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Integration of Ecology, Larval Phenotypes, and Mate-Recognition Signals with Molecular and Morphological Data Indicate Taxonomic Inflation in *Nyctibatrachus* (Anura: Nyctibatrachidae)

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Taxonomic studies over the past decade of the endemic Night Frog genus *Nyctibatrachus* (originally described in 1882) from Peninsular India have more than tripled, from 11 at the turn of this century to 36 by 2017. Despite these revisionary contributions, it is still challenging for field biologists to identify night frog species reliably, due to a near-complete absence of diagnostic, discrete character states or trait values. Worse, many questionably diagnosed night frog species' status has ostensibly been "supported" by phylogenies derived from sparsely sampled gene-trees that are based on a single locus or a handful of markers—with topology and arbitrary genetic distance thresholds of 3–6% used to support new species descriptions. We sought to re-evaluate and validate the species boundaries of six currently nominated species of *Nyctibatrachus* of the *aliciae* group (*N. aliciae, N. periyar, N. deveni, N. pillaii*), *N. vasanthi,* and *N. poocha* clade using a comprehensive integrative taxonomic approach that integrates classical taxonomy, molecular species delimitation analysis, statistical analysis of morphological characters of adults and larvae, analyses of bioacoustics, and natural history information. Our results indicate that recent descriptions of *Nyctibatrachus deveni, N. periyar,* and *N. pillaii* represent cases of taxonomic inflation (over-splitting), because the evidence cited in support of their recognition is irreproducible, subjective, and devoid of strong statistical support. We demonstrate the need for multidimensional species delimitation approaches in the celebrated Western Ghats biodiversity hotspot paleo-endemic genus *Nyctibatrachus* and suspect that this concerning trend of over-splitting amphibian species based on limited data and untenable support may be applicable to other amphibian groups.

OVER the last two decades, systematic biology has undergone major advances in the numbers and kinds of methods available for delimiting species boundaries (Camargo and Sites, 2013; Carstens et al., 2013). Criteria for species delimitation typically involve methods employed to translate species concepts into a nominal, Linnaean nomenclatural arrangement (genus–species name pairs) logically consistent with modern species concepts (Wiley, 1978; Frost and Hillis, 1990; de Queiroz, 1997, 1998, 2007). With over 7,200 species, anuran amphibians (frogs and toads) are among the most diverse of terrestrial vertebrates. Moreover, their numbers are steadily increasing (AmphibiaWeb, 2022; Frost, 2022). With the advent of molecular data and advanced statistical phylogenetics, the past two decades have witnessed a surge in new amphibian taxonomic descriptions, especially in the tropics, which have been noted for harboring markedly high species diversity (Scott, 1976; Wiens and Donoghue, 2004; Wiens et al., 2009; Giam et al., 2012; Pyron and Wiens, 2013; Brown, 2014). However, the last two decade's unquestionably accepted assumption that a significant portion of that diversity could be (Stuart et al., 2006) or must be (Bickford et al., 2007) phenotypically cryptic has gone largely untested with independent sources of data (Padial et al., 2010).

Molecular sequence data and statistical methods for distance-based analyses of DNA sequences (predominantly relying on one or, at most, a handful of genetic markers) have been pivotal in species delimitation but have fallen under increased levels of criticism, particularly when evaluating the expectation of the presence of morphologically cryptic species hidden within widespread or allopatrically distributed taxa (Will et al., 2005; Taylor and Harris, 2012; Collins and Cruickshank, 2013). Subsequently, this debate recently has focused on approaches for distinguishing speciation-level processes of diversification from population-level geographic structure, typified by divergent lineages, typically inferred empirically across geographical boundaries (Avise, 2000; Pfenninger and Schwenk, 2007; Coates et al., 2018). However, many recent investigations use only a limited number of available procedures (Esselstyn et al., 2012; Carstens et al., 2013), lack a unified general analytical framework, and have fallen short of fundamental hypothesis-testing analytical procedures. The limitations of many incomplete investigations have been uncovered by more comprehensive reinvestigations in recent times (Leaché et al., 2009; Vieites et al., 2009; Chan et al., 2017, 2020; Sukumaran and Knowles, 2017).

For any standard, two-step (A) proposition of hypothesized species boundaries ("discovery" stage), and subsequent (B) testing of hypothesized species boundaries ("validation" stage) procedure, it is the data-driven, statistically defensible species delimitation (Sites and Marshall, 2003; Welton et al., 2013; Freudenstein et al., 2016; Chan et al., 2017) and analytically reproduceable framework (Fujita et al., 2012; Leaché et al., 2014) that has the potential to withstand healthy scientific skepticism. To aid the standardization of integrated taxonomy, three categories of variably characterized candidate species have been adopted: unconfirmed candidate species, confirmed candidate species, and deep conspecific lineages (Vieites et al., 2009). This standardiza-

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tion is a widely adopted one in recent years as part of deviating from traditional taxonomy (e.g., Wollenberg et al., 2011; Fouquet et al., 2014; Perl et al., 2014).

Analytical advances in statistical species delimitation in recent years have relied increasingly on genetic data, especially in groups for which additional data streams are unlikely to become available (Leavitt et al., 2016; Chan et al., 2020). Recent studies have concluded that barcoding approaches, that may adequately serve as a discovery stage in two-step species delimitation procedures, may not actually hold much promise for delineating closely related species in subsequent validation stages of species delimitation (Meyer and Paulay, 2005; Welton et al., 2013). Additionally, the perils of basing inferences of species delimitation (and resulting taxonomic changes) solely on mitochondrial-marker barcoding approaches have been recognized (Padial et al., 2010; Ahrens et al., 2016). One frequent artifact of proposed but unvalidated taxonomic units that are putatively considered as biological species is the problem of "taxonomic inflation," resulting in diversity estimates of taxonomic groups which may be overestimates of real species diversity (Sites and Crandall, 1997; Isaac et al., 2004; Robuchon et al., 2019; Chan et al., 2020, 2022a). Also, failure rates of using exclusively morphological data or single-marker barcoding confrm that neither should be used as a single information source (Hillis, 1987; Smith and Carstens, 2019). This awareness raises the need for a cultural change in the practice of revisionary taxonomy, which places an objective burden of proof on authors, necessitating statistical analyses of multiple data streams (Fujita et al., 2012; Chan et al., 2017, 2018, 2020; Jackson et al., 2017; Oliver et al., 2018). In recent years, integrative taxonomic approaches that combine multiple, independent, data or character sets (such as external morphological, internal anatomical, ecological, acoustic, and larval traits; apart from geographic considerations, sympatry versus allopatry, and inference of biogeographical range evolution), and rigorous statistical procedures, are becoming industry standard (Dayrat, 2005; Padial et al., 2010; Schlick-Steiner et al., 2010).

The tropical frog genus *Nyctibatrachus* (commonly called Wrinkled- or Night Frogs) currently contains 36 named species, all of which are endemic to the Western Ghats mountains of the Indian Peninsula (Biju et al., 2011; Garg et al., 2017; Krutha et al., 2017). They are the most diverse group in the family Nyctibatrachidae, which comprises two other genera, *Astrobatrachus* (1 species) and *Lankanectes* (2 species). Until the last decade, 16 species of *Nyctibatrachus* were recognized but this number almost doubled with the description of 12 new species (Biju et al., 2011), purportedly on the basis of one or more morphologically diagnostic traits in adults (even with the description of these traits, it was impossible to identify various species in the field). These descriptions set the precedent for an additional nine new species (Gururaja et al., 2014; Garg et al., 2017; Krutha et al., 2017) that were largely described based on the taxonomy of Biju et al. (2011). In addition, Van Bocxlaer et al. (2012) published a molecular phylogeny that ostensibly confirmed the taxonomic changes by Biju et al. (2011). One clade that proves extremely confusing for identification in the field comprises *N. aliciae* Inger, Shaffer, Koshy, and Bakde, 1984; *Nyctibatrachus deveni* Biju, Van Bocxlaer, Mahony, Dinesh, Radhakrishnan, Zachariah, Giri, and Bossuyt, 2011; *N. periyar* Biju, Van Bocxlaer, Mahony, Dinesh, Radhakrishnan, Zachariah, Giri, and Bossuyt, 2011; and *N. pillaii* Biju, Van Bocxlaer, Mahony, Dinesh, Radhakrishnan, Zachariah, Giri, and Bossuyt, 2011; which we will henceforth refer to as the *aliciae* group (= Van Bocxlaer et al.'s [2012] *aliciae* group). The type localities of these four species are geographically separated; the greatest aerial distance between such localities (*N. deveni* and *N. pillaii*) is ~230 km (see Fig. 1A).

As members of the *aliciae* group, these four species form a clade sister to *N. vasanthi* and *N. poocha* (Garg et al., 2017) and all are restricted to the mountain sub-ranges south of the Palakkad Gap, the most prominent geological discontinuity in the otherwise linear and continuous Western Ghats (Fig. 1A). *Nyctibatrachus aliciae*, the earliest described species in this clade, was described from Ponmudi, a spur hill in the southernmost Agasthyamala Hill Range (Inger et al., 1984). *Nyctibatrachus aliciae* and *N. pillaii* were described from the Agasthymala Hill Range, with the former from the western slopes and the latter from the east in Kakachi, whereas *N. periyar* and *N. deveni* were described from Periyar Tiger Reserve in the Pandalam Hills and Kaikatti in the Nelliyampathy Hills, respectively (Biju et al., 2011). *Nyctibatrachus vasanthi* was described from Kakachi in the Agasthyamala Hill Range (Ravichandran, 1997) whereas the type locality of *N. poocha* is Munnar in the High Ranges of the southern Western Ghats (Biju et al., 2011).

In our assessment, the four species constituting the *aliciae* group were described based on arbitrary genetic distance thresholds and subjective morphological character descriptions. Also, only sparse and disjunct populations were evaluated, which can potentially produce misleading genetic distances. Additionally, no other lines of evidence were considered (e.g., ecology, bioacoustics, larval characters, etc.). The results of our comprehensive study of the *aliciae* group, *N. vasanthi*, and *N. poocha* clade suggest that distance-based species delimitations that are predicated on sparse geographic sampling can produce spurious and untenable results that are not supported by other biologically relevant lines of evidence. Similarly, we demonstrate that subjective morphological traits can produce erroneous assumptions of species diagnosability and boundaries. Together with new data on adult morphology, reproductive behavior, habitat preferences, bioacoustics, molecular genetic information, and greatly improved biogeographic range/occurrence data, we re-evaluate the taxonomy of the *aliciae* group of night frogs to explore if the reason for the difficulty in field identification is an artifact of taxonomic inflation. We hypothesize that taxonomic inflation in the *aliciae* group is not an isolated case (e.g., Chan et al., 2020, 2022a; Abraham et al., 2021) and demonstrate that a more pluralistic approach that considers multiple lines of evidence can provide a more holistic, accurate, and statistically defensible inference of species boundaries.

