Phylogeny of the island archipelago frog genus *Sanguirana*: Another endemic Philippine radiation that diversified ‘Out-of-Palawan’

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Abstract

Recent higher-level frog phylogenetic analyses have included a few members of the endemic Philippine frog genus *Sanguirana*. Although the monophyly of the group has never been disputed, the recent phylogenetically-supported inclusion of the Palawan Wood Frog (*Sanguirana sanguinea*) in this clade was highly unexpected. In addition, species boundaries and relationships remain unclear and new species continue to be discovered. We estimate the phylogeny for this endemic Philippine genus using two mitochondrial gene regions and six nuclear loci and complete sampling for all known species. We use a time-calibrated Bayesian estimate of phylogeny and model-testing approach to biogeographic inference to infer ancestral areas and probable means of diversification. These analyses identify *Sanguirana* as an additional clade for which the biogeographic ‘Out-of-Palawan’ biogeographic scenario is unambiguously preferred. This study lends additional support to recent work suggesting that a substantial portion of Philippine vertebrate megadiversity originated via colonization of the archipelago from the Palawan microcontinent, with subsequent invasion of oceanic islands (e.g., range expansion over Huxley’s Modification of Wallace’s Line), numerous instances of overwater dispersal, and geographic radiation across the archipelago.

**1. Introduction**

Our limited understanding of the biogeographic patterns and evolutionary processes behind Southeast Asia’s exceptionally high levels of vertebrate diversity is both a crippling limitation for conservation biologists (Woodruff, 2010) and a remarkable opportunity for evolutionary biologists and biogeographers (Brown and Diesmos, 2009; Lohman et al., 2011; Brown et al., 2013). Studies of amphibian radiations (Setiadi et al., 2011; Blackburn et al., 2013) and terrestrial vertebrates (Brown and Diesmos, 2009), the Philippine Archipelago recently has been the focus of several integrative studies of amphibian radiations (Setiadi et al., 2011; Blackburn et al., 2013; Brown and Siler, 2013; Brown et al., 2013, 2015). One moderately sized clade is the endemic frog genus *Sanguirana* (formerly the *Rana everetti* Complex; Brown et al., 2000), which consists of seven described and morphologically diagnosable species and one or two undescribed “cryptic” species (Brown et al., 2000; Fuiten et al., 2011). Additional new species most likely await discovery in unsurveyed portions of the archipelago (Brown et al., 2000, 2013; Brown, 2015).

In a recent review, Brown et al. (2013) distinguished between the archipelago’s partially or fully characterized adaptive radiations (e.g., Blackburn et al., 2013; Brown et al., 2015) and the lugubrious pace of taxonomic work (Joppa et al., 2011), and country-specific, idiosyncratic, and variable policies of natural resource management (La Viña et al., 1997; Lim et al., 2008; Meijaard, 2011).

Home to numerous celebrated clades of co-distributed terrestrial vertebrates (Brown and Diesmos, 2009), the Philippine Archipelago has been the focus of several integrative studies of amphibian radiations (Setiadi et al., 2011; Blackburn et al., 2013; Brown and Siler, 2013; Brown et al., 2013, 2015). One moderately sized clade is the endemic frog genus *Sanguirana* (formerly the *Rana everetti* Complex; Brown et al., 2000), which consists of seven described and morphologically diagnosable species and one or two undescribed “cryptic” species (Brown et al., 2000; Fuiten et al., 2011). Additional new species most likely await discovery in unsurveyed portions of the archipelago (Brown et al., 2000, 2013; Brown, 2015).

