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Gene flow creates a mirage of cryptic species in a Southeast Asian spotted stream frog complex

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Abstract

Most new cryptic species are described using conventional tree- and distance-based species delimitation methods (SDMs), which rely on phylogenetic arrangements and measures of genetic divergence. However, although numerous factors such as population structure and gene flow are known to confound phylogenetic inference and species delimitation, the influence of these processes is not frequently evaluated. Using large numbers of exons, introns, and ultraconserved elements obtained using the FrogCap sequence-capture protocol, we compared conventional SDMs with more robust genomic analyses that assess population structure and gene flow to characterize species boundaries in a Southeast Asian frog complex (*Pulchrana picturata*). Our results showed that gene flow and introgression can produce phylogenetic patterns and levels of divergence that resemble distinct species (up to 10% divergence in mitochondrial DNA). Hybrid populations were inferred as independent (singleton) clades that were highly divergent from adjacent populations (7%–10%) and unusually similar (<3%) to allopatric populations. Such anomalous patterns are not uncommon in Southeast Asian amphibians, which brings into question whether the high levels of cryptic diversity observed in other amphibian groups reflect distinct cryptic species—or, instead, highly admixed and structured metapopulation lineages. Our results also provide an alternative explanation to the conundrum of divergent (sometimes nonsister) sympatric lineages—a pattern that has been celebrated as indicative of true cryptic speciation. Based on these findings, we recommend that species delimitation of continuously distributed "cryptic" groups should not rely solely on conventional SDMs, but should necessarily examine population structure and gene flow to avoid taxonomic inflation.

KEYWORDS

FrogCap, gene flow, hybridization, HYDE, phylogenetic network, PHYLONET, population genetics

1 | 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). While lineages that are obviously distinct can be easily diagnosed, the delimitation of cryptic species can be controversial. The rise in cryptic species discoveries has largely been driven by the expansive use of molecular data and new methods for analyses of increasingly large data sets, which have enabled us to elucidate genetic structure at an

unprecedented geographical scale, depth, and resolution. However, most new cryptic species have, to date, been identified or described using tree- and distance-based methods, which rely on phylogenetic arrangements and genetic divergence thresholds (Brown & Stuart, 2012; Fišer, Robinson, & Malard, 2018; Hillis, 2019). This is disconcerting because studies have demonstrated that phylogenetic estimation (and, by implication, most downstream species delimitation inferences) can be biased or misled by factors such as incomplete lineage sorting and gene flow (e.g., Jones, 2018; Leaché et al., 2015; Linkem, Minin, & Leaché, 2016; Long & Kubatko, 2018; Mendes & Hahn, 2018; Roch, Nute, & Warnow, 2019; Xu & Yang, 2016), thereby obfuscating the distinction between population structure and species divergence (Chan et al., 2017; Drillon, Dufresnes, Perrin, Crochet, & Dufresnes, 2019; Harrison & Larson, 2014; Luo, Ling, Ho, & Zhu, 2018; Maguilla & Escudero, 2016; McFadden et al., 2017; Morales & Carstens, 2018; Quattrini et al., 2019; Supple, Papa, Hines, McMillan, & Counterman, 2015; Surveswaran, Gowda, & Sun, 2018). As such, it remains unclear whether the purportedly high levels of hidden diversity within many cryptic species complexes consist of distinct, undescribed species or, instead, genetically structured metapopulation lineages that are not evolutionarily isolated. Nevertheless, these confounding factors are usually ignored when delimiting cryptic species, and empirical studies that consider these potentially confounding factors are the exception rather than the norm (Camargo, Morando, Avila, & Sites, 2012; Chambers & Hillis, 2020; Chan et al., 2017; Dufresnes et al., 2020; Morales & Carstens, 2018; Stanton et al., 2019). Therefore, understanding the effects that these processes may have on inferences of species delimitation are critical to avoid erroneous estimations of species diversity, particularly in biodiversity hotspots such as Southeast Asia, where cryptic species have been widely interpreted as being responsible for a large portion of this imperilled region's purportedly unrecognized biodiversity (Brown & Stuart, 2012; Inger, Stuart, & Iskandar, 2009; Koh et al., 2013; Sodhi, Koh, Brook, & Ng, 2004; Wilcove, Giam, Edwards, Fisher, & Koh, 2013).

Genomic methods can reveal genetic structure in unparalleled detail (e.g., Benestan et al., 2015; Chan et al., 2017; Lim et al., 2017; Schield et al., 2018), but accurately characterizing species boundaries within an evolutionary framework remains challenging. Gene flow among populations, and even species, can bias species tree estimation and produce incorrect topologies (Eckert & Carstens, 2008; Edwards, Potter, Schmitt, Bragg, & Moritz, 2016; Ginsberg, Humphreys, & Dyer, 2019; Hahn & Nakhleh, 2016; Hinojosa et al., 2019; Leaché, Harris, Rannala, & Yang, 2014; Solís-Lemus, Yang, & Ané, 2016). These errors can then be exacerbated in downstream species delimitation analyses that are predicated on the species tree, which is assumed to be correct (Talavera, Dincă, & Vila, 2013; 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 distinguishing between population-level structure and species divergence (Barley, Brown, & Thomson, 2018; Chan et al., 2017; Jackson, Carstens, Morales, & O'Meara, 2017; Leaché, Zhu, Rannala, & Yang, 2019; Luo et al., 2018; Sukumaran & Knowles, 2017; Supple et al., 2015).

Most species delimitation methods either disregard gene flow (distance-based methods) or assume that gene flow is absent (e.g., multispecies coalescent [MSC] methods; Jackson et al., 2017; Leaché et al., 2019). Consequently, one of the adverse effects of ignoring gene flow is that MSC methods tend to overestimate species numbers by interpreting population structure as species divergence (Chambers & Hillis, 2020; Leaché et al., 2019; Luo et al., 2018; Sukumaran & Knowles, 2017; Wagner, Härtl, Vogt, & Oberprieler, 2017; Wagner et al., 2020). To date, few methods have jointly estimated and modelled gene flow into the species delimitation framework; the exceptions, or methods that do characterize gene flow are computationally expensive for larger genomic data sets comprising > 3–5 populations (Jackson et al., 2017; Smith & Carstens, 2019). As an alternative, modular approaches that separately test for confounding effects can provide additional independent lines of evidence to differentiate between population- and species-level divergence (Chambers & Hillis, 2020; Chan et al., 2017; Dincă, Lee, Vila, & Mutanen, 2019; Dufresnes et al., 2020; Morales & Carstens, 2018; Zheng et al., 2017). Such analyses are not reliant on a single species tree, which can be challenging to estimate accurately (see references above) or may not even be present (Hahn & Nakhleh, 2016). Instead, modular approaches utilize population genetic markers, parameter estimates, or gene trees from thousands of loci to provide a more unbiased representation of phylogenetic variation (Blischak, Chifman, Wolfe, & Kubatko, 2018; Buerkle, 2005; Frichot, Mathieu, Trouillon, Bouchard, & François, 2014; Leaché et al., 2019). We employed such an approach to infer species boundaries in Southeast Asian spotted stream frogs of the *Pulchrana picturata* complex, which have been shown to potentially comprise numerous cryptic species (Brown & Siler, 2014).

