**Species delimitation in the grey zone: introgression obfuscates phylogenetic inference
and species boundaries in a cryptic frog complex (Ranidae: *Pulchrana picturata*)**

**Kin Onn Chan^{1,2,*}, Carl R. Hutter², Perry L. Wood, Jr.³, L. Lee Grismer⁴, Rafe M.
Brown²**

¹ Lee Kong Chian National History Museum, Faculty of Science, National University of
Singapore, 2 Conservatory Drive, Singapore 117377. Email: chankinonn@gmail.com

² Biodiversity Institute and Department of Ecology and Evolutionary Biology, University of
Kansas, Lawrence, KS 66045, USA. Email: carl.hutter@ku.edu; rafe@ku.edu

³ Department of Biological Sciences & Museum of Natural History, Auburn University,
Auburn, Alabama 36849, USA. Email: plw0013@auburn.edu

⁴ Herpetology Laboratory, Department of Biology, La Sierra University, 4500 Riverwalk
Parkway, Riverside, California 92505, USA. Email: lgrismer@lasierra.edu

**Corresponding author*

ABSTRACT

As molecular methods continue to elucidate genetic structure at increasingly finer resolutions, delimiting species in the grey zone of the speciation continuum is becoming more relevant in biodiversity research, especially in under-studied biodiversity hotspots such as Southeast Asia where new species are being described at an unprecedented rate. Obvious species at both ends of the speciation continuum have mostly been described and attention is now turning towards the “grey zone:” an intermediate stage in which species criteria are in conflict and boundaries between populations and species are less clear. This study demonstrates that widely-used criteria (phylogenetic placement, genetic divergence, phylogeny- and distance-based species delimitation methods) can overestimate species diversity/boundaries when introgression is present. However, a comprehensive species delimitation framework that considers spatial and genetic population structure, introgression, and the use of species delimitation methods based on parameter estimation, can provide a more accurate characterization of species boundaries in this grey zone. We applied this approach to a group of Southeast Asian frogs from the *Pulchrana picturata* Complex that exhibits continuous morphological variation and high genetic divergences. Results showed that introgression was rampant among Bornean populations, which led to phylogenetic discordance and an overestimation of species. We suspect that our results do not form an isolated case; and that introgression among cryptic populations, occurring continuously across a wide geographic area (e.g., the topographically complex island of Borneo, and Earth’s major continents), may be more common than previously thought.

Keywords: gene flow, hybridization, isolation-by-distance, landscape genetics, population genetics, population structure

INTRODUCTION

Species delimitation plays a pivotal role in biodiversity research with potential cascading effects in conservation and other applied sciences (Devitt, Wright, Cannatella, & Hillis, 2019; Stanton et al., 2019). This is particularly germane in highly threatened biodiversity hotspots such as Southeast Asia where the rate of species discovery is high and species richness is still severely underestimated (Brown & Stuart, 2012; Koh et al., 2013; Sodhi, Koh, Brook, & Ng, 2004; Wilcove, Giam, Edwards, Fisher, & Koh, 2013). The rise in new species discoveries is largely driven by the use of molecular approaches, with increased attention on phenotypically cryptic and recently diverged lineages that often occur in the “grey zone” of the speciation continuum—an intermediate state where alternative species concepts are in conflict, resulting in indistinct species boundaries (De Queiroz, 2007; Fišer, Robinson, & Malard, 2018; Roux et al., 2016). However, recent studies have shown that characterizing species boundaries in this zone can be complicated due to a number of factors such as incomplete lineage sorting, gene flow, and introgression (Chan et al., 2017; Drillon, Dufresnes, Perrin, Crochet, & Dufresnes, 2019; Harrison & Larson, 2014; Supple, Papa, Hines, McMillan, & Counterman, 2015). Nevertheless, relatively few empirical studies in Southeast Asia have applied genomic data to assess these processes during the practice of species delimitation (Chan et al. 2017).

Although genomic approaches have allowed the characterization of genetic structure at unprecedented detail (e.g. Benestan et al., 2015; Chan et al., 2017; Lim et al., 2017; Schield et al., 2018), the distinction between populations and species can remain nebulous in the grey zone. Continuous genetic variation may appear as discrete population clusters that are spatially autocorrelated if geographic sampling is discontinuous or when the clustering model does not account for continuous processes such as isolation by distance (Bradburd, Coop, & Ralph, 2018). Furthermore, gene flow among such populations, and even species, can bias species tree estimation and produce incorrect topologies (Eckert & Carstens, 2008;

Leaché, Harris, Rannala, & Yang, 2014; Solís-Lemus, Yang, & Ané, 2016). This error can then be exacerbated in downstream species delimitation analyses that are predicated on the species tree, which is assumed to be correct (Xu & Yang, 2016; Yang & Rannala, 2010). Additionally, performing species delimitation analysis on genome-scale data faces the problem of computational scalability (Bryant, Bouckaert, Felsenstein, Rosenberg, & Roychoudhury, 2012; Fujisawa, Aswad, & Barraclough, 2016; Ogilvie, Heled, Xie, & Drummond, 2016) and the problem of distinguishing between population-level structure and species divergence (Jackson, Carstens, Morales, & O'Meara, 2017; Leaché, Zhu, Rannala, & Yang, 2018; Luo, Ling, Ho, & Zhu, 2018; Sukumaran & Knowles, 2017). These challenges demonstrate that species delimitation in the grey zone of the speciation continuum can be complicated, heavily impacted by sampling gaps and biases, and ideally should involve a comprehensive and careful examination of genetic structure, while taking into account spatial and evolutionary processes.