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the possibly non-adaptive, “geographic” radiations (e.g., Setiadi et al., 2011; Brown and Siler, 2013). The latter, loosely defined category (Brown et al., 2013) includes clades with one or more representative species on each island bank, or Pleistocene Aggregate Island Complex (PAIC; Brown and Diesmos, 2009; Fig. 1). In these clades, species have been characterized as ecologically similar with little to no evidence of a phenotype-environment correlation or within-island, ecologically associated diversification (Brown et al., 2000, 2013; Brown and Siler, 2013). Of particular interest are clades that show a mixture of diversification patterns, with single ecological generalist species on some islands, but evidence of intra-island diversification, habitat specialization, and multiple sympatric species on other islands (Setiadi et al., 2011; Brown et al., 2013, 2015).

We estimate the phylogeny and biogeographic history of one such group: Philippine endemic stream frogs of the genus Sanguirana (Brown et al., 2000; Fuiten et al., 2011). Aside from being one of only two amphibian genera endemic to the country (the other being Platymantis; Brown et al., 2015), Sanguirana is interesting because of its pattern of species distributions. The genus consists of at least eight species (Fuiten et al., 2011), with all taxa restricted to individual PAICs (Brown et al., 2000). Several of the species (S. albotuberculata, S. everetti, S. sanguinea, and S. sp. [undescribed]) possess exclusive, non-overlapping distributions isolated to single PAICs or island subsets within PAICs, but the remainder (S. aurantipunctata, S. igorota, S. luzonensis, and S. tipanan) features some degree of sympatry in the mountains of Luzon Island (Figs. 1 and 2; Brown et al., 2000).

Here we infer the biogeographic history of this group and ask whether any of the previously hypothesized, shared mechanisms of diversification for Philippine terrestrial vertebrates (Brown et al., 2013) apply to Sanguirana. Our multilocus phylogenetic estimate and ancestral inference of biogeography identifies the genus as yet another clade with a biogeographic history consistent with the ‘Out-of-Palawan’ biogeographic scenario (Blackburn et al., 2010; Esselstyn et al., 2010; Siler et al., 2012; Brown et al., 2013). As such, the genus Sanguirana represents the first amphibian clade for which the Palawan Arc Hypothesis (Blackburn et al., 2010; Siler et al., 2012) is a tractable explanation for the initial isolation, biogeographic origin, and subsequent diversification of one of the archipelago’s more spectacular and completely endemic radiations (Brown and Diesmos, 2009; Brown et al., 2013).

2. Materials and methods

2.1. Taxon sampling and data collection

Ingroup sampling included 161 individuals collected from 47 localities, including all known species of Sanguirana (Fig. 1; Appendix A, Supplemental Material). We selected closely related outgroup species Glandirana minima and Hylarana erythraea, on

Fig. 1. Map of the Philippine archipelago (A) with sampling color coded by species (see key). Lighter blue shadings indicate shallow seas and the underwater bathymetric contour forming the major Pleistocene Aggregate Island Complexes (PAICs; Brown and Diesmos, 2009). Hypothesized relationships of Sanguirana species (B), depicted by the consensus tree resulting from the post-burnin posterior distribution from the Bayesian analysis of the concatenated dataset. Strongly supported nodes (>0.95 Bayesian PP; >70% ML) are denoted with black dots) and moderately supported nodes (0.90–0.95 Bayesian PP) are indicated with gray dots. Bayesian analysis of nuclear genes only (C) resulted in weakly-supported differences in branching order among species but resolve the paraphyly of S. luzonensis (with respect to S. igorota and S. tipanan); (D) deep coalescence as one possible explanation for incongruence between mtDNA and nDNA gene lineages. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
the basis of uncontroversial higher-level phylogenetic relationships of the family Ranidae (Wiens et al., 2009), and sequenced a total of 6098 nucleotides from two mitochondrial gene regions and six nuclear loci (Table 1). Primers, PCR methods, thermal profiles, and sequencing protocols follow our recent phylogenetic studies (Brown and Siler, 2013; Brown et al., 2015). We visualized products on 1.5% agarose gels and used ABI BigDye Terminator v3.1 sequencing chemistry (Life Technologies, Grand Island, NY) for cycle sequencing. PCR products and sequencing reactions were purified using ExoSAP-IT and Sephadex cleanups, respectively (Amersham Biosciences, Piscataway, NJ). Purified products were analyzed with an ABI Prism 3730xl Genetic Analyzer, and sequences were assembled and edited using Geneious vR8.1.7 (Kearse et al., 2012). All novel sequences were deposited in GenBank (Appendix A, Supplemental Material).

