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Cryptic genetic diversity in "widespread" Southeast Asian bird species suggests that Philippine avian endemism is gravely underestimated

David J. Lohman [a,b,*], Krista K. Ingram [c], Dewi M. Prawiradilaga [d], Kevin Winker [e], Frederick H. Sheldon [f], Robert G. Moyle [g], Peter K.L. Ng [a], Perry S. Ong [h], Luan Keng Wang [a], Thomas M. Braile [e,i], Dwi Astuti [d], Rudolf Meier [a]

[a] *Department of Biological Sciences, National University of Singapore, 14 Science Drive 4, Singapore 117543, Republic of Singapore*
[b] *Department of Biology, The City College of New York, The City University of New York, Convent Avenue at 138th Street, New York, NY 10031, USA*
[c] *Department of Biology, Colgate University, 13 Oak Drive, Hamilton, NY 13346, USA*
[d] *Division of Zoology, Research Centre for Biology-LIPI, Jl. Raya Bogor Km. 46, Cibinong-Bogor 16911, Indonesia*
[e] *University of Alaska Museum, 907 Yukon Drive, Fairbanks, AK 99775, USA*
[f] *Museum of Natural Science, Louisiana State University, 119 Foster Hall, Baton Rouge, LA 70803, USA*
[g] *Biodiversity Research Center, University of Kansas, 1345 Jayhawk Boulevard, Lawrence, KS 66045, USA*
[h] *Institute of Biology, University of the Philippines-Diliman, Quezon City 1101, Philippines*
[i] *National Museum of the Philippines, Padre Burgos St., Manila 1000, Philippines*

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ABSTRACT

Mistakenly classifying morphologically cryptic endemic species as populations of widespread species potentially interferes with the conservation of biodiversity because undetected endemics that are imperilled may lack appropriate protection. It also impedes the reconstruction of the evolutionary history of a taxon by obscuring the number and distributional limits of species. Here, we present genetic and phylogenetic evidence corroborated by morphology that Philippine populations of seven widespread, non-migratory passerine birds might represent unrecognized, distinct species. An extrapolation based on this finding suggests that the proportion of endemic bird species in the Philippines could be much higher than currently estimated. This high degree of cryptic diversity in a well-studied, volant taxon implies that large numbers of unrecognized species can be expected in less thoroughly studied groups. We predict that genetic investigations of insular populations of widespread species will frequently reveal unrecognized island endemics, and because of the vulnerability of island habitats and their biota, these taxa may be particularly susceptible to extinction.

1. Introduction

Species richness, endemism, and habitat loss are frequently used to designate and prioritize areas for conservation (Myers et al., 2000; Prendergast et al., 1993). Conservation planning therefore depends on accurate species-level taxonomy (Mace, 2004). When a single nominal species actually includes more than one biologically distinct species, alpha diversity is underestimated and conservation priorities are improperly assigned because endemic species are overlooked. The importance of discovering such cryptic diversity within widespread species is now well recognized (Dulvy and Reynolds, 2009; Marks, 2010; Zou et al., 2007) and is frequently facilitated through the incorporation of genetic data in biodiversity research (Balke et al., 2009; Bickford et al., 2007; Meier, 2008).

Cryptic species-level diversity occurs not only in poorly understood taxa. Even comparatively well-studied groups such as birds occasionally yield multiple well-differentiated lineages within a widespread species (Moyle et al., 2005; Sheldon et al., 2009; Zou et al., 2007). Here, we examine whether the unexpectedly low proportion of endemic bird species found in the Philippines may be due to undiscovered species-level differentiation. Endemism in Philippine birds is currently thought to be just 31%, which is substantially lower than in Philippine land mammals (64%) and amphibians (77%) (Kennedy et al., 2000; Ong et al., 2002). Although lower levels of endemism might be expected in volant animals, this dearth of avian endemics is surprising considering that the Philippines has been separated from other land masses for millions of years (Hall, 2001), that many resident forest bird species rarely disperse over water (Moore et al., 2008), and that only one-third of Philippine birds are migratory or oceanic (Kennedy et al., 2000).

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* Corresponding author at: Department of Biology, The City College of New York, The City University of New York, Convent Avenue at 138th Street, New York, NY 10031, USA. Tel.: +1 212 650 8456; fax: +1 212 650 8585.
E-mail address: dlohman@ccny.cuny.edu (D.J. Lohman).