MATERIALS AND METHODS

We consider the currently recognized nominal species in the *aliciae* group (*N. aliciae*, *N. periyar*, *N. deveni*, *N. pillaii*), *N. vasanthi*, and *N. poocha* clade (Biju et al., 2011; Gururaja et al., 2014; Garg et al., 2017; Krutha et al., 2017) as putative species to serve in the first stage (i.e., the discovery step) of an industry-standard two-stage species delimitation approach (Fujita et al., 2012; Freudenstein et al., 2016; Chan et al., 2017; Hillis, 2019). These studies have used Sanger data,

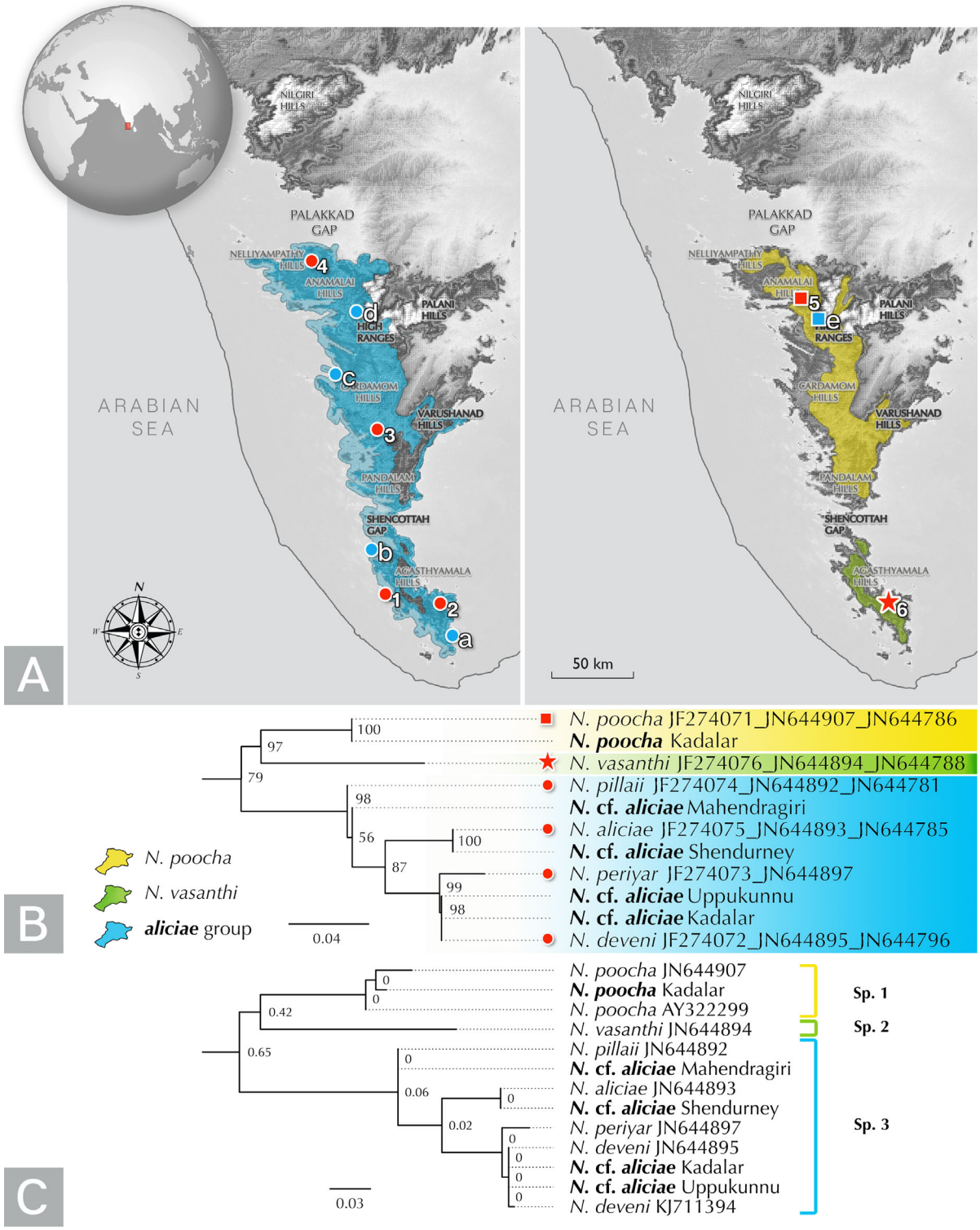

Fig. 1. (A) Map of the southern Western Ghats of India showing estimated distributional range and collection and observation localities of species of *Nyctibatrachus* belonging to: (left) the *aliciae* group (circles) and (right) *N. poocha* (squares) and *N. vasanthi* (star). Red = type locality for which genetic samples are available on GenBank; blue = collection locality of specimens and tissues used in this study. Collection localities from current and previous studies: a. Mahendragiri, b. Shendurney, c. Uppukunnu, d. and e. Kadalar, 1. Ponmudi, 2. Sengaltheri, 3. Vallakadavu, 4. Nelliyampathy, 5. Valparai, 6. Kakachi. Samples downloaded from GenBank have respective accession numbers provided. (B) Maximum likelihood phylogeny of the *aliciae* group, *N. poocha*, and *N. vasanthi* derived from 2172 bp comprising two mitochondrial (16S and ND1) and one nuclear gene (TYR); newly sequenced individuals from intermediate populations used in this study marked in bold. (C) Species delimitation using mPTP analysis, based on a 16S rRNA ML phylogeny, for which 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 Sp. 1 (= *N. poocha*), Sp. 2 (= *N. vasanthi*), and Sp. 3 (= *N. aliciae* + *N. periyar* + *N. deveni* + *N. pillaii*) as distinct species.

consisting of one or two gene regions, and identified genetically divergent individuals, purportedly justified to represent new species by genetic cut-offs of 7–9%, as measured by uncorrected *p*-distances of the 16S gene. Because the specific status of these taxa has not been validated via discrete character state differences or other independent sources of data, we consider them as unconfirmed candidate species (Vieites et al., 2009) and thus implement a suite of validation analyses described below to validate their species boundaries.

Fieldwork and sampling.—We conducted field studies in intervening areas, separating the type localities (Fig. 1A) of all six species of Night Frogs (of the *aliciae* group, *N. vasanthi*, and *N. poocha* clade), and collected two adult specimens of each named taxon from each type locality (except for *N. pillaii*, for which we collected individuals from adjacent sites [within a radius of 50 km] outside the protected area network). Additionally, we observed and collected adult ($n = 5$) and larval specimens from intermediate sites between pairs of type localities, from elevational ranges between 140–1,450 m above sea level (Supplemental Appendix 1; see Data Accessibility). We photographed tadpoles and adults in life, then staged the tadpoles (Gosner, 1960), euthanized both tadpoles and adults in 5% lidocaine, preserved their tissues for genetic material, and fixed voucher specimens in 10% neutral-buffered formalin. After two days in formalin, adult specimens were transferred to 70% ethanol.

Morphological data collection.—To ensure consistency, definitions of measurements (rounded to nearest 0.1 mm) and terminology for adult frogs follow Biju et al. (2011), after independently reviewing their mensural characters with the type series deposited by Biju et al. (2011). Specimens were vouchered and deposited in the Natural History Museum, Trivandrum (TNHM), India (Supplemental Appendix 1; see Data Accessibility). We also measured all original type specimens of all six unconfirmed candidate species in the museum of the Zoological Survey of India, Western Ghats Field Research Station, Kozhikode, India (ZSI/WGFRS). We recorded larval morphological measurements (see Supplemental Appendix 2 for list of characters; see Data Accessibility) to the nearest 0.1 mm, with Mitutoyo calipers, following Altig and McDiarmid (1999), from preserved specimens. Characters such as skin texture, shape of the tips of terminal phalanges, and extent of interdigital webbing of the foot were critically reconsidered for all specimens, with a goal of quantitatively describing variation in proportions, shape, and presence/absence of distinct structures.

Phenotypic data analyses.—Raw morphological measurements were obtained both from individuals documented in Biju et al. (2011: table 2) and our collection (Supplemental Appendix 3; see Data Accessibility). Morphological analyses were based on a standardized and statistically defensible framework established by Chan and Grismer (2021). Due to differences in body size and to eliminate bias caused by ontogenetic variation, each character (except SVL [snout–vent length]) was scaled to the same size of each species by adjusting their shape according to allometry (Thorpe, 1983; Lleonart et al., 2000). Measurements were adjusted for allometric growth (to adjust for intrapopulational variation) using a modification of the following equation outlined by Thorpe (1975): $X_{adj} = \log(X) - \beta[\log(SVL) - \log(SVL_{mean})]$ where X_{adj} = size corrected variable; X = unadjusted dependent variable; β = regression coefficient or slope of the relationship between $\log(X)$ and $\log(SVL)$; SVL_{mean} = species-specific average SVL calculated separately for each species (as opposed to SVL_{mean} averaged across all species as originally intended by Thorpe [1975]). All logarithmic transformations are performed at base 10. This adjustment is implemented through the allom() function in the R package GroupStruct (Chan and Grismer, 2021). All downstream analyses were performed on the adjusted values. We then performed principal component analysis (PCA) to find the best minimum-dimensional representation of external phenotypic variation in the data and to further determine whether continuous morphological variation could form the basis of statistically detectable group structure. Principal components with eigenvalues of 1.0 or more were retained in accordance with Kaiser's criterion (Kaiser, 1960). The R package "hypervolume" (Blonder et al., 2014) was used to construct hypervolumes using Gaussian kernel density estimation to estimate the probability density function of the retained principal components. An ANOVA was performed on the same dataset to determine whether the means of morphological characters differed significantly among populations, followed by a Tukey HSD test to determine specifically which population pair of character means differed after adjusting for multiple comparisons (Abdi and Williams, 2010). All morphological analyses were performed and visualized in R (R Core Team, 2019).

Molecular data collection and analyses.—We extracted total genomic DNA from five specimens of *Nyctibatrachus* (four samples of *N.* cf. *aliciae* included previously unsampled intermediate locations, as did a single *N. poocha*) with Qiagen DNeasy extractions. A ~570 bp segment of mitochondrial DNA, corresponding to a portion of the ribosomal subunit (16S rRNA; Frost et al., 2006), was amplified with standard polymerase chain reaction (PCR; Palumbi, 1996). Raw reads were aligned using default parameters of the MAFFT algorithm (Katoh and Standley, 2013), and quality trimmed, filtered, error corrected, and implemented in Geneious Prime® version 2019.1.3 (Kearse et al., 2012). Resulting alignments were subsequently refined (adjusting ambiguous sites) by eye in the Geneious program and new sequences were deposited in GenBank under accession numbers MN496458–MN496462. A combined/concatenated dataset of 2,172 bp was assembled from Biju et al.'s (2011) original dataset (sequences of specimens from type localities), augmented with our newly collected data. We also included three samples of *Lankanectes* (the endemic Sri Lankan sister genus of *Nyctibatrachus*) and *Astrobatrachus* (another Western Ghats endemic nyctibatrachid) as outgroups. Our final alignment consisted of 570 bp 16S fragments generated in our study and single exemplar sequences from GenBank with complete 16S, ND1, and TYR (tyrosinase) data from all described species of *Nyctibatrachus*.

A maximum likelihood (ML) phylogeny was estimated from the 16S mtDNA dataset, using RAxML v7.2.8 (Stamatakis, 2006; substitution model: GTR + G; 200 independent best-tree searches; rapid-bootstrapping algorithm with 500 replicates). To obtain an overview of intra- vs. interspecific genetic variation, pairwise uncorrected 16S *p*-distances were calculated in PAUP* 4.0b10 (Swofford, 2001) and visualized as a sequence of ridgeline plots using R (for individual values,

see Table S1; see Data Accessibility). Our uncorrected *p*-distance matrix of the 16S gene includes, apart from the *aliciae* group + *N. vasanthi* + *N. poocha* clade, *Nyctibatrachus minimus* (a miniature species outside of the *aliciae* group + *N. vasanthi* + *N. poocha* clade), *Lankanectes* (the sole sister genus within the subfamily Nyctibatrachinae), and *Astrobatrachus* (the sole sister subfamily in the family Nyctibatrachidae).

Molecular species delimitation.—Previous studies merely employed arbitrary distance thresholds, and no model-based species delimitation analyses were performed. As such, we performed a model-based single-locus species delimitation analysis using the program mPTP ver. 0.2.4 (multi-rate Poisson Tree Processes), which has been shown to be at par with other popular distance-based methods (Kapli et al., 2017). In order to obtain more robust and comprehensive estimates of intra- versus interspecific divergences, we performed this analysis on a larger phylogeny that included our sequences (sampled from populations in intermediate sites not included in Biju et al. [2011]) and representative species of other clades in *Nyctibatrachus*. 16S rRNA sequences for the additional species that were obtained from GenBank (Biju et al., 2011: table 4) and an ML phylogeny was estimated using RAxML v7.2.8. The minimum branch length was first calculated from the aligned sequences, followed by the mPTP analysis using the RAxML phylogeny. Two independent MCMC chains were executed (10,000,000 generations each), with sampling done every 50,000 generations. Convergence between MCMC runs was assessed by examining the combined likelihood values of each run. We present, however, only results pertinent to this study, the *aliciae* group + *N. vasanthi* + *N. poocha* clade (full results in Fig. S1; see Data Accessibility).

Acoustic data and analyses.—We recorded advertisement calls (Bee et al., 2013) of males from all sites (one male each from each site was genotyped to ensure correspondence to assigned species) visited with a Roland Edirol R-09HR portable stereo recorder, in uncompressed .wav format at a sampling rate of 44.1 kHz and 16-bit resolution. Recording segments varied from 5–15 calls (depending on logistics and safety) and were made from a distance of 50–150 cm. We then visualized recordings using the SEEWAVE R package (Sueur et al., 2008; R Core Team, 2019), and temporal and spectral properties were analyzed in Raven©Pro 1.5 (Bioacoustics Research Group, Cornell Lab of Ornithology, 2012). Call analyses included generating audiospectrograms, oscillograms, and a standardized Fast Fourier transformation (FFT), with a frame length of 512 Hz, a time-grid overlap of 99%, and Hann windows. We adopted a common, simple definition of a call and defined calls as an entire assemblage of acoustic signals produced in a given sequence (Duellman and Trueb, 1986; Köhler et al., 2017). We measured dominant frequencies and call durations (call period minus inter-call interval) for sampled taxa. Ambient temperature was noted during call recordings, and temperature around the frog was measured using a Fluke 62 MAX InfraRed Thermometer. Calls are deposited in Cornell University's Macaulay Library of Animal Sounds under accession numbers ML237488–ML237496.