2.2. Sequence characteristics, alignment, and phylogenetic analyses

Initial alignments were produced in MAFFT v7.221 (Katoh and Standley, 2013) with slight manual adjustments. To assess gene congruence, we estimated gene trees with Bayesian and likelihood analyses and compared estimates from these independent partitions for the presence of strongly supported conflict between gene regions. Finding none, we concatenated data for subsequent analyses. We then assembled two datasets: one with all genes included (but with missing nuclear data for many individuals [Table 1]; Appendix A, Supplemental Material) and another reduced to samples with near-complete nuclear gene sampling. Because exploratory comparative analyses yielded the same strongly supported nodes, we felt justified in including all available data in subsequent phylogenetic analyses.

Partitioned Bayesian analyses were conducted in MrBayes v3.2.5 (Ronquist and Huelsenbeck, 2003). We partitioned the ribosomal RNA mitochondrial sequences by region (12S, tRNAval, and 16S), nuclear DNA was partitioned by locus, with gene-specific protein-coding regions partitioned by codon position (Table 1). The Akaike Information Criterion (AIC), as implemented in jModelTest v2.1.7 (Darriba et al., 2012), was used to select the best model of nucleotide substitution for each partition (Table 1). We ran four MCMC analyses, each with four Metropolis-coupled chains, an incremental heating of 0.02, and an exponential distribution with 25 as the rate parameter prior on branch lengths. All analyses were run for...
20 × 10^2 generations, with parameters and topologies sampled every 1000 generations. We assessed stationarity with Tracer v1.6 (Rambaut and Drummond, 2007) and convergence with AWTY (Wilgenbusch et al., 2004). Stationarity was achieved after 5 × 10^6 generations (first 25% samples), which we discarded as burn-in.

Partitioned maximum likelihood (ML) analyses were conducted in RAxMLHPC v8.0.0 (Stamatakis, 2014) on the concatenated dataset using the same partitions (Table 1) but with the GTR+Γ model for each. One hundred replicate inferences were performed, each initiated with a random starting tree. Nodal support was assessed with 1000 bootstrap pseudoreplicates. Alignments and resulting topologies were deposited in Dryad (doi: 10.5061/dryad.6394c).

### 2.3. Divergence time estimation and biogeographic inference

We used BEAST v1.8.2 (Drummond et al., 2012) to estimate divergence times. We unlinked clock models and original substitution rates per gene/partition, and employed the following priors for two calibration strategies: (1) we applied a range of empirically observed amphibian divergence rates (1.4% Mya^-1, 95% HPD = 0.8–1.9%) following Sanguila et al. (2011) as the rate calibration for the mitochondrial genes in our data set; (2) for comparison, we applied the time estimate of the approach of the oceanic Philippine lineages (1.4% divergence between species Mya^-1/2, 95% HPD = 18.3–10.2) inferred when using our geological calibration (Fig. 1C), involves a possibly non-monophyletic Philippine lineages from Luzon (S. tipanan, S. everetti, S. luzonensis, and S. albotuberculata + S. igorota) with near-complete mitochondrial and nuclear sequences to estimate the divergence times among the major clades. When our amphibian mitochondrial molecular clock (1.4% divergence between species Mya^-1) was applied, we estimated the oldest ingroup node as 12.1 Mya (95% HPD = 10–6; Hall, 1998) to provide a crude estimate of the origin of the oceanic Philippine lineage. We then combined four separate runs for each calibration (25% burn-in for each) and considered effective sample sizes adequate if >200. We diagnosed convergence with Tracer v1.6 and used TreeAnnotator v1.8.2 to generate our final maximum clade credibility topology.

