Genomic differentiation in an endemic Philippine genus (Aves: *Sarcophanops*)due to geographic isolation on recently disassociated islands

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Phylogeographic studies of Philippine vertebrates have demonstrated that genetic variation is broadly partitioned by Pleistocene island aggregation. Contemporary island discontinuity is expected to influence genetic differentiation, but remains relatively undocumented perhaps because the current episode of island isolation started relatively recently. We investigated inter- and intra-island population structure in a Philippine endemic bird genus (*Sarcophanops*) to determine if genetic differentiation has evolved during the recent period of isolation. We sequenced thousands of genome-wide RAD markers from throughout the Mindanao group to assess fine-scale genetic structure across islands. Specifically, we investigated patterns of gene flow and connectivity within and between taxonomic and geographic bounds. A previous assessment of mitochondrial DNA detected deep structure between *Sarcophanops samarensis* and sister species, *S. steerii*, but was insufficient to detect differentiation within either species. Analysis of RAD markers, however, revealed structure within *S. samarensis* between the islands of Samar/Leyte and Bohol. This genetic differentiation likely demonstrates an effect of recent geographic isolation (post-LGM) on the genetic structure of Philippine avifauna. We suggest that the general lack of evidence for differentiation between recently isolated islands is a failure to detect subtle population structure due to past genetic sampling constraints, rather than the absence of such structure.

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Abstract

Phylogeographic studies of Philippine vertebrates have demonstrated that genetic variation is broadly partitioned by Pleistocene island aggregation. Contemporary island discontinuity is expected to influence genetic differentiation, but remains relatively undocumented perhaps because the current episode of island isolation started relatively recently. We investigated inter- and intra-island population structure in a Philippine endemic bird genus (*Sarcophanops*) to determine if genetic differentiation has evolved during the recent period of isolation. We sequenced thousands of genome-wide RAD markers from throughout the Mindanao group to assess fine-scale genetic structure across islands. Specifically, we investigated patterns of gene flow and connectivity within and between taxonomic and geographic bounds. A previous assessment of mitochondrial DNA detected deep structure between *Sarcophanops samarensis* and sister species, *S. steerii*, but was insufficient to detect differentiation within either species. Analysis of RAD markers, however, revealed structure within *S. samarensis* between the islands of Samar/Leyte and Bohol. This genetic differentiation likely demonstrates an effect of recent geographic isolation (post-LGM) on the genetic structure of Philippine avifauna. We suggest that the general lack of evidence for differentiation between recently isolated islands is a failure to detect subtle population structure due to past genetic sampling constraints, rather than the absence of such structure.

Introduction

The Philippine Archipelago is recognized as one of the most biologically diverse hotspots in the world (Myers et al., 2000) largely due to a complex geologic and climatic history that has catalyzed the evolution of endemic biodiversity (Brown et al., 2013). Due to cyclic sea level change, the extent of land above water in the Philippine Archipelago has varied dramatically throughout its geologic history. Specifically, changing climate regimes during the Last Glacial Maximum (LGM; 19-25 kyr bp) resulted in lower global sea levels, consequently uncovering shallow land bridges between islands. This network of shallow land bridges dramatically increased connectivity across the archipelago (Heaney, 1985), forming clustered groups of interconnected islands, or Pleistocene Aggregate Island Complexes (PAICs; Diesmos et al., 2002; Brown et al., 2013). Of the more than 7,000 islands found in the present-day Philippine Archipelago (Kennedy et al., 2000), nearly all were reduced to six large PAICs (Luzon, Palawan, Mindoro, Negros-Panay, Mindanao, Sulu; Heaney, 1985)

Endemic Philippine avifauna generally adheres to the patterns of geographic and phylogenetic structure predicted under the PAIC model, at least when additional complexities such as topography, paleoclimatic factors, and colonization history are acknowledged (Hosner, Nyári & Moyle, 2013; Hosner et al., 2014; Sánchez-González, Hosner & Moyle, 2015). That is, populations present on a particular PAIC (e.g. Mindanao PAIC), are likely to be closely related to one another, but genetically distinct from populations confined to different PAICs during the LGM (e.g. Luzon PAIC; Sánchez-González & Moyle, 2011). Although broad strokes at understanding Plio-Pleistocene diversification across the archipelago have been possible for some time, the power to detect more recent, fine-scale differentiation has been limited by DNA sequencing depth. Furthermore, much of the work on Philippine biodiversity has focused on the patterns and processes shaping diversity throughout the archipelago, despite the fact that not all lineages have distributions spanning its entirety. Hence, the generation of recent population genetic structure owing to Holocene isolation on individual islands within the same PAIC remains theoretical. Here, we investigate the effect of individual islands in generating genetic differentiation in the endemic Philippine broadbills (Aves; Family: Eurylaimidae; Genus: *Sarcophanops*), in which all extant lineages occur on one previously connected landmass (the Greater Mindanao PAIC, which now comprises many

