ORIGIN AND DIVERSIFICATION OF PHILIPPINE BULBULS

BY

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ABSTRACT

We examine the origin and diversification of Philippine bulbuls using a phylogenetic framework. We used maximum likelihood and Bayesian methods to construct trees from DNA sequences of two mitochondrial and two nuclear genes obtained from 11 Philippine bulbul species as well as 32 other Asian and African taxa. We found eight independent colonization events of bulbuls to the Philippines, including one clade comprising Philippine members of the genus *Ixos* that underwent extensive diversification within the archipelago. Each Philippine clade of bulbuls invaded either the Palawan region or the oceanic islands of the Philippines, but not both. Genetic data reveal at least 5 lineages that warrant recognition as full species. This study underscores how Philippine avian diversity is currently underestimated and highlights the need for further phylogenetic studies in other Philippine bird groups.

I. INTRODUCTION

Oceanic island archipelagos provide a unique microcosm for studies of diversification. The stark geographic boundaries and comparatively simple floras and faunas remove many confounding factors present in continental regions. The Philippine archipelago, comprising over 7,000 islands, is one of the largest on Earth, yet relatively little is known about the origins and diversification history of most of its endemic flora and fauna. A basic question of all archipelagoes, and the Philippines in particular, is the relative importance of colonization and *in situ* diversification in forming insular biotas. Among Philippine vertebrates, reptiles, amphibians, and mammals show high levels of endemism, suggesting a substantial role for diversification within the archipelago (Kennedy *et al.*, 2000; Ong *et al.*, 2002). This is supported by phylogenetic studies of select clades within these groups (e.g., Evans *et al.,* 2003; Jansa *et al.,* 2006; McGuire and Kiew, 2001). In contrast, birds show relatively low levels of endemism and suggest, in keeping with their volant nature, that colonization may be a more important driver of species diversity. That is, origins of the Philippine bird fauna involved more assembly whereas other vertebrate faunas involved greater diversification.

The Philippine archipelago has had a complex geological history but the major stages are now fairly well understood as summarized in Hall (2002) and Yumul *et al.* (2008). The western islands of Palawan and Mindoro are composed of continental crust that rifted from the margins of southern China and rafted to their current

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position. Most or all of this land was probably submerged at some point during the transit across the South China Sea. Parts of Mindanao – the Zamboanga Peninsula and the Daguma Range in the southwest – also have continental origins. These fragments were in isolation for a long period of time but they have been in contact with central Mindanao since the middle Miocene. The rest of the archipelago is oceanic, arising *de novo* since the late Cretaceous and throughout the Cenozoic from a complex system of island arcs between the Eurasian and the Philippine Sea plates. Individual fragments have moved in a dynamic fashion, changing direction and pace, but this belt of oceanic islands has been moving in a general northwesterly direction since the early Cenozoic. During the last glacial maximum in the Pleistocene, sea levels are estimated to have been 120 m below current levels exposing land connections between some Philippine islands (Heaney, 1986; Voris, 2000; Fig. 1).

Early workers traced the origins of Philippine bird fauna to the Sunda, Palearctic and Australasian regions (e.g., Dickerson, 1928).   The Sunda region is considered the chief colonization source (Dickinson *et al.,* 1991) and the Palawan group of islands and the Sulu archipelago are seen as important invasion routes to the Philippines (Diamond and Gilpin, 1983). The dominant framework in the study of diversification of Philippine fauna in the last two decades has been that Pleistocene islands during the last glacial maximum define the limits of homogenous populations, each of which is a center of endemism (Heaney, 1986). These patterns of colonization and diversification have been studied in a phylogeographic context in birds for a few
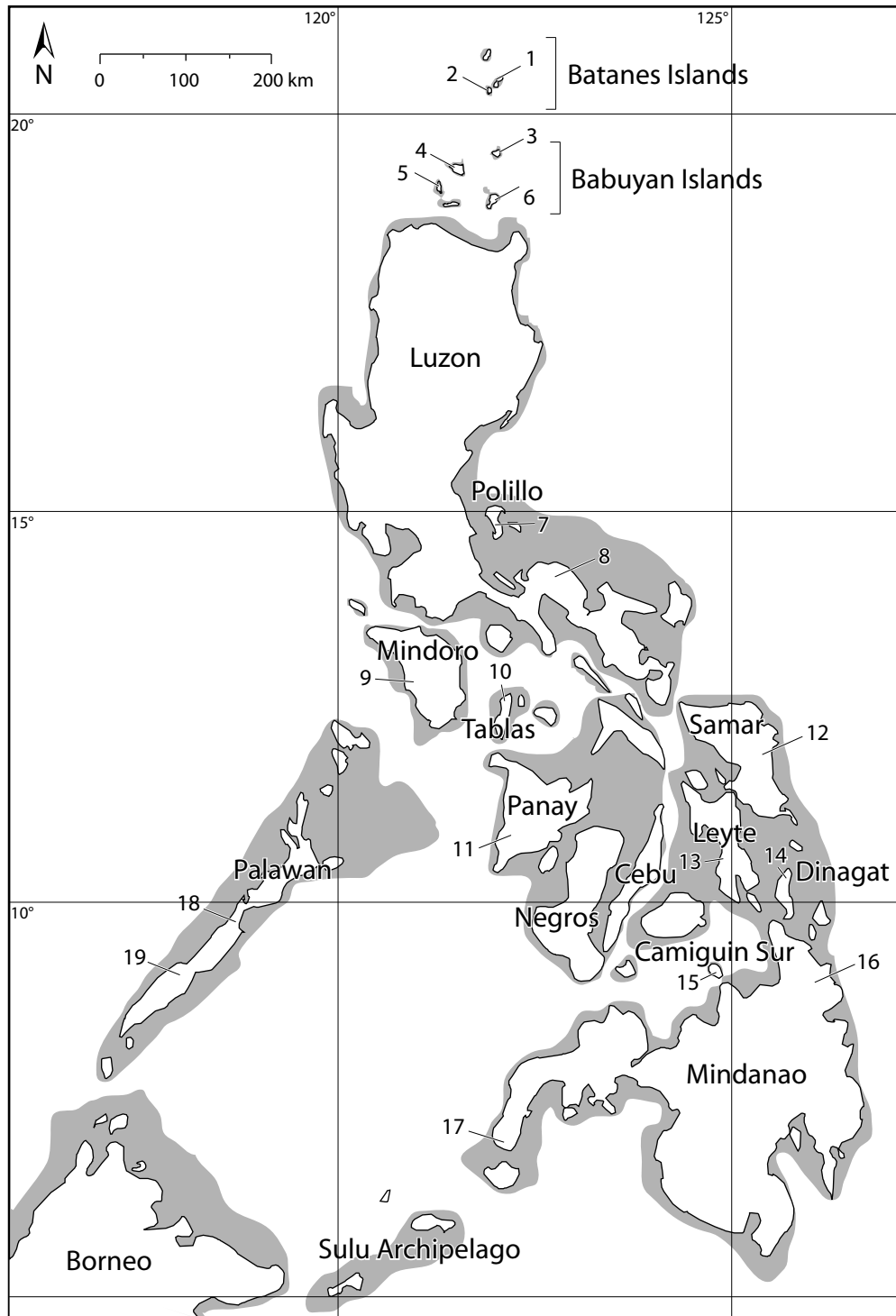
Figure 1. Location of sampling localities. Numbers correspond to those in Table 1. Light gray areas show late Pleistocene islands based on Heaney (1986).

species (Jones and Kennedy, 2008a, b) but no thorough investigations have been carried out at higher taxonomic levels.