We implemented this approach to delimit species boundaries in Spotted Stream Frogs of the *Pulchrana picturata* complex (Brown & Guttman, 2002; Brown & Siler, 2014). Currently, *P. picturata* is a single species that exhibits considerable but continuous morphological variation throughout its distribution range in southern Thailand, Peninsular Malaysia, Sumatra, and Borneo (Brown & Guttman, 2002; Frost, 2019). High levels of genetic structure and mitochondrial divergence (up to 10%) have been detected among strongly-supported and geographically circumscribed clades (Brown & Siler, 2014), suggesting that this complex could comprise multiple cryptic species. Moreover, instead of being nested within the Bornean clade, one population from Borneo was recovered within the Thailand, Peninsular Malaysian, and Sumatran clade with high support (supplementary figure S3 in Brown and Siler, 2014), indicating that introgression could be affecting phylogenetic inference. In this study, we used a newly developed suite of genomic markers and target-

capture protocol (FrogCap; Hutter et al., 2019) to obtain more than 12,000 informative loci consisting of exons, introns, and ultraconserved elements (UCEs) from representative populations across the distributional range of *P. picturata* to infer evolutionary relationships and determine whether deep divergences among clades and observed geographically-structured genetic variation corresponds with statistically-defensible hypothesized species boundaries. As validation, we tested for the presence of introgression and evaluated the effect of this phenomenon on phylogenetic inference and species delimitation. This study contributes to the nascent body of literature examining the performance of species delimitation procedures, from the perspective of natural populations, geographically-structured phylogenomic data, and an empirical study system set in one of the world's most dramatic and variable land-and-seascape geographic context and where the field of biogeography had its inception: Southeast Asia and landmasses of Sundaland.

MATERIALS AND METHODS

Sampling and sequencing

A total of 24 samples were genotyped using the FrogCap sequence capture marker set (Ranoidea V1 probe set; Hutter et al., 2019) including four samples from distantly related genera (*Boophis tephraeomystax*, *Mantidactylus melanopleura*, *Cornufer guentheri*, and *Abavorana luctuosa*), six samples of closely related congeners (*Pulchrana banjarana*, *P. siberu*, and *P. signata*), and 14 ingroup samples of the *P. picturata* complex from throughout its distribution range in Peninsular Malaysia, Sumatra, and Borneo. For assurances of taxonomic and nomenclatural clarity, we included a sample from the type locality [Mount Kinabalu, Sabah; *sensu* Brown and Guttman's (2002) lectotype designation]. Tissue samples were obtained from the museum holdings of the University of Kansas Biodiversity Institute,

Kansas (KU), Field Museum of Natural History, Chicago (FMNH), and La Sierra University Herpetological Collection, California (LSUHC; Table S1). Genomic DNA was extracted using the automated Promega Maxwell® RSC Instrument (Tissue DNA kit) and subsequently quantified using the Promega Quantus® Fluorometer. Library preparation was performed by Arbor Biosciences and briefly follows: (1) genomic DNA was sheared to 300–500 bp; (2) adaptors were ligated to DNA fragments; (3) unique identifiers were attached to the adapters to later identify individual samples; (4) biotinylated 120mer RNA library baits were hybridized to the sequences for an incubation period of 19 hours and 23 minutes; (5) target sequences were selected by adhering to magnetic streptavidin beads; (6) target regions were amplified via PCR; and (7) samples were pooled and sequenced on an Illumina HiSeq PE-3000 with 150 bp paired-end reads (Hutter et al., 2019). Sequencing was performed at the Oklahoma Medical Research Foundation DNA Sequencing Facility.

Bioinformatics and data filtering

The full bioinformatics pipeline for filtering adapter contamination, assembling markers, and exporting alignments are available at CRH's GITHUB (pipeline V2: <https://github.com/chutter/FrogCap-Sequence-Capture>). Raw reads were cleaned of adapter contamination, low complexity sequences, and other sequencing artefacts using the program FASTP (default settings; Chen, Zhou, Chen, & Gu, 2018). Next paired-end reads were merged using BBMERGE (Bushnell, Rood, & Singer, 2017). Cleaned reads were then assembled *de novo* with SPADES v.3.12 (Bankevich et al., 2012) under a variety of k-mer schemes. Resulting contigs were then matched against reference probe sequences with BLAST, keeping only those that uniquely matched to the probe sequences. The final set of matching loci was then aligned on a marker-by-marker basis using MAFFT.

Alignments were trimmed and saved separately into functional datasets for phylogenetic analyses and data type comparisons. These datasets include (1) Exons: each alignment was adjusted to be in an open-reading frame and trimmed to the largest reading frame that accommodated >90% of the sequences; alignments with no clear reading frame were discarded; (2) Introns: each previously delimited exon was trimmed out of the original contig and both remaining intronic regions were concatenated; (3) Exons-combined: exons from the same gene were concatenated and treated as a single locus (justifiable under the assumption that as they might be linked); and (4) UCEs. We applied internal trimming to the intron and UCE alignments using the program trimAl (automatic1 function; Capella-Gutiérrez et al., 2009). All alignments were externally trimmed to ensure that at least 50 percent of the samples had sequence data present.