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The biota of the Philippines is regarded as one of the planet's most diverse and imperilled (Heaney and Mittermeier, 1997), causing this archipelago to be classified as a biodiversity hotspot (Myers et al., 2000). The mistaken inclusion of cryptic endemic species in widespread taxa can have important consequences for species survival and the strategic designation of protected areas in the Philippines. To test the hypothesis that Philippine populations of widespread Southeast (SE) Asian birds may include cryptic endemic species (Peterson, 2006), we studied 10% of non-endemic, resident Philippine passerine species ($N = 7$) to examine patterns of intraspecific genetic diversity and phylogeography across SE Asia.

Few studies have examined patterns of genetic diversity and speciation in SE Asia despite its dynamic geographic complexity (Sathiamurthy and Voris, 2006), its historical importance in the development of biogeographical study (Wallace and Daito, 1865), and the extreme threats faced by its biota (Sodhi et al., 2004). In part, legal and logistical difficulties impede studies that require sample collection across this geographically and culturally diverse region (Madhusudan et al., 2006). With tissue samples from 16 countries gathered over 18 years by 54 collectors and held in 13 institutions (Supplementary Table 1), we were able to assemble the most extensive multi-species dataset to date for Southeast Asian birds. Here, we examine regional patterns of dispersal and genetic diversity within morphologically defined species.

2. Material and methods

2.1. Taxon sampling

We studied seven focal species fitting the following criteria: (1) confined to Asia and widespread in SE Asia; (2) amenable to sampling with mist nets; and (3) represented in the world's avian tissue collections with multiple samples from different localities. Based on these criteria, we studied seven species: *Arachnothera longirostra* ($N = 45$); *Copsychus saularis* ($N = 51$); *Dicaeum trigonostigma* ($N = 10$); *Lalage nigra* ($N = 9$); *Nectarinia jugularis* ($N = 20$); *Pycnonotus goiavier* ($N = 35$); *Rhipidura javanica* ($N = 40$). All species but *L. nigra* have described endemic Philippine subspecies, which is not surprising considering that nearly 80% of all resident, non-endemic Philippine passerines have endemic subspecies (Kennedy et al., 2000). We included as outgroups all congeneric species with available tissue samples; we also included all available cytochrome *c* oxidase subunit I (COI) and cytochrome *b* (cytb) sequences from GenBank for focal and congeneric outgroup species. Seventy-eight percent of our samples came from vouchered specimens. Information for each sample, including voucher location, is provided in Supplementary Table 1.

2.2. DNA sequencing

DNA was extracted from liver, muscle, or blood with a Qiagen DNeasy Blood & Tissue Kit. Two mitochondrial gene regions were amplified and sequenced from all individuals: 655 bp of the COI DNA barcoding region and an additional fragment of *ca.* 1230 bp that included the entire cytb gene and approximately 30–40 bp flanking each end of this gene. The dataset for *C. saularis* and its outgroups lacked sufficient variability for the reconstruction of a robust phylogenetic hypothesis, and a third mitochondrial gene, NADH hydrogenase subunit 2 (ND2) was sequenced from all *Copsychus* specimens. The entire cytb gene was amplified in two overlapping fragments, generally with the primer pairs Passer-cytb-F1B/Passer-cytb-R1 and Passer-cytb-F3/Passer-cytb-R2, but other primer pairs were used on recalcitrant samples (Supplementary Table 2). The improved avian DNA barcoding primer pair Passer-F1/Passer-R1 was used to amplify COI from all samples (Lohman et al., 2009), and the primer pairs L5215/CopsyND2-R1 and CopsyND2-F2/H6313 were used to amplify the entire ND2 gene in two overlapping fragments (Supplementary Table 2). Each PCR reaction consisted of 2.5 µl 10× TaKaRa ExTaq buffer with 20 mM $MgCl_2$, 1.2 µl of each 10 mM primer, 1 µl 100 mM dNTPs, 0.2 µl TaKaRa ExTaq polymerase, 16.9 µl H_2O, with 2 µl template DNA, for a total reaction volume of 25 µl, and was amplified with the following thermal cycler conditions: 3 min at 95 °C followed by 40 cycles of 1 min at 94 °C, 40 s at 58 °C (COI) or 53 °C (cytb), and 1.5 min at 72 °C, and finally 5 min at 72 °C. The resulting products were cleaned with SureClean (BioLine), cycle-sequenced with BigDye Terminator 3.1 (ABI), cleaned with CleanSEQ (Agencourt), and sequenced in both directions on an ABI 3130xl DNA analyzer.