RESULTS

Adult morphology: traditional character state "differences".—Our re-evaluation of Biju et al.'s (2011) morphological descriptions of the four species in the *aliciae* group, along with *N. poocha* and *N. vasanthi*, revealed that minimal or no variation in any quantified dimension can be reproduced using their non-diagnostic character state comparisons, which constituted the diagnoses of newly named taxa (Table 1). In contrast to the purportedly diagnostic (= discrete, or non-overlapping, fixed differences among nominal taxa) character states that could not be reproduced, little, or no, difference among populations named as *N. aliciae*, *N. pillaii*, *N. periyar*, or *N. deveni* (Biju et al., 2011) is evident to us using either solely their specimens (these species' type material) or their specimens together with our additional specimens from the same localities. We present our assessment of each reported "diagnostic" character difference originally reported by Biju et al. (2011), below:

1. Adult male SVL upper limit for *N. aliciae*, *N. periyar*, and *N. pillaii* was reported to be ~25 mm, whereas we found a marginally smaller maximum SVL of 24.2 mm in *N. deveni*. Biju et al. (2011) categorized *N. periyar* as a "medium-sized" species and categorized the other three species as "small-sized" even though the maximum SVL of all specimens from the four groups falls within a range of 24–25 mm (see Table 1). Given the < 1 mm difference in body size and clear pitfalls inherent in inference of the evolutionary process of speciation based solely on one tail end of a continuously distributed trait such as body size, we invalidate the allegedly diagnostic character state difference of "medium-sized" versus "small-sized."
2. We found major discrepancies in Biju et al.'s (2011) morphological description of *N. pillaii*, its phylogenetic placement, reporting of type specimens, and diagnostic traits, depicted in their photographic figures. We have determined that the holotype of *N. pillaii* (ZSI/WGRC/V/A/808) from Kakachi is in fact a subadult of *N. vasanthi* (identified by the species' characteristic linear-ridge skin structure on the limbs and reticulated lateral skin folds; see species diagnosis below; Fig. 2B). Photographs of both live and preserved specimens of *N. pillaii* (Biju et al., 2011: figures 47, 48) are actually of *N. vasanthi*. Thus, we removed the measurements/values from the holotype specimen from our PCA analysis because including these data would clearly confound analysis of morphological data with interspecific variation. We note that Biju et al. (2011) reported the GenBank sequence JF274074 as *N. pillaii*, which is derived from the specimen voucher SDB 40286 from Sengalthery, Tamil Nadu (Biju et al., 2011: tables 2, 4) on the eastern slopes of the Agasthyamala Hills (this sequence falls sister to the population of *N. aliciae* from Ponmudi in the ND1 gene tree; not shown), and is, thus, a member of the *aliciae* group. We conclude that it is possible Biju et al. (2011) sequenced tissues from eastern populations of *N. aliciae*, but mistakenly designated a verifiably distinct subadult *N. vasanthi* as the holotype (whose sequence has not been included in the Van Bocxlaer et al. [2012] phylogeny). We thus find no support for the distinctiveness or taxonomic validity of *N. pillaii* due to the phylogenetic placement of *N. pillaii* and its conflicting morphological characteristics.
3. Biju et al.'s (2011) "finger and toe disc" characters correspond to no reproducible variation. The character combination of "third finger disc with dorso-terminal

Table 1. Morphological diagnoses by Biju et al. (2011) of the different unconfirmed candidate species (named but unvalidated) of *Nyctibatrachus* in the *aliciae* group + *N. vasanthi* + *N. poocha* clade. According to Biju et al. (2011), all species can be distinguished from known congeners by the following combination of characters. Species names and characters in bold letters.

Character	*N. aliciae*	*N. deveni*	*N. periyar*	*N. pillaii*	*N. vasanthi*	*N. poocha*
1. Adult size	small male adult size (SVL 20.5–25.6 mm)	small male adult size (SVL 23.5±0.7 mm, n = 5)	medium adult size (male SVL 24.2–25.2 mm, n = 2, female SVL 29.9 mm, n = 1)	small adult male size (SVL 20.0–25.4 mm, n = 9)	small to medium male adult size (SVL 21.9–27.6 mm, n = 7)	medium adult male size (SVL 25.3–32.2 mm, n = 7)
2. Finger and toe disc development	finger and toe discs well developed (FDIII 0.6±0.1 mm, FWIII 0.3±0.1 mm, n = 8; TDIV 0.9±0.1 mm, TWIV 0.4±0.1 mm, n = 8)	finger and toe discs well developed (FDIII 0.6±0.0 mm, FWIII 0.3±0.0 mm, n = 5; TDIV 0.7±0.0 mm, TWIV 0.3±0.3 mm, n = 5)	finger and toe discs well developed (FDIII 0.8±0.0 mm, FWIII 0.4±0.0 mm, n =2; TDIV 0.8±0.0 mm, TWIII 0.1±0.0 mm, n = 2)	finger and toe discs well developed (FDIII 0.6±0.1 mm, FWIII 0.3±0.1 mm, n = 9; TDIV 0.8±0.1 mm, TWIV 0.3±0.1 mm, n = 9)	finger and toe discs well developed (FDIII 0.6±0.1 mm, FWIII 0.3±0.0 mm, n = 7; TDIV 0.8±0.1 mm, TWIV 0.4±0.1 mm, n = 7)	finger and toe discs well developed (FDIII 0.7±0.1 mm, FWIII 0.3±0.1 mm, n = 7; TDIV 1.0±0.1 mm, TWIV 0.3±0.0 mm, n = 7)
3. Finger and toe disc characters	third finger disc with dorso-terminal groove, cover notched distally, fourth toe disc with dorso-terminal groove, cover bifurcate distally	third finger disc with dorso-terminal groove, cover notched distally, fourth toe disc with dorso-terminal groove, cover bifurcate distally	third finger disc with dorso-terminal groove, cover notched distally, fourth toe disc with dorso-terminal groove, cover bifurcate distally	third finger and fourth toe discs with dorso-terminal groove, cover bifurcate distally	third finger and fourth toe discs with dorso-terminal groove, cover bifurcate distally	third finger and fourth toe discs with dorso-terminal groove, cover bifurcate distally
4. Dorsal skin	weakly wrinkled dorsal skin with prominent granular projections	dorsal skin with prominent granular projections	N/A	N/A	N/A	N/A
5. Snout ridge	well-developed ridge extending from the lip over the tip of the snout to between the nostrils, at which point it bifurcates, producing an inverted 'Y'	well-developed ridge extending from the lip over the tip of the snout to between the nostrils, at which point it bifurcates, producing an inverted 'Y'	well-developed ridge extending from the lip over the tip of the snout to between the nostrils, at which point it bifurcates, producing an inverted 'Y'	well-developed ridge extending from the lip over the tip of the snout to between the nostrils, at which point it bifurcates, producing an inverted 'Y'	well-developed ridge extending from the lip over the tip of the snout to between the nostrils, at which point it bifurcates, producing an inverted 'Y'	well-developed ridge extending from the lip over the tip of the snout to between the nostrils, at which point it bifurcates, producing an inverted 'Y'
6. Interdigital foot webbing	webbing medium, reaching before the third subarticular tubercle on either side of toe IV	webbing medium, reaching above the third subarticular tubercle on either side of toe IV	webbing medium, reaching just above the third subarticular tubercle on either side of toe IV	webbing medium, reaching beyond the third subarticular tubercle on either side of toe IV	webbing medium, reaching before the third subarticular tubercle on either side of toe IV	webbing medium, reaching the third subarticular tubercle on either side of toe IV

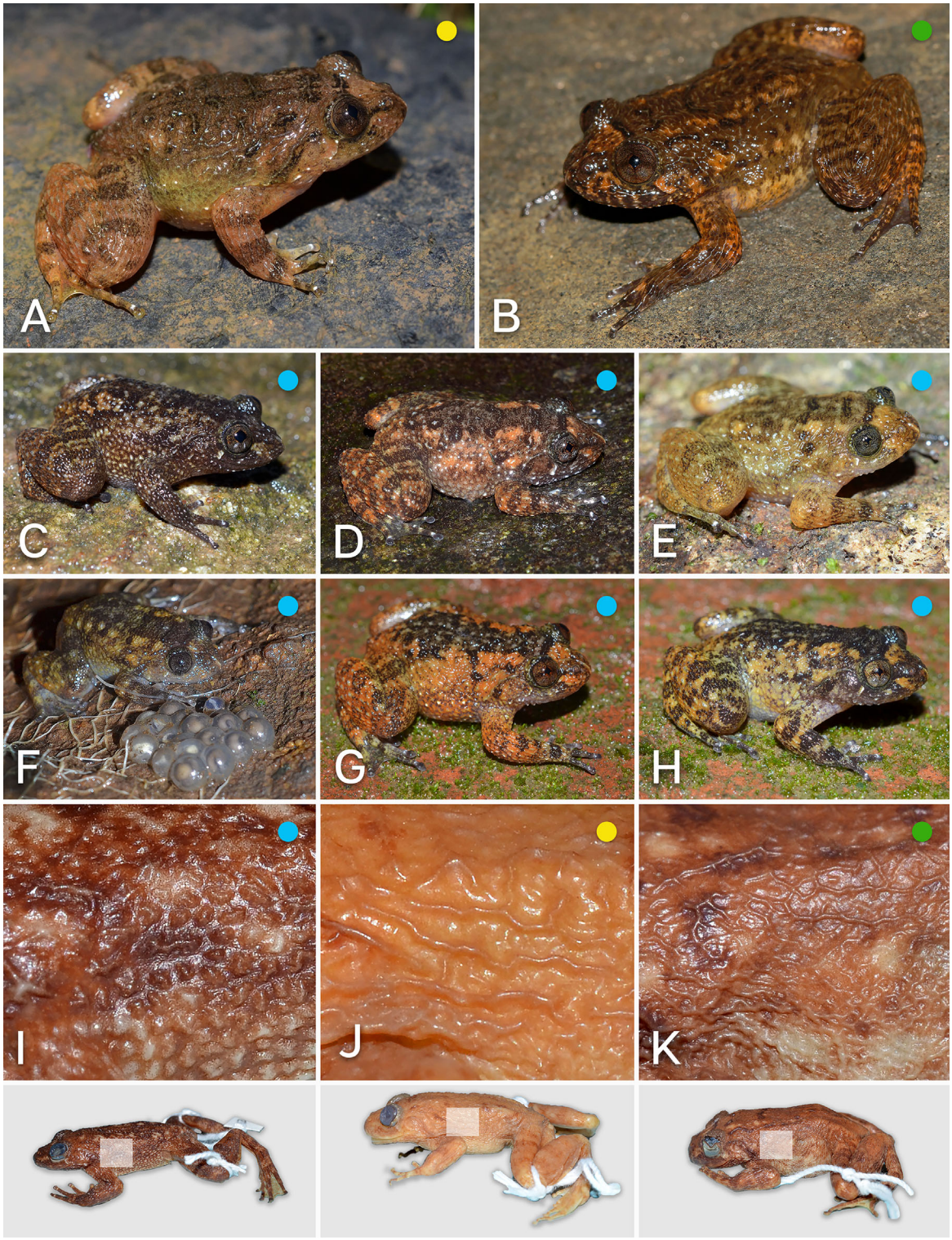

Fig. 2. Live adult males of (A) *Nyctibatrachus poocha*, (B) *N. vasanthi*, and (C) *N. aliciae* from Ponmudi (type locality), (D) *N. pillaii* from Kakachi (type locality), (E) *N.* cf. *aliciae* from Konni, (F) *N. periyar* from Gavi (photograph inverted for comparison), (G) *N. deveni* from Kadalar, and (H) *N. deveni* from Nelliyampathy (type locality). Dorsolateral skin texture respectively of (I) *N. aliciae*, (J) *N. poocha*, and (K) *N. vasanthi*.

groove, cover notched distally, fourth toe disc with dorso-terminal groove, cover bifurcating distally" is entirely subjective, cannot be qualitatively confirmed in the same original specimens, or quantitatively characterized with expanded sampling. We find these structures to be uniform in all four species of the *aliciae* group (Fig. S2; see Data Accessibility). Additionally, Biju et al. (2011), having mistakenly designated a subadult *N. vasanthi* as the holotype for *N. pillaii*, provided the finger and toe disc character of *N. vasanthi* in their description of *N. pillaii*: "third finger and fourth toe discs with dorso-terminal groove, cover bifurcate

distally." Our study of both the paratype specimens of *N. pillaii* and individuals of the *aliciae* group from our own collection (collected from the various type localities), including those from unprotected areas near the type locality show that all four described species possess the same finger and toe disc character state, which we document here (Fig. S2; see Data Accessibility) to provide final correction of these compounded errors. Given that neither the species "Diagnoses" presented in the original Biju et al. (2011) paper nor our confirmation of (the absence of) variation among all type series show any differences, we find that *N. aliciae*, *N. pillaii*, *N. periyar*, and *N. deveni* have the same "finger and toe disc" character "state" (Fig. S2; see Data Accessibility).