In addition to analysing the unfiltered datasets, we also filtered the data by removing loci with low phylogenetic information, which can introduce noise and increase systematic bias (McLean, Bell, Allen, Helgen, & Cook, 2019). We used parsimony-informative-sites (PIS) as a proxy for hierarchical structure and phylogenetic information; and removed the lower 50% of loci that contained the least PIS. All datasets were analysed separately to assess phylogenetic congruence. Summary statistics, partitioning, and concatenation of data were performed using the program AMAS (Borowiec, 2016) and custom R scripts.

SNP extraction

To obtain variant data across the target samples, we used GATK v4.1 (McKenna et al., 2010) and followed the recommended best practices when discovering and calling variants (Van der Auwera et al., 2013), using a custom R pipeline available on Carl R Hutter's GitHub (<https://github.com/chutter/FrogCap-Sequence-Capture>). To discover potential variant data

(e.g. SNPs, InDels), we used a consensus sequence from each alignment from the target group as a reference and mapped the cleaned reads back to the reference markers from each sample. We used BWA (“bwa mem” function; Li, 2013) to map cleaned reads to the reference markers, adding the read group information (e.g. Flowcell, Lane, Library) obtained from the fastq header files. We next used SAMTOOLS (H. Li et al., 2009) to convert the mapped reads SAM file to a cleaned BAM file, and merged the BAM file with the unmapped reads as required to be used in downstream analyses. We used the program PICARD to mark exact duplicate reads that may have resulted from optical and PCR artifacts and reformatted the dataset for variant calling. To locate variant and invariant sites, we used GATK4 to generate a preliminary variant dataset using the GATK program *HaplotypeCaller* to call haplotypes in the GVCF format for each sample individually.

After processing each sample, we used the GATK *GenomicsDBImport* program to aggregate the samples from the separate datasets into their own combined database. Using these databases, we used the *GenotypeGVCF* function to genotype the combined sample datasets and output separate “.vcf” files for each marker that contains variant data from all the samples for final filtration. Next, to filter the .vcf files to high quality variants, we used the R package *vcfR* (Knaus & Grünwald, 2017) and selected variants to be used in downstream analyses that had a quality score > 20, where we also filtered out the top and bottom 10% of variants based on their depth and mapping quality to avoid potentially problematic sites.

To assemble different datasets for different programs, we further filtered the datasets to be used in various programs. First, to create a SNP output file for downstream analyses (see below) we selected the best SNP from each marker alignment to ensure independence of the selected SNP. The best SNP was determined through the following criteria: (1) we only considered sites that were variable and heterozygous; (2) had 10% or less missing samples for

that site; and (3) we randomly selected the best SNP from the top 25% ranked by genotype quality score and depth.

Phylogenetic estimation and discordance

We used concatenation, species-tree summary, and distance-based methods for phylogenetic estimation. For our concatenated analysis, we used the maximum likelihood program IQ-TREE v1.6 (Chernomor, Von Haeseler, & Minh, 2016; Nguyen, Schmidt, Von Haeseler, & Minh, 2015) and, because of the unprecedented number of loci retrieved with FrogCap, we performed an unpartitioned analysis using the GTR+GAMMA substitution model. Branch support was assessed using 5,000 ultrafast bootstrap replicates (UFB; Hoang et al., 2017) and nodes with UFB >95 were considered strongly-supported. A summary-based species tree analysis was performed using ASTRAL-III (Zhang, Rabiee, Sayyari, & Mirarab, 2018) because this approach has one of the lowest error rates when the number of informative sites are high and has also been shown to produce more accurate results compared to other summary methods under a variety of conditions including high levels of incomplete lineage sorting (ILS) and low gene-tree estimation error (Davidson, Vachaspati, Mirarab, & Warnow, 2015; Mirarab et al., 2014; Molloy & Warnow, 2017; Ogilvie et al., 2016; Vachaspati & Warnow, 2015, 2018). As input for our ASTRAL analysis, individual marker gene trees were estimated using IQ-TREE, with the best-fit substitution model for each locus determined by the program ModelFinder (Kalyaanamoorthy, Minh, Wong, von Haeseler, & Jermin, 2017). Finally, the same set of gene trees was used to estimate species trees using the distance-based method ASTRID, which has been shown to outperform ASTRAL when many genes are available and when ILS is very high (Vachaspati & Warnow, 2015).

Phylogenetic analyses were performed separately on each dataset and we used the program DiscoVista (Sayyari, Whitfield, & Mirarab, 2018) to assess phylogenetic discordance by comparing the relative frequencies of all three topologies surrounding a particular focal branch, in instances in which topological discordance was observed in summary species-tree procedures.