Currently, *Pulchrana. picturata* is considered a single species that exhibits notable but nondiscrete (continuous) morphological variation throughout its distribution in the Malay Peninsula, Sumatra, and Borneo (Brown & Guttman, 2002; Frost, 2020). High levels of genetic structure and up to 10% mitochondrial divergence (16S rRNA) 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 a separate clade consisting of populations from Thailand, Peninsular Malaysia, and Indonesia with high support (Figure S3 in Brown & Siler, 2014), alluding to the possibility that gene flow may have biased phylogenetic inference in that study using only a handful of loci (Brown & Siler, 2014).

Accordingly, we undertook the present study, using a newly developed target-capture protocol specifically designed for anurans (FrogCap; Hutter et al., 2019) and obtained 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 determine whether deep divergences among

clades and observed geographically structured genetic variation correspond with statistically defensible cryptic species boundaries. Specifically, we test for gene flow among genetically structured populations and assess its effects on inferences of phylogenetic and species boundaries to determine whether species delimitation based on phylogenetic arrangement and genetic divergence can accurately estimate cryptic species diversity.

2 | MATERIALS AND METHODS

2.1 | Sampling and sequencing

Our sampling design is predicated on a Sanger-based molecular phylogenetic analysis with comprehensive geographical sampling by Brown and Siler (2014). Based on their multilocus phylogeny (Figure S3 in Brown & Siler, 2014), we strategically selected samples from each notably divergent clade, making sure to include samples from different geographical populations to adequately capture the genomic diversity within this species complex. A total of 24 specimens were genotyped using the FrogCap sequence capture marker set (Ranoidea V1 probe set; Hutter et al., 2019) including 10 outgroup specimens (*Boophis tephraeomystax*, *Mantidactylus melanopleura*, *Cornufer guentheri*, and *Abavorana luctuosa*, *Pulchrana banjarana*, *P. siberu* and *P. signata*), and 14 ingroup specimens 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 using the MyBaits V3 protocol and briefly follows: (a) genomic DNA was sheared to 300–500 bp; (b) adaptors were ligated to DNA fragments; (c) unique identifiers were attached to the adapters to later identify individual samples; (d) biotinylated 120mer RNA library baits were hybridized to the sequences for an incubation period of 19 hr and 23 min; (e) target sequences were selected by adhering to magnetic streptavidin beads; (f) target regions were amplified via PCR (polymerase chain reaction); and (g) 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.

2.2 | Bioinformatics and data filtering

The full bioinformatics pipeline for filtering adapter contamination, assembling markers, and exporting alignments are available at CRH's GITHUB (bioinformatics-pipeline_stable-V1: 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 (default settings; Bushnell, Rood, & Singer, 2017). Cleaned reads were then assembled de novo with SPADES version 3.12 (settings: "--careful --hap-assembly --expect-gaps"; 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 markers was then aligned on a marker-by-marker basis using MAFFT (Katoh & Standley, 2013).

Alignments were trimmed and saved separately into functional data sets for phylogenetic analyses and data type comparisons. These data sets include (a) 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; (b) Introns: each previously delimited exon was trimmed out of the original contig and both remaining intronic regions were concatenated; (c) 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 (d) UCEs. We applied internal trimming to the intron and UCE alignments using the program TRIMAL (automatic1 function; Capella-Gutiérrez, Silla-martínez, & Gabaldón, 2009). All alignments were externally trimmed to ensure that at least 50% of the samples had sequence data present at the alignment edges.

In addition to analysing the unfiltered data sets, 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 (PISs) as a proxy for hierarchical structure and phylogenetic information, and removed the lower 50% of markers that contained the fewest PISs. All data sets 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.

2.3 | SNP extraction

To obtain variant data across the target samples, we used GATK version 4.1 (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 (variant-pipeline_stable-V1: 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 (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 artefacts and reformatted the data set for variant calling. To locate variant and invariant sites, we used GATK4 to generate a preliminary variant data set 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 data sets into their own combined database. Using these databases, we used the *GenotypeGVCF* function to genotype the combine sample data sets 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, and we also filtered out the top and bottom 10% of variants based on their depth and mapping quality to avoid potentially problematic sites.

2.4 | Phylogenetic estimation and discordance

We used maximum-likelihood (ML) analysis of concatenated data and coalescent-based methods for phylogenetic estimation. For our ML analysis, we used the program IQ-TREE version 1.6 (Chernomor, Von Haeseler, & Minh, 2016; Nguyen, Schmidt, Von Haeseler, & Minh, 2015) and, because of the large number of markers 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, Chernomor, von Haeseler, Minh, & Le, 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, & Jermiin, 2017). Because species boundaries have not been adequately characterized, individual specimens were not assigned to species. 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 the Intron, Exon, Exons-combined, and UCE data sets 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.

2.5 | Species delimitation framework

We use a unified species concept that considers species to be separately evolving metapopulation lineages (de Queiroz 2005). Our primary criterion for assessing lineage independence is the cessation of gene flow among constituent populations. Although gene flow does not preclude speciation (Feder, Egan, & Nosil, 2012; Sousa & Hey, 2013), lineages that exchange genes should necessarily exhibit diversification in other aspects to demonstrate that they are on a separate evolutionary trajectory before they can be categorized as a distinct species (He et al., 2019; Jónsson et al., 2014; Martin et al., 2013). Therefore, we consider continuously distributed populations that demonstrate contemporary gene flow and no other notable forms of divergence to be a metapopulation lineage of the same species.

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 haplotypes derived from originally inferred, strongly supported multilocus inferences (Brown & Siler, 2014) and reanalysis of 16S rRNA data in this study—see supplementary material in Brown and Siler (2014) for information and GenBank accession numbers of 16S samples. 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 infer putative species boundaries. These single-locus methods have been shown to be effective at delimiting candidate species with uneven sampling (Blair & Bryson, 2017). We used default settings for the ABGD analysis and estimated an ML phylogeny with IQ-TREE based on the 16S marker, to use as input for the MPTP analysis. The minimum branch length was automatically detected using the *minbr_auto* function. Two Markov chain Monte Carlo (MCMC) chains were executed using 10,000,000 iterations with samples saved every 50,000 iterations. Finally, for comparison with previous 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, which are explained in detail below.

2.5.1 | Population clustering

We performed dimension-reduction analysis on our single nucleotide polymorphism (SNP) data set to infer and visualize population

clusters which might correspond to inferred putative species. A principal component analysis (PCA) was performed to obtain an orthogonal linear transformation of the data using the R package *adegenet* (Jombart & Ahmed, 2011). Additionally, a t-Distributed Stochastic Neighbour-Embedding (t-SNE) method was used to reveal structure at multiple scales (van der Maaten & Hinton, 2008). The t-SNE method is an improvement on traditional linear dimensional reduction methods such as PCA and multidimensional scaling because it is nonlinear and is better at capturing structure and presence of clusters in high-dimensional data (Li, Cerise, Yang, & Han, 2017; van der Maaten & Hinton, 2008). The t-SNE analysis was performed using the R package *Rtsne* (Krijthe, 2015) under the following settings: dims = 3, perplexity = 5, theta = 0.0, max_iter = 1,000,000.

2.5.2 | Population structure

Next, we examined population structure by calculating ancestry coefficients using a program based on sparse non-negative matrix factorization (SNMF). This method is comparable to other widely used programs such as ADMIXTURE and STRUCTURE, but is computationally faster and robust to departures from traditional population genetic model assumptions such as Hardy–Weinberg equilibrium (Frichot et al., 2014). Ancestry coefficients were estimated for 1–5 ancestral populations (*K*) using 100 replicates for each *K*. The cross-entropy criterion was then used to determine the best *K* based on the prediction of masked genotypes. The SNMF analysis was implemented through the R package lea (Frichot & François, 2015).