4. The character combination of "well developed ridge extending from the lip over the tip of the snout to between the nostrils, at which point it bifurcates, producing an inverted 'Y' " is minimally variable. This is not a characteristic trait for any specific species or clade of *Nyctibatrachus*, but a common character in all species in the genus (see Biju et al., 2011). Thus, the inverted 'Y' ridge character on the snout that is common for all species of *Nyctibatrachus* cannot be considered a valid diagnostic character in the sense that its presence/absence varies among species or can be used to distinguish one unconfirmed candidate species from another.
5. The only skin texture diagnosis offered by Biju et al. (2011) is for *N. aliciae* and *N. deveni*, which have the "dorsal skin with prominent granular projections." Our observations of the museum type material and of specimens from our collections reveal that this trait is shared by all individuals of all four purported species of the *aliciae* group (Fig. 2I) and, as such, cannot be used to distinguish any one species from any/all others.
6. Interdigital webbing of the foot is a highly variable character among individuals of the same population (at a single locality), as well as between left and right feet of the same individual in some instances, and so, qualitative characterizations (e.g., one specimen more or less webbed than another) of degree of webbing are unreliable for distinguishing species (or populations). We found that interdigital webbing attachment of the right foot differs from that of the left on a single individual of *N. periyar* from Uppukunnu (Fig. 3A; TNHM (H) NY5), where the webbing reaches only up to the third subarticular tubercle on either side of the right Toe IV, while it reaches the base of the toe disc on both sides of the left Toe IV. We also find two individuals of *N. aliciae* from the same population in Ponmudi with complete webbing (i.e., webbing reaching just below the toe disc on either side of toe IV; Fig. 3B; TNHM (H) NY6) and medium webbing (i.e., webbing reaching just beyond the third subarticular tubercle on one side, and just above the same on the other side of toe IV; Fig. 3C; TNHM (H) NY3). Considering the high levels of intraspecific and within-individual variation in interdigital webbing of the foot (thus its unreliability as a discrete interspecific diagnostic/taxonomic character in the *aliciae* group), we are again forced to disregard the comparative account in Biju et al.'s (2011) description of variation in interdigital webbing of the foot of *N. deveni* as diagnostic of the species. The authors stated that webbing reaches above the third subarticular tubercle on either side of Toe IV (vs. *N. periyar* that has webbing 'up to the third subarticular tubercle on either side of Toe IV').
7. Biju et al. (2011) described the canthus rostralis as "indistinct" in *N. deveni*, versus "rounded" in *N. periyar*, but provided no detail on this character for *N. pillaii*. This character was not reported for *N. vasanthi*, but it was characterized as "indistinct" in *N. poocha*. Our observation of the series including the types and our own fixed specimens for all species in the *aliciae* group, *N. vasanthi*, and *N. poocha* clade show the canthus rostralis as being rounded and the loreal region as concave and downward facing. Lacking any perceivable difference, we consider the dichotomy of Biju et al. (2011; "indistinct" [degree of prominence] versus "rounded" [shape or degree of angularity between dorsal and lateral surfaces]) to be subjective, non-diagnostic, and of no use for distinguishing any populations/species in this complex.

In conclusion, all seven of the purportedly diagnostic character states for one or more species are found to not vary (uniform across all species), to vary intraspecifically, and/or form continous ranges of variation, with overlapping and subjective character states (e.g., "medium" versus "small") assigned to individual species, but with no clear distinction in states among proposed species.

Tadpole morphology.—We measured tadpoles from different sites of *Nyctibatrachus aliciae* (Ponmudi), *N. deveni* (Nelliyampathy), *N. poocha* (Valparai and Munnar), and *N. vasanthi* (Pandipath and Pandimotta). We used 16S data to genetically confirm the identity of tadpoles to these four described species/populations. We describe the tadpole of *N. aliciae* and *N. deveni* as a single population/taxonomic entity because the larvae of these proposed unconfirmed candidate species are morphologically identical, and our traditional, character-based comparisons ("diagnoses") and multivariate analyses of mensural variation in adults demonstrate no discernable differences between these populations.

Nyctibatrachus aliciae: This description is based on two specimens (one from Ponmudi and the other from Nelliyampathy) in Stage 27 (Fig. 4A; Supplemental Appendix 1 and 2; see Data Accessibility). At this stage, larvae of this species have a pale brown body and an off-white tail that has off-white caudal fins. Dorsal surfaces of body with few scattered dark-brown spots; tail covered with irregular dark brown speckles, which extend into caudal fins, albeit at a smaller size. Both ventral and ventrolateral body surfaces with golden or silver iridocytes, in irregularly spaced clustered rows. Eyes black and gold, of moderate size, positioned dorsolaterally, not visible in ventral view. In lateral view, the body is slightly depressed, with snout rounded. Narial openings closer to tip of snout than distance from eye. Spiracle sinistral, ventrolaterally positioned at midbody; lateral line nonevident. Dorsal caudal fin larger than lower. Oral disc small (Fig. 4D), consisting of anterior and posterior labia, anteroventrally positioned, floral-shaped, its periphery framed by large marginal papillae; small submarginal papillae cover entire inner rim of oral disc. Large, finger-like papillae cover entirety of posterior labium and its fringes, forming a

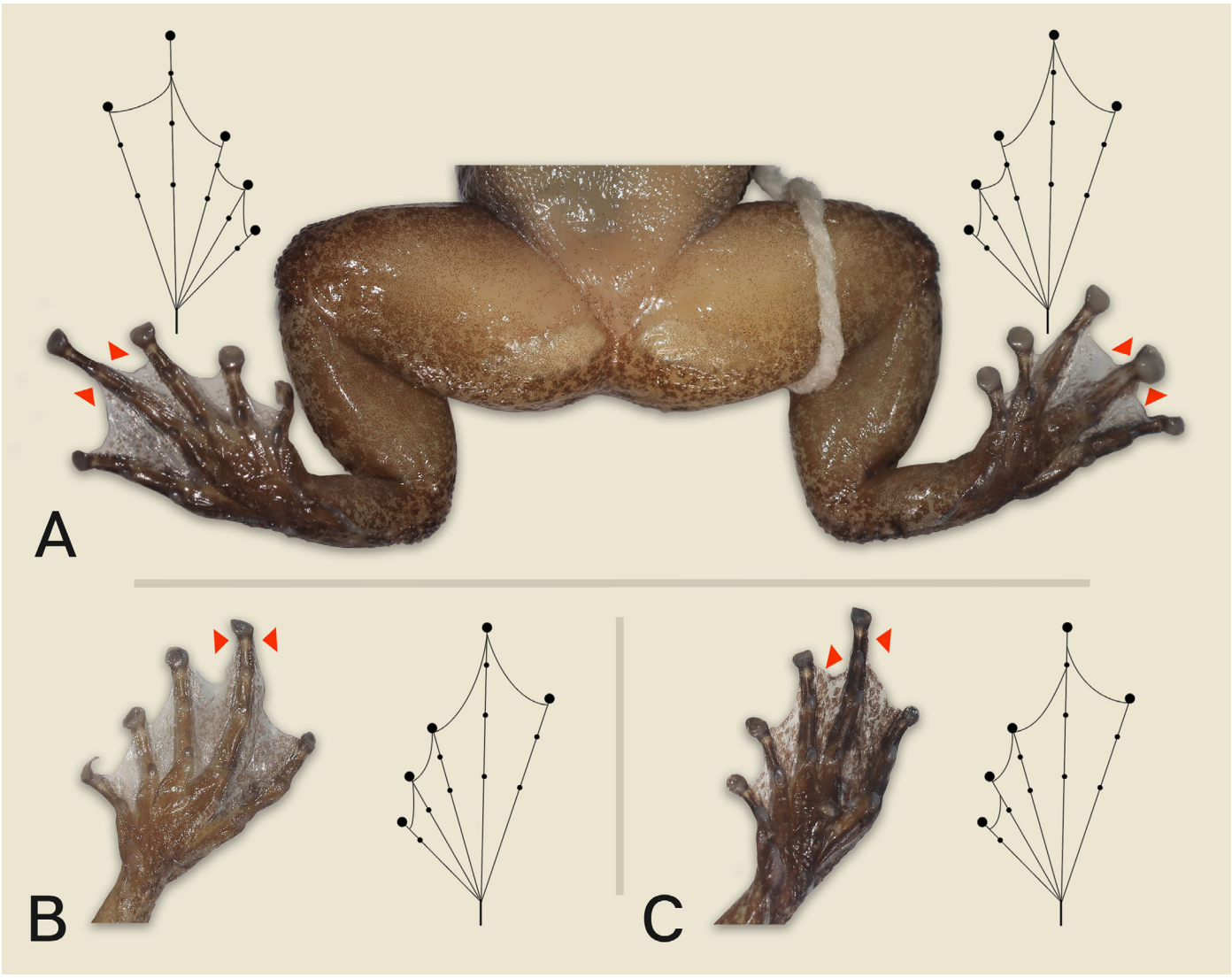

Fig. 3. Variability in foot webbing in the *aliciae* group: (A) webbing attachment on the right foot (left) reaches up to the third subarticular tubercle on either side of toe IV of the right foot, while webbing attachments touch the intercalary tubercle on either side of toe IV of the left foot; (B) complete webbing, where webbing attachments touch the intercalary tubercle on either side of toe IV of the left foot; and (C) medium webbing, where webbing attachment from toe V reaches up to the intercalary tubercle below the toe disc of toe IV and the webbing attachment from toe IV to toe III begins at the third subarticular tubercle of toe IV of the left foot. (A) Male *N. periyar* from Uppukunnu; (B, C) two individual *N. aliciae* males from the same population in Ponmudi.

single row on inner margin of the anterior labium. Labial tooth rows absent, keratinized jaw sheaths serrated and saw toothed. Upper jaw sheath wide, U-shaped; lower jaw sheath V-shaped. Measurements (mm) of two specimens: total length 53.9, 53.7; body length 15.1, 16; tail length 37, 37.9; body width 9.9, 10.8; body height 6, 7; maximum tail length 6.4, 7.5; lower tail fin height 1.3, 1.8; upper tail fin height 2.1, 2.7; eye–snout distance 3.6, 5; snout–spiracle distance 7.9, 7.9; internarial distance 1.2, 2.3; interorbital distance 4.2, 4.5; and eye diameter 2, 2.2. Advanced metamorphic stages of the tadpole are shown in Figure 5.