Species delimitation framework

We used a two-step approach to species delimitation, involving independent “discovery” and, subsequent “validation” stages (Hillis, 2019). For our discovery stage, putative evolutionary lineages were inferred from mitochondrial haplotypes derived from strongly-supported multilocus inferences based on 16S rRNA data (Brown & Siler, 2014). We then used sequence-based (Automatic Barcode Gap Discovery, ABGD; Puillandre, Lambert, Brouillet, & Achaz, 2012) and phylogeny-based (Multi-rate Poisson tree processes, mPTP; Kapli et al., 2017) species delimitation methods to putative species boundaries. These single-locus methods have been shown to be effective at delimiting species with uneven sampling (Blair & Bryson, 2017). We used default settings for the ABGD analysis and estimated a maximum likelihood phylogeny with IQ-TREE based on the 16S marker, to use as input for the mPTP analysis. Two MCMC chains were executed using 10,000,000 iterations with samples saved every 50,000 iterations. Finally, for comparison with Sanger-generation studies, we examined mitochondrial divergences among reciprocally monophyletic putative species pairs, by comparing distributions of uncorrected p-distances.

Putative species were then validated using genomic data. We performed rigorous examinations of population structure, clustering, introgression, and genetic divergence among populations, which are explained in detail below.

239

240 *Population clustering.*—We performed dataset dimension-reduction analysis on our SNP
 241 dataset, to infer and visualize population clusters which might correspond to inferred putative
 242 species. A principal component analysis (PCA) was performed to obtain an orthogonal linear
 243 transformation of the data using the R package *adeigenet* (Jombart & Ahmed, 2011).
 244 Additionally, a t-Distributed Stochastic Neighbour-Embedding (t-SNE) method was used to
 245 reveal structure at multiple scales (van der Maaten & Hinton, 2008). The t-SNE method is an
 246 improvement to traditional linear dimensional reduction methods such as PCA and
 247 multidimensional scaling because it is non-linear and is better at capturing structure and
 248 presence of clusters in high-dimensional data (W. Li, Cerise, Yang, & Han, 2017; van der
 249 Maaten & Hinton, 2008). The t-SNE analysis was performed using the R package *Rtsne*
 250 (Krijthe, 2015) under the following settings: dims=3, perplexity=5, theta=0.0,
 251 max_iter=1000000.

252

253 *Population structure.*—Next, we examined population structure by calculating ancestry
 254 coefficients using the program sNMF. This method is comparable to other widely-used
 255 programs such as ADMIXTURE and STRUCTURE, but is computationally faster and avoids
 256 Hardy-Weinberg equilibrium assumptions (Frichot, Mathieu, Trouillon, Bouchard, &
 257 François, 2014). Ancestry coefficients were estimated for 1–10 ancestral populations (K)
 258 using 100 replicates for each K. The cross-entropy criterion was then used to determine the
 259 best K based on the prediction of masked genotypes. The sNMF analysis was implemented
 260 through the R package LEA (Frichot & François, 2015).

261 Non-spatial clustering methods including sNMF, STRUCTURE, and ADMIXTURE
 262 assume that allele frequencies of individuals within a cluster are equal, regardless of their

geographic location. This assumption doesn't account for differentiation caused by continuous processes, such as isolation-by-distance (IBD) and can, consequently, overestimate the number of discrete clusters, especially when geographic sampling is sparse—as is the case, in many empirical studies. Therefore, we also performed a spatially-aware model-based clustering analysis (*conStruct*), which also considers IBD as an explanation for genetic variation (Bradburd et al., 2018). The same SNP dataset was used to represent allele frequencies, and geographic coordinates for each sample were converted into a pairwise great-circle distance matrix using the R package *fields* (Nychka, Furrer, Paige, & Sain, 2017). Our *conStruct* analysis was performed with spatial and non-spatial models, using 200,000 MCMC iterations; traceplots were examined to assess convergence. A cross-validation approach was then used to compare different K values between spatial and non-spatial models. Posterior distributions of parameters were estimated using a training partition consisting of 90% randomly-selected loci. The predictive accuracy of each value of K was then measured using log-likelihoods of the remaining loci, averaged over the posterior. A total of 8 replicates were used to assess each value of K.

To confirm whether IBD contributed to genetic variation, we implemented a distance-based redundancy analysis (dbRDA), which has been shown to be an improvement over traditional Mantel tests because it uses a principal coordinates analysis to linearize the response variable, thereby removing violations of linearity (Guillot & Rousset, 2013; Kierepka & Latch, 2015). Genetic distances were represented by pairwise population G_{st} (Nei, 1973), which was calculated using the R package *mmod* (Winter, 2012). Geographic distances were transformed into distance-based Moran's eigenvector maps (dbMEM) and used as an independent variable (Legendre, Fortin, & Borcard, 2015). The dbRDA analysis was then performed using the *capscale* function in the R package *vegan* (Oksanen et al., 2017). Statistical significance was assessed using 999 permutations.

288

289 *Introgression.*—Admixture among populations were confirmed using Bayesian hybrid-index
 290 analysis and the python program HyDe. A hybrid-index analysis calculates the proportion of
 291 allele copies originating from parental reference populations (Buerkle, 2005), whereas a
 292 HyDe analysis detects hybridization using phylogenetic invariants based on the coalescent
 293 model with hybridization (Blischak, Chifman, Wolfe, & Kubatko, 2018). Different
 294 combinations of plausible parental populations were tested, based on results from our
 295 population structure and preliminary species delimitation analyses.