Bulbuls (Pycnonotidae) are ubiquitous residents of the tropical and semi-temperate Old World and are ideal subjects to study colonization and diversification patterns in the Philippines. Bulbuls are found throughout Southeast Asia, with high species ririchness in the Sunda region (e.g., 22 species on Borneo). They include some of the commonest species in SE Asian forests, and comprise a sizable proportion of the species diversity of frugivores. Despite supposed strong dispersal ability, as evidenced by their presence throughout the oceanic Philippines, the family is almost completely truncated by Wallace's line: only a single genus, *Thapsinillas*, occurs in the Moluccas. The family is widespread throughout the Philippine archipelago— occurring even on very small and remote islands. The Philippines harbors 11 bulbul species that are placed in five genera. We follow the taxonomy of Fishpool and Tobias (2005) except for the taxonomic treatment of *Iole palawanensis* where we follow Dickinson (2003). Six species of bulbuls are endemic to the Philippines: five are endemic to oceanic islands of the Philippines (*Pycnonotus urostictus, Ixos philippinus, I. rufigularis, I. everetti* and *I. siquijorensis*) and one occurs only in the Palawan region (*Iole palawanensis*). Four species are shared with the Sunda region, of which three are found in the Philippines only on Palawan (*Pycononotus atriceps, P. plumosus*, and *Alophoixus bres*) and the other is distributed throughout the oceanic Philippines (*Pycnonotus goiavier*) except the Batanes-Babuyan island chain (Fig. 1).

The other non-endemic species, *Microscelis amaurotis*, occurs only on small islands in the Batanes-Babuyan island group north of Luzon and has a range that extends north through Taiwan and the Ryukyu archipelago to northern Japan and the Korean peninsula. Many of these species include multiple described subspecies and some workers have suggested that some of these races merit recognition as full species (Fishpool and Tobias, 2005; Allen, 2006; Peterson, 2006).

A recently published molecular phylogeny of bulbuls (Moyle and Marks 2006) recovered two main lineages—one exclusively African and the other mostly Asian. Their study included only five Philippine species and all but one were widespread species sampled from localities outside of the Philippines. In the present study, we use the Moyle and Marks (2006) phylogenetic framework for bulbuls to examine the origin and diversification of Philippine members of the family. In particular, we address the following questions: 1) How many independent colonization events spawned the Philippine bulbul radiation? 2) Do temporal patterns of colonization history suggest clustered dispersal events, potentially facilitated by specific historical factors, or random sweepstakes dispersal? 3) Do genetic data reflect Palawan Island's recent land connection to the islands of the Sunda Shelf? 4) What general patterns of colonization and diversification can be inferred from the genealogical history of the Philippines' bulbul fauna?

II. MATERIALS AND METHODS

We used DNA sequence data for 28 species from Moyle and Marks' (2006) study of bulbul systematics that used the following markers: the entire second and third subunits of mitochondrial nicotinamide adenine dinucleotide dehydrogenase (ND2 and ND3) and the seventh intron of the nuclear Beta-Fibrinogen (Fib7). We expanded this dataset by adding 46 individuals and sequencing an additional nuclear gene, the fifth intron of the Transforming Growth Factor β2 (TGF5), for all samples. In total, ingroup sampling (Table 1) consisted of 74 individuals representing 43 recognized bulbul species (Fishpool and Tobias, 2005). All 11 species that occur in the Philippines, including 18 of the 25 named subspecies, were sampled. For most subspecies, we sampled 1 individual but we added additional individuals if they were collected from different localities or from outside the Philippines. Our ingroup sampling included all bulbul species that occur on Borneo, with the exception of *Alophoixus finschii* and *Pycnonotus nieuwenhuisii*. Two African species (*Pycnonotus barbatus* and *Pycnonotus nigricans*) and one Malagasy species (*Hypsipetes madagascariensis*) that were previously shown to form part of the largely Asian clade (Moyle and Marks, 2006) were also included in the ingroup. We chose *Bleda canicapillus, Criniger calurus, Phyllastrephus icterinus* and *Calyptocichla serina* as outgroup taxa because they belong to the African radiation sister to the mostly Asian clade of bulbuls (Moyle and Marks, 2006, Johansson *et al.,* 2007).

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Table 1. Samples included in the study, all vouchered in their respective institution: LSUMNS – Louisiana State University Museum of Natural Science; KUNHM – University of Kansas Natural History Museum; FMNH – Field Museum of Natural History; SP – Sabah Parks museum collection. Map code refers to code in Figure 1 that shows location of where samples were collected.

Species	Subspecies	Sample #	Source	Locality, Map Code
Ingroup				
Spizixos semitorques	–	B20554	LSUMNS	Captive
Pycnonotus zeylanicus	–	B23321	LSUMNS	Sabah, Malaysia
Pycnonotus melanoleucos	–	B50353	LSUMNS	Sabah, Malaysia
Pycnonotus atriceps 1	–	B36320	LSUMNS	Sabah, Malaysia
Pycnonotus atriceps 2	–	12317	KUNHM	Sarawak, Malaysia
Pycnonotus atriceps 3	–	12641	KUNHM	Puerto Princesa, Palawan, Philippines, 18
Pycnonotus montis	–	B51038	LSUMNS	Sabah, Malaysia
Pycnonotus cyaniventris	–	B36425	LSUMNS	Sabah, Malaysia
Pycnonotus jocosus	–	10347	KUNHM	China
Pycnonotus xanthorrhous	–	11153	KUNHM	China
Pycnonotus sinensis	–	10313	KUNHM	China
Pycnonotus barbatus	–	B39494	LSUMNS	Ghana
Pycnonotus nigricans	–	B34228	LSUMNS	South Africa
Pycnonotus leucogenys	–	F347847	FMNH	Pakistan
Pycnonotus eutilotus	–	B36313	LSUMNS	Sabah, Malaysia
Pycnonotus urostictus	urostictus	18039	KUNHM	Luzon, Philippines, 8
Pycnonotus urostictus	atricaudatus	14222	KUNHM	Samar, Philippines, 12
Pycnonotus urostictus	philippensis	14052	KUNHM	Dinagat, Philippines, 14
Pycnonotus urostictus	basilanicus	18181	KUNHM	Zamboanga, Mindanao, Philippines, 17
Pycnonotus flavescens	–	17746	KUNHM	Sabah, Malaysia
Pycnonotus goiavier	gourdini	B36351	LSUMNS	Sabah, Malaysia
Pycnonotus goiavier	samarensis	14223	KUNHM	Samar, Philippines
Pycnonotus plumosus	hutzi	17712	KUNHM	Sabah, Malaysia
Pycnonotus plumosus	hutzi 1	B23354	LSUMNS	Sabah, Malaysia
Pycnonotus plumosus	hutzi 2	17707	KUNHM	Sabah, Malaysia
Pycnonotus plumosus	hachisukae	22149	SP	Pulau Balambangan, Sabah, Malaysia
Pycnonotus plumosus	cinereifrons 1	12660	KUNHM	Puerto Princesa, Palawan, Philippines, 18
Pycnonotus plumosus	cinereifrons 2	12806	KUNHM	Mantalingahan Range, Palawan, Philippines, 19

(continued on next page)

Table 1. (continued from previous page)