2.3. Phylogenetic analyses

Each gene was aligned separately for each bird genus. Alignments of COI sequences, which contained no indels, were performed with Sequencher 4.6 (Gene Codes Corp.). Approximately 40 bases preceding the 5′ end of cytb (comprising the 3′ end of the ND5 gene and an intergenic region of variable length) and approximately 30 bases following the 3′ end of cytb (comprising part of the Thr-tRNA gene and an intergenic region of variable length) were also sequenced. Each of these flanking regions was aligned separately using MUSCLE (Edgar, 2004) implemented in Seaview (Galtier et al., 1996); cytb was aligned in Sequencher. All genes were concatenated using TaxonDNA 1.5 (Meier et al., 2006) for subsequent analyses.

Phylogenetic analyses were conducted using several optimality criteria. Bayesian phylogenetic analyses were performed with MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). The data were divided into four partitions: each codon position of the protein encoding genes was a separate partition, and the combined regions flanking the cytb gene comprised the fourth partition. MrModeltest 2.2 (Nylander, 2004) was used to select an appropriate evolutionary model for each partition using Akaike's Information Criterion (Supplementary Table 3). Parameter values for the substitution models were estimated from the data and allowed to vary independently between partitions. Two runs of four chains (one cold and three heated, temp = 0.25) were run simultaneously for 6 million generations, and trees were sampled every 100 generations. Changes in the posterior probabilities of up to twenty splits were plotted over the generations of the analysis with the computer program "Are We There Yet?" (Nylander et al., 2008) to confirm that the chains had probably converged. Only the *C. saularis* tree topology failed to stabilize after 6 million generations, but it did converge after a second analysis of 10 million generations. After completion of each analysis, the first 25% of the sampled trees was discarded before a majority-rule consensus tree was calculated from the remaining trees.

Maximum likelihood bootstrap trees were constructed with GARLI 0.96 (Zwickl, 2006). The appropriate evolutionary model for each unpartitioned dataset was determined with MrModeltest (Supplementary Table 3), and all model parameters were estimated from the data. Each bootstrap replicate automatically terminated after the search algorithm progressed 10,000 generations without improving the tree topology by a log likelihood of 0.01 or better. A majority-rule consensus tree of the 500 bootstrap replicate trees was calculated with PAUP∗ 4.0b10 (Swofford, 2002).

Parsimony bootstrap support for the phylogenetic estimates was assessed with T.N.T. 1.1 (Goloboff et al., 2008). After increasing the maximum number of saved trees to 5000, we performed a bootstrap analysis using symmetric resampling (Goloboff et al., 2003) implementing a traditional search with 33% change probability (5000 replicates). The results were summarized as absolute frequencies.

2.4. Genetic analyses

Uncorrected proportional genetic distances (*p*-distances) among samples were calculated with PAUP∗, and within-group and between-group *p*-distances were calculated with MEGA 4.0 (Tamura et al., 2007). Standard errors of distances calculated with MEGA were obtained through 1000 bootstrap replicates. In the absence of suitable fossils and known vicariance or dispersal events that could be used to date the intraspecific divergences in these species, we approximated the divergence time of extant Philippine populations by dividing cytb *p*-distances between Philippine and non-Philippine birds by 0.021. The mean, minimum, and maximum *p*-distances were divided by this value to obtain a range of plausible dates. The evolutionary rate of 2.1% sequence divergence per million years was recently validated by comparing 74 bird fossil and biogeographic calibration points of varying ages with cytb sequence divergence (Weir and Schluter, 2008). The approximate dates estimated with this method fell within the range calculated with a relaxed clock, uncorrelated lognormal model (Sheldon et al., 2009).