We used BioGeoBEARS (Matzke, 2013) to gain insight into potential speciation modes and to estimate ancestral geographical ranges. We considered a total of six models (Matzke, 2013): Dispersal-Extinction-Cladogenesis (DEC; Ree and Smith, 2008), Ronquist’s (1997) Dispersal-Vicariance Analysis (DIVA; Ronquist, 1997), and the BayArea model (Landis et al., 2013); and an additional parameter j that was considered for each model to evaluate founder-event speciation, which could be associated with long distance dispersal and subsequent divergence of colonizing lineages (Matzke, 2013). We then estimated biogeographic ancestral ranges and transitions thereof under the best-fit model (selected with AIC).

### 3. Results

#### 3.1. Phylogeny

Bayesian and likelihood analyses (Figs. 1 and 2) of the complete dataset strongly support a monophyletic Philippine Sanguirana (MrBayes posterior probabilities [PP] = 1.00, Maximum Likelihood bootstrap support [MLBS] = 100). Sanguirana sanguinea from Palawan Island is the sister taxon to the remaining clade of oceanic Philippine lineages (PP = 0.99, MLBS = 80; Fig. 1). Within this clade of oceanic Philippine species are three strongly supported taxa whose relationships to each other are not resolved with strong support: (1) the Luzon endemic S. aurantipunctata, (2) the Mindanao clade of S. alboboterculata + S. everetti (PP = 1.00, MLBS = 100), and (3) a clade in which an undescribed species from the West Visayan islands (Figs. 1 and 2; PP = 0.99, MLBS = 93) is sister to a clade of species from Luzon (S. igorota, S. luzonensis, and S. tipanan; PP = 0.73, MLBS = 88). The relationships among S. igorota, S. luzonensis, and S. tipanan remain poorly resolved (Fig. 1). Weakly supported differences between the mitochondrial gene tree (not shown; identical to the concatenated tree in Fig. 1B) and a separate nuclear topology (Fig. 1C), involves a possibly non-monophyletic S. luzonensis with respect to S. igorota and S. tipanan (Fig. 1).

#### 3.2. Biogeographic inference and time frame for diversification

We subsampled 49 individuals (at least two samples/clade from the 161-individual dataset) with near-complete mitochondrial and nuclear sequences to estimate the divergence times among the major clades. When our amphibian mitochondrial molecular clock (1.4% divergence between species Mya^-1) was applied, we estimated the oldest ingroup node as 12.1 Mya (95% HPD = 16.9–7.7), which approximates the divergence (14.1 Mya, 95% HPD = 18.3–10.2) inferred when using our geological calibration (10–6 Mya, Hall, 1998). The estimated divergence times for the oceanic clade were nearly identical (molecular clock: 7.8 Mya, 95% HPD = 10.6–5.2; geology: 7.9 Mya. 95% HPD = 9.5–6.4). Divergence times within the oceanic Philippines were similarly coincident, and dated during the late Miocene to Pliocene (Fig. 2).

The best-fit biogeographic model selected by AIC weights was Matzke’s (2013) likelihood implementation of the DIVA model (i.e., two-parameter model; dispersal allowed but penalized
relative to vicariance; Ronquist, 1997), which incorporates dispersal to a geographic area not observed in the parental lineages (DIVALIKE+J; Table 2). The ancestral range estimate based on this model inferred a process of vicariance for diversification between the ephemerally accreted (or, at least, adjacent) Palawan microcontinent + proto-Luzon block (Hall, 1998; Brown and Diesmos, 2009), which then separated as the latter slipped farther north along the Philippine trench (Fig. 2; Hall, 1998; Brown et al., 2013). Subsequently, because the oceanic Philippines arrived at its current configuration within the last eight million years (Hall, 1998; Brown and Diesmos, 2009), we infer that speciation during this period was associated with repeated bouts of overwater dispersal and colonization between isolated PAICs (Luzon to Mindanao ~7.3 Mya; Luzon to Western Visayas ~5.9 Mya; Fig. 2).