Species	Subspecies	Sample #	Source	Locality, Map Code
Pycnonotus simplex	–	17777	KUNHM	Sabah, Malaysia
Pycnonotus brunneus	–	B36341	LSUMNS	Sabah, Malaysia
Pycnonotus erythropthalmos	–	B36378	LSUMNS	Sabah, Malaysia
Alophoixus pallidus	–	10111	KUNHM	China
Alophoixus ochraceous	–	B38614	LSUMNS	Sabah, Malaysia
Alophoixus bres	*gutturalis 1*	B38567	LSUMNS	Sabah, Malaysia
Alophoixus bres	*gutturalis 2*	17701	KUNHM	Sabah, Malaysia
Alophoixus bres	*gutturalis 3*	12389	KUNHM	Sarawak, Malaysia
Alophoixus bres	*frater 1*	12607	KUNHM	Puerto Princesa, Palawan, Philippines, 18
Alophoixus bres	*frater 2*	12784	KUNHM	Mantalingahan Range, Palawan, Philippines, 19
Alophoixus phaeocephalus	–	B38568	LSUMNS	Sabah, Malaysia
Setornis criniger	–	B23359	LSUMNS	Sabah, Malaysia
Tricholestes criniger	–	B38601	LSUMNS	Sabah, Malaysia
Iole olivacea 1	–	B51043	LSUMNS	Sabah, Malaysia
Iole olivacea 2	–	17765	KUNHM	Sabah, Malaysia
Iole palawanensis 1	–	12618	KUNHM	Puerto Princesa, Palawan, Philippines, 18
Iole palawanensis 2	–	12813	KUNHM	Mantalingahan Range, Palawan, Philippines, 19
Ixos mcclellandii	–	10269	KUNHM	China
Ixos malaccensis	–	B51130	LSUMNS	Sabah, Malaysia
Ixos philippinus	*philippinus 1*	11006	KUNHM	Polillio, Philippines, 7
Ixos philippinus	*philippinus 2*	17947	KUNHM	Luzon, Philippines, 8
Ixos philippinus	*guimarasensis*	15281	KUNHM	Panay, Philippines, 11
Ixos philippinus	*saturatior 1*	14301	KUNHM	Leyte, Philippines, 13
Ixos philippinus	*saturatior 2*	14250	KUNHM	Samar, Philippines, 12
Ixos philippinus	*mindorensis*	12566	KUNHM	Mindoro, Philippines, 9
Ixos rufigularis	–	18213	KUNHM	Zamboanga, Philippines, 17
Ixos siquijorensis	*cinereiceps*	14448	KUNHM	Tablas, Philippines, 10
Ixos everetti	*everetti 1*	14184	KUNHM	Samar, Philippines, 12
Ixos everetti	*everetti 2*	14286	KUNHM	Leyte, Philippines, 13
Ixos everetti	*catarmanensis*	12956	KUNHM	Camiguin Sur, Philippines, 15
Microscelis amaurotis	*amaurotis*	B16985	LSUMNS	Honshu, Japan
Microscelis amaurotis	*batanensis 1*	10907	KUNHM	Babuyan Claro, Philippines, 3

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Table 1. (continued from previous page)

Species	Subspecies	Sample #	Source	Locality, Map Code
Microscelis amaurotis	*batanensis 2*	17882	KUNHM	Batan, Philippines, 1
Microscelis amaurotis	*batanensis 3*	17926	KUNHM	Sabtang, Philippines, 2
Microscelis amaurotis	*fugensis 1*	10971	KUNHM	Calayan, Philippines, 4
Microscelis amaurotis	*fugensis 2*	15261	KUNHM	Dalupiri, Philippines, 5
Microscelis amaurotis	*camiguinensis*	10888	KUNHM	Camiguin Norte, Philippines, 6
Hemixos flavala	–	15140	KUNHM	Myanmar
Hemixos cinereus	–	B38659	LSUMNS	Sabah, Malaysia
Hemixos castanonotus	–	10273	KUNHM	China
Hypsipetes leucocephalus	–	10337	KUNHM	China
Hypsipetes madagascariensis	–	F393308	FMNH	Madagascar
Outgroup:				
Phyllastrephus icterinus	–	B27097	LSUMNS	Cameroon
Bleda canicapillus	–	B39389	LSUMNS	Ghana
Criniger calurus	–	B27104	LSUMNS	Cameroon
Calyptocichla serina	–	11614	KUNHM	Equatorial Guinea

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We extracted genomic DNA from pectoral muscle or liver tissue using the DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA) following the manufacturer's protocol. We amplified target DNA fragments by performing polymerase chain reaction (PCR) using a combination of external and internal primers (Table 2). We purified PCR products with ExoSAP-IT (US78201, Amersham Biosciences, Piscataway, NJ). Purified PCR products were then sequenced with ABI Prism BigDye Terminator chemistry (Ver. 3.1; Applied Biosystems, Foster City, CA) using the same primers used for PCR. Cycle sequencing products were purified through precipitation in 70% ethanol and then analyzed on an ABI 134 Prism 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA). We used Sequencher 4.7 (Gene Codes Corp., Ann Arbor, MI) to reconcile complementary gene sequence contigs and align sequences across taxa.

Maximum likelihood (ML) heuristic tree searches were performed using GARLI 0.96 (Zwickl, 2008) on the combined 4-gene dataset. We selected the ML tree from 10 replicate searches. Node support was assessed by conducting ML heuristic searches on 500 bootstrap replicates of the data. We performed Bayesian analysis using Mr. Bayes 3.1 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) with the data divided into five partitions: one partition for each codon position in the combined ND2 and ND3 dataset; and one partition for each of the nuclear introns. All parameters (except topology) were unlinked between partitions. We ran four Markov chains for 2 million generations. Markov chains were sampled every 100

generations, yielding 20,000 parameter point estimates. Likelihood scores were

examined for stationarity and all prior samples were discarded as burn in. We

conducted similar ML and Bayesian analyses on each intron and on the combined

mitochondrial gene dataset (ND2 + ND3). We used MrModeltest 2.2 (Nylander,

2004) in conjunction with PAUP* 4.0b10 (Swofford, 2002) to select the appropriate

model of sequence evolution under the Akaike Information Criterion (AIC) for the

entire dataset and its subsets.

Table 2. Primers used in this study.

Gene	Primer name	Sequence (5' to 3')	Reference
ND3	L10755	GACTTCCAATCTTTAAAATCTGG	Chesser, 1999
	L11151	GACTGCGACAAAATCCCATTCCA	Chesser, 1999
ND2	L5215	TATCGGGCCCATACCCCGAAAAT	Hacket, 1996
	L5758	GGCTGAATRGGMCTNAAYCARAC	Johnson & Sorenson, 1998
	H6313	CTCTTATTTAAGGCTTTGAAGGC	Johnson & Sorenson, 1998
	H1064	CTTTGAAGGCCTTCGGTTTA	Drovetski et al., 2004
	500L	GAATAGGACTAAACCAAACAC	Filardi & Moyle, 2005
	SWH	CCTAGGTGGGAGATRGAGGA	Sheldon et al., 2005
	503L	TAGGMCTWAAYCARACMC	this study
	328L	CCRTTYCACTTYTGATTCCC	this study
	479L	TTTCTACCGCCCTAGGAGGG	this study
Fib7	FIB-BI7L	TCCCCAGTAGTATCTGCCATTAGGGTT	Prychitko & Moore, 1997
	FIB-BI7U	GGAGAAAACAGGACAATGACAATTCAC	Prychitko & Moore, 1997
	FIB-BI7Lb	CAGTGCTCTATTATGTACTTTAC	Sheldon et al., 2005
	FIB-BI7Lc	TGTAATYASGTATTAAGCAC	this study
	FIB-BI7Uc	AGAAGAYRGGMGYTCAGTTG	this study
	FIB-BI7Ld	CATGGTGCACTCAGAAAGTAAAGC	this study
	FIB-BI7Ud	GCTAAAYGTCTGTGCAGTTCCC	this study
TGF5	TGF5	GAAGCGTGCTCTAGATGCTG	Bures et al., 2002
	TGF6	AGGCAGCAATTATCCTGCAC	Bures et al., 2002
	TGF5alt	ATCTGGGGTGATTCCCACATGC	Moyle et al., 2009
	TGF6alt	ACTCAAGTGTWTTTCTCCAGGTCC	Moyle et al., 2009

We performed Shimodaira-Hasegawa (SH) Tests using PAUP* 4.0b10 (Swofford, 2002) and GARLI 0.96 (Zwickl, 2008) to test monophyly of currently recognized species that were recovered as non-monophyletic in the ML and Bayesian analyses.