islands). The two species of Philippine broadbill, *Sarcophanops steerii* and *S. samarensis*, occur in non-overlapping ranges on Mindanao and the Eastern Visayas (incl. Samar, Leyte, and Bohol Islands), respectively. We used restriction-site associated DNA sequencing (RAD-seq) to produce a genome-wide panel of thousands of single nucleotide polymorphisms (SNPs), which allows for assessment of subtle population genomic structure across islands that were part of the same PAIC as recently as the LGM. Inferring differentiation at this evolutionary timescale has not, to our knowledge, been documented in Philippine avifauna. Focusing on a genus (*Sarcophanops*) endemic to a single PAIC enables us to: (1): examine inter- or intra-island population structure within *Sarcophanops* species to get a glimpse into genetic connectivity of avifauna endemic to the Mindanao PAIC, and (2) expand our understanding of the population history of these enigmatic taxa.

Materials & Methods

We obtained tissue samples (N = 22) of *Sarcophanops* from across their distribution in the Philippines, and used two individuals from *Serilophus lunatus* as outgroup (Table 1; Fig. 1). All tissue samples are frozen and/or ethanol-preserved muscle tissue and have associated voucher specimens housed in the Biodiversity Institute at the University of Kansas. We used a modified RAD-seq (Miller et al., 2007) protocol to prepare genomic libraries of putatively-neutral loci from across the genome. Briefly, we ligated custom adapters with barcodes (Andolfatto et al., 2011) to all samples, which were pooled and subsequently purified with AMPure magnetic beads (Agencourt). We used a Pippin Prep (Sage Science) to size select fragments between 500 and 600 bp. We then purified the library again with magnetic beads, performed a brief polymerase chain reaction (PCR) in duplicate (14 cycles), and performed a final purification before dual-indexing the samples (with standard Illumina indices) for multiplexing. The multiplexed library was pooled with libraries from unrelated projects and sequenced across three lanes of an Illumina HiSeq2500 flow cell.

To assemble loci de novo and create SNP datasets from our sequencing data, we used the STACKS (Catchen et al., 2013) pipeline (more details available in Supp Mat). We used *ustacks* with the default settings. In *cstacks*, we tested various numbers of mismatches allowed between stacks when assembling loci (N = 1-7; Table S2). We then used the *sstacks* module with default settings. Finally, we used the *populations* module of STACKS to filter SNP loci and create two datasets: (1) a 50% coverage matrix (50CM; requiring a SNP to be represented in ≥ 50% of individuals), and (2) a 75% coverage matrix (75CM). We required all loci to have a minimum read depth of five and maximum observed heterozygosity < 50% to reduce inclusion of paralogs. We also assessed how changing the minimum read depth could affect population genetic estimates and insured good coverage across the genome by using the BLAST+ utility (Camacho et al., 2009), requiring a minimum of 70% sequence identity across at least 25 bp, and a maximum e-value of 0.001 to define a match.

We used RAxML v8 (Stamatakis, 2014) to identify phylogenetic relationships among individuals using a concatenated matrix of all full-length sequences. We first estimate an appropriate model of sequence evolution (GTR + I + G in this case) based on the Bayesian Information Criterion (BIC) using PAUP v.4.0.151 (Swofford, 2002). In RAxML, we estimated a maximum likelihood tree and assessed support using 1000 rapid bootstrap replicates. We used the programs STRUCTURE (Pritchard et al., 2000) and DAPC (Jombart, Devillard & Balloux, 2010) to investigate population genetic structure for the 75CM dataset. For both analyses, we subset our datasets to include only one SNP per locus (two replicates each) to minimize potential linkage effects. We ran STRUCTURE initially to infer lambda with the number of populations (k) limited to one. Next, we used a constant lambda, the admixture model with

correlated allele frequencies, and a number of likely k values (k = 1–5, five runs for each k). We defined the burn-in period as the first 100,000 MCMC generations with a subsequent 100,000 iterations sampled. To determine the most likely number of genetic clusters, we used the ΔK method of Evanno *et al.* (2005). We also performed the same analysis on *S. samarensis* alone to look for population structure within the Visayan islands. DAPC analyses were performed in R (R Core Team, 2013), using the package 'adegenet' (Jombart, 2008; Jombart & Ahmed, 2011). For DAPC, the most likely number of populations was determined based on BIC values.