296 We implemented the hybrid-index analysis on our SNP dataset using the R package
 297 *gghybrid* (Bailey, 2018) after removing loci with a minor allele frequency >0.1 in both
 298 parental reference sets. A total of 10,000 MCMC iterations were performed with the first
 299 50% discarded as burnin. The HyDe analysis was performed on sequence data. First,
 300 admixture at the population level was assessed using the *run_hyde* script that analyses all
 301 possible four-taxon configurations consisting of an outgroup (*Pulchrana signata*) and a triplet
 302 of ingroup populations comprising two parental populations (P1 and P2) and a putative
 303 hybrid population (Hyb). Next, analysis at the individual level was performed using the
 304 *individual_hyde* script to detect hybridization in individuals within populations that had
 305 significant levels of genomic material from the parental species. Finally, we performed
 306 bootstrap resampling (500 replicates) of individuals within hybrid populations to obtain a
 307 distribution of gamma values to assess heterogeneity in levels of introgression.

308

309 *Genealogical divergence index.*—Finally, we used the genealogical divergence index (*gdi*) to
 310 determine whether putative species boundaries corresponded to species-level divergences
 311 (Chan & Grismer, 2019; Leaché et al., 2018). First, an A00 analysis in BPP was used to

estimate the parameters τ and θ with the *thetaprior* = 3 0.002 e (Flouri, Jiao, Rannala, Yang, & Yoder, 2018). Two separate runs were performed and converged runs were concatenated to generate posterior distributions for the multispecies coalescent parameters that were used subsequently to calculate *gdi* following the equation: $gdi = 1 - e^{-2\tau/\theta}$ (Jackson et al., 2017; Leaché et al., 2018). Population A is distinguished from population B using the equation $2\tau_{AB}/\theta_A$, whereas $2\tau_{AB}/\theta_B$ is used to differentiate population B from population A. Populations are considered distinct species when *gdi* values are >0.7 , and low *gdi* values (<0.2) indicate two populations belong to the same species. Values of $0.2 > gdi < 0.7$ indicate ambiguous species status (Jackson et al., 2017; Pinho & Hey, 2010). Because BPP performs best on neutrally evolving loci, we conducted the analysis only on our intron dataset. In order for the analysis to be computationally tractable, we further filtered these data to include only loci with full taxon representation.

RESULTS

Data collection, phylogeny estimation, and topological discordance

Summary statistics for retained loci are presented in Table 1. In general, almost 12,000 intronic and exonic markers were obtained; UCEs numbered 625 and were on average the longest (713 bp), whereas exons were shortest (212 bp). After exons from the same gene were identified and combined, a total of 2,186 markers remained (average length 617 bp). Introns exhibited the most informative sites, with more than 2.6 million variable sites and over 950,000 PIS (Table 1).

Two different topologies (T1 and T2) were obtained across all phylogenetic analyses and datasets (Fig. 1). In general, geographic populations (Peninsular Malaysia, Sumatra, Borneo) formed highly supported monophyletic clades with the exception of two Bornean

samples (ND_7479, ND_7056), which we designated as putative hybrids. For most datasets (Exons-combined, Introns, UCEs), these two samples were recovered as the first-branching lineages within the Peninsular Malaysia + Sumatra clade, with high support across all analyses (T1). However, for the Exon dataset, one of those samples (ND_7479) was recovered as the first-branching lineage of the Bornean clade, with high support across all analyses (T2). Complete details of all phylogenetic trees from analyses of each datasets are provided in Supplementary material.

The relative frequency of alternate topologies surrounding a discordant branch revealed that the number of gene trees supporting the main topology was only slightly more (<3%) than those supporting an alternate topology, indicating a high level of discordance and a lack of overwhelming support for a particular topology (Fig. 2). These outcomes were most evident in datasets that had relatively fewer markers (Exons-combined, 2,186; UCEs, 625) and in which the primary topology was supported by not more than 20 additional gene trees.

Putative species boundaries

The topology of the mitochondrial phylogeny estimated for the mPTP analysis was the same as the topology from analyses of our Exons dataset (T2; Figs. 1, 3A). Excluding the outgroup (*Pulchrana signata*), the mPTP analysis inferred a total of five species (Fig. 3A). The first species (Sp1) comprised samples from Peninsular Malaysia, Sumatra, and one of the putative hybrids (Hybrid 1; ND_7056). Putative species Sp2 included samples from eastern Borneo (FMNH 230864, ND_8281; Fig. 1), which were the sister lineage to True *P. picturata* (exemplified by topotype ID_7750 from Mount Kinabalu). Other Bornean populations were split into two distinct clades but these were not strongly-supported as distinct species (average support value 0.62) and were therefore considered a single putative species (Sp3).

The mPTP also delimited the putative Hybrid 2 as a distinct species with strong support. These five putative species (True *picturata*, Hybrid 2, Sp1, Sp2, Sp3) were also delimited by the ABGD analysis, again with strong support. A comparison of mitochondrial p-distances showed that the level of divergences within Sp1 (including Hybrid 1) and Sp3 were relatively low at $\leq 3\%$ (Fig. 3B); in comparison, divergences among putative species were high ($>5\%$).

Validation using genomic data

Population structure.—A total of 11,490 unlinked SNPs were obtained and used for clustering (PCA, t-SNE), population structure (sNMF, conStruct), and introgression (Bayesian hybrid index, HyDe) analyses. In the PCA analysis, the outgroup (*Pulchrana signata*) and populations from Peninsular Malaysia and Sumatra formed two distinct clusters that were distantly separated—markedly more so than Bornean populations, which showed less separation (Fig. 4A). The t-SNE analysis showed similar results but with more diffusion within clusters (Fig. 4B).