To estimate relative timing of colonization events we produced an ultrametric tree and erected 95% confidence intervals for node heights using BEAST version 1.4.8 (Drummond and Rambaud, 2007). We chose not to place dates on the ultrametric tree because reliable calibration points are not available for bulbuls. We used the same data partitioning scheme and models of sequence evolution as the Bayesian analysis on the concatenated data. We also constrained topologies in the MCMC run to the Bayesian consensus tree obtained in the first analysis. Rate homogeneity along branches of the phylogeny was tested using a likelihood ratio test (Hasegawa *et al.,* 1985). A strict molecular clock hypothesis for the dataset was rejected ($p < 0.001$) thus a relaxed clock model (Drummond *et al.,* 2006) with uncorrelated rates drawn from a lognormal distribution was selected. We ran an MCMC for 10 million generations and logged parameters every 100 generations. We discarded the first 6.5 million generations as burn-in.

III. RESULTS

A. Sequence attributes

The final dataset had a total alignment length of 2,907 bp of combined DNA sequences from ND2, ND3, Fib7 and TGF5 (Table 3). We sequenced partial or entire genes for all samples except for the TGF5 intron of *Pycnonotus montis* and *Iole olivacea 1*. 1,118 characters (38%) were parsimony informative (Table 3). All sequences were deposited in GenBank (Accession Nos. XXXXX-XXXXX). Aligned ND2 and ND3 sequences contained no stop codons and appeared to be of mitochondrial origin. Overlapping fragments contained no conflicts, base composition was relatively homogenous across taxa, codon positions contained expected relative divergences (3 > 1 > 2), and resulting relationships contained no highly suspect arrangements. Aligned Fib7 and TGF5 sequences contained several inferred insertions or deletions (indels), which were mapped on the inferred tree to provide additional support for phylogenetic relationships (Johnson *et al.* 2001; Moyle, 2004; Moyle and Marks, 2006). Models of sequence evolution used for the ML and Bayesian analyses were: the general time reversible model with gamma-distributed rates among sites and invariant sites (GTR + I + G) for the concatenated data, the combined mitochondrial genes and each of their partition by codon position; and the general time reversible model with gamma-distributed rates among sites (GTR + G) for each of the introns (Table 3).

Table 3. Sequence attributes and models of sequence evolution used for ML and Bayesian analyses.

	ND2	ND3	Fib7	TGF5
Aligned length	1041	351	919	596
Sequence length	339-1041	351	545-868	316-592
Number variable	613	197	341	198
Number informative	557	182	229	150
Models				
ML (combined data)	GTR + I + G			
ML (individual loci)	GTR + I + G*		GTR + G	GTR + G
Bayesian (combined data – partitions)	Codon Pos 1[a]: GTR + I + G Codon Pos 2[a]: GTR + I + G Codon Pos 3[a]: GTR + I + G		GTR + G	GTR + G
Bayesian (individual loci)	Codon Pos 1[a]: GTR + I + G Codon Pos 2[a]: GTR + I + G Codon Pos 3[a]: GTR + I + G		GTR + G	GTR + G

[a] combined ND2 and ND3 sequence

B. Phylogenetic relationships

Maximum likelihood (not shown) and Bayesian (Fig. 2) analyses of the entire dataset produced well-resolved and mostly congruent topologies. Minor incongruences between the Bayesian consensus tree and the ML tree were characterized by low bootstrap support in the ML tree. Topologies from ML and Bayesian analysis of individual genes and the combined mitochondrial data (not shown) were almost entirely consistent with the Bayesian consensus tree inferred from the concatenated data. The only highly supported node incongruent with Fig. 2 was inferred from the mitochondrial gene dataset and placed *Ixos philippinus guimarasensis* sister to the clade formed by *I. p. mindorensis* and *I. siquijorensis*.

The topology recovered from our analyses was very similar to that inferred by Moyle and Marks (2006) because it was based in large part on the same data. Three main clades were inferred in this mostly Asian lineage of bulbuls. The first clade (Clade A,
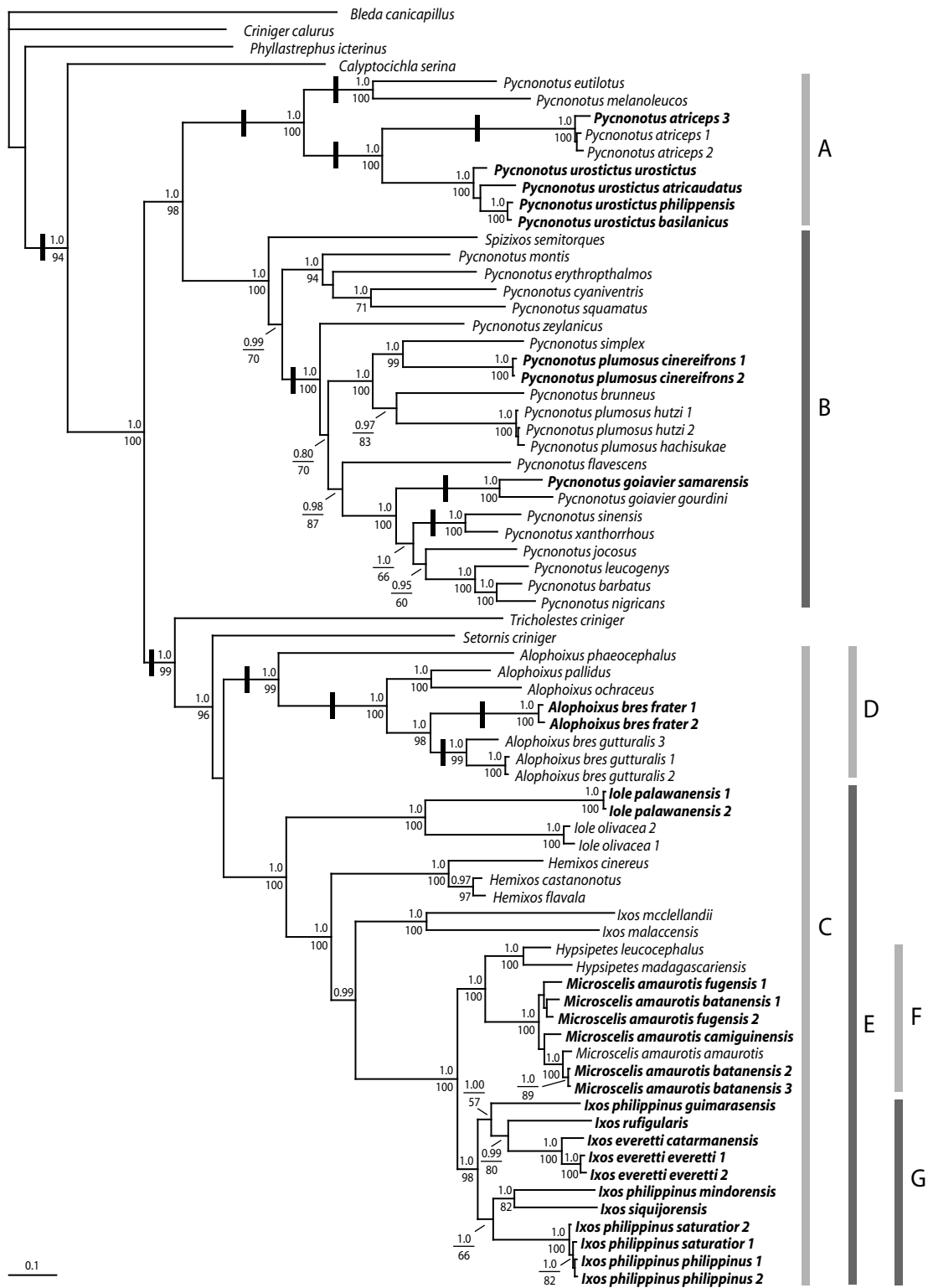
Figure 2. Bayesian consensus tree inferred from the combined sequences of the mitochondrial genes ND2 and ND3 and the nuclear introns Fib7 and TGF5. Philippine samples appear in bold. Numbers above nodes indicate Bayesian posterior probability > 95%, numbers below indicate ML bootstrap support > 50%. Black bars indicate the inference of indels in the introns.