Results

Sequencing coverage across individuals was variable (Table 1), with a median ~1.6 million reads per individual (sd = 822,669 reads). From these reads we recovered ~25,000 RAD-tags per individual (sd = 8533). The 50% and 75% coverage matrices had 1,737 and 885 loci, respectively, corresponding to 4,310 and 2,271 SNPs (Table S1). All raw sequence data from RAD-seq are available at the NCBI Sequence Read Archive, accessioned under BioProject XXX (ID #s pending). Genetic differentiation, measured by F_{ST}, between sampling localities within a given species was generally low ($F_{ST} < 0.15$), but high between species ($F_{ST} > 0.30$; Table 2). Genetic structure was most apparent across species (i.e. between *S. samarensis* and *S. steeri*) in phylogenetic (Fig. 1) and population genetic (Fig. 2) analyses. Phylogenetic analysis in RAxML supported a deep split between species, but relationships within *S. samarensis* were largely ambiguous with respect to island. However, we did find Bohol was recovered as monophyletic in the 50CM tree. Population genetic analyses recovered a similar pattern overall pattern, but some differences are observed, likely due to RAxML analyses being based on a concatenated dataset. In STRUCTURE, the ΔK method most strongly supported two genetic clusters, separating Visayan and Mindanao individuals (Fig. 2). We also ran STRUCTURE on only the Visayan individuals and recovered a strong break between Bohol and Samar + Leyte, but did not recover any further genetic partitioning (i.e. no split between Samar and Leyte). When running DAPC, we observed three distinct clusters corresponding to individuals from Mindanao, Bohol, and Samar + Leyte (Fig. 2).

Discussion

When comparing diversification in *Sarcophanops* to other endemic fauna from the Mindanao PAIC, we observe that many taxa show a similar pattern of differentiation. For example, in *Cyrtodactylus* geckos (Welton et al., 2010) and *Crocidura* shrews (Esselstyn, Timm & Brown, 2009) the Visayan and Mindanao populations form independent genetic clusters, which was consistent with our phylogenomic and population genetic analyses which recover a deep split between the Mindanao (*S. steerii*) and Visayan (*S. samarensis*) species. Recently published findings based on Bayesian species delimitation of mitochondrial DNA sequence data also revealed the same deep split between Mindanao and Visayan species (Hosner et al., 2018), but failed to identify a signature of divergence within *S. samarensis* as we found here. The well-supported phylogenetic split between the Mindanao and Visayan species in both the mtDNA and nuclear DNA suggest they remained isolated during the LGM, despite the fact all these islands formed a single contiguous island, the Mindanao PAIC. Possibly, this isolation relates to the role of environmental suitability. Based on paleoclimate projections, (Hosner et al., 2014) found that the shallow Leyte Gulf—the land bridge uniting the northern and southern islands of the Mindanao PAIC—was unsuitable for most species in their study and still acted as a barrier to gene flow despite increased

land connectivity. Although we did not perform niche modeling in this study, the Leyte Gulf could have also been unsuitable habitat for *Sarcophanops*, thus facilitating the divergence of Mindanao and Visayan populations.

In our study, RAD-seq data revealed fine-scale inter-island diversification within the Visayan broadbills, which was not evident in mtDNA alone (Hosner et al., 2018). This suggests the shallow split between Bohol and Samar + Leyte is rather recent, most likely post-LGM. SNP-based genetic structure (Fig. 2) revealed a high probability of two distinct populations within *S. samarensis*: Samar + Leyte, and Bohol. This geographic partitioning is particularly interesting given the current taxonomic treatment of *S. samarensis*, which contains no subspecific taxa from the Visayas. In Mindanao, there are two described subspecies, but we recovered only one *S. steerii* population in the RAD-seq dataset, with slight evidence to support separation of the Zamboanga population, as seen in the mtDNA dataset.

