Gilpin et al. 1976, Mayr 1996). Island systems are convenient for evolutionary studies because these natural laboratories have simple biotas compared to continental systems and many species distributions are clearly demarcated by water barriers. The discrete range limits provide testable hypotheses regarding the mode of speciation across geographic barriers (Case and Cody 1987).

The speciation process is governed by the extent of gene flow between populations. Genetically isolated populations will diverge over time due to a combination of drift and selection (Wright 1978). The dominant paradigm of avian diversification is allopatric speciation: vicariance and/or dispersal events splitting lineages into multiple populations that subsequently diverge (Mayr 1996, Coyne and Price 2000, Grant and Grant 2009, Carling, Lovette et al. 2010, Brelsford 2011). Because birds are reticent to cross water barriers, gene flow is assumed to be negligible among populations on oceanic islands, resulting in effective pre-mating isolation (Grant and Grant 1992, Price and Bouvier 2002, Mendelson 2003). However recent evidence confirms that gene flow can occur at any stage of the speciation process, even on oceanic islands (Petren, Grant et al. 2005, Warren, Bermingham et al. 2012). Sympatry after allopatric divergence is known as secondary contact. Quantification of post-divergence gene flow is especially important in avian systems because birds have some of the greatest potential for hybridization; hybrid inviability between bird species may take as long as 20 million years to evolve (Wilson, Maxson et al. 1974, Prager and Wilson 1975, Fitzpatrick 2004). When

populations begin exchanging genes after a period of allopatry it has little-known consequences for biodiversity.

Theoretical and empirical studies of secondary contact between independently evolving populations illustrate a range of possible outcomes, from reinforcement to fusion (Barton 2001, Mayr and Diamond 2001, Mank, Carlson et al. 2004, Hermansen, Saether et al. 2011, Pettengill and Moeller 2012). Significant phenotypic and behavioral differences may reinforce divergence when species come into contact (Grant and Grant 2009, Hermansen, Saether et al. 2011). Secondary contact can create new independent lineages by increasing additive genetic variation in the contact zone if hybrid individuals have higher fitness. (Fitzpatrick 2004, Rheindt and Edwards 2011). Conversely secondary contact between distinct groups can homogenize differences accrued in allopatry. An extreme example is an introgressive sweep: the merging of two distinct populations into one. Studying the the genetic consequences of secondary contact is especially important in light of shifting species boundaries induced by climate change.

Secondary contact has been investigated in several island groups (Grant and Grant 2009, Brown, Jordan et al. 2011, Warren, Bermingham et al. 2012). An increasingly well-studied system for investigating the processes of insular diversification is the Philippine archipelago (Brown and Diesmos 2009). The archipelago harbors many endemic species but their geographic and phylogenetic origins vary across taxa. The Philippines' well-documented geologic history and diverse geography (Hall 1998, Hall 2002) provide an ideal backdrop for examining both inter- and intra-island diversification. The country contains islands that vary in size and complexity, and deep-water barriers circumscribe many species limits. This geographic complexity may have contributed to the high levels of biodiversity and endemism in the

archipelago and its designation as a biodiversity hotspot (Myers, Mittermeier et al. 2000, Brown and Diesmos 2009).

Luzon Island, the largest in the Philippines, has many endemic species and subspecies (Kennedy, Gonzales et al. 2000). It is topographically complex with a narrow land bridge separating the southern tip, the Bicol Peninsula, from the rest of the island. The peninsula is a volcanic arc that rafted into proto-Luzon approximately 3.5 MYA (Heaney, Balete et al. 1999). Modern-day Luzon contains dramatic atmospheric and environmental gradients that have given rise to heterogeneous microclimates and ecological communities. Its steep mountain ranges, lowland valleys, and geographic bottlenecks delineate avian, reptilian, amphibian, and mammalian ranges. However, these ranges are not coterminous across taxa (Esselstyn, Garcia et al. 2008, Siler, Diesmos et al. 2011, Welton, Diesmos et al. 2012, Hosner, Nyari et al. 2013), emphasizing the need for molecular phylogenetic studies of more endemic Philippine radiations. Time-calibrated phylogenetic studies can be compared to geologic and tectonic events to unearth the processes leading to the various spatial patterns of evolutionary diversification within Luzon.

Two co-occurring species within the avian genus *Orthotomus* (tailorbirds) provide the opportunity to examine avian diversification on Luzon Island. Tailorbirds are small, insectivorous passerines that occur throughout Southeast Asia. The evolutionary relationships of this group have been uncertain, until Sheldon et al. (2012) published a molecular phylogeny of lowland tailorbirds. This study suggested the Philippine Tailorbird (*O. castaneiceps*) was paraphyletic, so the two subspecies (*O. c. castaneiceps* and *O. c. chloronotus*) were raised to the rank of full species. The Sheldon et al. study reconstructed *O. derbianus* as the sister taxa to *O. chloronotus*. *Orthotomus chloronotus*, endemic to North and Central Luzon, is now known as the Trilling Tailorbird. The Gray-backed Tailorbird, *O. derbianus*, also a Luzon endemic, occurs in

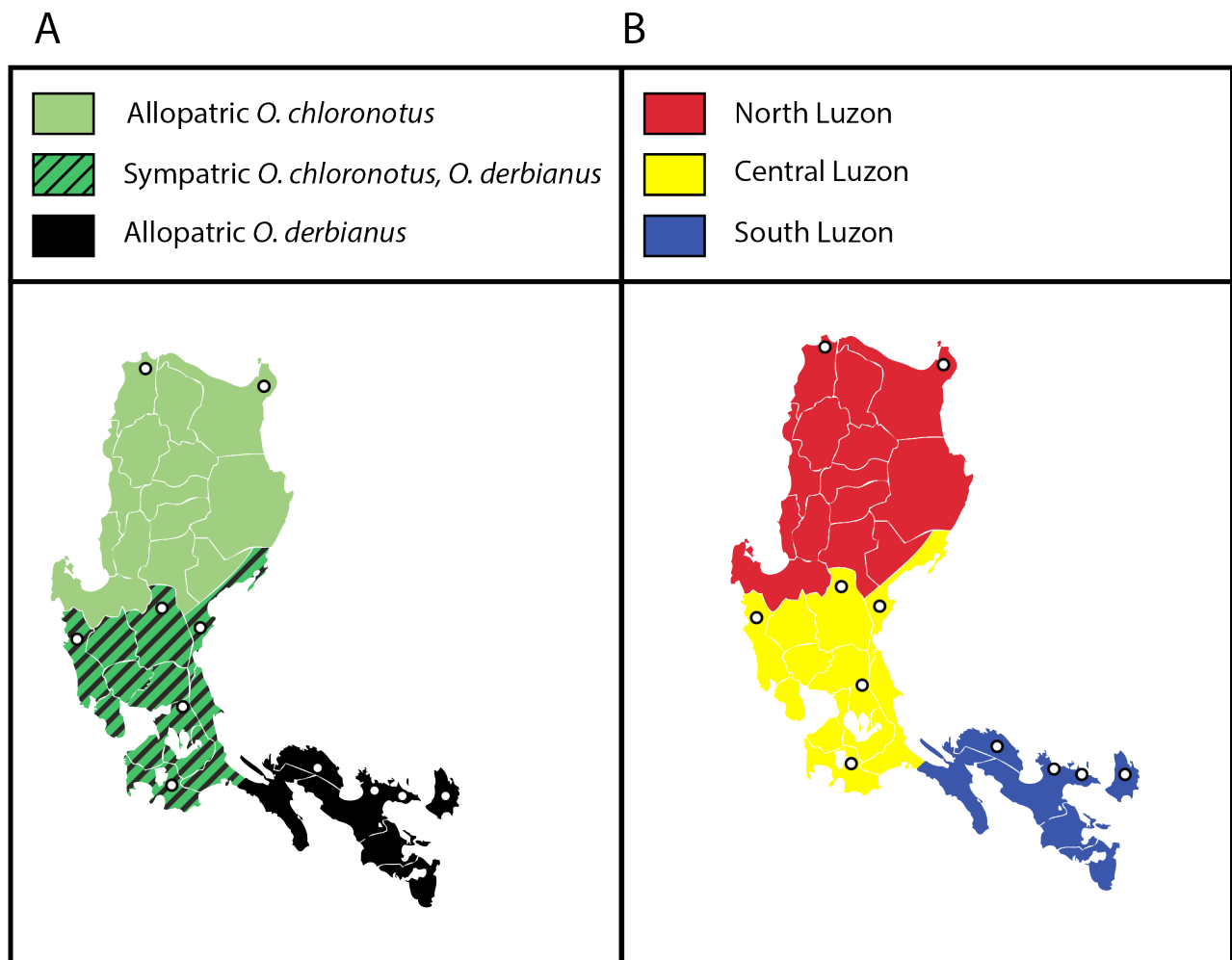
Central and Southern regions of the island. Due to phenotypic distinctiveness *O. chloronotus* and *O. derbianus* are considered separate species by most taxonomists: *O. chloronotus* is green-backed while *O. derbianus* is gray-backed. This plumage difference implies an independent geographic origin of green-backed and gray-backed birds and that Central Luzon's sympatric populations resulted from secondary contact. Avian sister taxa on islands are exceedingly rare, and any potential cases of this phenomenon deserve further study (Diamond 1977, Coyne and Price 2000).