Fig. 2) included four species of the genus *Pycnonotus* (*P. eutilotus*, *P. melanoleucos*, *P. atriceps*, and *P. urostictus*) and received high node support. This set of species was clearly distinguished from the other *Pycnonotus* species by a long deletion (ca. 203 bp) in the Fib7 gene.   Two of the species (*P. atriceps* and *P. urostictus*), which both occur in the Philippines but are allopatric, were most closely related to each other and share a 1-bp indel in TGF5. Monophyly of each species was well supported. Little genetic divergence (hereafter, genetic divergence refers to uncorrected p-distance in the ND2 data, unless otherwise stated) was detected between Bornean and Palawan individuals of *P. atriceps* (Table 4). On the other hand, genetic divergence between subspecies of *P. urostictus* varied from as high as 0.047 to as low as 0.008 (Table 4). A 38-bp deletion in Fib7 was common to *P. u. atricaudatus* and *P. u. urostictus*.

Table 4. Uncorrected p-distances in mitochondrial ND2 gene sequences of selected individuals.

Taxon pairs	*p*-distance
Pycnonotus atriceps 1 and *P. atriceps 3*	0.019
Pycnonotus atriceps 1 and *P. urostictus urostictus*	0.148
Pycnonotus urostictus atricaudatus and *P. u. basilanicus*	0.047
Pycnonotus urostictus philippensis and *P. u. basilanicus*	0.008
Pycnonotus plumosus cinereifrons 1 and *P. simplex*	0.120
Pycnonotus plumosus hutzi 1 and *P. p. hachisukae*	0.004
Pycnonotus goiavier samarensis and *P. g. gourdini*	0.071
Alophoixus bres frater 1 and *A. b. gutturalis 1*	0.109
Alophoixus bres gutturalis 1 and *A. b. gutturalis 3*	0.058
Iole palawanensis 1 and *Iole olivacea 2*	0.169
Microscelis amaurotis fugensis 1 and *M. a. batanensis 2*	0.044
Microscelis amaurotis amaurotis and *M. a. batanensis 3*	0.016
Ixos philippinus guimarasensis and *I. p. mindorensis*	0.122
Ixos philippinus guimarasensis and *I. rufigularis*	0.116
Ixos philippinus mindorensis and *I. siquijorensis*	0.106
Ixos philippinus saturatior 1 and *I. p. mindorensis*	0.110
Ixos rufigularis and *I. everetti everetti 1*	0.101
Ixos everetti everetti 1 and *I. e. catarmanensis*	0.034
Ixos philippinus saturatior 1 and *I. p. philippinus 1*	0.003

The second main clade (Clade B, Fig. 2) was sister to Clade A and consisted of all the other species of *Pycnonotus* and included *Spizixos semitorques*. In this clade a polyphyletic *P. plumosus* was recovered with high ML bootstrap support and posterior probabilities, although an SH Test performed could not reject monophyly of the species ($p = 0.165$). The Philippine population of this species *P. p. cinereifrons* was sister to *P. simplex* while its Bornean populations, *P. p. hutzi* and *P. p. hachisukae,* were sister to *P. brunneus*. The genetic divergence between *P. p. cinereifrons* and its sister taxon was very high (0.120) while the two Bornean subspecies *P. p. hutzi* and *P. p. hachisukae* showed little genetic difference between them (Table 4). Another Philippine species in Clade B showed substantial genetic divergence (0.071) between the Philippine subspecies *P. goiavier samarensis* and the Sunda race *P. g. gourdini* (Table 4). A 7-bp indel in Fib7 and a 4-bp indel in TGF5 were shared by both samples of *P. goiavier*.

The third main clade (Clade C, Fig. 2), formed by 8 genera (*Tricholestes, Setornis, Hypsipetes, Alophoixus, Iole, Ixos, Hemixos* and *Microscelis*) had strong node support and was sister to the clade formed by Clade A and Clade B. The study of Moyle and Marks (2006) placed Clade A as the most basal branch among the three main clades and this arrangement had high posterior probability (99%) but only moderately low ML bootstrap support (70%) in their study. The monotypic genus *Tricholestes* from the Malay Peninsula and the Greater Sundas branched basally in Clade C with strong

node support. Another monotypic genus, *Setornis* of the Greater Sundas diverged

next but this node had low support.   Aside from these two basal monotypic genera,

Clade C contained two main sub-groups: a well-supported clade of the genus

Alophoixus (Clade D, Fig. 2) and a lineage formed by the genera *Iole, Hemixos, Ixos,*

Hypsipetes and *Microscelis* (Clade E, Fig. 2), previously lumped together in the

genus *Hypsipetes*. In Clade E, all genera were recovered as monophyletic with the

exception of the genus *Ixos* (see below).

In Clade D, relationships among taxa of the genus *Alophoixus* were well-resolved. *A.*

phaeocephalus was sister to all other species in the genus. A monophyletic *A. bres*

was sister to a clade formed by *A. pallidus* and *A. ochraceus*. The inference of three

distinct indels in the Fib7 gene provided additional support to the recovered topology.

However, a 13-bp deletion shared by *A. pallidus, A. ochraceus* and *A. bres gutturalis*

was not shared with the Palawan taxon *A. bres frater*. This Palawan taxon was

genetically distinct from the Bornean *A. b. gutturalis* (Table 4). These two

populations were further distinguished by three apomorphic indels that were present

only in one population but not in both. Even within Borneo, considerable genetic

divergence was found between Sarawak and Sabah samples (Table 4). This pattern of

genetic differentiation between bird populations within Borneo has also been noted in

Enicurus leschenaulti (Moyle *et al.,* 2005) and *Copsychus saularis* (Sheldon *et al.,* in

press).

In Clade E, the genus *Iole* was the most basal lineage. Within this genus, the Palawan-endemic *I. palawanensis* showed deep genetic divergence (0.169, Table 4) from the Bornean taxon *I. olivacea*. The genus *Hemixos* and a clade formed by *Ixos mclellandii* and *Ixos malaccensis* were next to branch out sequentially from the base of Clade E. While this relationship received high posterior probability, ML bootstrap support was very low. Moyle and Marks (2006) inferred a sister relationship between *Hemixos cinereus* and *Ixos malaccensis* with moderately high node support.

The youngest lineage in Clade E included two sister clades: one formed by the genera *Hypsipetes* and *Microscelis* (Clade F) and the other comprising all Philippine species of the genus *Ixos* (Clade G). In Clade F, *Microscelis amaurotis*, was sister to a clade formed by two species of the genus *Hypsipetes.* Considerable genetic differences were found among different island populations of *M. amaurotis* within the Philippines but little genetic divergence was seen between populations from the Batanes Islands and Honshu, Japan (Table 4). Genealogical relationships between island populations of this species were not well resolved.