2.5. Extrapolation procedure

Extrapolations of the number of potential cryptic bird species were based on the species included in Kennedy et al. (2000). Of the 572 bird species occurring in the Philippines, 180 are endemic or near-endemic (Kennedy et al., 2000). Because migratory species are unlikely to undergo genetic differentiation in isolation, extrapolations focused on non-migratory, non-endemic passerines with Philippine distributions not restricted to the peripheral Batan, Babuyan, or Sulu islands. Of 240 Philippine passerine species, 108 are endemic, 53 are migratory, 4 are introduced, and 3 are geographically restricted. The remaining 72 are resident and non-endemic (Supplementary Table 4).

We suspect that birds in other orders that avoid flying over water may show similar patterns. To estimate these numbers, we performed analogous calculations on the number of non-endemic, non-migratory species in each of the following orders: Apodiformes, Caprimulgiformes, Columbiformes, Coraciiformes, Gruiformes, Piciformes, and Psittaciformes.

3. Results and discussion

3.1. Phylogenetic and genetic patterns

Parsimony, maximum likelihood, and Bayesian methods all produced congruent phylogenetic hypotheses for each species, and Philippine populations were always distinct from conspecific populations outside of the Philippines (Fig. 1, Supplementary Fig. 1). The only consistent phylogeographic pattern among the species was the presence of a strongly supported clade containing exclusively Philippine individuals, suggesting a lack of gene flow in or out of the Philippine archipelago over time spans ranging from approximately 1.5–4.6 My (Table 1).

For most species, intraspecific genetic distances were unusually large. The smallest COI *p*-distance in all possible intraspecific comparisons between Philippine and non-Philippine samples exceeded 3% in five of the seven species we examined: *A. longirostra*, 8.8%; *R. javanica*, 5.6%; *P. goiavier*, 4.9%; *C. saularis*, 3.9%; *N. jugularis*, 3.2%; *D. trigonostigma*, 1.4%; *L. nigra*, 0.9% (Table 1). Although genetic distances merely approximate differentiation between species (Meier et al., 2006; Meier et al., 2008) and alone are not diagnostic of species limits (Winker, 2009), these distances are consistent with species-level differentiation among birds (Kerr et al., 2007; Meier et al., 2008). Further, levels of intraspecific variation overlapped with congeneric inter-specific variation in five of the seven species examined (Table 1). Closely related species occasionally share identical COI (Kerr et al., 2009, 2007; Meier et al., 2008) or cytb (Johns and Avise, 1998) sequences, and thus small genetic distances cannot be taken as evidence of conspecificity.

This genetic and phylogenetic evidence suggesting that Philippine populations are distinct species is corroborated by morphology. Morphologically-defined endemic Philippine subspecies are recognized in all but one focal species, *L. nigra*, which is the least genetically differentiated species in this study (Dickinson, 2003; Peterson, 2006). These endemic subspecies are: *A. longirostra flammifera*; *C. saularis deuteronymus* and *C. s. mindanensis*; *D. trigonostigma sibuyanicum* and *D. t. cinereigulare*; *N. jugularis jugularis* and *N. j. obscurior*; *P. goiavier goiavier*, *P. g. samarensis*, and *P. g. suluensis*; and *R. javanica nigritorquis*. If one used a combination of monophyly, morphological distinctiveness as recognized by current subspecific taxonomy, and a 3% COI distance as a threshold for highlighting possible unrecognized species, six putatively new endemic Philippine species are revealed in our sample of seven widespread "species". We feel that further work will support these as full species but we refrain from formally recognizing them pending additional data (*e.g.*, Collar, 2007). These species are found in the genera *Arachnothera, Copsychus, Nectarinia*, *Pycnonotus* (two endemic lineages), and *Rhipidura*, which represent four separate passerine families.

3.2. Species-specific patterns

Several species-specific phylogeographic findings are noteworthy. First, *C. saularis* is paraphyletic with regard to the Madagascar endemic *C. albospecularis* (Fig. 1B, Supplementary Fig. 1B), which has prompted the elevation of Philippine populations to the species *C. mindanensis* (Sheldon et al., 2009). Second, *P. goiavier* was the only species in our sample with significant phylogenetic structure within the Philippines. Individuals from Mindanao formed a monophyletic group distinct from other Philippine *P. goiavier* individuals (Fig. 1G, Supplementary Fig. 1F). Mindanao samples were further differentiated by a unique 1 bp insertion flanking the 5′ end of cytb and a minimum pairwise COI distance of 3.2% between birds from Mindanao and elsewhere. Philippine individuals of *D. trigonostigma* were differentiated from non-Philippine individuals by an insertion of 10 bp flanking the 3′ end of cytb.