Figure 1:

A) Distribution of *Orthotomus chloronotus* and *O. derbianus*

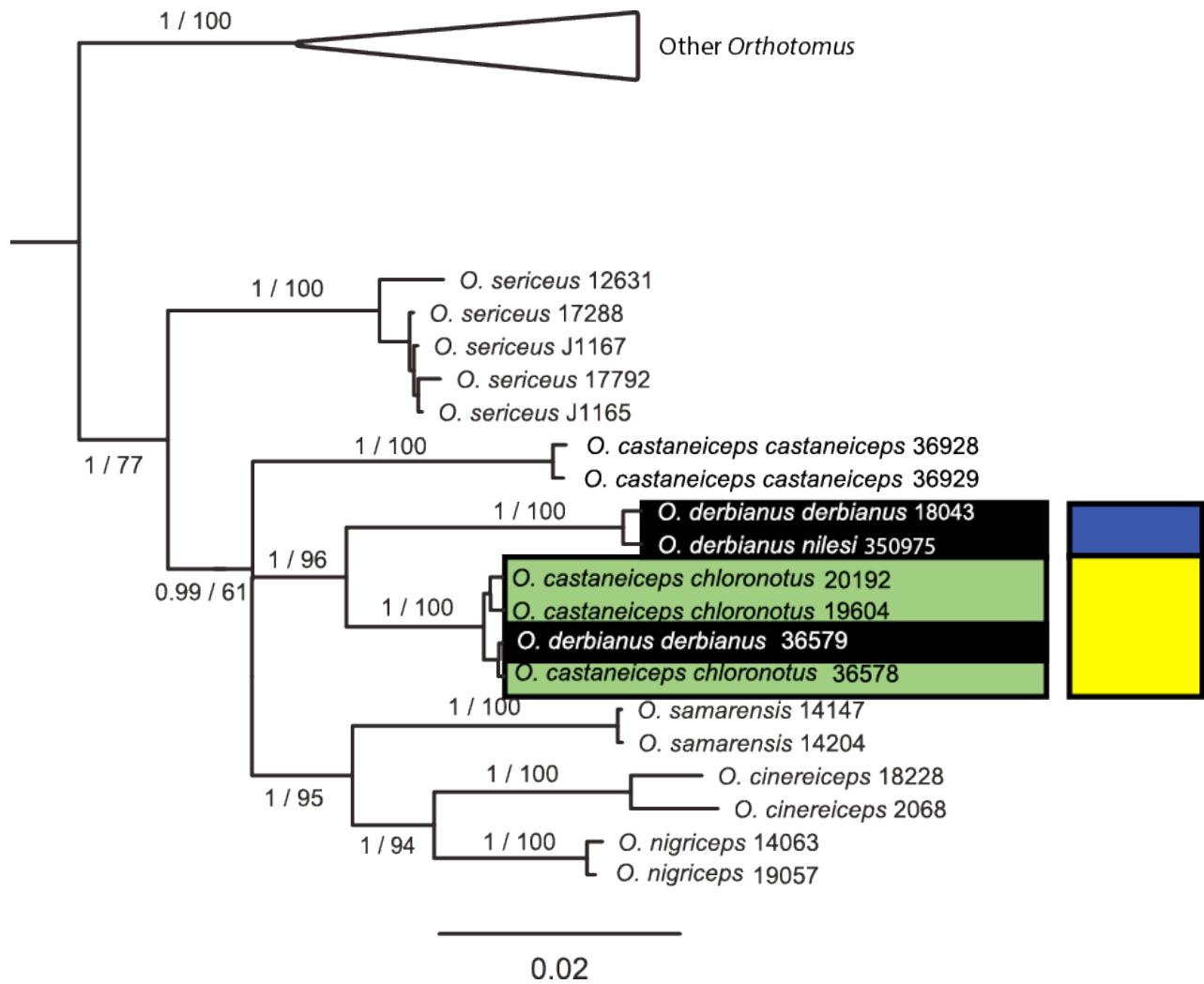
B) Geographic areas of Luzon

Sampling localities in white



Because conflicting literature has alternately described these birds as interbreeding or evolutionarily independent (Delacour and Mayr 1946, Gilliard 1950, Parkes 1971), the Luzon endemics' recently discovered sister relationship provide an ideal system to study the genetic consequences of secondary contact in island birds. Sheldon et al. found substantial mitochondrial distance between the two species (9%), evidence of prolonged isolation. But one individual from the sympatric zone was misclassified. A gray-backed bird was found in the same mitochondrial clade as those with green backs. Additionally a small number of museum specimens exhibit an intermediate green-gray plumage (Parkes 1971, Gilliard 1950, pers. obs. Appendix). Genotypic and phenotypic intermediacy is a classic sign of hybridization, raising questions about the current level of reproductive isolation between these taxa.

Figure 2: Sheldon et al. 2012 phylogeny of Philippine tailorbirds



Phenotype	Geography
 <i>chloronotus</i>	 North
 <i>derbianus</i>	 Central
	 South

Study design: To further examine the evolutionary relationship between *O. chloronotus* and *O. derbianus* and assess the extent of their genetic isolation, data was collected for a molecular reconstruction of relationships across Luzon Island. Multiple gene regions (mitochondrial, autosomal, sex-linked) were sequenced from spatially distributed populations to evaluate the relative support for three evolutionary histories that leave a distinct genetic signal: 1) Hybridization—interbreeding following allopatry; 2) reproductive isolation—no interbreeding after allopatry; and 3) panmixia—interbreeding with no evidence of past allopatry. Hybridization results in admixture of green and gray parental alleles in the sympatric zone, full reproductive isolation clusters birds according to green or gray plumage, and panmixia groups green- and gray-backed birds without regard to plumage. These relationships may differ between different populations, i.e. a pair of populations may be hybridizing while another set of two populations are diverging.

Hybridization, recent divergence, and population structure with contemporaneous migration are not always distinguishable (Holder, Anderson et al. 2001, Yu, Than et al. 2011). This is problematic because many recently-diverged populations continue to exchange genes and if gene flow is truly diminished, slow mutation rates make recent divergence difficult to detect. Gene flow after divergence is assumed to be negligible in current tests of recent divergence because it is computationally difficult to implement and it contradicts traditional views of allopatric speciation, especially on islands. Frameworks are emerging to tease apart these processes (Hey and Nielsen 2007, Joly, McLenachan et al. 2009, Kubatko 2009, Liu, Yu et al. 2009, Kubatko, Gibbs et al. 2011, Pettengill and Moeller 2012) but they were not used in this study due to insufficient data for the number of pairwise comparisons in populations required for exhaustive explorations of gene flow accounting for both geography and phenotype.

II. MATERIALS AND METHODS

DNA sequencing: Ingroup sampling consisted of 47 individuals (25 green-backed, 18 gray-backed, 4 intermediate) from eleven provinces across Luzon (Figure 1, Table 7). All DNA samples were extracted from tissue samples associated with vouchered museum specimens. Tissue samples came from the University of Kansas Natural History Museum (KUNUM), the the Field Museum (FMNH), and the Cincinnati Museum Center (CMC). In order of decreasing relatedness, for outgroups I used two individuals of *O. castaneiceps* from the western Visayan island of Panay and one *O. frontalis* from Dinagat (Sheldon, Oliveros et al. 2012). For FMNH and CMC-vouchered specimens (40 and 2 individuals, respectively), genomic DNA was extracted from frozen or alcohol-preserved muscle tissue using the noncommercial guanidine thiocyanate method of (Esselstyn, Garcia et al. 2008). DNA from nine buffer-preserved tissues provided by FMNH was extracted by digestion with proteinase K at 55°C for 12h, one round of phenol-chloroform purification and one round of chloroform purification. All samples of purified DNA were precipitated in 100% EtOH, washed in 70% EtOH, left to dry until the EtOH was fully evaporated and subsequently dissolved in Tris-ETDA buffer.