2.5.3 | Gene flow

Admixture among populations was confirmed using Bayesian hybrid-index analysis and the python program HYDE. A hybrid-index analysis calculates the proportion of allele copies originating from parental reference populations (Buerkle, 2005), whereas HYDE detects hybridization using phylogenetic invariants based on the coalescent model with hybridization (Blischak et al., 2018). Different combinations of plausible parental populations were tested, based on results from our population structure and preliminary species delimitation analyses. We implemented the hybrid-index analysis on our SNP data set using the R package *gghybrid* (Bailey, 2018) after removing loci with a minor allele frequency > 0.1 in both parental reference sets. A total of 10,000 MCMC iterations were performed with the first 50% discarded as burnin. The HYDE analysis was performed on sequence data from the intron and exon data sets (the UCE data set had insufficient sites). Sites with missing/ambiguous bases were ignored using the --ignore_amb_sites function. First, admixture at the population level was assessed using the *run_hyde* script that calculates all possible four-taxon configurations consisting of an outgroup (*Pulchrana signata*) and a triplet of ingroup populations comprising two parental populations (P1 and P2) and a putative hybrid population (Hyb). Next, analysis at the individual level was performed using the *individual_hyde* script to detect hybridization in individuals within populations that had significant levels of genomic material from the parental species. Finally, we performed bootstrap resampling (500 replicates) of individuals within hybrid populations to obtain a distribution of gamma values to assess heterogeneity in levels of gene flow.

2.5.4 | Genealogical divergence index

Finally, we used the genealogical divergence index (*gdi*) to determine whether putative species boundaries corresponded to species-level divergences (Chan & Grismer, 2019; Leaché et al., 2019). First, BPP (Yang & Rannala, 2010) was used to estimate the parameters τ and θ (A00 analysis) with the *thetaprior* = 3 0.002 e and *tauprior* = 3 0.004 (Flouri, Jiao, Rannala, Yang, & Yoder, 2018). Species assignments were based on putative species boundaries inferred from the discovery step. Because BPP performs best on neutrally evolving loci, we conducted the analysis only on our intron data set. For the analysis to be computationally tractable, we further filtered these data to include only loci with full taxon representation (1,515 loci). Two separate runs were performed (100,000 MCMC iterations each) 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., 2019). 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).

3 | RESULTS

3.1 | 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 data sets (Figure 1). Although gene tree estimation error can bias summary-based methods such as ASTRAL and ASTRID (Molloy & Warnow, 2017; Nute, Chou, Molloy, & Warnow, 2018; Roch & Warnow, 2015), our results from concatenation and summary analyses were congruent across all data sets, indicating that gene tree estimation error did not bias species tree

TABLE 1 Summary statistics of data sets used for phylogenomics and species delimitation analyses

Data set	No. of loci	Mean length (base pairs)	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

EC, exons combined; PIS50, top 50% loci with highest parsimony-informative-sites; UCE, ultraconserved elements.

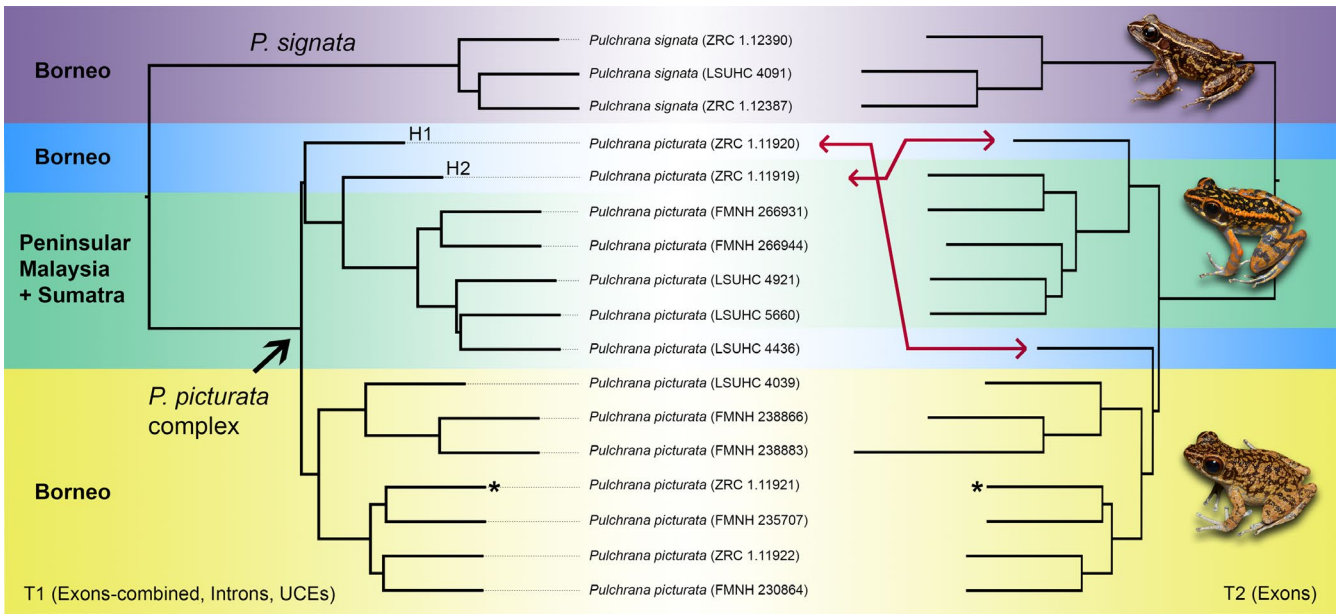

FIGURE 1 Two species tree summary topologies (T1, T2), inferred by ASTRAL-III, based on the unfiltered Exons-combined (2,186 markers), Introns (11,935), UCEs (625; left) and Exons data sets (11,978). All nodes were supported by 1.0 local posterior probabilities and placements of discordant samples (putative hybrids: H1, H2) are indicated by red arrows. IQ-TREE and ASTRID analyses produced the same topologies for the corresponding data sets. *Topotype specimen for *Pulchrana picturata*. See Supporting Information for trees with full taxon representation (including outgroups). Inset photos by A. Haas (top and bottom) and K.O.C. (middle) [Colour figure can be viewed at wileyonlinelibrary.com]

estimation. In general, regional populations (Peninsular Malaysia, Sumatra, Borneo) formed highly supported clades except for two Bornean samples (ZRC 1.11919 and ZRC 1.11920 from Sarawak), which we designated as putative hybrids (H1 and H2; Figure 1) based on their anomalous placement within the Peninsular Malaysia + Sumatra clade. For most data sets (Exons-combined, Introns, and UCEs), these two samples were recovered as the first-branching lineages within the Peninsular Malaysia + Sumatra clade, with high support across all analyses (topology T1; Figure 1). However, for the Exon data set, one of those samples (ZRC 1.11920) was recovered as the first-branching lineage of the Bornean clade, with high support across all analyses (topology T2; Figure 1). Complete details of all phylogenetic trees from analyses of each data sets are provided in the Supporting Information.

The relative frequency of alternative 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 alternative topology, indicating a high level of discordance and a lack of overwhelming support for a particular topology (Figure 2). These outcomes were most evident in data sets 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.