Philippine bulbuls, including *P. goiavier*, may be prone to cryptic diversification in the Philippines. Oliveros and Moyle (2010) found that two other Pycnonotidae "species" with Philippine populations were not monophyletic. Palawan and Bornean populations of *Pycnonotus plumosus* were genetically very divergent and did not form a clade, prompting elevation of the Palawan populations to a new species. Similarly, the smallest clade with all *Ixos philippinus* individuals also contained three other *Ixos* species, leading the authors to divide *I. philippinus* into three endemic species.

There were no consistent intraspecific phylogeographic patterns among the seven focal "species" across the Sunda shelf, which implies that dispersal patterns were not contemporaneous across the land bridges that joined Java, Sumatra, Borneo and the Malay Peninsula as recently as 10,550 y BP (Sathiamurthy and Voris, 2006). For example, every geographically defined population of *A. longirostra* was monophyletic, including birds from Singapore and from Johor Bahru at the tip of peninsular Malaysia, which are separated by less than 1 km of water (Supplementary Fig. 1A). In contrast, COI sequences from several Singaporean *R. javanica* were identical to individuals collected in East Kalimantan on the far side of Borneo, and *R. javanica* specimens from the entire Sunda shelf formed a single, undifferentiated clade (Fig. 1E, Supplementary Fig. 1G). Given the dramatic movements of land masses in SE Asia over the past several million years (Hall, 2001) and the fusion and separation of islands on the Sunda shelf