Table 1: Samples

Species	Voucher	Province	Locality
Outgroup:			
<i>Orthotomus frontalis</i>	KUNHM 14056	Dinagat	Dinagat Island, Municipality of Lorento
<i>O. castaneiceps</i>	KUNHM 15296	Antique	Panay Island, Municipality of San Remigio
<i>O. castaneiceps</i>	KUNHM 15330	Antique	Panay Island, Municipality of San Remigio
Ingroup:			
<i>O. chloronotus</i>	KUNHM 19604	Aurora	Luzon Island, Municipality of San Luis
	KUNHM 20192	Aurora	Luzon Island, Aurora Memorial National Park
	KUNHM 20236	Aurora	Luzon Island, Aurora Memorial National Park
	KUNHM 20255	Aurora	Luzon Island, Aurora Memorial National Park
	KUNHM 20302	Aurora	Luzon Island, Aurora Memorial National Park
	KUNHM 20307	Aurora	Luzon Island, Aurora Memorial National Park
	KUNHM 21773	Aurora	Luzon Island, Municipality of Casiguran
	PMNH 27132	Aurora	Luzon Island, Municipality of Baler, ASCOT campus
	KUNHM 25591	Ilocos Norte	Luzon Island, Municipality of Adams, Mt. Pao
	KUNHM 25596	Ilocos Norte	Luzon Island, Municipality of Adams, Mt. Pao
	KUNHM 25615	Ilocos Norte	Luzon Island, Municipality of Adams, Mt. Pao
	KUNHM 25626	Ilocos Norte	Luzon Island, Municipality of Adams, Mt. Pao
	KUNHM 25691	Ilocos Norte	Luzon Island, Municipality of Adams
	KUNHM 25711	Ilocos Norte	Luzon Island, Municipality of Adams
	KUNHM 25776	Ilocos Norte	Luzon Island, Municipality of Adams
	KUNHM 25801	Cagayan	Luzon Island, Municipality of Gonzaga Mt. Cagua
	KUNHM 25821	Cagayan	Luzon Island, Municipality of Gonzaga Mt. Cagua
	KUNHM 25827	Cagayan	Luzon Island, Municipality of Gonzaga Mt. Cagua
	KUNHM 25867	Cagayan	Luzon Island, Municipality of Gonzaga Mt. Cagua

Species	Voucher	Province	Locality
	KUNHM 25912	Cagayan	Luzon Island, Municipality of Gonzaga Mt. Cagua
	KUNHM 25932	Cagayan	Luzon Island, Municipality of Gonzaga Mt. Cagua
	KUNHM 25944	Cagayan	Luzon Island, Municipality of Gonzaga Mt. Cagua
	KUNHM 25976	Cagayan	Luzon Island, Municipality of Gonzaga Mt. Cagua
	KUNHM 25984	Cagayan	Luzon Island, Municipality of Gonzaga Mt. Cagua
	KUNHM 25987	Cagayan	Luzon Island, Municipality of Gonzaga Mt. Cagua
	PMNH 27048	Batangas	Luzon Island, Municipality of Lipa
	FMNH 454992	Nueva Vizcaya	Luzon Island, Municipality of Quezon
	FMNH 454993	Nueva Vizcaya	Luzon Island, Municipality of Quezon
	CMNH 36578	Zambales	Luzon Island, Municipality of Masinloc
<i>O. derbianus derbianus</i>	KUNHM 21753	Aurora	Luzon Island, Municipality of San Luis
	KUNHM 20163	Aurora	Luzon Island, Aurora Memorial National Park
	KUNHM 20208	Aurora	Luzon Island, Aurora Memorial National Park
	KUNHM 20250	Aurora	Luzon Island, Aurora Memorial National Park
	KUNHM 20326	Aurora	Luzon Island, Municipality of Baler ASCOT campus
	FMNH 472786	Rizal	Luzon Island, Municipality of Rodriguez
	CMNH 36579	Zambales	Luzon Island, Municipality of Masinloc
	KUNHM 18043	Camarines Norte	Luzon Island, Mt. Labo
	KUNHM 18088	Camarines Norte	Luzon Island, Mt. Labo
	FMNH 462049	Camarines Sur	Luzon Island, Municipality of Lagonoy
	FMNH 462050	Camarines Sur	Luzon Island, Municipality of Lagonoy
	FMNH 462051	Camarines Sur	Luzon Island, Municipality of Caramoan, Caramoan National Park
	FMNH 462052	Camarines Sur	Luzon Island, Municipality of Caramoan, Caramoan National Park

Species	Voucher	Province	Locality
	FMNH 462053	Camarines Sur	Luzon Island, Municipality of Caramoan, Caramoan National Park
<i>O. derbianus nielsi</i>	FMNH 350975	Catanduanes	Catanduanes Island, Municipality of Gigmoto
Intermediate	KUNHM 18038	Camarines Norte	Luzon Island, Mt. Labo
	KUNHM 19605	Aurora	Luzon Island, Municipality of San Luis
	KUNHM 19611	Aurora	Luzon Island, Municipality of San Luis
	KUNHM 20336	Aurora	Luzon Island, Municipality of Baler, ASCOT campus

I amplified and sequenced eleven distinct loci distributed across the nuclear and mitochondrial genomes using published primer sets (Table 2). Eleven unlinked markers spread across the avian genome were used: four sex-linked (Z-linked in avian systems), six autosomal, and two mitochondrial genes (mtDNA) concatenated into one locus. Target loci were amplified using a touchdown polymerase chain reaction (PCR) with an initial 120s at 95°C; five cycles of 20s at 95°C, 15s at 58°C, 30s at 70°C; five cycles of 20s at 95°C, 15s at 54°C, 30s at 70°C; ten cycles of 20s at 95°C, 15s at 50°C, 30s at 70°C; a final extension phase of 240s at 70°C; and a holding temperature of 12°C. Amplified PCR products were visualized on 2% high melt agarose gels stained with GelRed (Phenix Research Products), and successful reactions were cleaned with a 10% exonuclease 1–shrimp alkaline phosphatase (ExoSAP) solution. Cycle-sequencing took place with ABI Prism BigDye terminator chemistry with an initial 30s at 105°C and 25 cycles of 180s at 95°C, 15s at 50°C, 240s at 60°C; and a holding temperature of 4°C (Ver. 3.1; Applied Biosystems, Foster City, CA). These products were purified with 70% EtOH and a 40m spin down at 13000 RPM, and sequenced on an ABI Prism 3730xl Genetic Analyzer automated sequencer (Applied Biosystems, Foster City, CA). Contigs were aligned using Sequencher v4.9 (GeneCodes Corp., Ann Arbor, MI). All sequences were verified by visual inspection of chromatograms.

Phylogenetic analysis: Sequence data were divided into eleven partitions: six autosomal introns, four Z-linked introns, and two mitochondrial genes (first combined into a single partition). The most appropriate evolutionary models of nucleotide substitution were chosen for each partition using MrModeltest 2.3 (Nylander 2004), comparing models under the Akaike Information Criterion, using likelihood scores from PAUP* (Swofford 2002). Uncorrected p-

distance matrices for concatenated mitochondrial loci (ND2, ND3) and concatenated nuclear loci (all others) were calculated using PAUP* v4.0b10 .

Two independent maximum likelihood (ML) searches of ten runs were conducted in Garli 2.0 (Zwickl 2006) for 1) the concatenated eleven locus dataset including nuclear (nDNA) and mitochondrial (mtDNA) sequences, 2) the ten locus nuclear (nDNA) dataset, 3) the four-locus Z-linked nuclear dataset, and 4) the six-locus autosomal nuclear dataset. Clade support was evaluated with 100 nonparametric bootstrap replicates also conducted within Garli. Bayesian analysis (BA) using a Markov Chain Monte Carlo (MCMC) sampling algorithm was conducted in MrBayes 3.2.1 (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003, Ronquist, Teslenko et al. 2012). Two independent runs of 20 million generations were conducted with default chain-heating conditions. Parameters were sampled every 2000 generations. Additionally, each intron was analyzed separately, using identical ML search parameters and bootstrapping in Garli and BA settings in MrBayes, with the exception of running 10 million instead of 20 million generations for locus-by-locus Bayesian searches. The distribution of Bayesian posterior probabilities were examined with Tracer 1.5 (Drummond and Rambaut 2007), and convergence diagnosis required average standard deviation of split frequencies less than 0.01. Nonparametric bootstrap support was mapped onto the BA consensus tree using SumTrees v3.3.1, part of the DendroPy computing library v3.12.0 (Sukumaran and Holder 2010), and visualized with FigTree v1.3.1 (Drummond and Rambaut 2007).

Population genetic analysis: Genotypic data was phased into alleles using PHASE v2.0 (Stephens, Smith et al. 2001) as implemented in DnaSP (Rozas, Sánchez-DelBarrio et al. 2003, Librado and Rozas 2009). All subsequent analyses were conducted on phased alleles. F_{ST} , summary statistics of nucleotide diversity, and neutrality statistics (Tajima's D , Fu and Li's D^* ,

Fu and Li's F^*) were calculated using DnaSP (Tables 4 and 5) using proper specification of Z-linked and autosomal loci. A Mantel test in R tested for isolation by distance in each locus using uncorrected p-distances calculated in PAUP* and latitude-longitude coordinates for each sampling locality.

Population structure was investigated in Structure 3.2.4 (Pritchard, Stephens et al. 2000, Hubisz, Falush et al. 2009) for nuclear data, which were separated into three runs: autosomal, Z-linked, and the combined nDNA. All runs consisted of ten replicates of a 500000 step burn-in with 2 million subsequent steps, comparing likelihoods for assumptions of 1–6 populations. The number of population clusters were calculated using (Evanno, Regnaut et al. 2005)'s algorithm. Minimum spanning haplotype networks were created in PopART v1.2 beta (J. W. Leigh, available at <http://leigh.geek.nz/software.shtml>).