In the exclusively Philippine Clade G, a polyphyletic *Ixos philippinus* was evident with strong node support, although an SH Test performed could not reject that a monophyletic *Ixos philippinus* could as well explain the data ($p = 0.060$). Within *I. philippinus* three distinct lineages were inferred: *I. p. guimarasensis* from the western Visayas; *I. p. mindorensis* from the island of Mindoro; and a combined *I. p.*

philippinus and *I. p. saturatior*, from Luzon, eastern Visayas and Mindanao. *I. p. guimarasensis* was sister to a clade formed by *I. rufigularis* and *I. everetti* whereas *I. p. mindorensis* was sister to *I. siquijorensis*. The position of the third *I. philippinus* lineage was uncertain. *I. everetti* was monophyletic. These arrangements received high support from Bayesian posterior probabilities although a few received low or moderate ML bootstrap support. Genetic differences between these lineages were greater than 0.100 (Table 4).

IV. DISCUSSION

A. Taxonomy

Proposing a complete taxonomic revision of Asian bulbuls is beyond the scope of this study but some changes in the taxonomy of Philippine bulbuls are mandated based on these data. Individuals from two taxa, *Pycnonotus plumosus* and *Ixos philippinus*, did not form single, monophyletic clades. Although monophyly of each of these two taxa was not rejected by an SH Test, this test is considered very conservative (Goldman *et al.,* 2000; Buckley and Cunningham, 2002) and has a relatively low power of discrimination. Because the polyphyly of these species received strong nodal support from ML bootstrap proportions and posterior probabilities these two taxa warrant taxonomic changes, as follows. The Palawan population *P. p. cinereifrons* is genetically greatly divergent (0.136-0.138) from the Bornean taxa *P. p. hutzi* and *P. p. hachisukae,* and does not form a clade with them, but rather is sister to *P. simplex*. Morphological differences between populations on Borneo and Palawan are subtle (Fishpool and Tobias, 2005), but when combined with the large genetic differences and lack of monophyly, these data compel recognition of the Palawan population as a distinct species *Pycnonotus cinereifrons* (Tweedale, 1878). We lack samples of the other two subspecies, *P. p. porphyreus* and the nominate form, which occur in the western Greater Sunda Islands. However, based on geography it is unlikely that they are closely related to the Palawan subspecies and so it would be appropriate to retain them under *P. plumosus* until further data suggest otherwise.

Within the currently recognized taxon *Ixos philippinus*, at least three lineages warrant recognition as full species. One lineage, represented by the current subspecies *I. p. mindorensis*, is morphologically distinct, not closely related to the other subspecies of *I. philippinus*, and separated by a genetic distance of 0.106 from its closest relative, *Ixos siquijorensis*. A second lineage (*I. p. guimarasensis*) also is not related to other *I. philippinus* subspecies. Instead, it is recovered as sister to a clade comprising *Ixos rufigularis* and *Ixos everetti*. Two additional subspecies of *I. philippinus* included in our sampling, *I. p. saturatior* and *I. p. philippinus*, were recovered as sister to the clade of *I. p. mindorensis* and *I. siquijorensis*. Due to morphological differences (summarized in Fishpool and Tobias, 2005) and deep genetic divergences between these lineages, we recommend that the following taxa be recognized as full species: *Ixos mindorensis* (Steere, 1890), *Ixos guimarasensis* (Steere, 1890) and *Ixos philippinus* (J. R. Forster, 1795), with the last consisting of *I. p. saturatior* and the nominate form. The subspecies not included in our study, *I. p. parkesi*, known only from Burias Island, is best retained in *I. philippinus* until further review. Defining the limits of each of these species is beyond the scope of this study but a natural start to this effort is to use known distribution data of each subspecies (see Kennedy *et al.,* 2000).

We find the generic name *Ixos* inappropriate for the Philippine radiation of bulbuls in Clade G. The type species *I. virescens* Temminck, 1825 was not sampled but previous workers (Deignan, 1942; Dickinson and Gregory, 2002) have noted its close

affinity with *I. mclellandii* and *I. malaccensis*. To maintain monophyly of *Ixos* two options are available: return the species *guimarasensis, rufigularis, everetti, mindorensis, siquijorensis* and *philippinus* to the genus *Hypsipetes* or apply another generic name for this group. We prefer the first option for the sake of taxonomic stability.

Dickinson and Gregory (2002) noted similarities of the species *Microscelis amaurotis* with other members of the genus *Hypsipetes* but recommended placing it under its monotypic genus until molecular evidence clarifies its affinity. Our results, as well as that of Moyle and Marks (2006) support placement of the species in the genus *Hypsipetes*.

Individuals of *Alophoixus bres* from Palawan and Borneo were recovered as a clade. However, the Palawan form *A. b. frater* is diverged from Bornean samples by > 0.100 sequence divergence and, apart from its genetic distinctiveness, is easily distinguished morphologically. Fishpool and Tobias (2005) noted that *A. b. frater* has a slightly greener mantle compared with *A. b. gutturalis*. In addition, they differ by the following characters that were based on 21 skin specimens of *A. b. frater* and 3 specimens of *A. b. gutturalis* at the University of Kansas Natural History Museum: belly yellow in *A. b. frater* but pale brown in *A. b. gutturalis*; undertail coverts yellow in *A. b. frater* but rusty brown in *A. b. gutturalis;* flanks olive in *A. b. frater* while light brown in *A. b. gutturalis;* primaries and retrices dark brown edged olive in *A. b.*

frater but rusty brown edged rufous in *A. b. gutturalis*; throat of *A. b. frater* appears darker with more grey at base of throat feathers. Both genetic and morphological data support the recognition of *A. b. frater* (Sharpe, 1877) as a full species.

Considerable genetic divergence exists between named subspecies of other Philippine bulbul species. In *Pycnonotus urostictus,* pairwise distances greater than 0.040 separate the nominate form, *P. u. atricaudatus* and the group *P. u. philippinensis/P. u. basilanicus.* As noted above, the Bornean form *P. goiavier gourdini* is highly divergent (0.071) from the subspecies on Samar *P. goiavier samarensis.* Each population of *Microscelis amaurotis* in the Babuyan Islands show differences of 0.014–0.028 between islands, while in contrast, its nominate form on Honshu, Japan shows only 0.015–0.16 divergence from the subspecies *M. a. batanensis* in the Batanes Islands. The taxonomic treatment of these named forms requires further study of their morphology, voice and ecology.

B. Biogeography

The inferred phylogenetic relationships of Philippine bulbuls suggest eight independent colonization events of the Philippine islands – one each by *Pycnonotus atriceps*, *Pycnonotus urostictus*, *Pycnonotus plumosus cinereifrons*, *Pycnonotus goiavier*, *Alophoixus bres frater*, *Iole palawanensis*, *Microscelis amaurotis* and bulbuls of the genus *Ixos*. However this is not to say that the Philippine bulbul fauna was assembled only via colonization. We recovered a large clade of bulbuls endemic

to the Philippines that included a minimum of six species, indicating that substantial

in situ speciation also has contributed to contemporary bulbul diversity. The timings

of five invasions of the Philippines by bulbuls were evidently very old and may have

occurred synchronously as shown by an overlap in the 95% confidence intervals of

ages of nodes that gave rise to *Pycnonotus urostictus, P. plumosus, Alophoixus bres,*

Iole palawanensis and Philippine bulbuls of the genus *Ixos* (Fig. 3). This overlap is

much wider if the node leading to *A. bres* is excluded. This coincidence raises the

possibility of a single historical event influencing these splits such as a change in sea

levels or a substantial change in climate that severely fragmented habitats.