3.2 | Putative species boundaries

The topology of the 16S mitochondrial phylogeny estimated for the MPTP analysis was the same as the topology from analyses of our

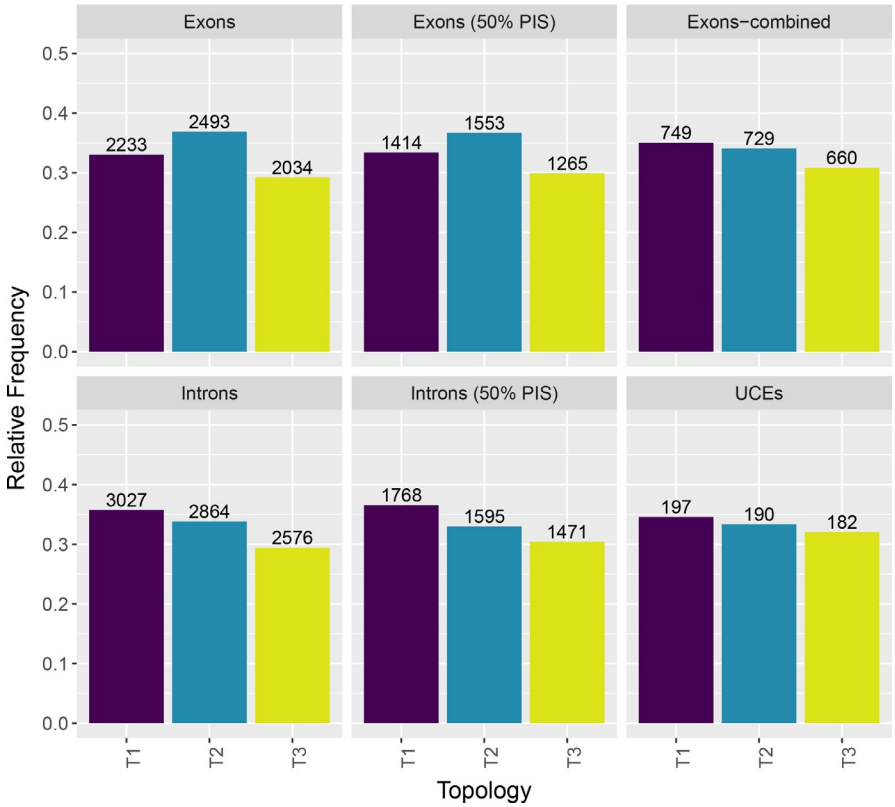

FIGURE 2 Relative frequencies of alternative gene tree topologies for each data set. Numbers on top of bars represent the actual number of gene trees supporting that particular topology. The T1 and T2 topologies are presented in Figure 1, whereas the T3 gene tree topology was not recovered in any of our phylogenetic species tree analyses [Colour figure can be viewed at wileyonlinelibrary.com]

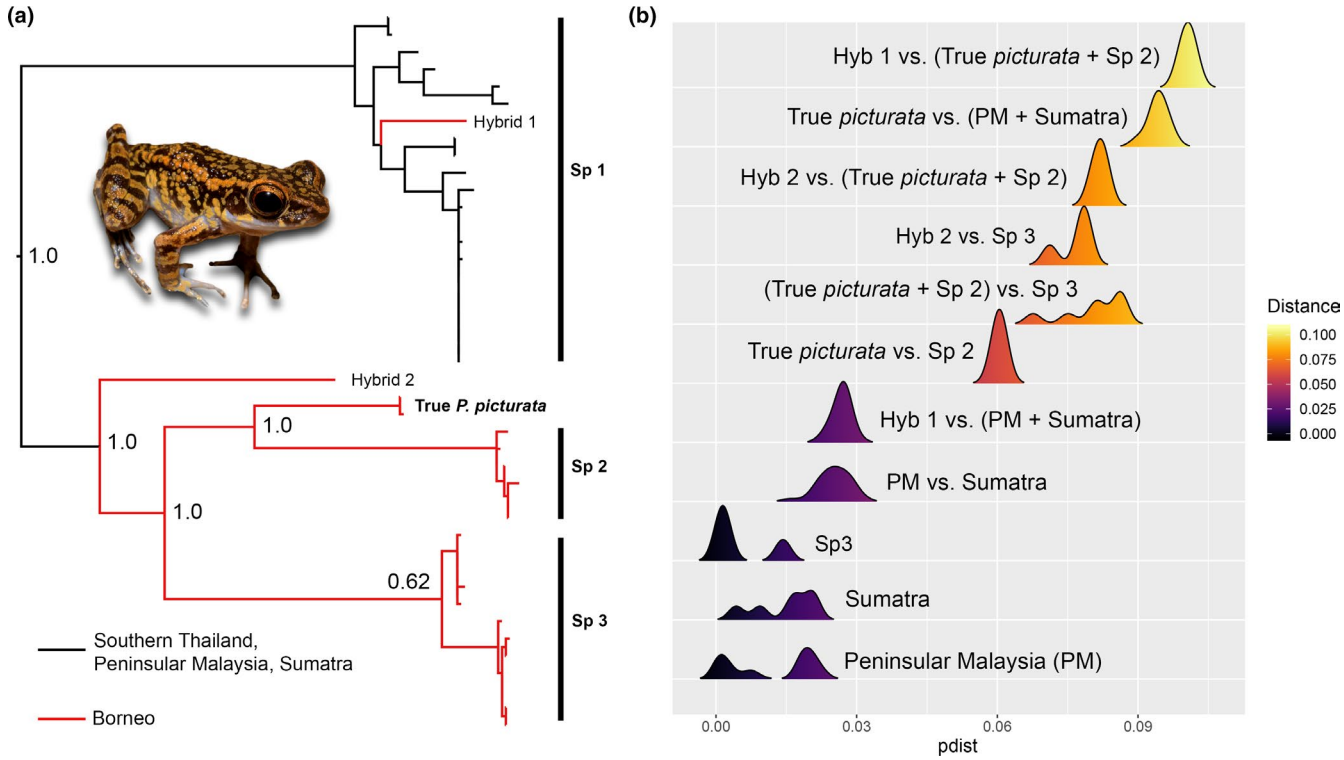

FIGURE 3 (a) Putative species delimitation using MPTP analysis, based on 16S rRNA data. Support values at nodes indicate the fraction of sampled delimitations in which a node was part of the speciation process. The analysis strongly supported the discovery-step delimitation of putative candidate lineages labelled here as Sp1, Sp2, Sp3, True *P. picturata*, and Hybrid 2 ("Hyb 2") as distinct species. The ABGD analysis produced the same candidate species discovery results. (b) Distribution of uncorrected *p*-distances among pairs of taxa/populations/samples, based on the 16S rRNA gene. Distributions labelled "Sp3," "Sumatra", and "Peninsular Malaysia (PM)" presumably represent intraspecific genetic variation. Inset photo by K.O.C [Colour figure can be viewed at wileyonlinelibrary.com]

Exons data set (topology T2; Figures 1, 3a). Excluding the outgroup (*Pulchrana signata*), the MPTP analysis inferred a total of five species (Figure 3a). The first species (Sp1) comprised samples from Peninsular Malaysia, Sumatra, and one of the putative hybrids (Hybrid 1; ZRC 1.11919 from Sarawak). Putative species Sp2 included samples from Sabah, Borneo (FMNH 230864 from Lahad Datu and ZRC 1.11922 from Tawau; Figure 1), which were the sister lineage to true *P. picturata* (exemplified by topotype ZRC 1.11921 from Mount Kinabalu, Sabah). 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). Our MPTP analysis also delimited the putative Hybrid 2 as a distinct species with strong support. These five putative species (True *P. picturata*, Hybrid 2, Sp1, Sp2, Sp3) were also delimited by the ABGD analysis 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 ≤ 3% (Figure 3b); in comparison, divergences among putative species were high (> 5%).