Nyctibatrachus vasanthi: This description is based on one specimen in Stage 27 (Fig. 4B; Supplemental Appendix 2; see Data Accessibility). Tadpole body blackish-brown with rusty shades due to presence of copper-colored iridocytes on lateral body surfaces. Tail off-white, paler, with off-white caudal fins and black horizontal band, formed from series of vertically barred, broken bands, extending from central caudal musculature, dorsally and ventrally, partially into caudal fins. Tail with irregular horizontal stripe on anterior half of tail, extending from posterior portion of body; posterior (caudal) portion with irregular horizontal stripe formed from prominent black vertical barred blotches, extending throughout both dorsal and ventral fin structures, with few copper-colored iridocytes, organized into irregularly spaced but clustered lines on ventrolateral body surface. Lateral line system evident in two series of minute golden spots: one on dorsolateral body surface, straight, continuing into the tail; second series circular, incomplete, encircling spiracle. Eyes moderate, black and gold, positioned dorsolaterally, not visible in ventral view. In lateral view, body dorsoventrally depressed; snout rounded. Narial openings located closer to eye than tip of snout. Spiracle sinistral, positioned ventrolaterally at midbody. Caudal fin rounded at tip; upper fin larger than lower. Oral disc small (Fig. 4E),

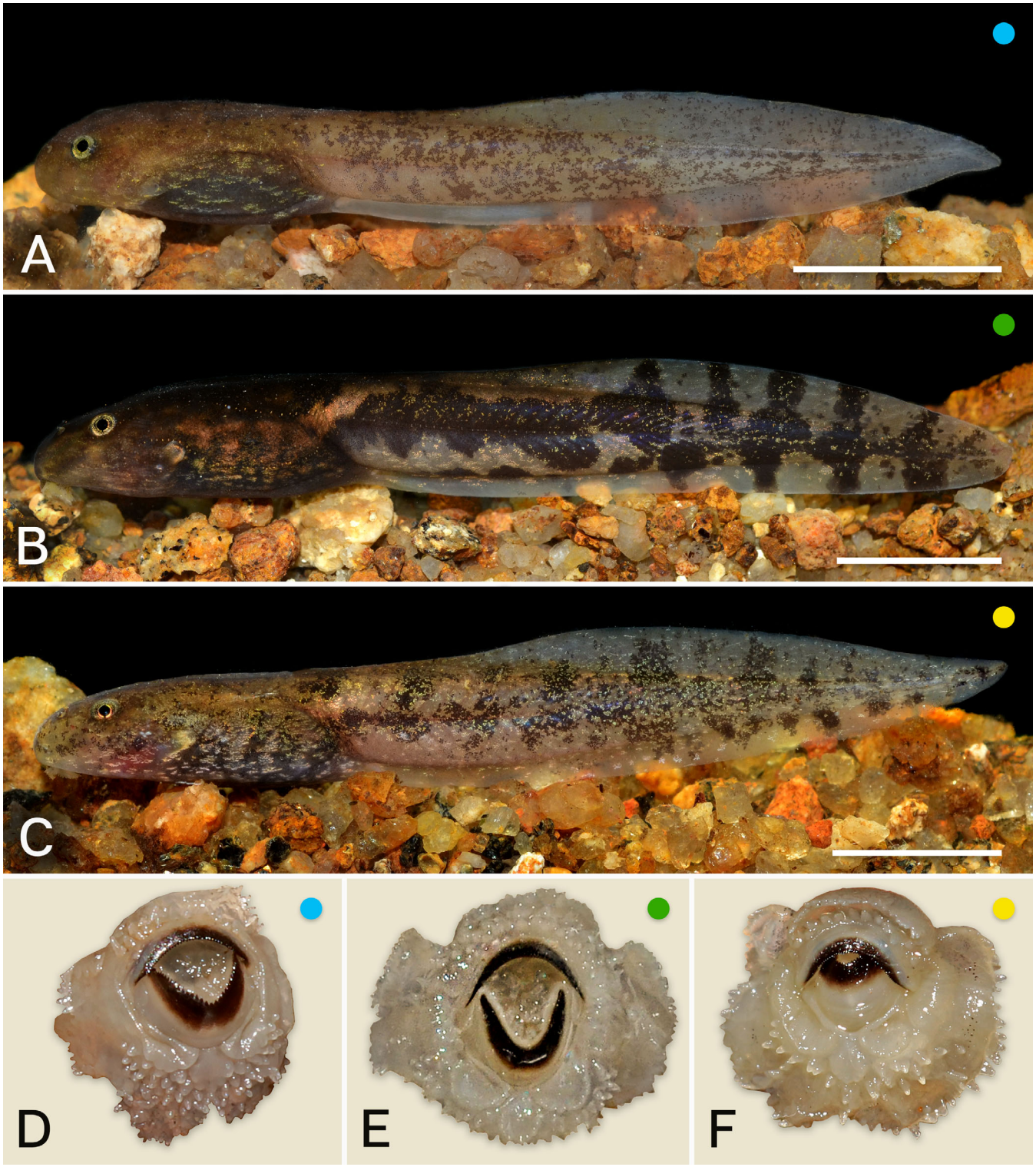

Fig. 4. Tadpoles in Stage 27 of (A) *Nyctibatrachus aliciae* (from type locality; TNHM T1), (B) *N. vasanthi* (from Pandipath, Kerala; TNHM T4), and (C) *N. poocha* (from Valparai, Tamil Nadu; TNHM T3). Scale bar = 10 mm. Oral disc structure of (D) *N. aliciae*, (E) *N. vasanthi*, and (F) *N. poocha*, for which there is little variation among the three species.

consisting of anterior and posterior labia, anteroventrally positioned, floral-shaped, its periphery framed by large marginal papillae; small submarginal papillae cover entire inner rim of oral disc. Large, finger-like papillae cover entirety of posterior labium and its fringes, forming a single row on inner margin of the anterior labium. Labial tooth rows absent, keratinized jaw sheaths serrated and saw toothed. Upper jaw sheath wide, U-shaped; lower jaw sheath V-shaped. Measurements (mm) of one specimen: total length 60.7, body length 16.3, tail length 43.9, body width 9.9, body height 8.1, maximum tail length 8.9, lower tail fin height 1.5, upper tail fin height 2.4, eye–snout

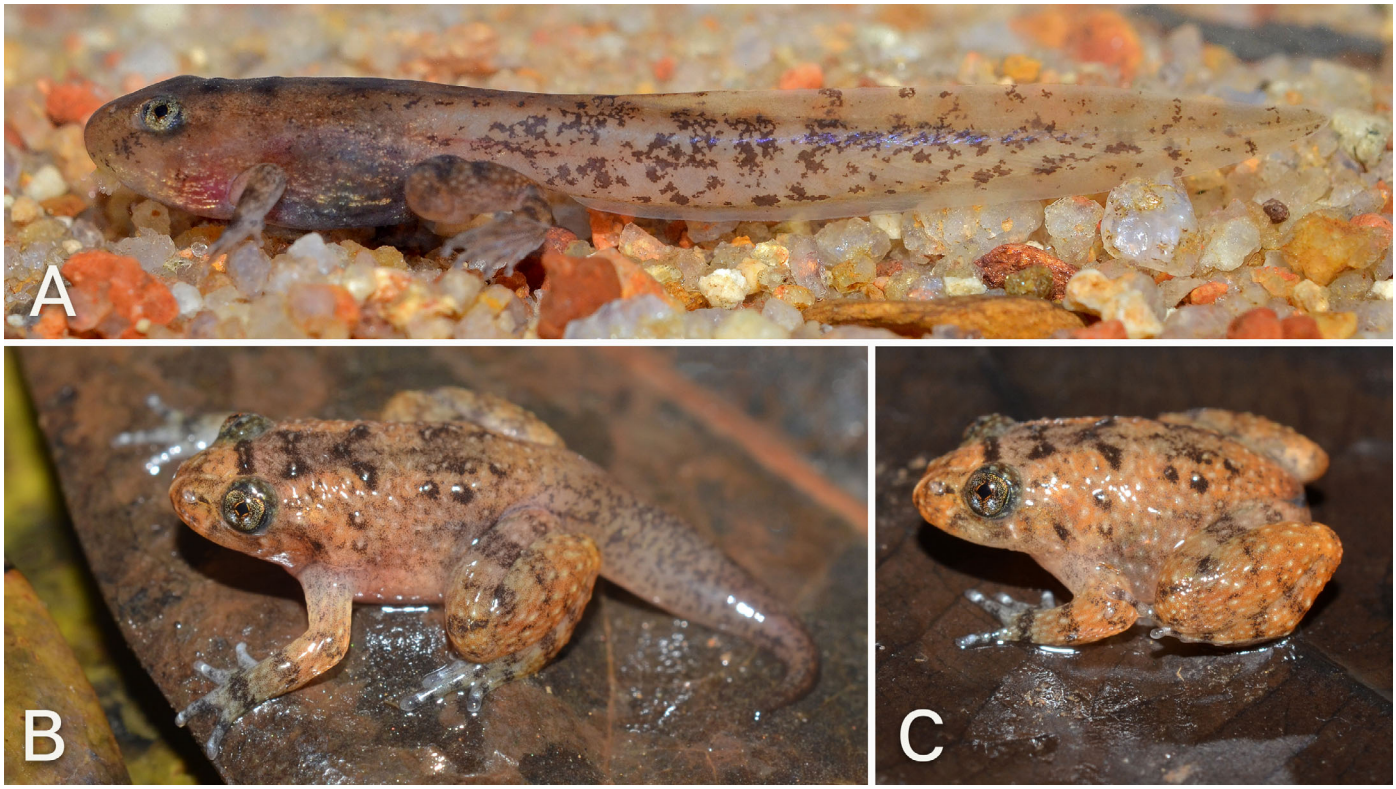

Fig. 5. Advanced metamorphic stages of *Nyctibatrachus aliciae*. (A) Stage 42, (B) Stage 43, (C) Stage 46.

distance 4.1, snout–spiracle distance 8.4, internarial distance 2.9, interorbital distance 5.4, and eye diameter 1.5.

Nyctibatrachus poocha: This description is based on two specimens (one from Munnar and the other from Valparai) preserved at Stage 27 (Fig. 4C; Supplemental Appendix 2; see Data Accessibility), at which this species' larvae have bronze-brown bodies with paler brown tails (pinkish-white in some individuals; observed in the wild, but not collected) and off-white caudal fins. Dorsal body surfaces covered in black spots; tail with large black broken bands, extending from central tail musculature dorsally and ventrally into fin structures. Body covered in silver iridocytes, which become more prominent on caudal fins. Silver iridocytes on ventrolateral body surfaces arranged into irregularly spaced clusters. Lateral line system evident as straight line, running dorsolaterally, continuing onto tail. Eyes, moderate, black and gold, with reddish hue on outer pupil margin, positioned dorsolaterally; not visible in ventral view. In lateral view, body dorsoventrally depressed; snout rounded. Narial openings located at a midpoint between tip of snout and eye. Spiracle sinistral, ventrolaterally positioned at midbody. Upper caudal fin larger than lower. Oral disc small (Fig. 4F), consisting of anterior and posterior labia, anteroventrally positioned, floral-shaped, its periphery framed by large marginal papillae; small submarginal papillae cover entire inner rim of oral disc. Large, finger-like papillae cover entirety of posterior labium and its fringes, forming a single row on inner margin of the anterior labium. Labial tooth rows absent, keratinized jaw sheaths serrated and saw toothed. Upper jaw sheath wide, U-shaped; lower jaw sheath V-shaped. Mean measurements (mm) of three specimens: total length 66, body length 20.3, tail length 45.9, body width 11.5, body height 9, maximum tail length 9.8, lower tail fin height 2.6, upper tail fin height 2.7, eye–snout distance 3.2, snout–spiracle distance 9.6, internarial distance 3, interorbital distance 5.9, and eye diameter 2.

Adult morphology: multivariate analyses of continuous variation.—We also observe considerable color variation in the *aliciae* group both among individuals from the same population, and across populations with no clear association (Fig. 2C–H). Base body color in individuals ranges from ochre brown to rusty brown to dark chocolate brown, with a variety of black or gray mottled patterns on dorsal surfaces and banded patterns on the limbs. In some individuals, a brighter version of the base body color extends as longitudinal dorsal bands from the back of the eyelids to the middle of the dorsum. In all individuals, the dorsal surface immediately behind all finger and toe discs sports a bright white bar. The edge of the subocular gland is either bright white or gray color in all individuals.