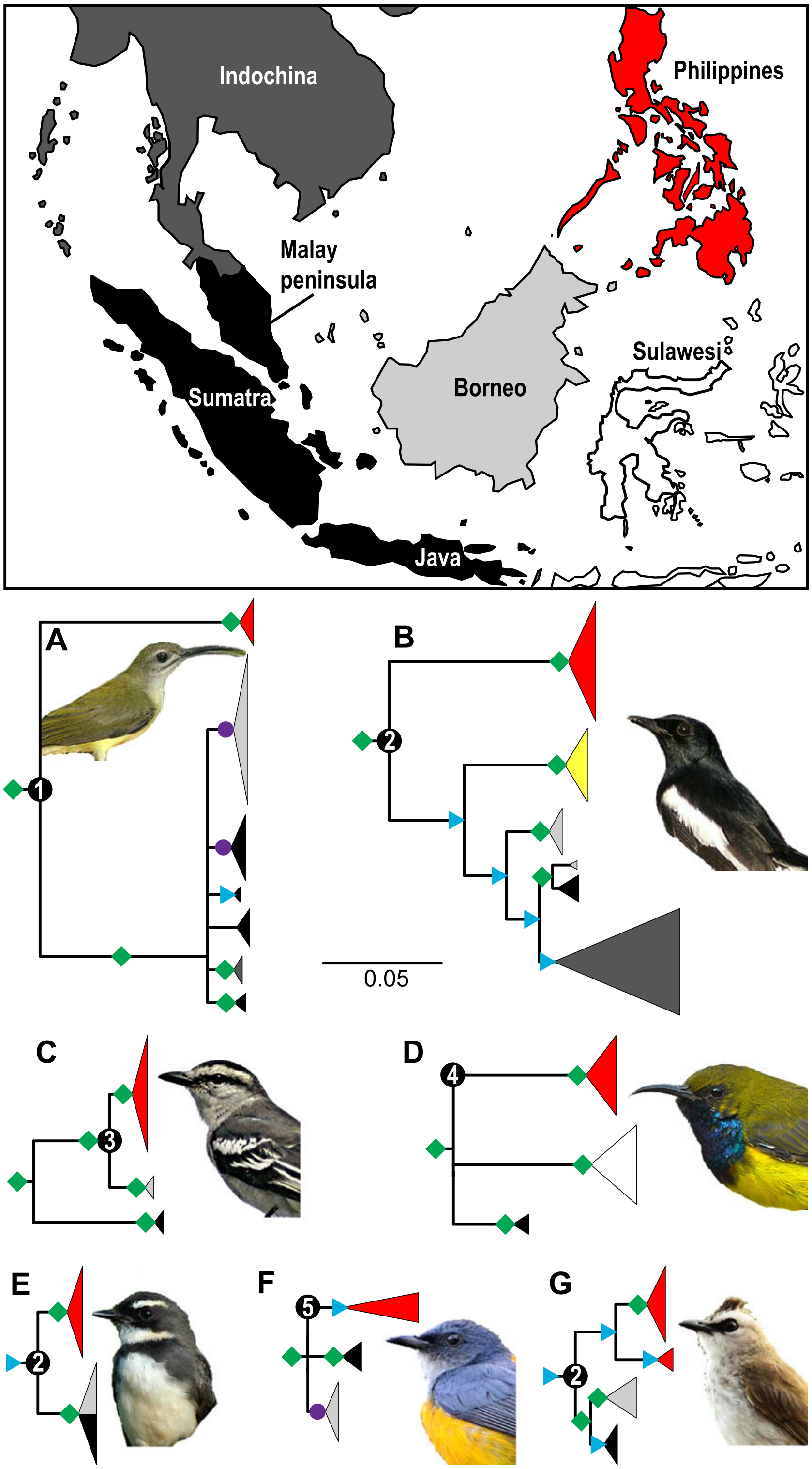

Fig. 1. Bayesian consensus trees of (A) *Arachnothera longirostra*; (B) *Copsychus saularis*; (C) *Lalage nigra*; (D) *Nectarinia jugularis*; (E) *Rhipidura javanica*; (F) *Dicaeum trigonostigma*; (G) *Pycnonotus goiavier*. Numbered nodes indicate approximate divergence between Philippine and non-Philippine clades (1) 4.6 ± 0.4 My; (2) 2.8 ± 0.3 My; (3) 1.6 ± 0.2 My; (4) 2.5 ± 0.2 My; (5) 1.5 ± 0.2 My. Clade support: ◆ = Bayesian posterior probability (B) ⩾99, maximum likelihood bootstrap (ML) ⩾95, and parsimony bootstrap (P) ⩾90; ▶ = B ⩾ 95, ML ⩾ 75, P ⩾ 70; ● = B ⩾ 90, ML ⩾ 50, P ⩾ 50; clades with B < 75 are collapsed. Colours of collapsed clades denote geographic locality of the samples as indicated by the map; the yellow *Copsychus* clade represents Malagasy *C. albospecularis*.

over the past several thousand years (Sathiamurthy and Voris, 2006), it seems unlikely that many common patterns will emerge across taxa. Even within the Philippines, patterns of island colonization and dispersal vary widely among species, and Philippine populations of several widespread bird species appear to have colonized the archipelago more than once (Jones and Kennedy, 2008; Oliveros and Moyle, 2010).

Samples from Sulawesi were available for a single species, *N. jugularis*. The three Sulawesi samples formed a strongly supported monophyletic group with six samples from the Solomon

Table 1
Genetic divergence at two mitochondrial loci in seven species of passerine birds widespread in SE Asia. Intraspecific and inter-specific divergence columns present the range of uncorrected pairwise (*p*-) distances in all possible pairwise comparisons between conspecific and congeneric samples, respectively. Values for the smallest Philippine/non-Philippine divergence represent the smallest distance between any Philippine and non-Philippine sample pair in all possible pairwise comparisons. Gray shading highlights overlap of intra- and inter-specific genetic distances, and bold smallest divergence values highlight values larger than 0.0224, the largest intraspecific distance found in a previous survey of intraspecific genetic diversity within bird species (Kerr et al., 2007). Including samples of *Copsychus albospecularis* in the *C. saularis* dataset (making nominal *C. saularis* paraphyletic) did not affect any of the values reported in this table.