4. Discussion

4.1. Phylogeny

Our phylogenetic estimate identifies weakly supported differences between mitochondrial and nuclear dataset partitions. These discrepancies involve the first-branching lineages within the apparently rapidly-radiating oceanic Philippines clade (Fig. 1B and C), and also the non-monophyly of S. luzonensis with respect to S. igorotae and S. tipanans (Fig. 1D). The latter conceivably could be explained by the presence of a cryptic species (Sierra Madre and Zambales mountains ranges of Luzon and possibly Marinduque Island; Fig. 1B), maintenance of ancestral polymorphism, deep coalescence of mitochondrial gene lineages, and/or the stochastic nature of lineage sorting in the coalescent (Fig. 1D). Distinguishing between these hypotheses is beyond the scope of the current dataset given the lack of support at some nodes and limited number of loci available.

4.2. ‘Out-of-Palawan’ biogeography in Philippine stream frogs

The analysis presented here identifies another Philippine radiation for which the Palawan Ark Hypothesis (Blackburn et al., 2010; Siler et al., 2012; Brown et al., 2013) best fits the available data. Because of low support for the clade ((S. everetti + S. albotuberculata) (S. sp. Negros-Panay (S. luzonensis (S. igorotae + S. tipanans + S. luzonensis))))), we are unable to infer the sequence of island colonizing the initial invasion of the oceanic portions of the archipelago (Blackburn et al. 2013). We thank the Biodiversity Monitoring Bureau (BMB) of the Philippine government for facilitating permits, M. Diesmos and A. C. Alcala for collaboration and logistical support, and the U.S. National Science Foundation (DEB 073199, 0334952, 0743491, 0804115, and 1418895) for supporting our research.

Table 2

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Much attention has been paid to hypotheses of common mechanisms of diversification in terrestrial vertebrates of the Philippines, an iconic Southeast Asian island archipelago (Woodruff, 2010; Lohman et al., 2011; Brown et al., 2013). In the Philippines, a disproportionate amount of this attention has focused on the ‘PAIC-paradigm,’ its impact in unrelated taxa, deviations from the model, and other recent (Pleistocene) explanations (reviews: Brown and Diesmos, 2009; Brown et al., 2013; Oaks et al., 2013). However, more recent inferences from increasingly robust multilocus molecular phylogenies (Esselstyn et al., 2010; Setiadi et al., 2011; Brown et al., 2015) suggest that alternate explanations are plausible, and clearly have contributed to establishing Philippine vertebrate megadiversity (Brown and Diesmos, 2009). These include within-island habitat connectivity/discontiguity, ecological divergence along altitudinal gradients, contingency in processes of insular community assembly, and the possibility paleo-transport of ancient isolated lineages on continental land fragments during the assembly of the archipelago (Hall, 1998; Blackburn et al., 2010; Brown et al., 2013). The Palawan Ark Hypothesis is one such explanation that may characterize the evolutionary history of more terrestrial biodiversity in the oceanic Philippines than has generally been appreciated (Esselstyn et al., 2010). Moving beyond the PAIC paradigm toward the acceptance of a plurality of shared mechanisms and a greater appreciation of overseas dispersal has been an inevitable and welcomed consequence of the accumulation new data from recent biodiversity surveys throughout the archipelago (Brown et al., 2013).

Acknowledgments

We thank the Biodiversity Monitoring Bureau (BMB) of the Philippine government for facilitating permits, M. Diesmos and A. C. Alcala for collaboration and logistical support, and the U.S. National Science Foundation (DEB 073199, 0334952, 0743491, 0804115, and 1418895) for supporting our research.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2015.10.010.

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