Results of our PCA analysis of adults show that the first four principal components have eigenvalues of more than 1.0 and together account for 75% of the total variance (Table S2; see Data Accessibility). The first principal component (PC1) has the highest loadings for snout length (from tip of snout to anterior orbital border), head length (from the rear of the mandible to tip of snout), foot length (from the base of the inner metatarsal tubercle to tip of the fourth toe), forearm length (from flexed elbow to base of the outer palmar tubercle), and eye length (horizontal distance between bony orbital borders), indicating that these characters explain most of the variation along the PC1 axis. The second component (PC2) possesses the highest loadings for eye length, snout–vent length, shank length, and thigh length. Ordination of

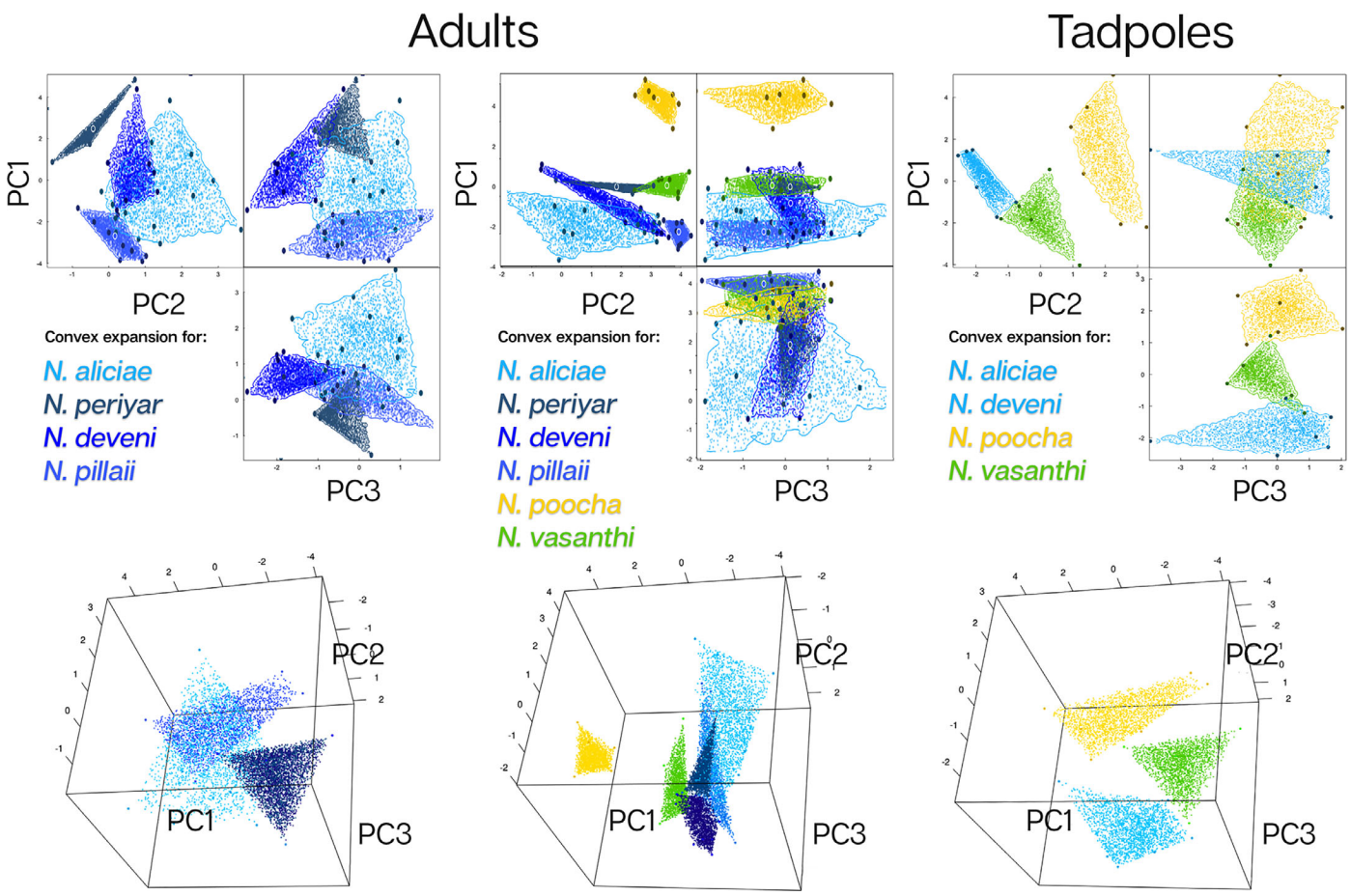

Fig. 6. PCA plots of morphological variables for adults of the *aliciae* group alone (left) and *aliciae* group, *N. poocha*, and *N. vasanthi* clade (center) and tadpoles (right) in 2D (top) and 3D (bottom). The principal components are visualized as hypervolumes constructed using kernel density estimation. Geometry of hypervolumes corresponds to a minimum convex hull (polytopes) that minimally encloses the data. Axes show the first three principal components and their proportion of variance. Note: The hypervolumes for *N. deveni* and *N. periyar* align completely, suggesting minimal morphological divergence.

the first two principal components shows overlap of all four species in the *aliciae* group along both axes (Fig. 6). Results from the PCA analysis indicate significant overlaps in morphological variation among *Nyctibatrachus aliciae*, *N. pillaii*, *N. periyar*, and *N. deveni* and that these putative species show no distinct separation in adult morphospace.

Tadpole morphology: multivariate analyses of continuous variation.—In our PCA analysis of the tadpoles of the *aliciae* group, *N. poocha*, and *N. vasanthi* clade, we could include only five specimens of *N. aliciae* (TNHM T1) and three of *N. deveni* (TNHM T2), representing populations from the extremities of the clade's range. The first seven principal components have eigenvalues ≥ 1.0, together accounting for 88% of the total variance (Table S3; see Data Accessibility). Principal component 1 exhibits loadings for total length, tail length (i.e., length from body terminus to tail tip), eye diameter, oral disc width, and tail muscle height at body-tail junction, indicating that these characters explain most of the variation along the PC1 axis. Principal component 2 possessed loadings for body length (i.e., length from snout to body terminus), body height, tail muscle width at body–tail junction, eye–snout distance, and distance of naris (center) from snout. Ordination of the first two principal components show non-overlap among *N. aliciae*, *N. poocha* (TNHM T3), and *N. vasanthi* (TNHM T4) along both axes, which leads us to interpret this variation as evidence in favor of three potentially diagnosable species (Fig. 6). We say this with the caveat that inclusion of missing taxa could change these results. In conclusion, and in contrast to adult morphology, we observe distinct separation in larval morphospace in *N. aliciae*, *N. poocha*, and *N. vasanthi*.

Phylogeny and genetic distance.—The results of our ML phylogenetic analysis (for which only the results of the *aliciae* group, *N. vasanthi*, and *N. poocha* clade are shown here) corroborate previous studies defining this clade as a monophyletic group consisting of *Nyctibatrachus aliciae*, *N. pillaii*, *N. periyar*, and *N. deveni*, along with sister species *N. poocha* and *N. vasanthi*. The placement of *N. vasanthi* in our tree agrees with results of Garg et al. (2017) but not those of Van Bocxlaer et al. (2012) with regard to this species' relationship with *N. poocha* (Fig. 1B). Both former studies contained only a single representative tip per proposed species in phylogenetic analyses, so we improve upon this singleton sampling with five additional tissue samples, representing previously unsampled intermediate geographic localities (Fig. 1A, a–d).

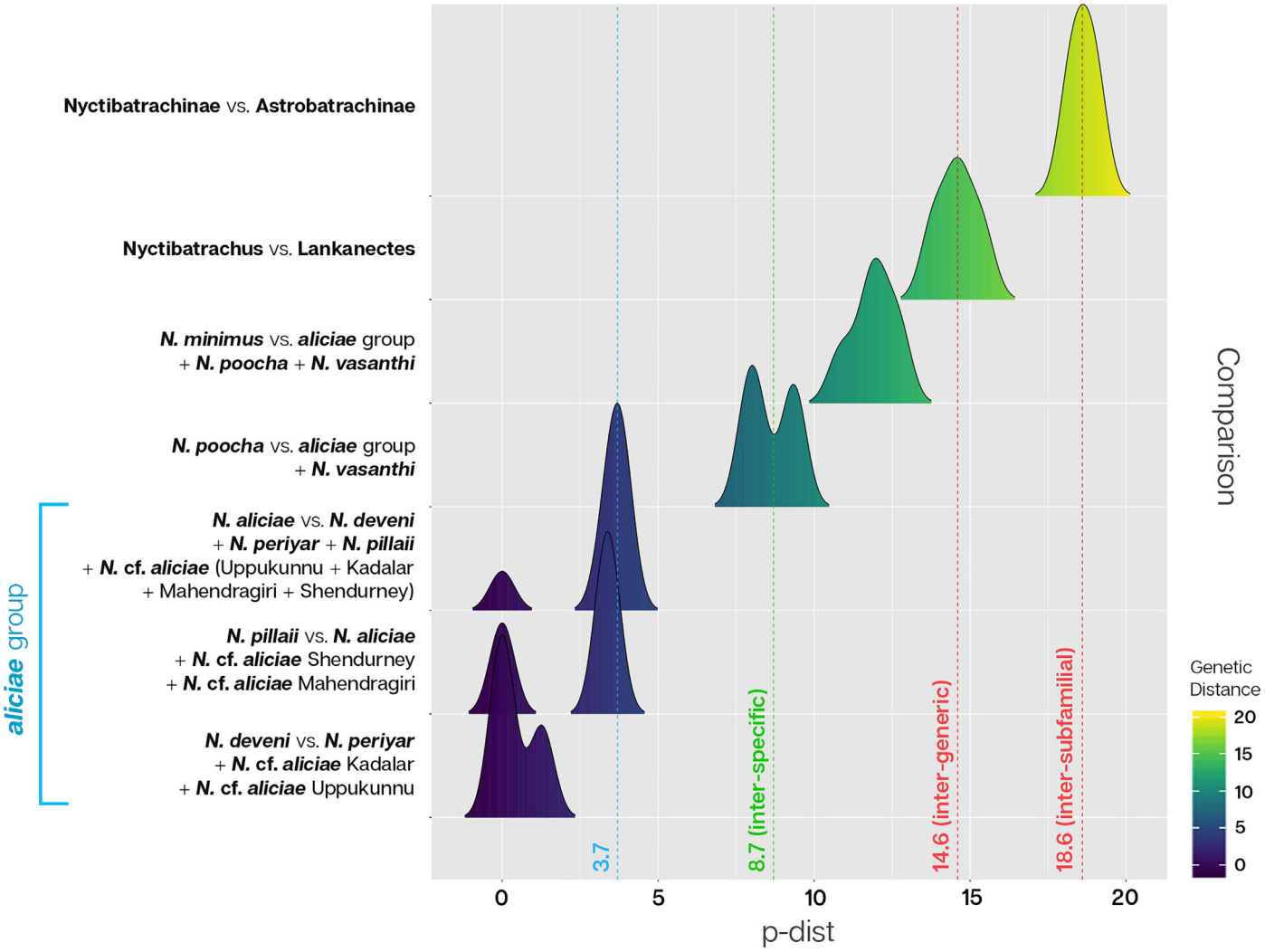

Fig. 7. Comparative uncorrected *p*-distances at the 16S rRNA gene for different taxonomic ranks in the family Nyctibatrachidae. For each particular rank comparison, the peak in each histogram represents the number of samples included, and the breadth represents range of genetic distances.

The average inter-subfamilial 16S rRNA pairwise divergence in Nyctibatrachidae (between Nyctibatrachinae and Astrobatrachinae) is 18.6% (Fig. 7). Within the subfamily Nyctibatrachinae, the average intergeneric divergence between *Nyctibatrachus* and *Lankanectes* is 14.6%. Within the *aliciae* group, *N. vasanthi*, and *N. poocha* clade, the average interspecific divergences between *N. vasanthi* and *N. poocha* is 8.3% and 8.7% among *N. poocha* + *N. vasanthi* and other samples of the *aliciae* group.

Within the *aliciae* group, the lowest interspecific pairwise divergence is 1.3% (between *N. deveni* and *N. periyar*) and the highest is 4.2% (between *N. pillaii* and *N. periyar*). The intraspecific divergence between individuals of *N. deveni* in Kadalar in the High Ranges (55 km aerial distance from the type locality) and Uppukkunu in the Cardamom Hills (680 m above sea level [ASL]; 78 km aerial distance from the type locality) is 0%, even though the type locality of *N. periyar* is also in the Cardamom Hills (815 m ASL; 44 km aerial distance from Uppukunnu); these localities include no significant shifts, perceivable breaks, or detectable gradients in habitat types, temperature, precipitation, or elevation.

Our exploration of uncorrected *p*-distances of the other two genes (TYR and ND1 [table 1 of Biju et al., 2011]) used in this study has yielded a similarly continuous range of divergences, smoothly scaling from intraspecific to "interspecific" (interpreted by Biju et al. [2011] and cited as support for taxonomic changes and new "species" descriptions [Fig. S2; see Data Accessibility]).

Molecular species delimitation.—Our mPTP analysis inferred a total of five species in the *aliciae* group (with three species), *N. vasanthi*, and *N. poocha* clade (Fig. 1C). The topology was similar to the overall concatenated ML topology (Fig. 1B), even with the inclusion of two additional 16S rRNA GenBank samples from different individuals. The first species (Sp. 1) comprises all the samples of *N. poocha*, while the second species (Sp. 2) is *N. vasanthi*. The third species identified by the analysis includes all the species of the *aliciae* group, including Biju et al.'s (2011) proposed species: *N. aliciae*, *N. periyar*, *N. pillaii*, and *N. deveni* (Fig. 1C).

Bioacoustic analysis of male mate-recognition signals.—Males of all species belonging to the *aliciae* group, *N. poocha*, and *N. vasanthi* clade have paired lateral vocal sacs (Fig. 8A, I).