The cross-entropy criterion of the sNMF analysis inferred K=3 and K=4 as the best-predicted numbers of ancestral populations, with K=3 being only marginally better (Fig. 4C). At K=3, populations from Peninsular Malaysia and Sumatra (Sp1) were clustered as a single population with no admixture (Fig. 5A). Similarly, populations from far east Borneo (true *picturata* + Sp2) also formed a single, non-admixed cluster. Other Bornean populations (Sp3, Hybrid 1, Hybrid 2) exhibited a cline of admixture with the two putative hybrid samples being the most admixed. At K=4, the putative hybrid samples were characterized as highly admixed and Sp3 formed a distinct non-admixed group (Fig. 5A).

The *conStruct* analysis also inferred K=3 and K=4 as ideal numbers of ancestral populations, with K=4 slightly better. Model comparison demonstrated that the spatial model

fitted the data slightly better than the non-spatial model at $K=3$, but the two had similar scores at $K=4$ (Fig. 4D). This was corroborated by the dbRDA analysis (p -value = 0.2736; R^2 = 0.2236), indicating that IBD was not a significant factor affecting genetic variation. In general, these assignments of individuals to population clusters were similar to results from the sNMF analysis, but with higher levels of admixture (Fig. 5B). Notably, our True *picturata* clade and Sp2 showed relatively high levels of admixture, whereas Sp3 had dissimilar levels of admixture. One Sp3 sample from far west Borneo was considerably admixed, while the other two samples from east Borneo were not (Fig. 5B).

Introgression and species delimitation.—Based on results from our population clustering and structure analyses, we inferred Sp1 and either Sp3 or True *picturata*+Sp2 to be potential parental populations, due to their dominant representation in ancestry coefficients. When Sp1 and True *picturata*+Sp2 were designated as parental references, the genome of Sp3 and the putative hybrid samples showed a mixture of alleles from both parent taxa (Fig. 6A). A similar result was achieved when Sp1 and Sp3 were designated as parental populations and, in both scenarios, the hybrid index of the putative hybrids was considerably higher (Fig. 6B).

The HyDe analysis at the population level produced a similar, but more nuanced, characterization of hybridization. Using different ingroup configurations, significant hybridization was detected in Hybrid, Sp2, and Sp3 populations (Table 2). Our Sp2 population showed the lowest level of hybridization ($\Gamma=0.9$), whereas Hybrid and Sp3 populations displayed moderate to high levels of hybridization ($\Gamma=0.2$ – 0.8). Furthermore, this analysis showed that hybridization was not limited to Sp1 and True *picturata* as parental populations, but also between Hybrid/True *picturata*, Sp1/Sp2, Sp1/Sp3, Hybrid/Sp2, and True *picturata*/Sp3. Analysis at the individual level showed the Hybrid

population to be a mixture of True *picturata*, Sp1, Sp2, and to a lesser extent Sp3, whereas individuals from Sp3 were a mixture of True *picturata*, Hybrid, Sp2, and Sp1. Individuals from Sp2 were the least admixed (Gamma=0.9; Table 2).

Our *gdi* analysis was performed on a reduced subset of 1,515 SNPs, but with full taxon representation. Additionally, to avoid bias, two putative hybrid samples were removed from this dataset due to their phylogenetic uncertainty and high levels of introgression. Our results indicate that populations from Peninsular Malaysia and Sumatra (Sp1) are distinct species, supported by high confidence (Fig. 6C; mean *gdi*=0.91). However, the specific status of all other populations (those from Borneo) remain uncertain (mean *gdi* 0.55–0.59), and so we conservatively consider them conspecific at the present time.

DISCUSSION

Phylogenetic conflict

As the phylogenomic era unfolds, increasing numbers of studies have shown that analysis of additional numbers of markers does not necessarily increase phylogenetic congruence or branch support, especially for clades of rapidly evolving species, characterized by shallow diversification events. On the contrary, many phylogenomic studies have demonstrated that increasing the amount of data may also increase phylogenetic incongruence (Dávalos, Cirranello, Geisler, & Simmons, 2012; Dell’Ampio et al., 2014; Galtier & Daubin, 2008; Jeffroy, Brinkmann, Delsuc, & Philippe, 2006; Philippe et al., 2011, 2017; Reddy et al., 2017); and that evolutionary relationships associated with rapid or young radiations remain difficult to resolve, even with orders of magnitude more data than a few decades ago (Edelman et al., 2019; Mclean et al., 2019; Meiklejohn, Faircloth, Glenn, Kimball, & Braun, 2016; Rosenberg, 2013; Whitfield & Lockhart, 2007). Experimental and probeset design

considerations are further complicated by the rapid development of target capture methods, which have produced a myriad of different genomic markers including UCEs, exons, and introns (Bi et al., 2012; Faircloth et al., 2012; Folk, Mandel, & Freudenstein, 2015). Recent studies have shown that the efficacy of different types of genomic markers can vary (Chen, Liang, & Zhang, 2017; Yao et al., 2015) and our study further demonstrates how different markers can produce strongly supported but conflicting phylogenetic signals (Figs. 1, 2). Our finding that phylogenetic incongruence among marker types was only detected in the hybrid samples suggests that the genomic landscape of introgression is heterogeneous and may have variable and unpredictable effects on different regions of the genome (Edelman et al., 2019). Our results showed that the topology inferred from exons corresponded better with genomic validation analyses in placing one of the hybrid samples within the Bornean clade, potentially alluding to an association between the genomic landscape of introgressed ancestry and exonic regions of the genome (Edelman et al., 2019; Folk, Soltis, Soltis, & Guralnick, 2018; Kim, Huber, & Lohmueller, 2018).