Coalescent analysis: Migrate v3.3.2 (Beerl and Felsenstein 1999, Beerli 2009) estimated migration patterns using coalescent methods. Recombination is a violation of Migrate's model, so the four-gamete test (Hudson and Kaplan 1985) was used to test for historic signs of recombination. When detected, the locus was truncated to the fragment containing the most informative sites. For known females (hemizygous at Z), only one Z-linked allele was included per locus. Mutations rate for each locus was estimated by Watterson's per-sequence estimator, and inheritance scalars were set to 1.0 for autosomal loci, 0.75 for Z-linked loci, and 0.25 for mtDNA. Immigration rates followed a stepping stone model, estimating migration between North/Central Luzon and Central/South Luzon for a total of 4 estimated migration rates. Priors for θ (effective population size) were uniform with range 0.0–0.1, and priors for migration rate were drawn from a uniform distribution with range 0.0–4500. Two independent runs of four

chains ran in parallel incrementally heated at 1.0, 1.2, 3.0, and 500000 recorded steps. There was a burn-in of 10000 steps.

III. RESULTS

Sequence attributes: Alignment of sequence data resulted in a concatenated dataset 6836 base pairs (bp) in length: 3548bp autosomal nuclear loci ranged from 324–818bp, Z-linked loci 420–535bp, and the combined mitochondrial locus was 1492bp long. For the autosomal loci, the total percentage of variable sites ranged from 0.92–3.18%. In Z-linked loci, variable sites were 0.35–0.42%. Mitochondrial genes had 9.99% variable sites (Table 2).

Table 2: Locus information

Locus	Length (bp)	Type, chromosome	Substitution model	GC content	Informative sites, %total	Source
CDC132	709	intron, 2	GTR+I	0.368	5, 0.71%	(Backström, Fagerberg et al. 2008)
G3PDH	324	intron, 1	HKY	0.525	3, 0.92%	(Fjeldså, Zuccon et al. 2003)
HMG2	535	intron, 4	GTR+I	0.585	17, 3.18%	(Backström, Fagerberg et al. 2008)
PepCK	672	intron, 20	GTR+I	0.399	13, 1.93%	(Sorenson, Oneal et al. 2003)
Rhodopsin	818	intron, 12	K2P+I	0.501	12, 1.47%	(Kimball, Braun et al. 2009)
Vimentin	489	intron, 2	JC	0.368	5, 1.02%	(Kimball, Braun et al. 2009)
CHD1Z	554	intron, Z	HKY+I	0.354	14, 2.53%	(Fridolfsson and Ellegren 1999)
GPBP	470	intron, Z	F81	0.359	4, 0.85%	(Backström, Brandstrom et al. 2006)
MUSK	485	intron, Z	F81	0.361	2, 0.41%	(Kimball, Braun et al. 2009)
PTCH	420	intron, Z	F81	0.419	2, 0.47%	(Borge, Webster et al. 2005)
ND2+ND3	1041+351	exons, mitochondrial	GTR	0.445	139, 9.99%	(Sorenson, Ast et al. 1999)

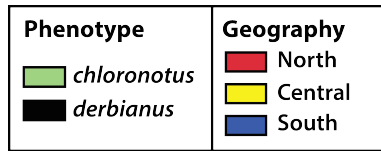
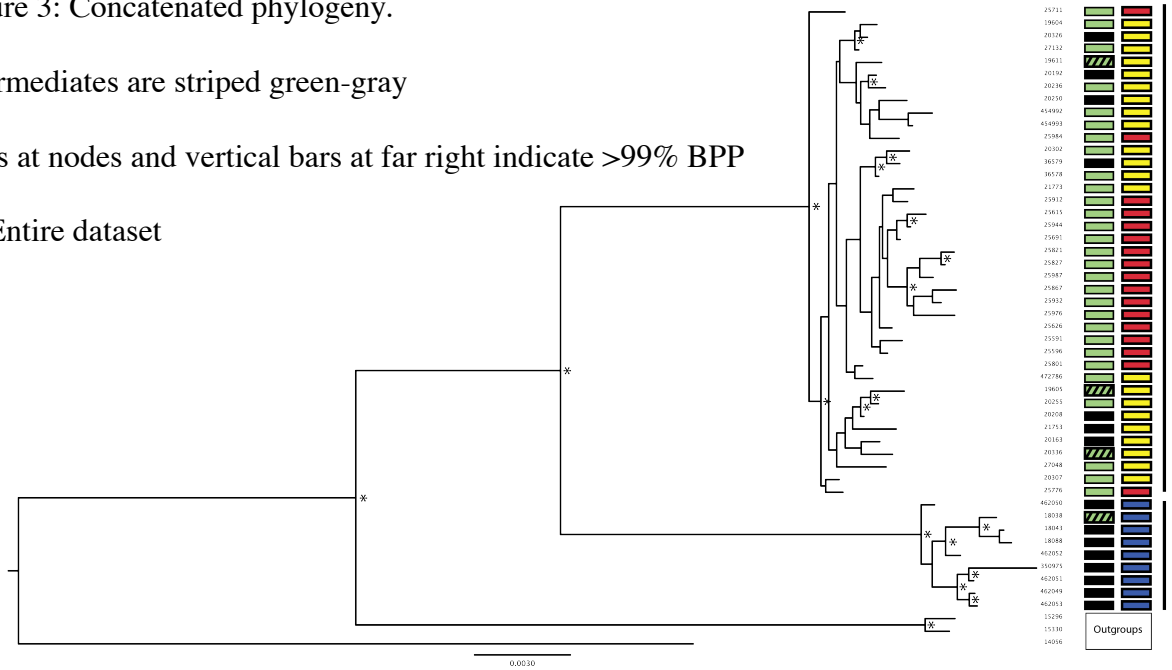
Gene tree and species tree reconstruction: For the concatenated dataset, Bayesian and ML analyses recovered a deep, well-supported divergence between North/Central Luzon (all green-backed and Central gray-backed birds) vs. Southern Luzon's isolated gray-backed birds (Figure 3). *Orthotomus derbianus nielsi*, an endemic subspecies of Luzon's satellite island Catanduanes, was nested within South Luzon in all analyses. No mitochondrial subdivision between the clade comprising North green-backed, Central green-backed, and Central gray-backed birds received meaningful node support. Substantial mtDNA divergence separated South gray-backed birds from all other samples, this locus provided most of the phylogenetic signal in the partitioned dataset. The clear divide between South Luzon vs. all others was not recovered in analyses of nuclear DNA (Figure 4). No geographic pattern was detected within the South samples, but four South samples were reciprocally monophyletic compared to the others, with the other four samples nested within the northern clade. Within this northern clade there was no significant nodal support grouping localities or plumage types. The autosomal phylogeny also supported four Bicol samples as sister to all others. There was no support for population splits within Luzon for Z-linked loci (Figure 5).

Figure 3: Concatenated phylogeny.