3.3 | Validation using genomic data

3.3.1 | Population structure

A total of 11,490 SNPs were obtained and used for clustering (PCA, t-SNE), population structure (SNMF), and gene flow (Bayesian hybrid index, HYDE) analyses. In the t-SNE and PCA analyses, Sp1, True *P. picturata* + Sp2, and Sp3 formed three distinct and distant clusters, whereas Hybrid 1 and Hybrid 2 were oriented between Sp1 and Sp3 (Figure 4a,b). The cross-entropy criterion of the SNMF analysis inferred $K = 2$ as the best-predicted number of ancestral populations (Figure 4c). Populations from Peninsular Malaysia and Sumatra (Sp1) were clustered as a single population with no admixture (Figure 5). Similarly, populations from far east Borneo (True *P. picturata* + Sp2) also formed a single, nonadmixed cluster. Other Bornean populations (Sp3, Hybrid 1, and Hybrid 2) exhibited a cline of admixture with the two putative hybrid samples being the most admixed. At $K = 3$, Sp3 formed a distinct group, while the putative hybrid samples were an admixture of all three populations (Figure 5).

3.3.2 | Gene flow and species delimitation

Based on results from our population clustering and structure analyses, we inferred Sp1 and either Sp3 or True *P. picturata* + Sp2 to be potential parental populations, due to their dominant representation in ancestry coefficients. When Sp1 and True *P. 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 (Figure 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 (Figure 6b).

Results for the HYDE analysis on the Exon and Intron data sets were largely congruent and produced similar, but more nuanced characterizations of hybridization (results for the HYDE analysis on the Intron data set is presented in Table 2; results for the Exon data set are given in Table S2). Using different ingroup configurations, significant hybridization was detected in all Bornean populations except for True *P. picturata* (Table 2). In agreement with the SNMF and hybrid index analyses, the individual-level analysis showed that the Hybrid and Sp3 populations were the most admixed (Gamma = 0.14–0.76; Table 2).

Our *gdi* analysis was performed on a reduced subset of 1,515 loci, but with full taxon representation. Additionally, to avoid bias, the two putative hybrid samples were removed from this data set due to their phylogenetic uncertainty and high levels of gene flow. Our results indicate that populations from Peninsular Malaysia and Sumatra (Sp1) are a distinct species, supported by high confidence

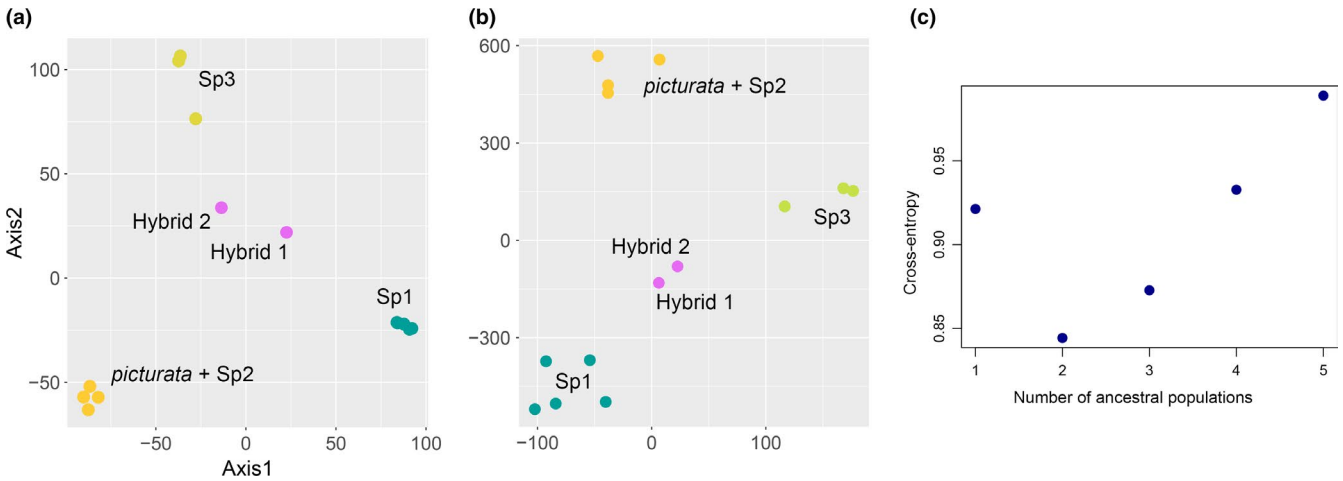

FIGURE 4 (a) Results of principal components analysis and (b) t-distributed Stochastic Neighbour Embedding (t-SNE) analysis, demonstrating population clustering after dimension-reduction of SNP data. (c) Cross-entropy results of $K = 1$–5 (lower cross-entropy scores correspond to the highest predictive accuracy) from the sparse non-negative matrix factorization (SNMF) analysis [Colour figure can be viewed at wileyonlinelibrary.com]

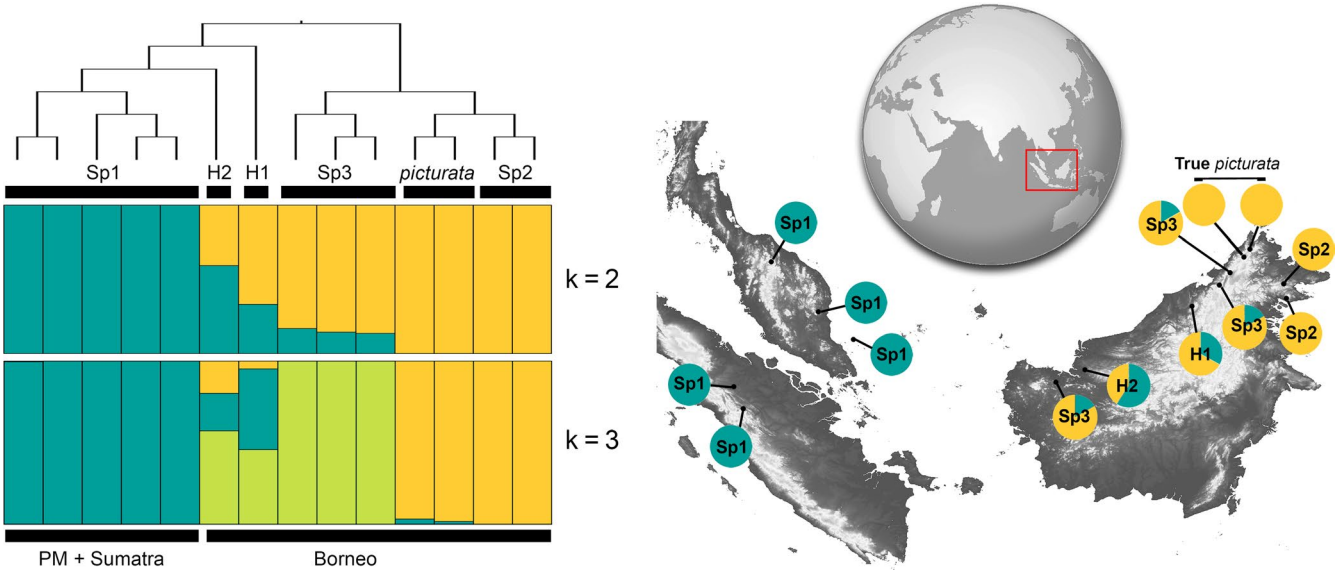

FIGURE 5 Left: barplots of admixture coefficients from the sparse non-negative matrix factorization (SNMF) analysis at $K = 2$ and $K = 3$, juxtaposed with a cladogram depicting the T1 topology (refer to Figure 1). Population labels correspond to putative species inferred from species discovery stage analysis of 16S rRNA. Right: distribution map depicting locations of each sample and pie charts of admixture ratios for the best-fit $K = 2$. The location of the study region is outlined in the red box on the global inset map [Colour figure can be viewed at wileyonlinelibrary.com]