The inadequacy of phylogeny- and distance-based species delimitation

Our results showed the manner by which highly introgressed individuals can be incoherently or misleadingly placed in a phylogeny. It is noteworthy that phylogenetic placements of hybrid samples in both topologies (T1, T2; Fig. 1) were not in agreement with patterns of spatial and genetic structure inferred from genomic validation analyses, which produced a more biogeographically cogent interpretation of the data. The prevalence of phylogenetic conflict in hard-to-resolve groups calls into the question the rationale of obtaining a single species tree when other alternative topologies are also frequently represented among categories of gene trees. Even if a single species tree is summarized, it may likely be an

inaccurate or only partial representation of evolutionary history, which can lead to incorrect inferences (Hahn & Nakhleh, 2016). In this study we demonstrated how basing a species delimitation framework on an ambiguous—albeit highly supported phylogeny affected by introgression can lead to an overestimation of species boundaries. With only a few Sanger markers, admixed individuals appeared to be highly divergent (Brown & Siler, 2014), even from their parental populations (up to 8% mitochondrial divergence) and, as a consequence, bias distance-based species delimitation methods (Fig. 3). In both mPTP and ABGD analyses, the Hybrid 2 sample was delimited as a distinct species with strong statistical support, while Hybrid 1 from Borneo was lumped with Sp1 (Peninsular Malaysia + Sumatra). However, genomic clustering analyses (PCA, t-SNE, structure, sNMF, *conStruct*) showed that Hybrid 1 was not tightly clustered with Sp1. Furthermore, our hybrid-index and HyDe analyses confirmed that multiple divergent populations (Hybrid, Sp2, and Sp3) were highly introgressed. We therefore advice caution when using distance- and/or phylogeny-based species delimitation methods for groups where introgression is known (or can be reasonably expected) to occur.

Characterizing cryptic species boundaries

All analyses showed a clear distinction between populations from Borneo and Peninsular Malaysia + Sumatra (Sp1). These results were further corroborated by the *gdi* analysis that inferred Sp1 as a distinct species. The timing of the diversification between Sp1 and Bornean populations (Chan & Brown, 2017) also coincides with the cyclical separation of Borneo from Peninsular Malaysia and Sumatra during the Pleistocene (Hall, 2012). In summary, all lines of evidence support the recognition of Sp1 as a distinct species that likely diverged in allopatry during the Pleistocene from a previously widespread ancestor, which occurred

throughout the emergent Sunda shelf connecting Borneo to Peninsular Malaysia and Sumatra. Genomic validation analyses also showed that Hybrid 1 should not be considered as Sp1, but rather part of the Bornean complex of introgressed populations. Our hybrid-index and HyDe analyses also demonstrate that, in addition to the Hybrid population, Sp3 and to a lesser extent Sp2 are also hybrid populations. Thus, we conclude that rampant introgression involving all Bornean populations formed a swarm which obfuscates both distance- and phylogeny-based species delimitation analyses. A total of four species were delimited in Borneo, all of which had high mitochondrial divergences (6–8%) consistent with interspecific divergences seen in other amphibian species (Fouquet et al., 2007; Vences, Thomas, van der Meijden, Chiari, & Vieites, 2005). However, none of the putative Bornean species could be validated by genomic data, which instead showed that genetic structure and genetic divergence among populations in Borneo can be better explained by introgression than by species divergence or IBD.

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Data accessibility

Relevant data generated from this project are available from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.zw3r2284d> [released upon publication].

Author contributions

RMB conceived of this project and, together with KOC and PLW, designed and implemented the study; CRH developed FrogCap resources, data processing, and SNP analysis pipelines; PLW oversaw sample preparation. KOC performed analyses, and composed the manuscript, with input from all authors, who approved this paper in its final form.

Figures and Tables

Fig. 1. Two species tree summary topologies (T1, T2), inferred by ASTRAL-III, based on the Exons-combined, Introns, UCE (left), and Exons datasets (right). All nodes were supported by 1.0 local posterior probabilities and placements of discordant samples (putative hybrids: H1, H2) are indicated by red arrows. IQ-TREE and ASTRID analyses produced the same topologies for the corresponding datasets. *=topotype specimen for *Pulchrana picturata*. Inset photos by A. Haas (top and bottom) and KOC (middle).

Fig. 2. Relative frequencies of alternate gene tree topologies for each dataset. Numbers on top of bars represent the actual number of gene trees supporting that particular topology. The T3 topology was not recovered in any of our phylogenetic analyses.