Intermediates are striped green-gray

Stars at nodes and vertical bars at far right indicate >99% BPP

A) Entire dataset



B) Luzon

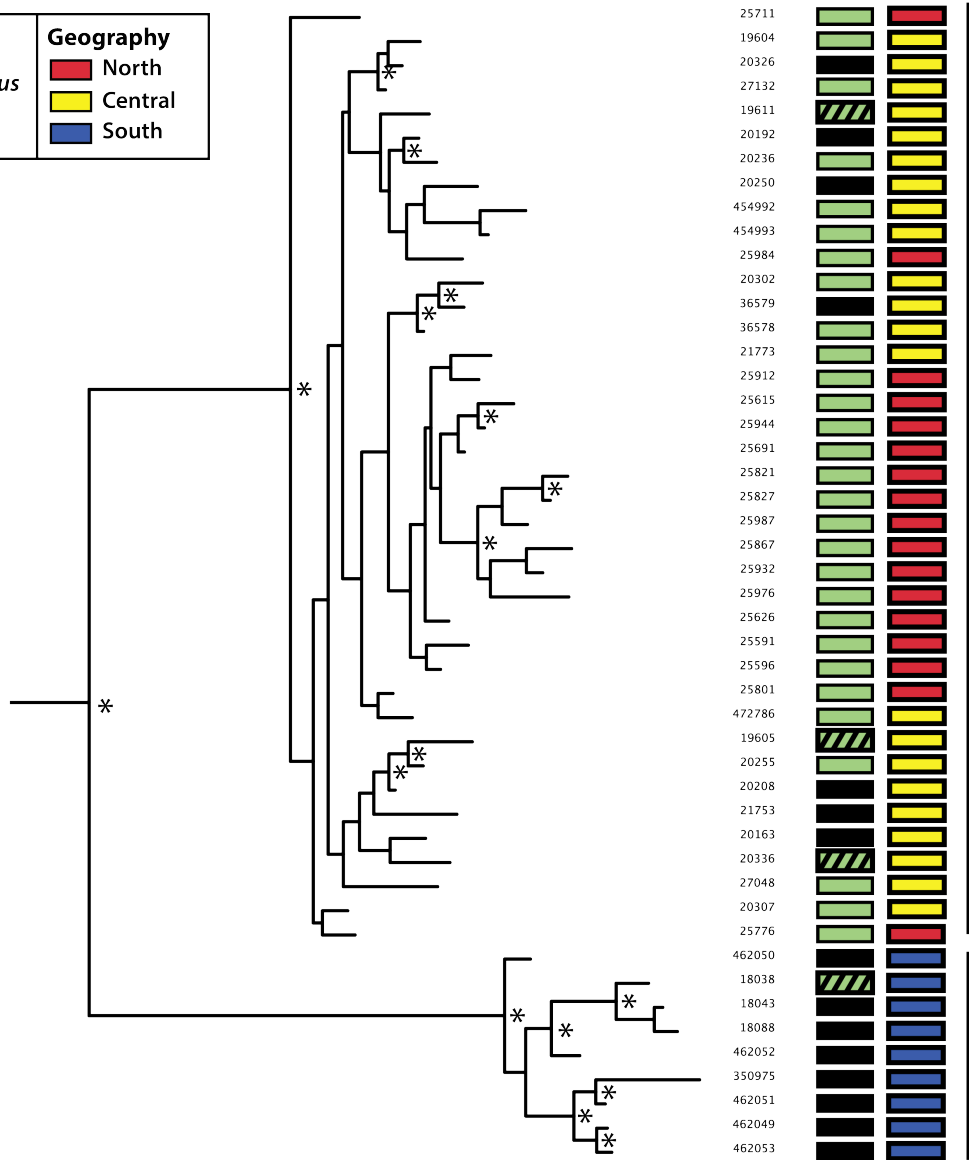
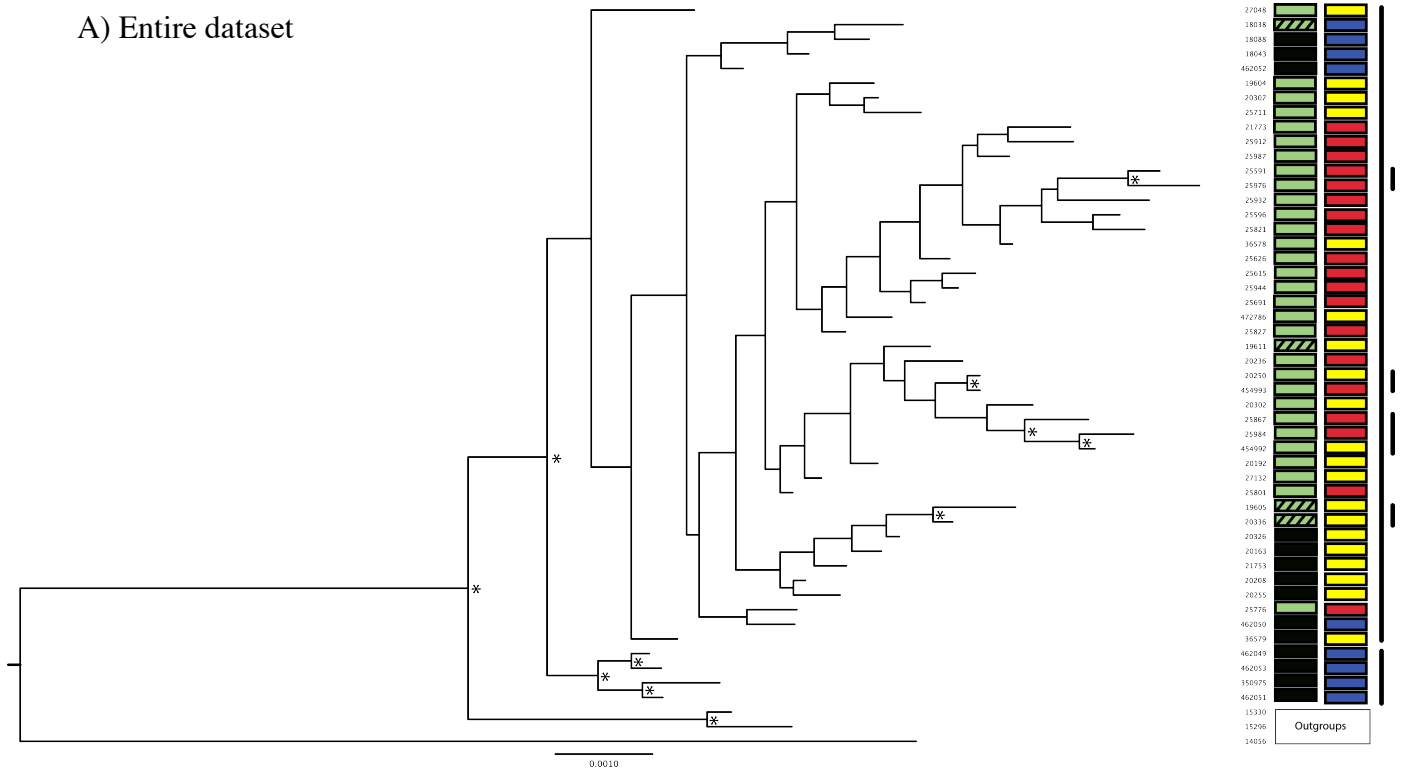