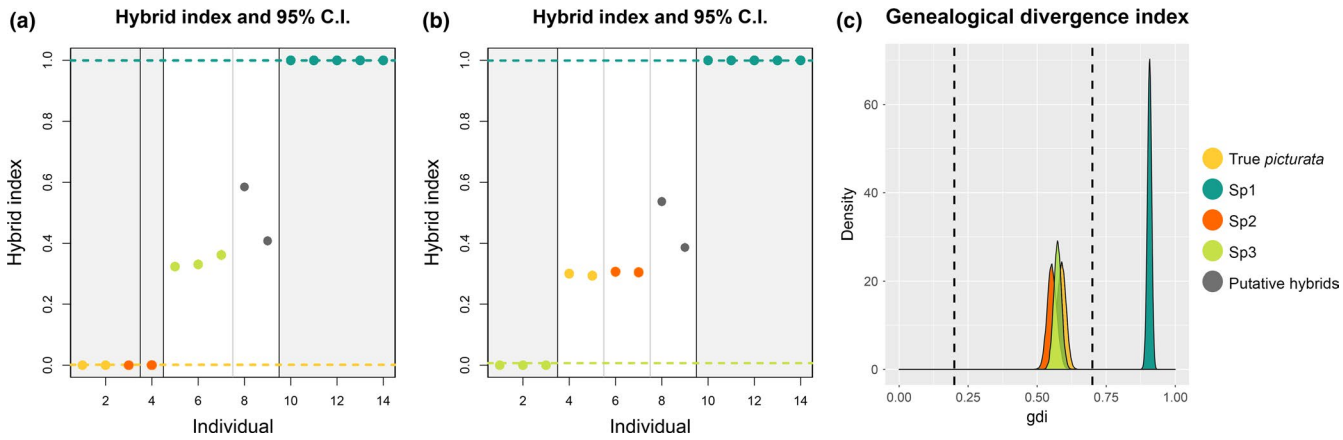

FIGURE 6 Bayesian hybrid-index plots, with Sp1, True *P. picturata* + Sp2 (a) and Sp1, Sp3 (b) as parental references. Dotted lines demarcate 95% confidence intervals. (c) Density plots of *gdi* values. We interpret species validation to be accomplished in cases of *gdi*> 0.7, whereas 0.2 < *gdi* < 0.7 indicate uncertain species status [Colour figure can be viewed at wileyonlinelibrary.com]

(Figure 7c; mean *gdi* = 0.91). However, the specific status of all other populations (those from Borneo) were uncertain (mean *gdi P. picturata* = 0.59; Sp2 = 0.57; Sp3 = 0.55), and so we conservatively consider them conspecific at the present time.

Because our results revealed high levels of gene flow among multiple populations, we also inferred a phylogenetic network that accounts for ILS and hybridization using the program PHYLONET version 3.8 (Wen, Yu, Zhu, & Nakhleh, 2018). To facilitate computation, we used all 625 single-locus gene trees from the UCE data set (outgroups removed) to infer a species network using the Minimizing Deep Coalescence (MDC) criterion, with the maximum number of reticulations set to five. A total of five runs were performed and all other parameters were set to default values. The best inferred network was congruent with results from the HYDE analyses and provided deeper insights at the individual level. Gene flow was detected among most Bornean populations and specifically, between H2 and a sample from Sumatra (FMNH 266944). Most gene flow occurred at the tips of the phylogeny, indicating that gene flow was largely recent or contemporary.

4 | DISCUSSION

4.1 | Confounding effects of gene flow

Our results showed that gene flow/introgression can produce confounding phylogenetic and divergence patterns that can be

TABLE 2 Results of HYDE analysis on the Intron data set at population and individual levels

P1	Hybrid	P2	Z score	*p* value	Gamma
Population-level					
Pic	Sp2	Sp3	7.477983	3.80E−14	0.910803628
Pic	Sp2	Sp1	4.311969	8.10E−06	0.966402726
Pic	Sp2	Hyb	4.1929	1.38E−05	0.963131316
Sp2	Sp3	Sp1	15.14088	0	0.749423257
Sp2	Sp3	Hyb	12.29148	0	0.731488927
Sp2	Hyb	Sp1	11.59205	0	0.158141765
Pic	Sp3	Sp1	15.02887	0	0.679736608
Pic	Sp3	Hyb	12.78638	0	0.633187133
Pic	Hyb	Sp1	9.005072	0	0.147015905
Sp3	Hyb	Sp1	7.826161	2.55E−15	0.148973935
Individual-level					
Pic	*Pulchrana picturata* (FMNH 230864)	Sp3	4.511414	3.22E−06	0.936427407
Pic	*Pulchrana picturata* (ZRC 1.11922)	Sp3	10.20986	0	0.89316752
Pic	*Pulchrana picturata* (FMNH 230864)	Sp1	1.605835	.05416	0.985399051
Pic	*Pulchrana picturata* (ZRC 1.11922)	Sp1	6.808204	4.97E−12	0.953101018
Pic	*Pulchrana picturata* (ZRC 1.11922)	Hyb	8.407745	0	0.93642339
Sp2	*Pulchrana picturata* (FMNH 238883)	Sp1	14.0391	0	0.757301878
Sp2	*Pulchrana picturata* (LSUHC 4039)	Sp1	17.64924	0	0.723858562
Sp2	*Pulchrana picturata* (FMNH 238866)	Sp1	13.5315	0	0.771335643
Sp2	*Pulchrana picturata* (FMNH 238883)	Hyb	12.21885	0	0.726729467
Sp2	*Pulchrana picturata* (LSUHC 4039)	Hyb	11.30665	0	0.756307021
Sp2	*Pulchrana picturata* (FMNH 238866)	Hyb	13.38228	0	0.709656215
Sp2	*Pulchrana picturata* (ZRC 1.11920)	Sp1	12.66585	0	0.457347509
Sp2	*Pulchrana picturata* (ZRC 1.11919)	Sp1	9.232075	0	0.073962378
Pic	*Pulchrana picturata* (FMNH 238883)	Sp1	14.55033	0	0.672953456
Pic	*Pulchrana picturata* (LSUHC 4039)	Sp1	16.81299	0	0.659010718
Pic	*Pulchrana picturata* (FMNH 238866)	Sp1	13.54099	0	0.710162089
Pic	*Pulchrana picturata* (FMNH 238883)	Hyb	12.87686	0	0.61531114
Pic	*Pulchrana picturata* (LSUHC 4039)	Hyb	12.74971	0	0.649178255
Pic	*Pulchrana picturata* (FMNH 238866)	Hyb	12.70818	0	0.633164258

(Continues)

TABLE 2 (Continued)

P1	Hybrid	P2	Z score	*p* value	Gamma
Pic	*Pulchrana picturata* (ZRC 1.11920)	Sp1	10.45553	0	0.408989678
Pic	*Pulchrana picturata* (ZRC 1.11919)	Sp1	7.067336	7.95E−13	0.06849511
Sp3	*Pulchrana picturata* (ZRC 1.11920)	Sp1	0.214591	.41504	0.949383896
Sp3	*Pulchrana picturata* (ZRC 1.11919)	Sp1	12.06656	0	0.111540831

p values <.05 indicate significant levels of hybridization. Population names follow putative species assignments. Pic, true *P. picturata*; Hyb, (H1 and H2).