Although all present-day islands in the eastern Visayas were connected at one point during the LGM, the narrow (0.8-1.6 km) and shallow (max. 20 m) San Juanico Strait separating Samar and Leyte probably extended terrestrial connectivity between these two islands longer relative to other neighboring islands in the Mindanao PAIC. Rising sea levels at the end of the Pleistocene would have isolated Bohol first, while prolonged connectivity between Samar and Leyte could have promoted gene flow, thus obscuring population genetic effects of inter-island diversification. Because little is known about the current population status of these birds, and because little appropriate forested habitat remains on Bohol in particular, understanding the genetic connectivity across the Visayan islands is an important contribution to properly addressing the conservation needs of this enigmatic genus.

Conclusions

Numerous studies have investigated the effect of PAICs in generating endemism in the Philippines (Brown et al., 2013). Yet, the nature of those studies has provided limited understanding of recent, between-island differentiation. Focusing on an endemic lineage restricted to a single and well-established island group, we were able to recover both deep and subtle genetic differentiation between islands. Because this differentiation was not well-supported in the "fast evolving" mtDNA, we suggest the two, previously undocumented Visayan lineages arose after the LGM and are therefore only detectable in a deep, genome-wide scan of thousands of loci using a method such as RAD-seq. This study represents a solid step forward in understanding genetic differentiation consistent with a post-LGM timeframe in a single PAIC. Furthermore, our results suggest that subtle differentiation within islands groups, particularly since the LGM, has been overlooked due to past genetic sampling constraints.

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251

Figure 1 (on next page)

RAxML trees for the 50CM (left) and 75CM (right) datasets.

Bootstrap support was assessed based on 1000 rapid bootstrap replicates; nodes with less than 50% bootstrap support have no node label. Visayan samples are shown in shades of blue, and Mindanao samples are shown in shades of yellow.