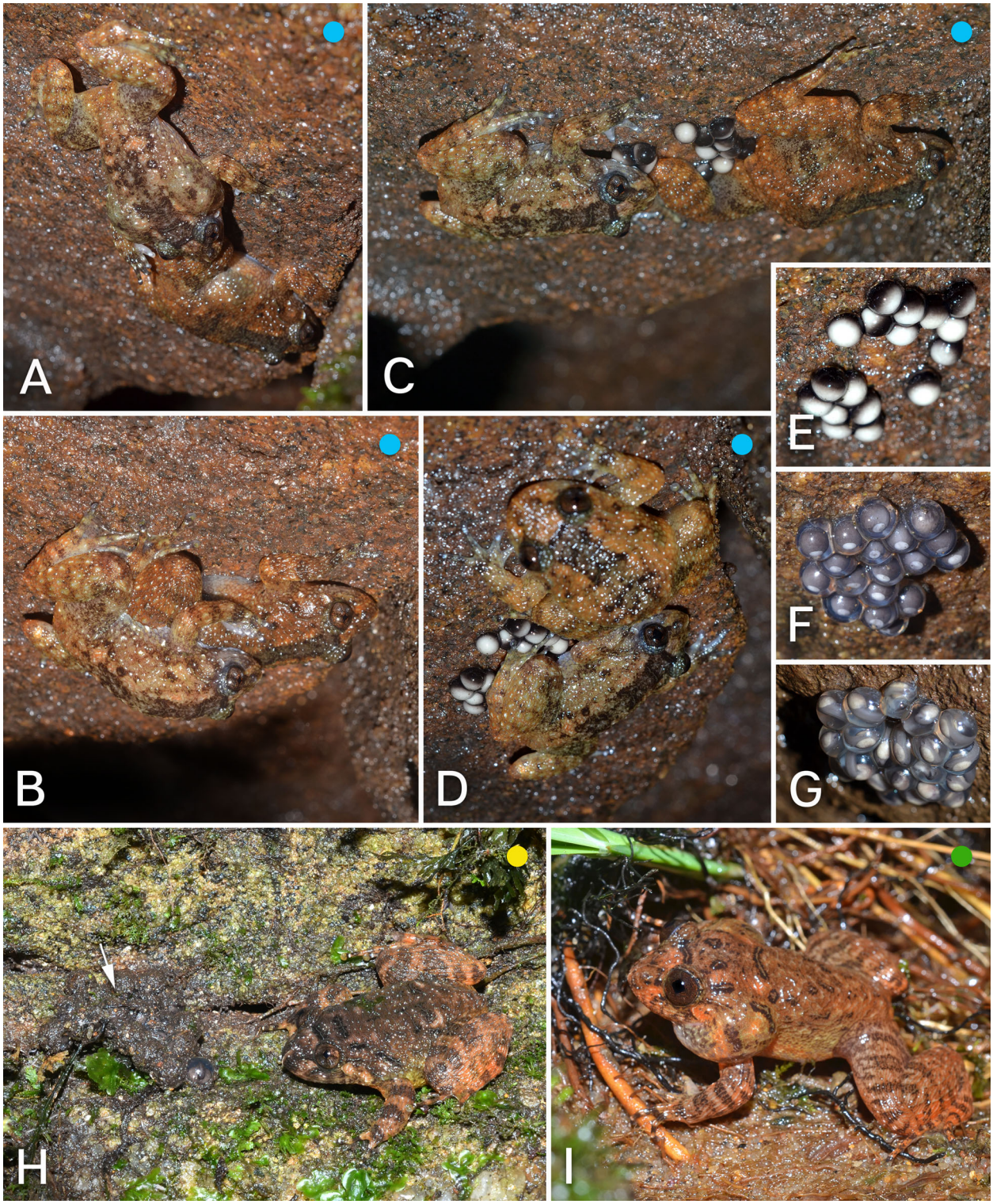

Fig. 8. Breeding behavior of *Nyctibatrachus aliciae*. (A) Male calling with female beside him; (B) male mounting female in loose amplexus; (C) male withdraws backwards off the female while she lays eggs simultaneously; (D) male sits on freshly laid clutch and rotates over it; (E) freshly laid egg clutch with exposed jelly layer, attached upside down to boulder; (F) egg clutch with embryos at Stage 10; and (G) clutch of Stage 19 embryos. Egg clutch protection in *Nyctibatrachus poocha*: (H) male guarding clutch on a mossy rock overlooking a forest stream (arrow = egg clutch covered with rotting vegetative debris); (I) vocalizing male *Nyctibatrachus vasanthi*.

Advertisement calls of all four species of the *aliciae* group are recorded from a range of elevations, and all species make the same repertoire of two call variants, which we call Type 1a and Type 1b (we call them call types even though we couldn't discern if they belong to different functional categories). In *N. aliciae* from the type locality on the slopes of Ponmudi (all calls recorded at ambient temperatures of 28.2±0.5°C), the Type 1a call consists of a rapid 'twoi' with a

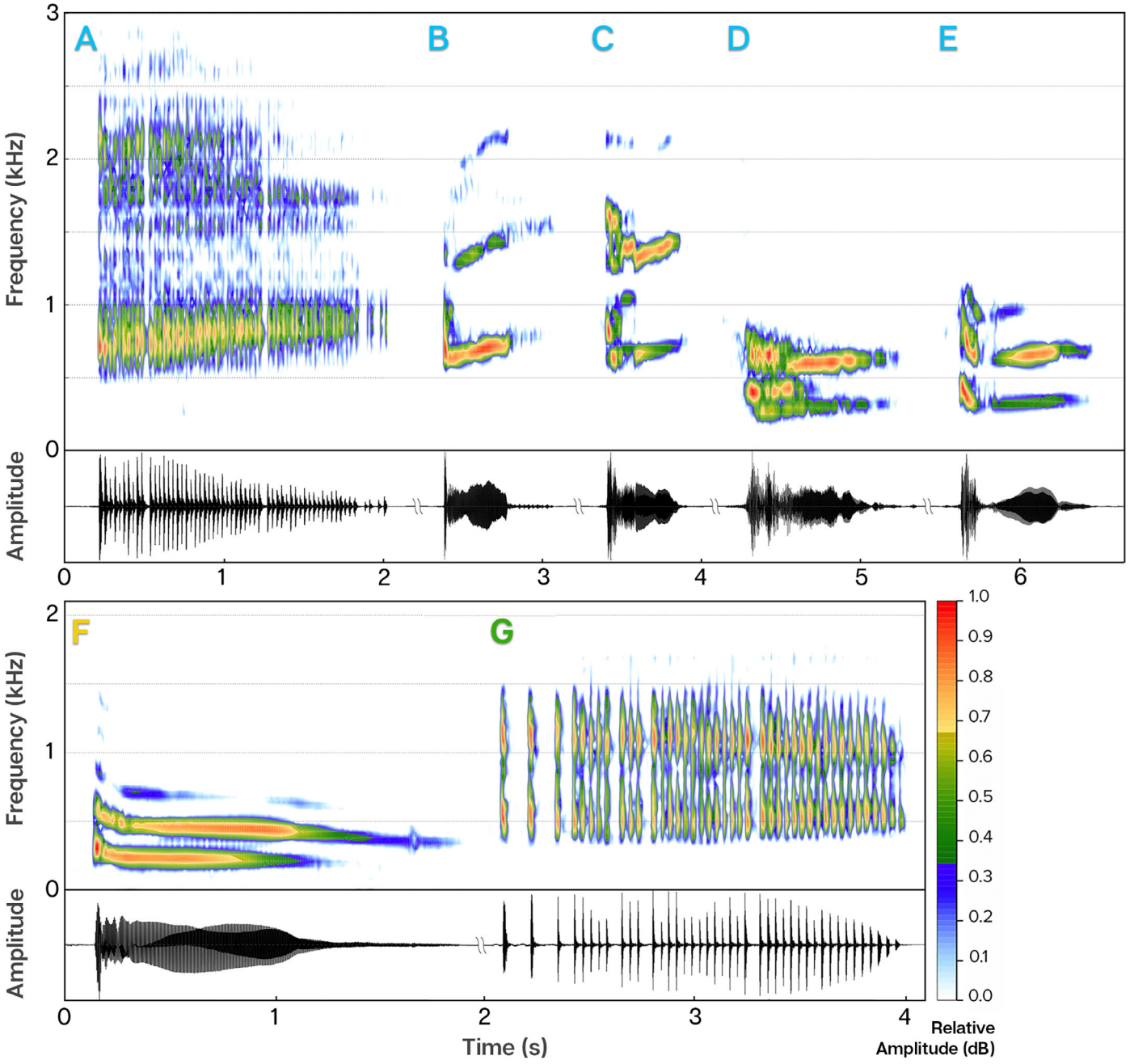

Fig. 9. Comparative spectrograms and corresponding oscillograms of advertisement calls of male *Nyctibatrachus* of the *aliciae* group, *N. poocha*, and *N. vasanthi* clade. Top: (A) call Type 1b of *N. aliciae* from Ponmudi; call Type 1a of (B) *N. aliciae* from Ponmudi; (C) *N. periyar* from Vallakadavu; (D) *N.* cf. *deveni* of Kadalar; and (E) *N. deveni* from Nelliyampathy. Bottom: advertisement call of (F) *N. poocha* and (G) *N. vasanthi*.

dominant frequency of 1,550 Hz and call duration averaging 0.31 s (mean = 0.31, range = 0.26–0.41, SD = 0.05, $n = 7$; Fig. 9B). The Type 1b call is an extended 'shirrrr,' which seems like a drawn-out variant of Type 1a, with spectral modulation and a dominant frequency of 1,378 Hz and call duration of 1.04 s ($n = 2$; Fig. 9A). The Type 1a call of *N. periyar* (recorded at 21.7°C) at the type locality of Vallakadavu is also a fast 'twoi,' with a dominant frequency of 1,550 Hz and call duration averaging 0.35 s (mean = 0.35, range = 0.15–0.33, SD = 0.05, $n = 23$; Fig. 9C), and the Type 1b call has a dominant frequency of 1,550 Hz and call duration of 0.94 s (mean = 0.94, range = 0.86–0.98, SD = 0.06, $n = 4$). For *N. deveni*, we could make recordings of only the Type 1a call of individuals, but from two sites: one at the type locality of Nelliyampathy and the other at Kadalar. The Nelliyampathy call (recorded at 25.9°C) has a dominant frequency of 1,575 Hz and call duration of 0.2 s ($n = 1$), while the Kadalar call (recorded at 21°C) has a dominant frequency of 1,525 Hz and call duration of 0.22 s (mean = 0.22, range = 0.20–0.26, SD = 0.02, $n = 9$; Fig. 9D). Type 1a calls for *N. pillaii* (recorded between 24.4±0.5°C) from Kakachi near the type locality of Sengaltheri has a dominant frequency of 3,375 Hz and call duration of 0.14 s (mean = 0.14, range = 0.13–0.16, SD = 0.01; $n = 4$; Fig. 9E).

Nyctibatrachus poocha (recorded at Kadalar at an ambient temperature of 18.5°C) produces only one type of advertisement call, a kitten-like 'paow,' with a dominant frequency of 1,875 Hz and average call duration of 0.28 s (mean = 0.28, range = 0.11–0.56, SD = 0.11; $n = 50$; Fig. 9F). The call of *N. vasanthi* (recorded both at Pandimotta in Shendurney Wildlife Sanctuary, and at the type locality in Kakachi, at an ambient temperature of 22.3±1.5°C) is typically a long 'shtrrrreeew,' with a dominant frequency of 2,437 Hz and an average call duration of 1.04 s (mean = 1.04, range = 0.89–1.25, SD = 0.15; $n = 7$; Fig. 9G).

The *aliciae* group is known to produce two different call variants, where the Type 1a call structure for *N. aliciae*, *N. periyar*, *N. deveni*, and *N. pillai*, comprising two harmonics at 1,550 Hz, is largely similar to each other albeit variation in frequency. The only call type of *N. poocha* also comprises two clearly defined harmonics, albeit at higher frequencies (1,875 Hz) than any of the unconfirmed candidate species of the *aliciae* group. To the human ear as well, the calls of the *aliciae* group and of *N. poocha* are markedly different, with the the *aliciae* group call being a 'twoi,' while the *N. poocha* call is a 'paow.' The sole call type of *N. vasanthi* is reminiscent of the drawn-out Type 1b call of the *aliciae* group, but it differs in the higher frequencies of the call (2,437 Hz vs. 1,464 Hz; $n = 2$) and shorter frequency intervals between the harmonics (Fig. 9A, G).