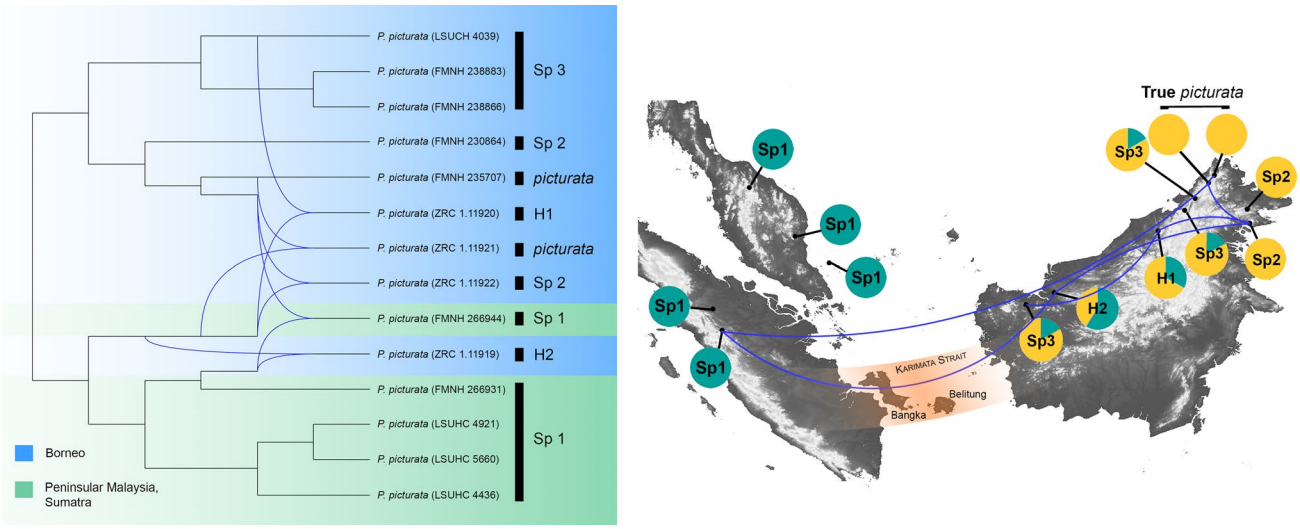

FIGURE 7 Left: results of the PHYLONET phylogenetic network analysis depicted using ICYTREE (Vaughan, 2017). Right: results from the SNMF analysis at $K = 2$. Blue lines connecting populations on the map correspond to blue lines depicting reticulations on the phylogenetic network. Dark orange shading represents the putative habitat corridor that facilitated gene flow between Sumatra and Borneo [Colour figure can be viewed at wileyonlinelibrary.com]

misleading when analysed using conventional species delimitation procedures. Two of the most highly introgressed hybrids (Hybrids 1 and 2) were from Borneo but were inferred in most analyses as independent lineages that were more closely related to the Peninsular Malaysia + Sumatra clade to the exclusion of the Bornean clade. Consequently, the hybrid samples were highly divergent from adjacent Bornean populations (7%–10% mitochondrial divergence), but remarkably similar (<3%) to allopatric populations from Peninsular Malaysia and Sumatra (Hybrid 1). High mitochondrial divergence could be due to mitochondrial gene flow, a phenomenon where introgressed mitochondrial DNA from another species reflects past introgressive events as opposed to lineage isolation (Ballard & Whitlock, 2004; Linnen & Farrell, 2007; Ruane, Bryson, Pyron, & Burbrink, 2014). Using a more robust population genomics approach, we showed that the genomic makeup of the hybrid samples contained relatively large proportions of alleles from Peninsular Malaysia/Sumatra (Sp1) lineages. These results also provide an alternative explanation for the conundrum of highly divergent (sometimes nonsister) sympatric/parapatric lineages (e.g., between H2 and Sp3)—a pattern that has been celebrated as an archetypal sign of genuine cryptic speciation (Brown, 2015; Cobos et al., 2016; Cooke, Chao, & Beheregaray, 2012; Grismer et al., 2015; Ladner & Palumbi, 2012; McLeod, 2010). Such anomalous patterns are not uncommon in amphibians and are present in virtually every Southeast Asian frog family that has been touted to harbour pronounced cryptic diversity: Bufonidae (Chan & Grismer, 2019), Dicroglossidae (Matsui et al., 2016; McLeod, 2010), Ichthyophiidae (Nishikawa et al., 2012), Megophryidae (Chen et al., 2018, 2017; Rowley et al., 2015), Ranidae (Lu, Bi, & Fu, 2014; Stuart, Inger, & Voris, 2006) and Rhacophoridae (Chan, Grismer, & Brown, 2018; Poyarkov et al., 2015). Our results demonstrate that high levels of genetic divergence between sympatric lineages could be an artefact of introgression as opposed to divergence via natural selection.

Although distinct highly divergent sympatric or parapatric cryptic species do undoubtedly exist (Pulido-Santacruz, Aleixo, & Weir, 2018), they usually consist of relatively old lineages that (a) are highly fragmented and whose phylogeographical structure was facilitated by environmental changes (repeated contraction and

expansion of refugia; Grismer et al., 2015); (b) diverged in isolation, followed by subsequent secondary contact (Chan & Brown, 2019); or (c) exhibit varying levels of niche partitioning, for example through contrasting phenologies (Amato et al., 2007; Scriven, Whitehorn, Goulson, & Tinsley, 2016) or small-scale habitat segregation (Muangmai, Von Ammon, & Zuccarello, 2016). However, the purported existence of high numbers of undescribed sympatric/parapatric cryptic species in numerous Southeast Asian amphibian complexes is mostly represented by relatively young (<5 million years old; e.g., Chen et al., 2017), widespread, continuously occurring lineages that are ecologically similar. Taking these characteristics into account (and disregarding the possibility of sympatric speciation, which remains a controversial and hotly debated topic; Foote, 2018), we hypothesize that our results may not be an isolated case, and that other young and highly divergent sympatric lineages (e.g., Brown, 2015; McLeod, 2010) could possibly be explained by introgression. Therefore, a re-analysis of such cases using more robust methods that assesses spatial population structure and gene flow is warranted.

4.2 | Cryptic species as a window on diversity—or slippery slope towards taxonomic inflation?