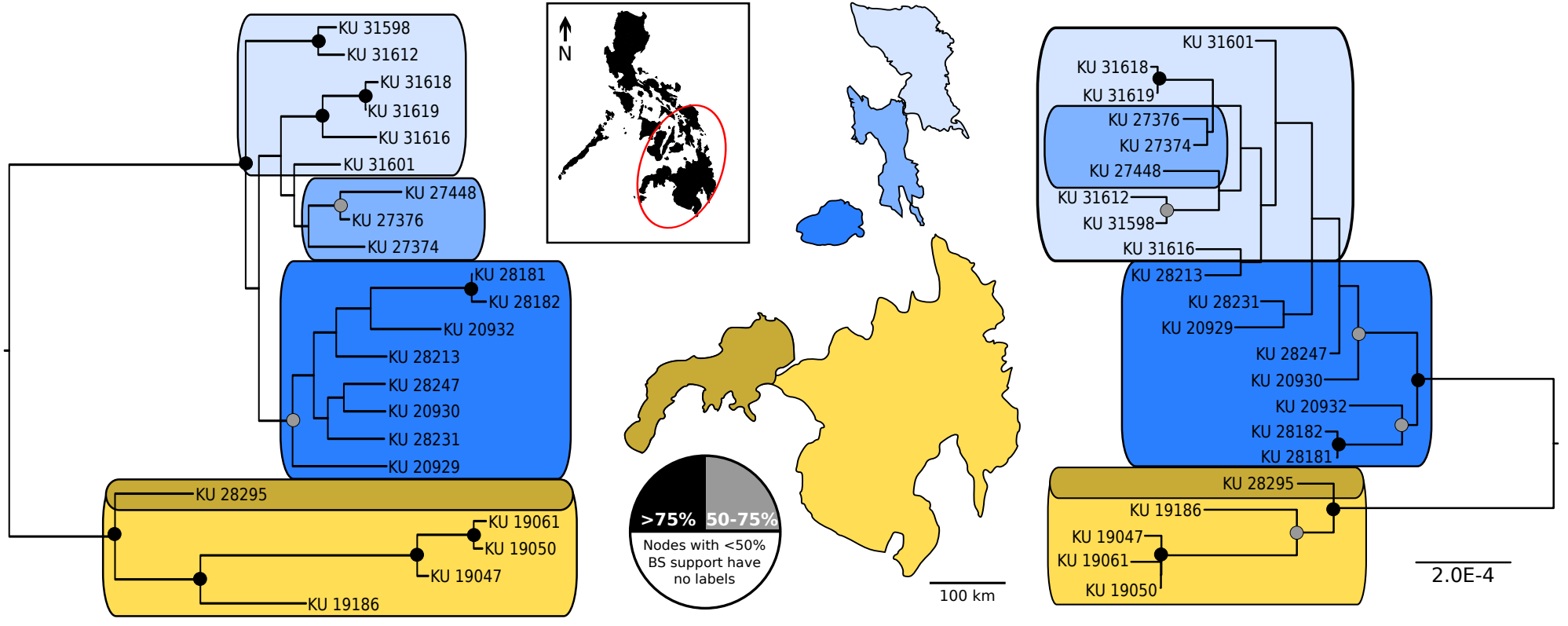

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Figure 2(on next page)

DAPC and STRUCTURE results for 75CM.

Again, Mindanao samples are shown in shades of yellow, and Visayan samples are shown in shades of blue. The STRUCTURE plot on the bottom (blue and yellow), shows the break between *S. steerii* and *S. samarensis*. The STRUCTURE plot on the right only includes individuals from *S. samarensis* and shows the clear break between Bohol (top, dark blue) and Samar + Leyte (bottom, light blue). For both STRUCTURE plots, each bar represents the probability of population assignment for a single individual.