Figure 4: Nuclear phylogeny

Intermediates are striped green-gray

Stars at nodes and vertical bars at far right indicate >99% BPP

A) Entire dataset



Phenotype	Geography
<i>chloronotus</i>	North
<i>derbianus</i>	Central
	South

B) Luzon

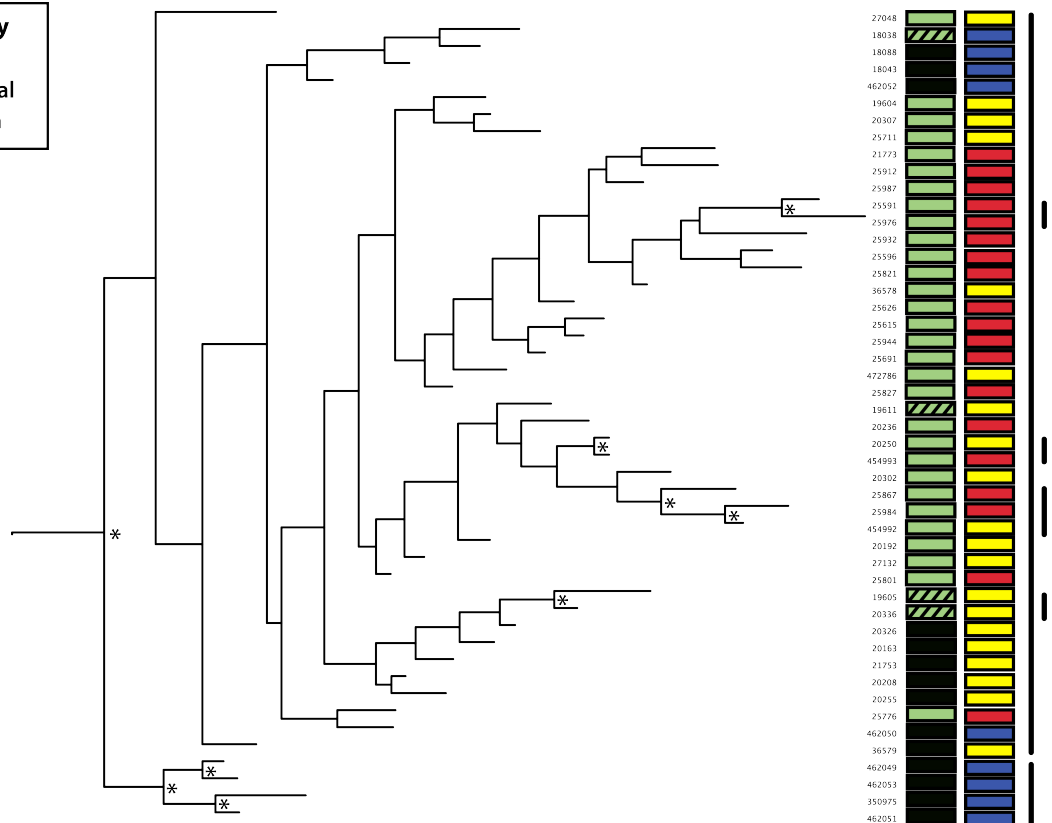
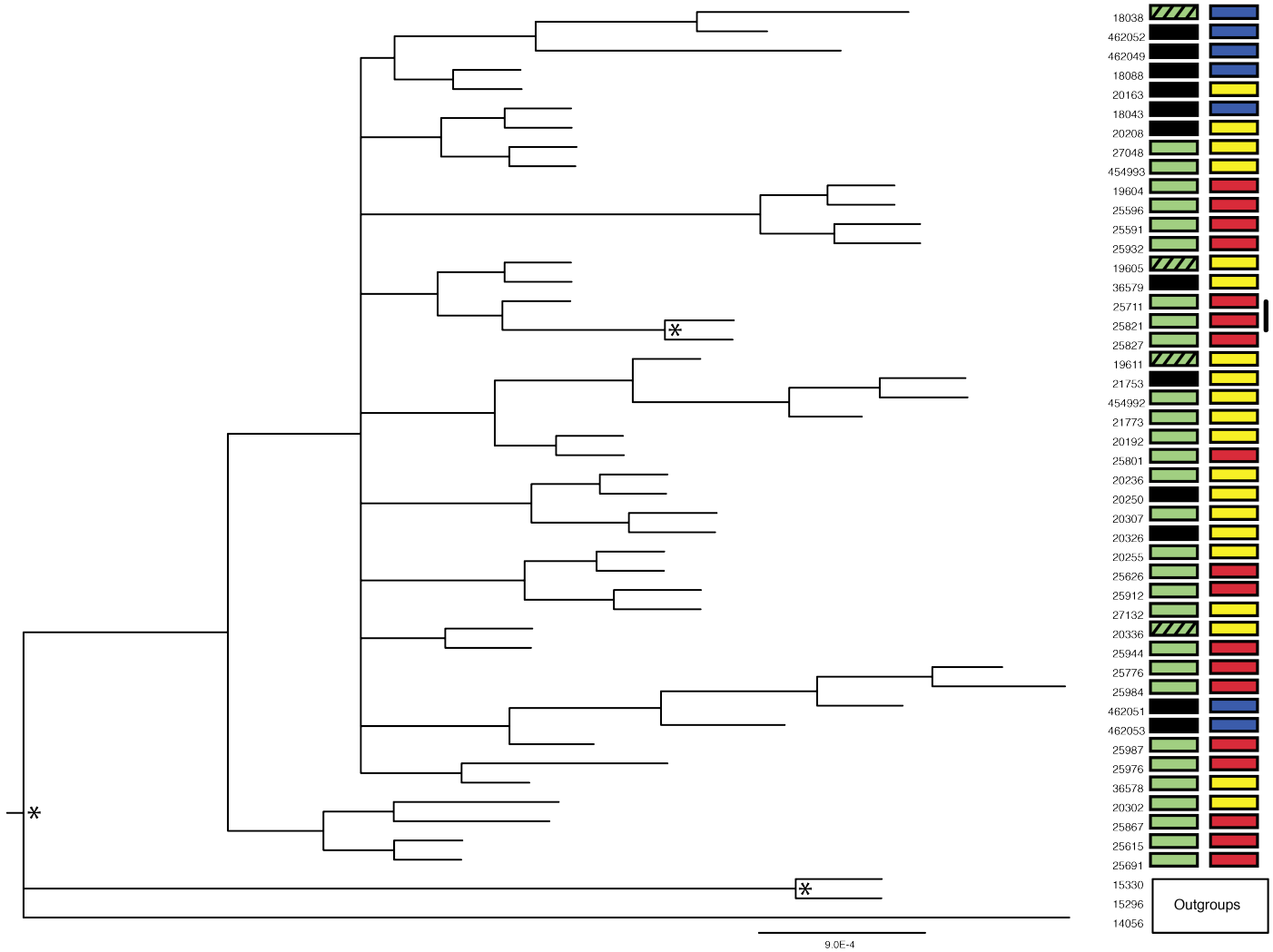


Figure 5: Z-linked phylogeny

Intermediates are striped green-gray

Stars at nodes and vertical bars at far right indicate >99% BPP



Phenotype	Geography
Light Green	North
Black	Central
Black	South

Population genetic analysis: No evidence of selection or rapid population size changes was detected using Tajima's D , Fu's F , Fu and Li's D^* , or Fu and Li's F^* test statistics for any allelic loci. There was evidence of population genetic differentiation roughly delimiting North, Central Luzon, and South Luzon in autosomal DNA but not in Z-linked DNA (Figures 6, 7); North/Central Luzon contained several private haplotypes not recovered from the Southern Bicol Peninsula. Isolation by distance across the whole sample was significant for nDNA and mtDNA. (Evanno, Regnaut et al. 2005)'s algorithm was used with Structure output to infer three populations for autosomal DNA, and one population for Z-linked DNA. For haplotype networks for autosomal VIM, Z-linked MUSK, and mtDNA ND3 see Figure 8. There is a large amount of admixture in Z-linked and autosomal loci across populations and phenotype, but the deep mitochondrial split consisted of all South Luzon gray-backed birds. Missing haplotypes are not shown, and there was a large distance between the South population that was collapsed. F_{ST} is shown for two combinations of data: one that splits populations by phenotype and geography, and another that follows Structure output with only geography (Table 3). F_{ST} was lowest between Central green- and gray-backed birds and all pairwise comparisons with South Luzon had higher population structure than any pairwise comparisons within North/Central Luzon.

Table 3: Four population F_{ST}

Population 1	Population 2	CDC	G3P	HMG	PEP	RHO	VIM	Mito	CHD Z	Musk Z	GPBP Z	PTCH Z
Bicol	North	0.07	0.052	0.31	0.17	0.371	0.276	0.97	0.36	0.108	0.198	0.075
Bicol	Central <i>chloronotus</i>	0.11	0.078	0.25	0.08	0.503	0.222	0.96	0.27	0.145	0.382	0.071
Bicol	Central <i>derbianus</i>	0.09	0.09	0.22	0.09	0.435	0.11	0.96	0	0.185	0.413	0.091
North	Central <i>chloronotus</i>	0	0.162	0.07	0.07	0.031	0	0.11	0.03	0.001	0.148	0
North	Central <i>derbianus</i>	0	0.256	0.17	0.01	0	0.032	0.09	0.36	0.019	0.172	0.03
Central <i>chloronotus</i>	Central <i>derbianus</i>	0	0.021	0	0	0.038	0.002	0.02	0.27	0	0.043	0

Table 4: Three population F_{ST}

Population 1	Population 2	CDC	G3P	HMG	PEP	RHO	VIM	Mito	CHD Z	Musk Z	GPBP Z	PTCH Z
North	Bicol	0.07	0.0521	0.314	0.17	0.371	0.276	0.97	0.36	0.108	0.2	0.075
North	Central	0	0.2107	0.124	0.04	0.003	0.003	0.11	0.15	0.187	0.15	0
Bicol	Central	0.1	0.0805	0.221	0.09	0.466	0.159	0.96	0.14	0.025	0.39	0.08

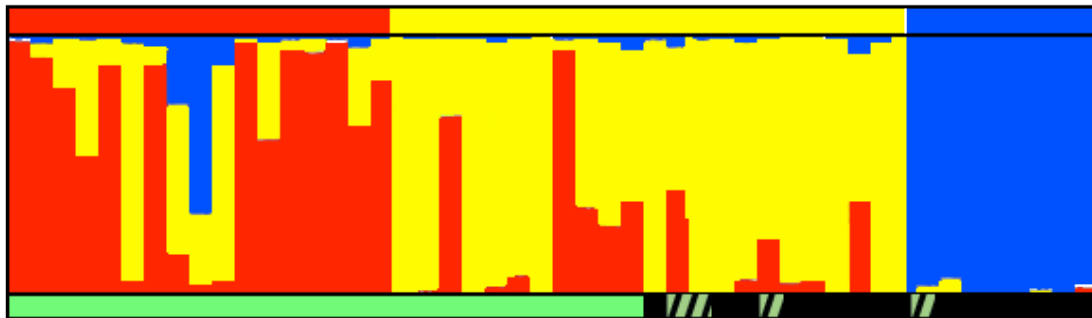
Figure 6: Autosomal structure results

Green and black bars at bottom represent phenotype

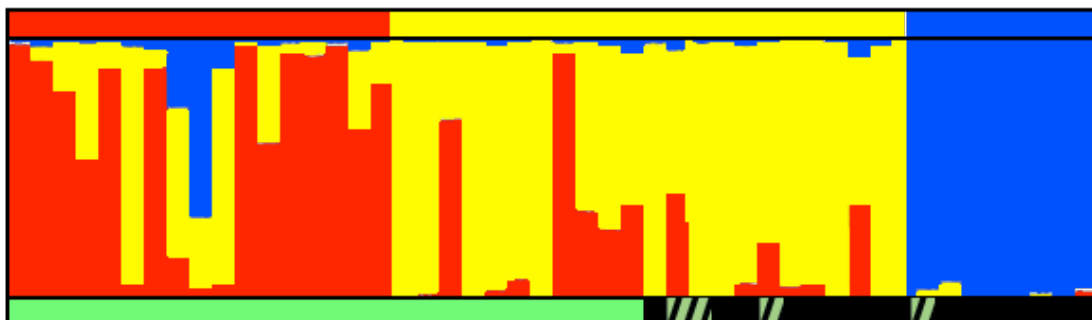
Striped green and black indicate intermediates