1. Patterns of colonization

Each of the major clades (A, B, and C) include taxa that colonized most of the entire

Philippine archipelago (Fig. 3). However, within each of these clades, one lineage

colonized either the Palawan region only or the oceanic Philippines region only, but

not both. Colonization of each region occurred only once in both Clades A and B,

and twice in Clade C. This pattern of invasion suggests that no colonization of the

oceanic Philippines by bulbuls took place through Palawan, assuming no extinctions

have occurred. This pattern is notable because the Palawan region has for a long time

been considered a stepping stone region to the Philippines (Dickerson, 1928; Inger,

1954; Darlington, 1957; Diamond and Gilpin, 1983; Dickinson *et al.,* 1991). In

bulbuls, the Palawan region did not serve as an "umbilicus" of Philippine fauna, as

Diamond and Gilpin (1983) called it; rather it was a biogeographic cul-de-sac.

A

Bleda canicapillus
Criniger calurus
Phyllastrephus icterinus
Calyptocichla serina
Pycnonotus eutilotus
Pycnonotus melanoleucos
Pycnonotus atriceps 3 (Palawan)
Pycnonotus atriceps 1
Pycnonotus atriceps 2
Pycnonotus urostictus urostictus (Luzon)
Pycnonotus urostictus atricaudatus (Samar)
Pycnonotus urostictus philippensis (Dinagat)
Pycnonotus urostictus basilanicus (Zamboanga)

Clade A

Spizixos semitorques
Pycnonotus montis
Pycnonotus erythropthalmos
Pycnonotus cyaniverntris
Pycnonotus squamatus
Pycnonotus zeylanicus
Pycnonotus simplex
Pycnonotus plumosus cinereifrons 1 (Palawan)
Pycnonotus plumosus cinereifrons 2 (Palawan)
Pycnonotus brunneus
Pycnonotus plumosus hutzi 1
Pycnonotus plumosus hutzi 2
Pycnonotus plumosus hachisukae
Pycnonotus flavescens
Pycnonotus goiavier samarensis (Samar)
Pycnonotus goiavier gourdini
Pycnonotus sinensis
Pycnonotus xanthorrhous
Pycnonotus jocosus
Pycnonotus leucogenys
Pycnonotus barbatus
Pycnonotus nigricans

Clade B

Tricholestes criniger
Setornis criniger
Alophoixus phaeocephalus
Alophoixus pallidus
Alophoixus ochraceus
Alophoixus bres frater 1 (Palawan)
Alophoixus bres frater 2 (Palawan)
Alophoixus bres gutturalis 3
Alophoixus bres gutturalis 1
Alophoixus bres gutturalis 2
Iole palawanensis 1 (Palawan)
Iole palawanensis 2 (Palawan)
Iole olivacea 2
Iole olivacea 1
Hemixos cinereus
Hemixos castanotus
Hemixos flavala
Ixos mcclellandii
Ixos malaccensis
Hypsipetes leucocephalus
Hypsipetes madagascariensis
Microscelis amaurotis fugensis 1 (Calayan)
Microscelis amaurotis batanensis 1 (Babuyan Claro)
Microscelis amaurotis fugensis 2 (Dalupiri)
Microscelis amaurotis camiguinensis (Camiguin Norte)
Microscelis amaurotis amaurotis
Microscelis amaurotis batanensis 2 (Batan)
Microscelis amaurotis batanensis 3 (Sabtang)
Ixos philippinus guimarasensis (Panay)
Ixos rufigularis (Zamboanga)
Ixos everetti catarmanensis (Camiguin Sur)
Ixos everetti everetti (Samar)
Ixos everetti everetti (Leyte)
Ixos philippinus mindorensis (Mindoro)
Ixos siquijorensis (Tablas)
Ixos philippinus saturatior 2 (Samar)
Ixos philippinus saturatior 1 (Leyte)
Ixos philippinus philippinus 1 (Polillio)
Ixos philippinus philippinus 2 (Luzon)

Clade C

0.02

B

Pycnonotus atriceps
Pycnonotus urostictus

Pycnonotus plumosus cinereifrons
Pycnonotus goiavier

Alophoixus bres frater
Iole palawanensis

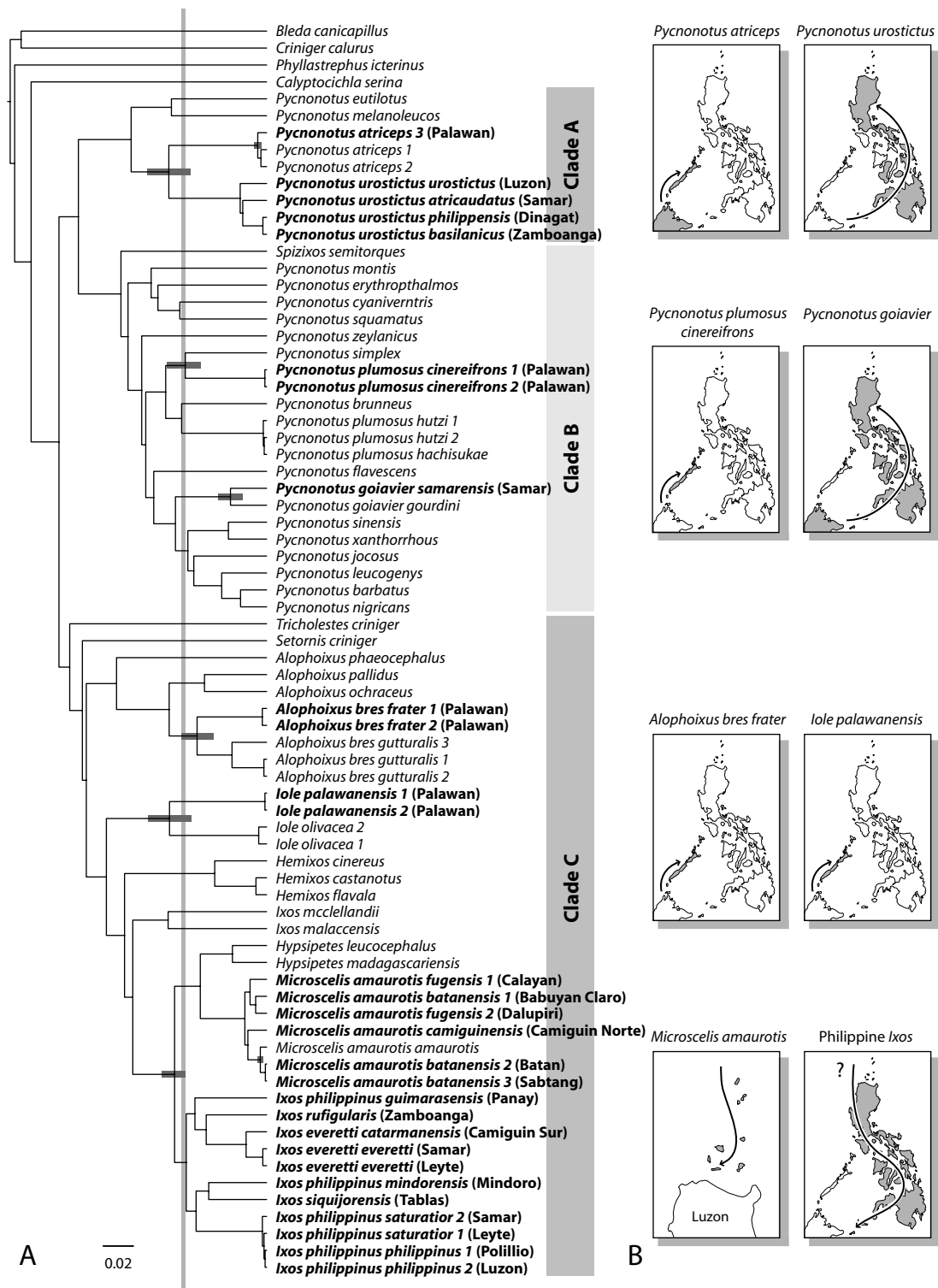Microscelis amaurotis
Luzon
Philippine *Ixos*
?