Elevational stratification.—We have never succeeded in documenting, nor are we aware of any records for, *Nyctibatrachus vasanthi* or *N. poocha* below 700 m ASL. Thus, both species apparently are elevationally/ecologically isolated from one another by the Shencottah Gap, which divides the Agasthyamala Range from the Nelliyampathy Hills-High Ranges-Cardamom Hills-Pandalam Hills complex. However, we observe species of the *aliciae* group across elevations of 80–1,450 m ASL, with continuous populations from Nelliyampathy in the north to Mahendragiri in the south spanning 270 km north to south, with populations even in the Shencottah Gap at 80 m ASL (Fig. 1).

Natural history and breeding behavior.—*Nyctibatrachus aliciae* is found among rocky substrates along forested streams, with males typically vocalizing upside down from the undersides of boulders; males also call from crevices and cracks along seepages. All three unconfirmed candidate species of the *aliciae* group occur in the same sets of habitats (and precisely same microhabitats) throughout the 270 km-long geographical range of this group.

We find no differences in the breeding behavior of *N. aliciae*, *N. periyar*, or *N. deveni* for which we made detailed observations. This account of breeding behavior of *N. aliciae* is made at Ponmudi at an elevation of 820 m ASL. At 2132 h on 20 June 2018, a male was making Type 1b calls continuously from under an overhanging boulder on the stream's edge, directly above the water column. The male stopped calling briefly when a female approached him. At 2137 h, he resumed making calls after the female moved towards the potential oviposition spot (Fig. 8A), stopped calling, and mounted her from her rear quarters, clasping her dorsally on her back in what is not truly inguinal amplexus. Then they remained in that posture until 2202 h, when he slowly moved his hands towards the female's pectoral region (Fig. 8B). At 2205 h, the female began depositing pigmented eggs on the roof of the boulder, soon after making a quivering gesture and with the male separating backwards from the female (Fig. 8C). At 2216 h, with oviposition of 17 eggs completed, the female rested adjacent to the clutch. At 2218 h, the male climbed onto the clutch and moved in a spinning manner over it (Fig. 8D), where the femoral glands came in contact with most of the eggs, but we are uncertain if this has any bearing on fertilization or larval development. By 2230 h, the female moved away from the vicinity, leaving the male and the egg clutch. We have observed males guarding clutches (egg jelly is always clear and never covered with any substrate) at various developmental stages (Fig. 8E, F, G) deposited as a result of amplexus with different females, at similar habitats throughout the distribution range of *N. aliciae*, *N. pillaii*, *N. periyar*, and *N. deveni*. Clutch sizes observed in different pairs ranged from 11 to 19.

We observed several male *Nyctibatrachus poocha* calling from moss-covered or bare slopes along stream banks. We noted males guarding egg clutches deposited at a height of 1.5 to 2 m from the flowing stream, the jelly layer of which was covered in either dust from decaying vegetative debris or mud depending on the oviposition site chosen (Fig. 8H). At all sites where egg clutches were observed, we noted guarding males close beside them (Fig. 8H), many of them vocalizing. Clutch sizes ranged from 6 to 17.

Our observations of *Nyctibatrachus vasanthi* reveal that the males call from the openings of either small crevices between rocks or crab tunnels on sandy banks along hill streams, unless in antagonistic interactions when they call and engage in combat in the open (Fig. 8I). We also observed sand-covered egg clutches on a dried and fallen reed of *Ochlandra* above the stream near a burrow with a vocalizing male that we suspect was guarding the clutches, but this needs to be further verified.

Taxonomic revision.—Based on the evidence provided, we place *N. deveni* syn.n. and *N. periyar* syn.n. in synonymy with *N. aliciae*; and *N. pillaii* syn.n. in synonymy with *N. vasanthi* (the holotype of *N. pillaii* [ZSI/WGRC/V/A/808] is a subadult of *N. vasanthi*).

Nyctibatrachus aliciae Inger, Shaffer, Koshy, and Bakde, 1984

Nyctibatrachus deveni Biju, Van Bocxlaer, Mahony, Dinesh, Radhakrishnan, Zachariah, Giri, and Bossuyt, 2011 syn.n.
Nyctibatrachus periyar Biju, Van Bocxlaer, Mahony, Dinesh, Radhakrishnan, Zachariah, Giri, and Bossuyt, 2011 syn.n.

Diagnosis.—*Nyctibatrachus aliciae* can be distinguished from congeners by the following combination of characters: (1) small male adult size (SVL 20.1–26.5 mm; $n = 31$), small adult female size (SVL 22.5–30.5 mm; $n = 5$); (2) skin on dorsal and lateral surfaces of the body and appendages with irregularly shaped granular projections (Fig. 2I), and elongated granular projections on dorsal surface of appendages; (3) advertisement call comprising both a fast 'twoi' call and an extended 'shirrrr' call; (4) tadpole with loosely scattered dark brown speckles throughout a uniformly paler base colored body and tail; and (5) eggs laid in small exposed clutches typically on rocky overhangs above streams.

Distribution.—Occurs from the Nelliyampathy Hills all the way to the southern parts of the Agasthyamala Range along an elevational range of 80–1,450 m ASL.

Nyctibatrachus vasanthi Ravichandran, 1997

Nyctibatrachus pillaii Biju, Van Bocxlaer, Mahony, Dinesh, Radhakrishnan, Zachariah, Giri, and Bossuyt, 2011 syn.n.

Diagnosis.—*Nyctibatrachus vasanthi* can be distinguished from known congeners by the following combination of characters: (1) small to medium male adult size (SVL 24.5–29.8 mm, $n = 6$); (2) skin on dorsal surfaces of the body and appendages with fine sinewy linear ridges running parallel to each other, and the lateral surface of the body with fine reticulated ridges (Fig. 2K); (3) advertisement call comprising a 'shtrrrreeew' call; (4) large tadpole with large black blotches over a coppery brown to pale pinkish-brown body and tail, with black vertical bars; and (5) eggs laid in clutches covered with decomposed vegetation or sand/mud typically on mossy/rocky or vegetative substrate above streams.

Distribution.—Restricted to the Agasthyamala Range along an elevational range of 800–1,700 m ASL.

DISCUSSION

Our results derived from revisiting the relationships between described species in the *Nyctibatrachus aliciae* group, *N. vasanthi*, and *N. poocha* clade, in conjunction with our natural history observations, ecological/microhabitat preferences, bioacoustics, morphometric analyses of adults and larvae, and new data from name-bearing type specimens of all proposed, but unconfirmed candidate species in this clade do not support the recognition of four distinct species within the *aliciae* group (Biju et al., 2011). We are aware of the limitations of using fragments of the mitochondrial 16S rRNA gene for species delimitation exercises which have recently been demonstrated to be less effective than full-length sequences in drawing phylogenetic and taxonomic inferences (Chan et al., 2022b). Our use of a fragment of this gene, instead of the complete sequence, for comparative analyses was to complement the sequences of the older Van Bocxlaer et al. (2012) study. Our mPTP analysis delimited only three species in the *aliciae* group, *N. vasanthi*, and *N. poocha* clade (i.e., all four proposed taxa within the *aliciae* group were lumped into a single species); along with *N. vasanthi* and *N. poocha*, we are able to reproduce support for only three putative species, albeit with moderate support. Nevertheless, the zero values associated with the *aliciae* group provide statistical evidence to the contrary—namely that branches within this clade are associated with the coalescent as opposed to speciation processes. The moderate support delimiting *N. poocha*, *N. vasanthi*, and the *aliciae* group could relate to the sampling available for *N. poocha* and *N. vasanthi*; or, alternatively, these too may require taxonomic reconsideration. Resolution of these questions, the irreproducibility and subjectivity of *N. aliciae* group taxonomy, and resolution of the suspected taxonomic inflation of this clade will require more robust statistical species delimitation analyses with genomic-level data sampled from throughout the genome (Chan et al., 2017, 2020).

Another error involves a photograph of a live specimen of *N. periyar* (fig. 44A in Biju et al. [2011]), which is wrongly attributed to the holotype. In fact, the individual in this figure with reticulated dorsal skin surface (as opposed to a granular one) unquestionably depicts a subadult *N. poocha*, based on its distinct skin texture (RA, pers. obs.; fig. 50A in Biju et al. [2011]). Finally, we also found misleading discrepancies in the tadpole descriptions by Biju et al. (2011). Although Biju et al. (2011) provided the purportedly first documentation of tadpole development (from egg to metamorph stage) of *N. aliciae*, based on our rearing of tadpoles of various species of *Nyctibatrachus* in the landscape, from embryonic to metamorphic stages (unpubl. data), we have unequivocally identified larvae in these images as *N. major* (fig. 5C in Biju et al. [2011]) and *N. beddomii* (fig. 5D, 5E, 5F, and 5G in Biju et al. [2011]) based on a combination of color patterns and size; we are convinced that the confusion resulting from these misidentification errors is to blame for the lengthy period of time that it took the community to identify these mistakes. In this paper, we have provided accurate and revised descriptions of *N. aliciae* group tadpoles (Figs. 4A, 9); descriptions of the tadpoloes of *N. major* and *N. beddomii* represent an important next step (RA, unpubl. data). Together, all of these examples demonstrate how dependence on traditional, qualitative morphology, even when combined with molecular data encompassing a handful of markers, can result in confusing and misleading taxonomy when the subjectivity of character states such as "small," "medium," and "large" are not reinforced with quantitative characterizations of variation, and phenotypic data are not critically evaluated for discrete variation—all of which are crucial for formulation of taxonomic diagnoses that are actually diagnostic (Welton et al., 2013).

Our surveys of mate-recognition vocalizations of males show limited regional variation, yet calls are overwhelmingly similar in harmonic and temporal structure (call types) for all unconfirmed candidate species throughout the range of the single cohesive taxonomic unit we refer to as the *aliciae* group. The variation may also be part of context-dependent syntax sequences as has been recently demonstrated for other frog species in the Western Ghats, including of a species of *Nyctibatrachus* in another clade (Bhat et al., 2022). However, our acoustic comparisons were also affected by limited sample sizes of calling individuals, that corresponded to specimens, due primarily to collecting restrictions (permit limitations). Thus, we cannot refute the possibility that our inability to correct for body size, upon which the dominant frequency is dependent, may be associated with variation among populations (Hoskin et al., 2009).

Finally, our natural history observations show that the different populations of the *aliciae* group are unified by life-history strategies and offspring care features such as egg clutches that are never covered up by debris, whereas *N. poocha* and *N. vasanthi* (albeit, based on a single observation of a clutch of sand-covered eggs near a vocalizing male's territory) cover their egg clutches with sand, mud, or vegetative debris. This parental care behavioral dichotomy may be a diagnostic trait that separates both these species from *N. aliciae*. Similar egg protection behavior has been noted in other clades of *Nyctibatrachus* (Gururaja et al., 2014), and our observations demonstrate that such behaviors are more widespread in the genus.

The use of genome-scale data and robust analyses are starting to reveal more cases of taxonomic inflation in Asian amphibians, particularly in groups that purportedly harbor high levels of cryptic diversity (Chan et al., 2020, 2022a). Besides genomic data, our study demonstrates that other lines of evidence based on natural history, ecology, bioacoustics, and statistically defensible morphological analyses can also be utilized to produce a more informed, comprehensive, and accurate inference of species boundaries. These robust and pluralistic approaches not only highlight the pitfalls of taxonomy that is predicated on distance-based methods and perfunctory morphological comparisons, but also alludes to the potential scale of taxonomic inflation in Asian amphibian systematics. Although high species diversity may reinforce the conservation status and perceived "importance" of a proposed biodiversity conservation hotspot, amphibian taxonomists must be conservative, hypothesis-based, data driven, and impervious to the criticism of taxonomic inflation (Conix, 2019). To be relevant to conservation biology, amphibian taxonomy must be minimally, or demonstrably, representative of biological diversity, perhaps particularly when involving the inference of microendemism limited to global biodiversity hotspots (Vieites et al., 2009; Lawson, 2013; Sukumaran and Knowles, 2017; Galtier, 2019; Chan et al., 2020).

DATA ACCESSIBILITY

Supplemental material is available at https://www.ichthyologyandherpetology.org/h2020139. Unless an alternative copyright or statement noting that a figure is reprinted from a previous source is noted in a figure caption, the published images and illustrations in this article are licensed by the American Society of Ichthyologists and Herpetologists for use if the use includes a citation to the original source (American Society of Ichthyologists and Herpetologists, the DOI of the *Ichthyology & Herpetology* article, and any individual image credits listed in the figure caption) in accordance with the Creative Commons Attribution CC BY License.

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