K = 3 most likely

A) K = 2



B) K = 3



Phenotype	Geography
█ <i>chloronotus</i>	█ North
█ <i>derbianus</i>	█ Central
	█ South

Figure 7: Z-linked structure results

Green and black bars at bottom represent phenotype

Striped green and black indicate intermediates

K = 1 most likely

A) K = 1



B) K = 2








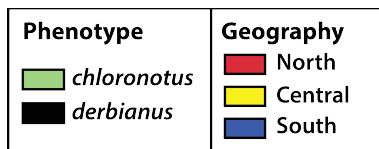
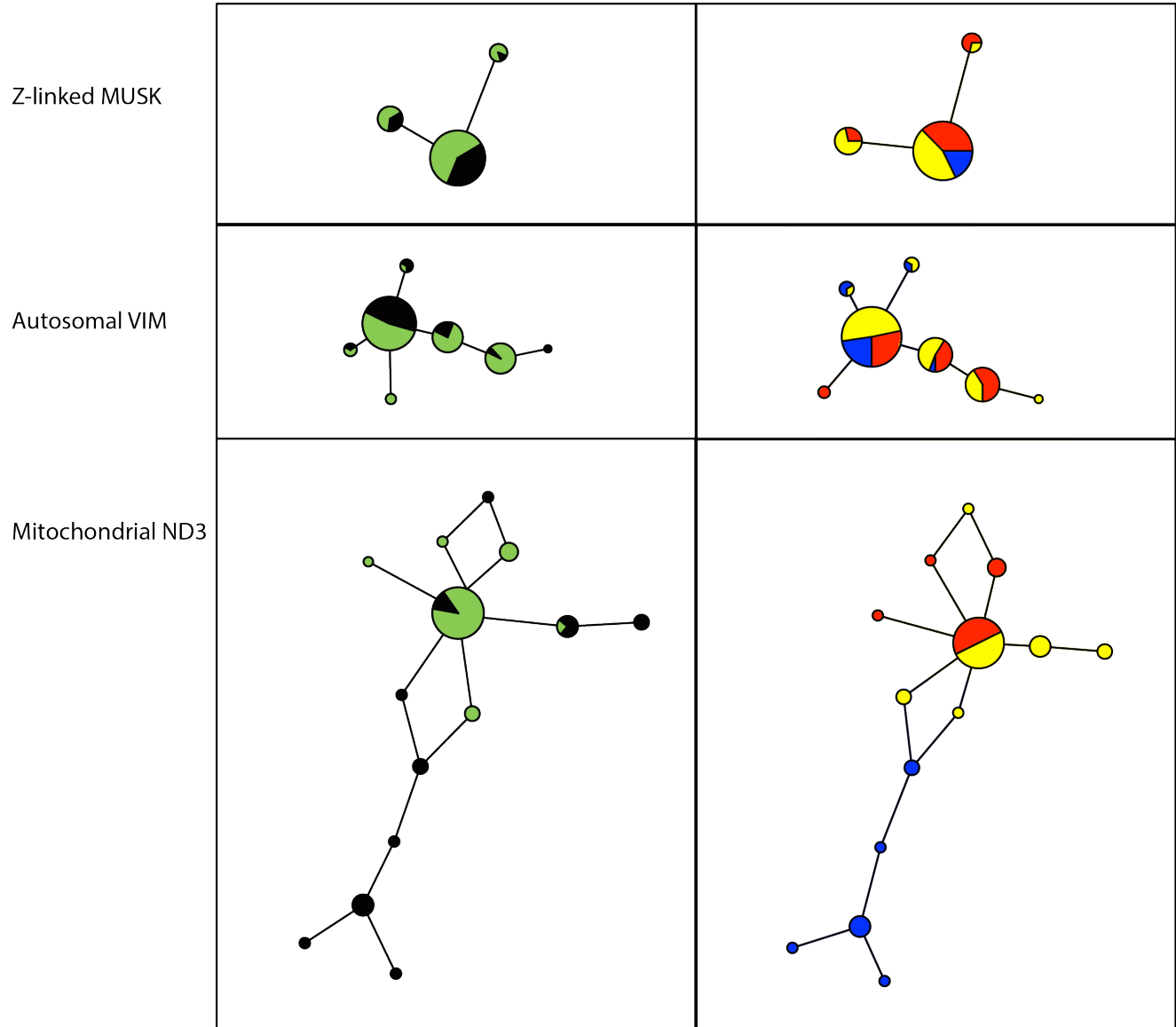
Phenotype	Geography
 <i>chloronotus</i>	 North
 <i>derbianus</i>	 Central
	 South

Figure 8: Haplotype networks



Migrate analysis: Because Structure reconstructed three populations roughly corresponding to North, Central, and South Luzon (Figure 6), these three geographic populations were defined for Migrate analyses. Migrate estimated mutation-scaled effective population sizes ($4N\mu$) of 0.01326 for the North, 0.02461 Central, and 0.01856 South. Watterson's estimator scaled mutation rate across loci and averaged 0.248 for Z-linked loci, 1.034 for autosomal loci, and 3.805 for the mitochondrial locus. The mode of parameters was: θ North = 0.00437, θ Central = 0.00257, θ South = 0.00217 (Figure 9). M , the mutation scaled migration rate m/μ for each pair of populations, was North→Central = 4191.0, Central→North = 745.0, South→Central = 156.7, Central→South = 273.3 (Figure 10). By multiplying M by θ , the effective number of migrants per generation is North→Central = 19.8, Central→North = 1.91, South→Central = 0.474, Central→South = 0.702.

Figure 9: Mutation-scaled population size

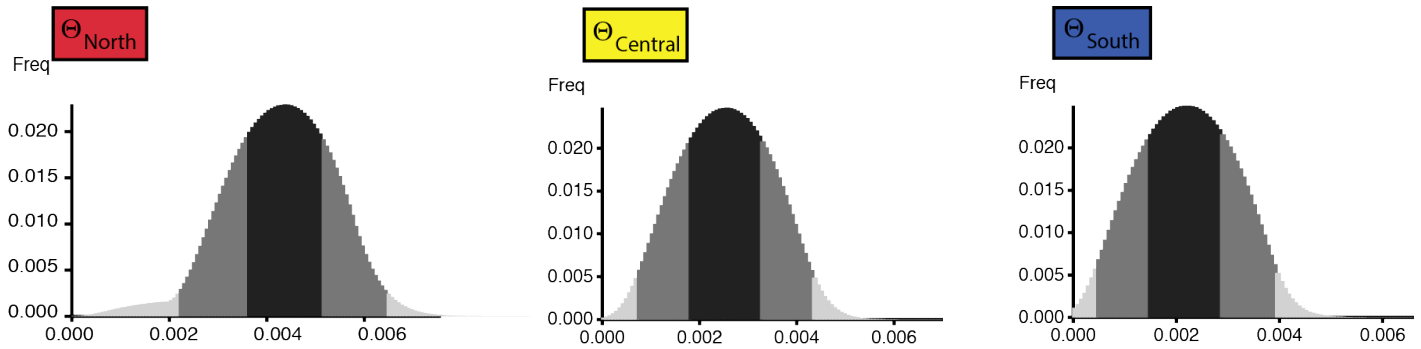
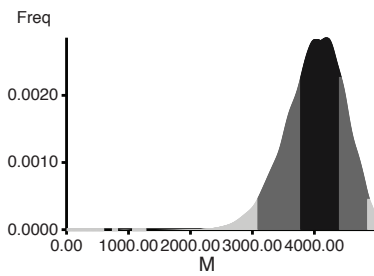


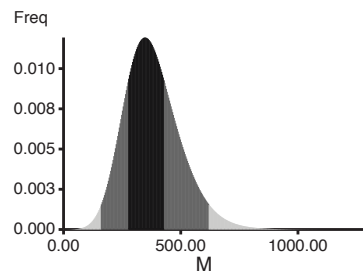
Figure 10: Stepping stone migration rates

Note difference in scale between North/Central and Central/South columns

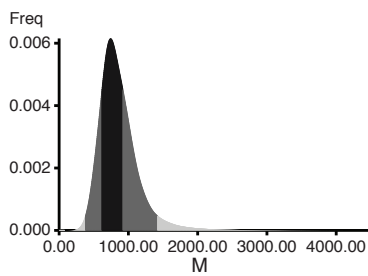
North to Central migration



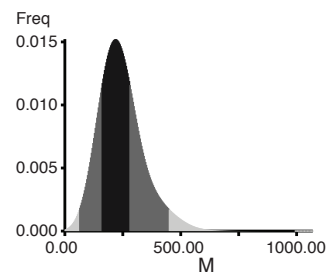
Central to South migration



Central to North migration



South to Central migration



Data overview:

South Luzon vs. North/Central Luzon: *Orthotomus chloronotus* and *O. derbianus* are not evolving independently from each other. Instead, allopatric gray-backed birds in South Luzon are distinct according to the faster evolving mitochondrial and autosomal haplotypes. Mitochondrial DNA, autosomal population structure, and migration analysis revealed the distinctiveness of gray-backed birds in South Luzon. In the concatenated phylogeny, South Luzon is reciprocally monophyletic from the rest of Luzon. In the nuclear phylogeny, four out of eight South Luzon birds were well-supported as sister to the rest of the dataset. Partitioning nuclear alleles into autosomal and Z-linked loci revealed their relative contributions to population structure: at the allelic level autosomal loci corroborated the mtDNA pattern: distinct population structure in South Luzon. The sampled Z-linked loci do not show population structure within South Luzon. Additionally, migration analysis inferred non-significant gene flow into and out of South Luzon.