Fig. 3. A. Putative species delimitation using mPTP analysis, based on 16S rRNA data. Support values at nodes indicate the fraction of sampled delimitations in which a node was part of the speciation process. The analysis strongly supported the discovery-step delimitation of Sp1, Sp2, Sp3, True *picturata*, and Hybrid 2 as distinct species. The ABGD analysis produced the same preliminary candidate species discovery results. **B.** Distribution of uncorrected *p*-distances based on the 16S rRNA gene. Inset photo by KOC.

Fig. 4. A. Results of the Principal Components Analysis and **B.** t-distributed Stochastic Neighbour Embedding (t-SNE) analysis demonstrating population clustering after dimension reduction of SNP data. **C.** sNMF cross-entropy results of K 1–10 (lower cross-entropy scores correspond to the highest predictive accuracy). **D.** cross-validation results from *conStruct* analysis, using non-spatial and spatial models (Ks of highest log-likelihood scores correspond to highest predictive accuracy).

Fig. 5. A. Barplots of admixture coefficients from the sNMF population structure analysis at K=3 and K=4 juxtaposed with a cladogram depicting our T1 topology. Population labels correspond to putative species inferred from species discovery stage analysis of 16S rRNA. Maps (right panels) depict locations of each sample and pie charts of admixture ratios for K=4. **B.** Results of spatial and non-spatial *conStruct* analysis and corresponding distribution map showing admixture ratios for K=4. H1 and H2 represent the putative hybrid samples. The location of the study region is outlined in the red box on the global inset map.

Fig. 6. Bayesian hybrid-index plots, with Sp1, True *picturata* + Sp2 (A) and Sp1, Sp3 (B) as parental references. Dotted lines demarcate 95% confidence intervals. **C.** Density plots of *gdi* values. We interpret species validation to be accomplished in cases of *gdi* > 0.7, whereas 0.2 < *gdi* < 0.7 indicate uncertain species status.

Table 1. Summary statistics of datasets used for phylogenomics and species delimitation analyses. EC=exons combined; PIS50=top 50% loci with highest parsimony-informative-sites. Branch lengths are in coalescent units.

Dataset	No. loci	Mean length	Total sites	Total var. sites	Total PIS
Intron-unfiltered	11,935	452	5,395,834	2,676,967	950,103
Exon-unfiltered	11,978	212	2,543,793	578,939	243,378
EC-unfiltered	2,186	617	1,349,664	286,927	121,681
UCE-unfiltered	625	713	445,346	103,021	37,368
Intron-PIS50	5,968	513	3,063,129	1,652,988	652,822
Exon-PIS50	5,989	378	1,673,499	428,542	190,302
EC-PIS50	1,093	870	950,907	212,555	92,220

Table 2. Results of HyDe analysis at population and individual levels. P-values <0.05 indicate significant levels of hybridization.

P1	Hybrid	P2	Zscore	Pvalue	Gamma
Population level					
Pic	Hyb	Sp1	6.9922	0.0000	0.2023
Hyb	Sp3	Pic	7.8896	0.0000	0.3450
Sp1	Hyb	Sp2	8.3316	0.0000	0.7772
Sp1	Hyb	Sp3	6.6488	0.0000	0.7480
Hyb	Sp3	Sp2	7.1718	0.0000	0.2626
Pic	Sp3	Sp1	9.1459	0.0000	0.7102
Pic	Sp2	Sp3	4.0127	0.0000	0.9072
Sp1	Sp3	Sp2	8.8885	0.0000	0.2360
Individual level					
Pic	ND_7056_Sarawak_Nanga_Ulai	Sp1	6.8507	0.0000	0.1177
Pic	ND_7479	Sp1	6.4388	0.0000	0.4719
Hyb	FMNH_238866_Sabah_Crocker	Pic	8.4826	0.0000	0.3501
Hyb	LSUHC_4039	Pic	6.6791	0.0000	0.3159
Hyb	FMNH_238883	Pic	8.4596	0.0000	0.3658
Sp1	ND_7056_Sarawak_Nanga_Ulai	Sp2	7.6828	0.0000	0.8783
Sp1	ND_7479	Sp2	6.8121	0.0000	0.4450
Sp1	ND_7056_Sarawak_Nanga_Ulai	Sp3	9.0282	0.0000	0.8301
Sp1	ND_7479	Sp3	-9999.0000	1.0000	-0.3707
Hyb	FMNH_238866_Sabah_Crocker	Sp2	8.4319	0.0000	0.2869
Hyb	LSUHC_4039	Sp2	4.9586	0.0000	0.2127
Hyb	FMNH_238883	Sp2	8.0984	0.0000	0.2790
Pic	FMNH_238866_Sabah_Crocker	Sp1	8.7067	0.0000	0.7282
Pic	LSUHC_4039	Sp1	10.0259	0.0000	0.6779
Pic	FMNH_238883	Sp1	8.6311	0.0000	0.7252
Pic	FMNH_230864	Sp3	3.2790	0.0005	0.9175
Pic	ND_8281_Sabah_Sg_Tawau	Sp3	4.7139	0.0000	0.8988
Sp1	FMNH_238866_Sabah_Crocker	Sp2	9.1245	0.0000	0.2333
Sp1	LSUHC_4039	Sp2	9.4740	0.0000	0.2589

872	Sp1	FMNH_238883	Sp2	8.0379	0.0000	0.2157
873						
874	Supplementary Material					
875	Table S1. List of samples used and sequenced in this study.					