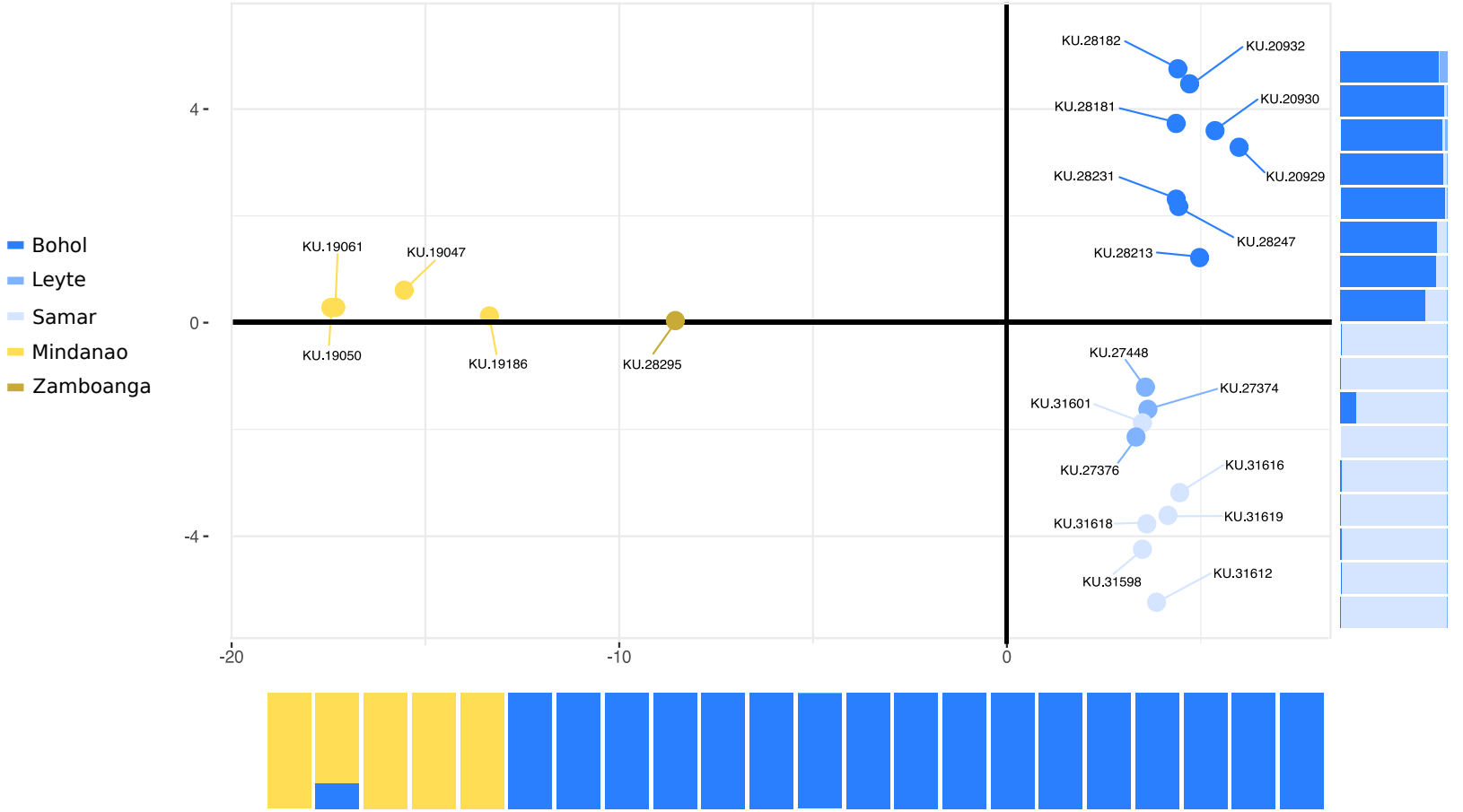

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Table 1(on next page)

List of samples used in this study and their associated sequencing statistics.

Table 1 – List of samples used in this study and their associated sequencing statistics.

Species	Museum #	Locality	# Reads	RAD-tags	Cov. Median	Cov. St.Dev.	% Missing 50% CM / 70% CM
Sarcophanops steerii	KU 19047	Mindanao	874532	20185	30	33.29	15.31/ 3.75
Sarcophanops steerii	KU 19050	Mindanao	991797	20902	35	37.70	13.46 / 3.52
Sarcophanops steerii	KU 19061	Mindanao	2408639	31817	72	83.53	3.46 / 0.48
Sarcophanops steerii	KU 28295	Mindanao	2600919	39135	74	80.55	14.20 / 13.43
Sarcophanops steerii	KU 19186	Zamboanga	1782408	26521	53	67.01	0.00 / 0.00
Sarcophanops samarensis	KU 20929	Bohol	2180969	32211	65	72.52	5.99 / 1.45
Sarcophanops samarensis	KU 20930	Bohol	1638944	23861	53	62.67	6.38 / 0.70
Sarcophanops samarensis	KU 20932	Bohol	1371645	26271	43.5	49.98	8.98 / 1.14
Sarcophanops samarensis	KU 28181	Bohol	712002	17718	26	29.03	15.85 / 5.02
Sarcophanops samarensis	KU 28182	Bohol	2285682	28961	65	78.28	3.25 / 0.84
Sarcophanops samarensis	KU 28213	Bohol	1197631	31888	37	38.54	13.53 / 3.57
Sarcophanops samarensis	KU 28231	Bohol	1981016	32462	60	68.49	4.69 / 0.97
Sarcophanops samarensis	KU 28247	Bohol	1465002	31170	44	51.05	6.26 / 0.48
Sarcophanops samarensis	KU 27374	Leyte	1722020	23775	56	65.06	3.62 / 0.00
Sarcophanops samarensis	KU 27376	Leyte	2833494	30055	88	98.85	3.36 / 0.00
Sarcophanops samarensis	KU 27448	Leyte	1525375	25388	46	58.30	3.81 / 0.00
Sarcophanops samarensis	KU 31598	Samar	450539	22184	15	14.55	23.32 / 2.03
Sarcophanops samarensis	KU 31601	Samar	808201	25697	23	28.23	14.32 / 0.31
Sarcophanops samarensis	KU 31612	Samar	1793799	39214	54	55.63	5.87 / 1.23
Sarcophanops samarensis	KU 31616	Samar	3347326	44974	87	100.42	6.17 / 1.50
Sarcophanops samarensis	KU 31618	Samar	889378	24240	26	31.44	9.35 / 0.35
Sarcophanops samarensis	KU 31619	Samar	986355	25079	32	35.91	7.87 / 0.13
Serilophus lunatus	KU 23405	Vietnam	3515364	55899	63	91.42	4.52 / 0.00
Serilophus lunatus	KU 23552	Vietnam	1302599	27169	35	43.52	12.44 / 0.00

1

Table 2(on next page)

Pairwise estimates of F_{ST} for the 75% and 50% coverage matrices above and below the diagonal, respectively.

Table 2 – Pairwise estimates of F_{ST} for the 75% and 50% coverage matrices above and below the diagonal, respectively.

	Bohol	Leyte	Samar	Mindanao	Zamboanga
Bohol		0.114	0.122	0.323	0.321
Leyte	0.116		0.106	0.369	0.452
Samar	0.114	0.116		0.340	0.355
Mindanao	0.310	0.346	0.333		0.204
Zamboanga	0.309	0.447	0.357	0.214	

1