North/Central Luzon: Mitochondrial DNA is unstructured autosomal haplotypes also delimit North and Central Luzon populations, but there is significant gene flow between them that does not correlate with plumage. These populations are not phylogenetically distinct, but autosomal population structure separates Central from North Luzon. The sampled Z-linked loci do not show population structure. There is significant migration between Central and North Luzon, and migration analysis inferred much higher migration from North→Central Luzon than from Central→North Luzon.

Central Luzon: Within Central Luzon there is no evidence of assortative mating correlated with plumage. There is no genetic differentiation separating green-backed from gray-backed birds in Central Luzon at the phylogenetic or population genetic level in mitochondrial,

autosomal, or Z-linked loci. Central Luzon has distinct population structure separating it from both North and South Luzon, but there is no population structure within Central Luzon. Due to this lack of structure, migration between plumages within Central Luzon was not analyzed.

IV. DISCUSSION

“An outsider would never realize how many interesting cases of evolutionary intermediacy are concealed in the seeming definiteness of the species and subspecies designations” (Mayr 1963). This study was spurred by the opportunity to investigate the role of secondary contact between recently diverged species. Widespread sampling across allopatric and sympatric populations allowed for finer-scale resolution of diversity in Luzon tailorbirds. The ancestral plumage of Luzon *Orthotomus* remains ambiguous, and this study has uncovered a much more complicated scenario than originally thought. Different levels of gene flow are contributing to divergence in South Luzon, high gene flow with the maintenance of population structure between North and Central Luzon, and free interbreeding within Central Luzon’s green- and gray-backed birds.

Divergent South Luzon: Allopatric gray-backed birds are in the process of diverging from the rest of the island. Genetic breaks coincide with the distributional limit of the sympatric zone, explaining the lack of green-backed birds in South Luzon. The average mitochondrial divergence of 9% between North/Central vs. South Luzon suggests a population split and exceeds the mtDNA divergence between many uncontroversial pairs of species (Johnsen, Rindal et al. 2010, Mila, Tavares et al. 2012). Under traditional hypotheses of the evolution of phylogeographic structure, the high mutation rate and 25% effective population size relative to autosomal DNA, mtDNA is expected to differentiate rapidly upon divergence. Most interpretations of low mitochondrial gene flow across sharp geographic boundaries are usually assumed to be the result of long-term barriers to migration, cryptic species boundaries, or secondary contact (Avise, Arnold et al. 1987, Avise 2009).

In slower evolving autosomal DNA, South Luzon's population structure will likely lead to better-resolved phylogenetic structure that will corroborate the mtDNA phylogeny (Avice, Arnold et al. 1987, Avice 2009). The presence of strong autosomal structure in the nDNA clade of 4/8 (far) Southern gray-backed birds supports the hypothesis that incomplete lineage sorting (ILS) is in the process of sorting Southern nuclear haplotypes from Central/North birds. This is corroborated by the per-generation migration rate in and out of South Luzon with regard to Central Luzon being less than 1, indicating that genetic drift is the driver of population differentiation in the South. Independent of South Luzon's original plumage composition, the low amount of South Luzon gene flow accounts for the fixation of the gray phenotype.

Structured populations in North and Central Luzon:

Taken together, there is evidence of some divergence between North and South Luzon but also high rates of gene flow. Migration is significant between these populations but K and F_{ST} calculations recovered enough differences in allele frequencies to infer two distinct clusters of populations in Hardy-Weinberg equilibrium. The migration from North Luzon into Central Luzon is tenfold higher than the rate from Central to North Luzon, suggesting that the Northern population is invading Central Luzon. These results explain the limit of the sympatric zone, and may be due to isolation by distance and/or hybridization

Significant isolation by distance (IBD) was found, which was expected because of the large gap (approx. 250km) between North and Central sampling localities. The presence of IBD, however, does not preclude other contributions to genetic structure such as geography or assortative mating. The effective number of migrants per generation between North and Central Luzon is greater than one in both directions, which indicates that immigration rates should be high enough to keep populations at similar allele frequencies. Lower migration into North Luzon

may also be explained by a hybridization scenario: lower gray-backed fitness, but there is no signal of expected differences in mtDNA or Z-linked introgression.

The major signal of hybridization upon secondary contact is the unknown barrier restricting Central dimorphism from South Luzon: phenotypically and in population structure. Since the avian sex-determining, hemizygous W chromosome is carried by females, there should be lower introgression in W-linked and mtDNA than Z-linked and autosomal DNA according to Haldane's rule (Haldane 1922). Because the hemizygous sex exhibits lower hybrid fitness, high mtDNA, moderate Z-linked, and low autosomal structure (Saetre, Borge et al. 2001, Carling and Brumfield 2008, Rheindt, Christidis et al. 2009). However the predicted genetic differences in known avian hybrid zones are reversed: strong population structure in autosomal introns was recovered between South and North/Central Luzon but there was no population structure in Z-linked loci. None of the expected patterns were found; instead there was higher population structure in autosomal alleles than mtDNA or Z-linked loci. This also violates theoretical patterns of hybridization, where levels of introgression follow distinct patterns according to each DNA classes (Turelli and Orr 1995, Orr 1997).

Hybridization may still be uncovered if there is variation in selective pressure: on hybrid/gray-backed fitness in North Luzon or on different classes of DNA. If a local mtDNA haplotype is favored, or if female and male hybrids have equal fitness, there would be low mitochondrial structure. To discover evidence of a selective sweep on a particular mtDNA haplotype, a rare mtDNA haplotype to compare to the common one must be uncovered. This may be found with denser sampling and sequencing more mtDNA genes, but if the selective sweep is complete there is no way to detect whether it occurred. The hybridization scenario invokes selection against hybrids and/or gray-backed birds, combined with local adaptation. This makes isolation by

distance the more parsimonious explanation for North/Central population structure and its high levels of admixture.

Panmictic Central Luzon: There was no evidence of structure within Central Luzon. It appears that there is no assortative mating according to phenotype; F_{ST} between Central green- and gray-backed birds ranged from 0.078–0.015. Although plumage differences suggest secondary contact, the area is panmictic with no genetic evidence of past differentiation. The mechanism maintaining the plumage polymorphism in the sympatric zone is unknown, and the answer depends on whether plumage is neutral or under selection. If females prefer males of a different plumage in Central Luzon the polymorphism will be maintained, but if there is no plumage based assortative mating, the gray phenotype will eventually disappear as a result of genetic drift and immigration from North Luzon. However without data on clines in plumage proportions throughout Central Luzon, assortative mating, or offspring fitness there is not enough data to implicate the selective or stochastic factors maintaining plumage dimorphism Central birds.

In conclusion: Luzon tailorbirds have genetic affinities that are not predicted by their plumage, align with a common a population bottleneck (South Luzon), and are unaffected by another (Mid-Sierra Filter Zone). Their previously undocumented population structure highlight the need for phylogeographic investigations of other closely related groups. Luzon's two tailorbird species are uniquely distributed because of their area of sympatry. Genetically, however, there are two allopatric populations: South and North/Central Luzon. The previously unknown genetic break in South Luzon is mirrored by several avian subspecies distributions on the island (Kennedy, Gonzales et al. 2000). There are no know geographic barriers, however, correlating with population structure between North and Central Luzon. Dimorphic populations

were sampled on either side of the Mid-Sierra Filter Zone, which bisects the Sierra Madre mountain range and is delimited by three river valleys of low forest cover. This variation in habitat circumscribes many species boundaries but does not affect *Orthotomus* gene flow within Central Luzon (Kennedy, Gonzales et al. 2000, Welton, Diesmos et al. 2012).

Traditionally, studies of avian colonization and diversification have focused on extrinsic promoters of biodiversity including geography, geology, and environmental change (Mayr 1963, Edwards, Kingan et al. 2005). Intrinsic factors such as behavior offer further explanations for evolutionary paradigms. Especially in hybridization studies, measuring migration in both sexes will help determine the fitness consequences of interbreeding. Additionally, strong breaks in mitochondrial DNA are usually assumed to be the result of long-term barriers to gene flow (Avice, Arnold et al. 1987, Avice 2009), but can also form in continuously distributed populations if individual dispersal distances are low. Empirical studies on *Orthotomus* ecology including migration and mating behavior will provide better resolution on the geographic origin of the derived phenotype. This study provides an important addition to both Philippine biogeography and evidence of cryptic evolutionary relationships within Luzon Island.

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