According to the most widely adopted definition, cryptic species (a) are genetically but not morphologically distinguishable; and (b) are, or have been, classified as a single nominal species (Bickford et al., 2007). Other researchers have specified that cryptic species should also be recently diverged, occur in sympatry, or exhibit reproductive isolation (Chenuil, Cahill, Délémontey, du Luc, & Fanton, 2019; Struck et al., 2018). Viewing speciation as a continuous, gradual and protracted process (Rosindell, Cornell, Hubbell, & Etienne, 2010; Sukumaran & Knowles, 2017), recently diverged lineages that are morphologically similar but genetically divergent may also be associated with the "grey zone" of the speciation continuum—a region of diversification in which there is conflict among operational species criteria (de Queiroz, 2005; Roux et al., 2016). Early diverging lineages in the grey zone can be referred to as incipient species, and we suspect that a number of previously identified cryptic species may fall within this category. These are lineages that have begun to diverge but still exchange genes or maintain signatures of recent gene flow (Marques et al., 2016; Schield et al., 2015; Supple et al., 2015). At this stage of speciation, species boundaries are ephemeral and incipient species can continue to diverge and eventually form distinct species (complete reproductive isolation), or merge back into a single species (Feder et al., 2012; Harrison & Larson, 2014; Mallet, 2008). Therefore, it is critical for cryptic species delimitation to be scrutinized for evidence that lineages are on diverging trajectories of ancestor–descendant series of populations, among which independent lineage status has been achieved via cessation of gene flow (Chan et al., 2017), postzygotic incompatibilities (Pulido-Santacruz et al., 2018), or prezygotic isolation mechanisms such as ecographic segregation (Dufresnes et al., 2020; Slager et al., 2020; Sobel & Streisfeld, 2015), environmental adaptation (Rundle & Nosil, 2005) and behavioural/mate recognition differentiation (Boake, Andreadis, & Witzel, 2000; Drillon et al., 2019; Köhler et al., 2017). These criteria are more robust, compatible with evolutionary theory and species concepts, and reflective of lineage separation; they should thus be included as part of a more informed, modern, multidisciplinary statistical species delimitation framework to avoid unnecessary taxonomic inflation.

Our study also suggests that hybridization (ancient, intermittent, or ongoing) may play a significant role in the evolutionary history and biodiversity of "cryptic" species (Taylor & Larson, 2019), particularly in the Sunda region where large land masses and island archipelagos have been periodically connected and separated due to climatic changes or geological events (Hall, 2013; Yumul, Dimalanta, Marquez, & Queaño, 2009; Yumul et al., 2004). Advancements in high-throughput sequencing has enabled us to move beyond classical criteria for species delimitation such as phylogenetic arrangements and divergence thresholds, and build towards a more process-based understanding of how species boundaries are formed and maintained (Smith & Carstens, 2019; Struck et al., 2018). This includes critical questions such as: (a) How prevalent is hybridization in phylogeographically structured species complexes? (b) How does hybridization affect species boundaries and biodiversity estimates? (c) Is hybridization context-dependent? (i.e., hybrid zones facilitated by landscape features or temporally induced by intermittent habitat corridors during past climatic/geological events). Although quantifying biodiversity is crucial to many fields in biology and conservation, we showed that tree- and distance-based criteria can be poor proxies for cryptic species divergence, thereby implying that general/global genetic distance thresholds (Fouquet et al., 2007; Vieites et al., 2009) should not be used as a primary criterion to assess lineage independence. Distance-based methods can still be useful to identify putative species, but more robust criteria should be implemented to validate cryptic species boundaries.

4.3 | Systematics and biogeography

All analyses in both discovery and validation steps showed a clear distinction between populations from Borneo (True *Pulchrana picturata*, Sp2, Sp3, H1 and H2) and Peninsular Malaysia + Sumatra (Sp1). This was further corroborated by the *gdi* analysis that inferred Sp1 as a distinct species from the true *P. picturata* from Borneo. The SNMF, hybrid index and HYDE analyses showed that H1 and H2 had substantial amounts of Sp1 alleles, thereby explaining their apparently anomalous phylogenetic placement. This highlights the importance of identifying and excluding strong hybrids from species delimitation (and probably species tree) analyses and, additionally, exposes the limitation of phylogeny-based classification that can yield paraphyletic groups in the presence of gene flow (Kumar et al., 2017; Ma et al., 2017). However, it is also possible that additional sampling, especially from central and southern Sumatra, as well as western Borneo, could change the species tree

topology. Regardless, our results unequivocally demonstrate the presence of marked gene flow among populations from Borneo and Sumatra, and the absence of gene flow with populations from Peninsular Malaysia.

The PHYLONET analysis further demonstrated that H2 introgressed specifically with a population from Sumatra (FMNH 266944). This, coupled with the absence of Bornean alleles in Sumatran and Peninsular Malaysian populations, alludes to a west to east ancestral admixture event(s) facilitated by a more southerly habitat corridor, probably along the Karimata Strait via the Bangka–Belitung arc, as opposed to a more northerly route through the Riau Archipelago (Figure 7)—a pattern that has also been documented in numerous other vertebrate groups (Inger & Voris, 2001; Mason, Helgen, & Murphy, 2019; Nijman & Nekaris, 2010). Although we were unable to estimate the timing of diversification in this study, a previous study estimated the diversification of major clades in the *Pulchrana signata/picturata* complex during the Pliocene (Chan & Brown, 2017), during which there was land connection between Borneo and Sumatra, and before the fragmentation of these land masses at the onset of the Pleistocene (Hall, 2013). Therefore, it is likely that ancient introgression between Sumatra and Borneo lineages occurred during the Pliocene and that the cessation of gene flow (and subsequent allopatric diversification) was caused by the inundation of land bridge corridors during the Pleistocene. Subsequently, cyclical Pleistocene glaciations exposed intermittent land bridges, which could have re-established gene flow. This is congruent with the patterns of introgression inferred by our phylogenetic network analysis, which showed the occurrence of ancient as well as more recent introgression.

Our results also showed that populations from the northeastern region of Borneo are distinct from populations in western and central Borneo. This east–west structure is an emerging (Lim et al., 2017; Mason et al., 2019) but understudied pattern that alludes to a possible biogeographical transition zone within Borneo. Studies employing broader and denser geographical sampling are urgently needed, especially in the region of Kalimantan, which represents a crucial sampling gap that has hampered efforts to provide a more comprehensive understanding of the region's biodiversity and evolutionary history.

In summary, our data indicate that only two distinct evolutionary lineages are present within the *P. picturata* complex. One lineage comprises populations from Peninsular Malaysia and Sumatra, and the other occurs in Borneo. Populations from western and central Borneo exchanged genes with Sumatran populations in the past and admixed lineages continued to breed with other Bornean populations, creating a confusing mirage of what might have been interpreted as "cryptic" species given the morphological similarities, inferred phylogenetic structure, and high genetic divergence among admixed populations. The genomes of the Hybrid, Sp2 and Sp3 populations in Borneo contain large numbers of alleles from the True *P. picturata*. Furthermore, they occur continuously across the landscape, exhibit signatures of contemporary gene flow, and show no evidence of pre- or postzygotic isolation. Thus, there is no evidence to indicate that they are on a separate evolutionary trajectory. We therefore consider all Bornean populations to be a conspecific metapopulation lineage under the name *P. picturata*. In contrast, Sp1 is sufficiently divergent from Bornean populations and although gene flow occurred from Sp1 into Borneo, our data did not show the converse. Therefore, in addition to allopatry, the evidence clearly indicates that Sp1 represents a reproductively isolated and separately evolving lineage that is distinct from the True *P. picturata* from Borneo and should be considered a novel species.

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AUTHOR CONTRIBUTIONS

R.M.B. conceived the project and, together with K.O.C. and P.L.W., designed and implemented the study; C.R.H. developed FrogCap resources, data processing, and SNP analysis pipelines; P.L.W. oversaw sample preparation; L.L.G. and I.D. provided genetic material and facilitated fieldwork. K.O.C. performed analyses, and composed the manuscript, with input from all authors, who approved this paper in its final form.

DATA AVAILABILITY STATEMENT

Raw sequence reads are available at the GenBank SRA (BioProject: PRJNA633673 [outgroup samples]; BioProject: PRJNA636105 [ingroup samples]). FrogCap bioinformatic scripts are available at https://github.com/chutter/FrogCap-Sequence-Capture. Supplementary data associated with this study can be obtained from the online version of this manuscript and dryad repository (https://doi.org/10.5061/dryad.zw3r2284d).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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