	Gene	Intraspecific divergence	Inter-specific divergence	Smallest philippines/non-philippines divergence
Arachnothera longirostra	COI	0–0.098	0.107–0.165	**0.088**
	cytB	0–0.149	0.096–0.143	**0.094**
Copsychus saularis	COI	0–0.060	0.031–0.117	**0.039**
	cytB	0–0.094	0.024–0.120	**0.052**
Dicaeum trigonostigma	COI	0–0.030	0.085–0.127	0.014
	cytB	0–0.040	0.084–0.163	0.019
Lalage nigra	COI	0–0.064	0.002–0.095	0.009
	cytB	0–0.061	0.065–0.108	0.021
Nectarinia jugularis	COI	0–0.061	0.088–0.134	**0.032**
	cytB	0–0.055	0.064–0.130	**0.044**
Pycnonotus goiavier	COI	0–0.062	0.010–0.160	**0.049**
	cytB	0–0.065	0.013–0.159	**0.053**
Rhipidura javanica	COI	0–0.068	0.065–0.198	**0.056**
	cytB	0–0.062	0.058–0.179	**0.057**

Islands, and this was sister to the Philippine clade (Supplementary Figure 1E). Unfortunately, no Bornean specimens were available for this species. Because of its unique geological history, endemism is high in Sulawesi (Evans et al., 2003), and additional sampling from this island may reveal that – like the Philippines – Sulawesi populations of “widespread” bird species are highly genetically distinct. In addition, studies of genetic differentiation between Solomon Island birds (Smith and Filardi, 2007) and conspecifics found elsewhere will illuminate patterns of dispersal across the Sahul shelf.

3.3. Estimation of Philippine avian endemism

Assuming that our sample is representative of Philippine populations of species that are considered widespread in SE Asia, the data suggest that current alpha taxonomy underestimates the true number of Philippine endemic bird species by at least 50%. This estimate is based on the observation that there are 72 non-endemic, resident Philippine passerine bird species (Kennedy et al., 2000) and on our finding of six new putative species from our sample of seven, suggesting that 62 of these (86% of 72%) might have cryptic Philippine lineages. It is unlikely that underestimation of regional endemism is confined to the perching birds (Passeriformes). We therefore extended this projection to terrestrial non-passerine bird orders that are unlikely to fly over water and which contain some endemic Philippine species (see section 2.5). If the percentage of species with cryptically distinct Philippine lineages is the same in these groups as in passerines, then 50 of these resident, non-migratory species (86% of 58%) are projected to have cryptic Philippine lineages. This suggests that, after additional taxonomic scrutiny, the percentage of endemic species in the entire Philippine avifauna could increase from its present 31% to approximately 50%. A recent molecular phylogenetic study of Asian bulbuls (Pycnonotidae) (Oliveros and Moyle, 2010) raised endemic subspecies to species status and split endemic species into multiple species, providing independent evidence that Philippine bird endemism is lower than currently estimated.

Studies of the factors contributing to endemicity and restricted geographical ranges in birds are few, but Philippine species currently known to be endemic are generally dependent on intact forests. This strongly suggests that our extrapolation is conservative, because all the species sampled in this study prefer disturbed habitats and are vagile, whereas many of the unsampled species are more prone to differentiation because they inhabit closed forest and disperse poorly.

Fewer than one-fifth of the world’s bird species are restricted to islands, but over 90% of documented avian extinctions are island endemics (Johnson and Stattersfield, 1990). This is because islands generally support smaller populations that are more prone to inbreeding and are more susceptible to natural disasters and anthropogenic disturbance including habitat loss and predation by introduced predators (Blackburn et al., 2004; Duncan and Blackburn, 2007). Conservation resources are allocated toward protecting endangered species – rarely subspecies – and the mistake of regarding island endemics as populations of widespread species has had dire consequences for endemic avifauna in other archipelagos (Hazevoet, 1996; Sangster, 2000).

4. Conclusions

Strongly supported monophyly, relatively large genetic distances, and morphological distinctiveness all suggest that a large fraction of the “non-endemic” Philippine avifauna is composed of unrecognized endemic species. Extrapolation from our data suggests that avian endemicity may eventually be revised upwards by as much as 50%. Among species, phylogeographic patterns across the Sunda shelf evince a variety of dispersal histories, but Philippine populations of all focal species were strongly monophyletic and seem to have diverged from other populations 1.5–4.6 Ma. Our findings suggest that insular populations of many widespread species may represent overlooked endemic species presently lacking recognition and protection. This oversight is particularly troubling because island populations are especially prone to extirpation (Biber, 2002; Groombridge, 2007) and because the Philippine archipelago has lost >75% of its forests in the past century (Ong et al., 2002).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biocon.2010.04.042.

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