Fig. 3 (A) Ultrametric tree generated from the concatenated dataset. Bars along nodes indicate 95% C.I. of node heights of genetic splits of Philippine and non-Philippine taxa. Grey vertical bar shows possible coincidence of five splitting events. (B) Maps showing current distribution of taxa and their inferred colonization route.

Evidence of colonization of the oceanic Philippines from the Sundas through Palawan is lacking in phylogenetic studies of other bird groups that occur across these two regions such as honey-buzzards of the genus *Pernis* (Gamauf and Haring, 2004), pygmy kingfishers (Moyle *et al.,* 2007) flycatchers of the genus *Ficedula* (Outlaw and Voelker, 2008), striped babblers of the genus *Stachyris* (Moyle *et al.,* 2009) and flowerpeckers (Nyári *et al.,* in review). In other vertebrates, Palawan did not act as a gateway to the rest of the Philippines in pit vipers of the genus *Trimeresurus* (Sanders *et al.*, 2004). Evidence exists for bird taxa reaching only as far as Mindoro through Palawan based on distribution data (e.g. *Ceyx erithaca*, *Spizaetus cirrhatus* and *Treron curvirostra*) and at least one study noted the same colonization pattern in fanged frogs of the genus *Rana* (Brown and Guttman, 2002). The only evidence from molecular phylogenetic studies we have found for colonization of the Philippine oceanic islands via Palawan is in fruit bats of the genus *Cynopterus* (Campbell *et al.,* 2004). The role of Palawan as a bridge to the rest of the Philippines appears to have been over-emphasized in previous literature as similarly noted by Jones and Kennedy (2008b).

Colonization of the oceanic islands of the Philippines by bulbuls appears to have followed two routes. The ancestors of two lineages, *Pycnonotus urostictus* and *Pycnonotus goiavier* likely invaded from the south through the Sulu archipelago because they are sister to Bornean taxa and are currently present in the Sulus. On the other hand, *Microscelis amaurotis* likely colonized the Philippines from the north.

The individual from Japan was embedded inside the Babuyan and Batanes radiation of the species but arrangements in this clade were not well supported. We also lack samples of seven other subspecies found in the rest of its range. The path taken by the ancestor of Philippine bulbuls of the genus *Ixos* (Clade G) is unclear. Members of its sister Clade F range from East Asia through mainland Asia to Madagascar; no member of this group occurs in the Sundas, suggesting the possibility of a northern invasion route. On the other hand, the southern path through the Sulu archipelago is also possible because the genus sister to Clades F and G, *Ixos* (Fig.3), includes Bornean residents. Moreover, greater bulbul diversity within Clade G is present in southern Philippines than its northern half (see below), a pattern that can be expected if the lineage has occupied the south for a longer time. It is unlikely that bulbuls colonized the Philippines from Wallacea, a migration route inferred in other vertebrates (Evans *et al.,* 2003; Esselstyn *et al.,* in review) and hypothesized in other birds (Dickerson, 1928). The only extant bulbuls in the area belong to the genus *Thapsinillas* and are morphologically similar to *Ixos everetti* and may be closely related to this taxon (Fishpool and Tobias, 2005). If this is the case, then *Thapsinillas* may have been an offshoot from the Philippine *Ixos* radiation. Further work on the phylogenetics and phylogeography of more species occurring throughout the Philippines is required to better understand the importance of each colonization route.

2. Diversification of Philippine bulbuls

The island of Palawan harbors four sympatric bulbul species, each of which is associated with an independent invasion from Borneo. This number comes as no surprise because there is no evidence of *in situ* diversification on Palawan in birds and very few examples in other vertebrates (e.g., Heaney, 1979). Genetic divergences between Palawan bulbuls and their Bornean sister taxa, with the exception of *Pycnonotus atriceps,* are deep and thus were likely caused by long periods of isolation. Invasion of Palawan by the ancestors of these highly diverged taxa likely occurred long before the Pleistocene, when land bridges are thought to have appeared between Palawan and Borneo (Heaney, 1986) and served as a passageway for invading fauna. Not only did the origin of the endemic Palawan bulbuls likely predate these land-bridge connections to Borneo, the substantial genetic divergences indicate that the populations did not interbreed during times of overland connection. Even if land connections between Palawan and Borneo were present during the Pleistocene drier grassland habitat may have served as a barrier to interbreeding of these forest species (Bird *et al.,* 2007).

In the oceanic islands of the Philippines the assembly of bulbul fauna took place through a mixture of invasion and *in situ* diversification. On the islands outside the Mindanao Pleistocene island complex and Cebu, the number of species present is equal to the number of Philippine bulbul clades that reached them. Thus, Luzon harbors three species, Panay, two and small oceanic islands like Tablas, Camiguin

Sur and the Batanes Islands, one. Upon reaching these northern and western islands, some lineages developed unique forms (e.g. *Ixos philippinus mindorensis* on Mindoro, *Ixos siquijorensis* on Tablas) but no further diversification occurred. However, on islands belonging to the Mindanao Pleistocene island complex and on the island of Cebu, the number of species present is greater than the number of bulbul clades that invaded them. The island of Mindanao, for instance, harbors five species of bulbuls whereas only three waves of invasions of the island occurred. Three species on this island, *Ixos rufigularis, Ixos everetti,* and *Ixos philippinus saturatior*, belong to the same clade. Higher diversity in the southern Philippines that is not seen in the rest of oceanic Philippines may have resulted because Mindanao had intermittent land connections with the large islands of Leyte, Samar and Bohol (Heaney, 1986; Voris, 2000) and these temporary links may have provided opportunities for vicariant events and back-colonizations between islands. An alternative hypothesis is that these three species arose in allopatry prior to the mid-Miocene when the Zamboanga peninsula, central Mindanao and eastern Mindanao were distinct islands (Yumul et al., 2008). This explanation is unlikely because it would require several additional long-distance dispersal events and molecular divergences between the taxa do not seem high enough for such an ancient scenario. The island of Cebu, on the other hand, supports two bulbul species belonging to Clade G, the endemic subspecies *Ixos siquijorensis monticola* and *Ixos philippinus saturatior*. We lack samples from Cebu so the mode of assembly of bulbuls on the island is uncertain. If *I. s. monticola* is more closely related to other forms of the *I.*

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siquijorensis than it is to *I. p. saturatior* as suggested by current taxonomy then Cebu was likely the subject of two waves of immigration from neighboring islands.

Within the oceanic islands of the Philippines diversification patterns do not follow boundaries of late Pleistocene islands. We lack extensive sampling from different islands in each island group but some results are clear from the data. For instance, Luzon and Mindanao bulbuls are indistinct in *Ixos philippinus;* whereas in *Pycnonotus urostictus*, Samar and Mindanao birds are as distinct as Samar and Luzon individuals. Diversification patterns not congruent with late Pleistocene island configurations have also been observed in other studies of birds (e.g., Jones and Kennedy, 2008a, 2008b) and exceptions noted in other vertebrates (Esselstyn, *et al.,* in review).

This study proposes to increase the number of bulbul species endemic to the Philippines from six to ten. The subspecies *Ixos siquijorensis cinereiceps* from Tablas Island will likely be split from the nominate form on Siquijor Island because considerable vocal and plumage differences have been noted (Allen, 2006). This result underscores how Philippine bird diversity is currently underestimated (see Peterson, 2006). Further molecular systematic studies on other bird families and with greater island sampling are recommended to better understand the history of avian diversification in this archipelago.

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