A little frog leaps a long way: compounded colonizations of the Indian Subcontinent discovered in the tiny Oriental frog genus *Microhyla* (Amphibia: Microhylidae)

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ABSTRACT

Frogs of the genus *Microhyla* include some of the world's smallest amphibians and represent the largest radiation of Asian microhylids, currently encompassing 50 species, distributed across the Oriental biogeographic region. The genus *Microhyla* remains one of the taxonomically most challenging groups of Asian frogs and was found to be paraphyletic with respect to large-sized fossorial *Glyphoglossus*. In this study we present a time-calibrated phylogeny for frogs in the genus *Microhyla*, and discuss taxonomy, historical biogeography, and morphological evolution of these frogs. Our updated phylogeny of the genus with nearly complete taxon sampling includes 48 nominal *Microhyla* species and several undescribed candidate species. Phylogenetic analyses of 3,207 bp of combined mtDNA and nuDNA data recovered three well-supported groups: the *Glyphoglossus* clade, Southeast Asian *Microhyla* II clade (includes *M. annectens* species group), and a diverse *Microhyla* I clade including all other species. Within the largest major clade of *Microhyla* are seven well-supported subclades that we identify as the *M. achatina*, *M. fissipes*, *M. berdmorei*, *M. superciliaris*, *M. ornata*, *M. butleri*, and *M. palmipes* species groups. The phylogenetic position of 12 poorly known *Microhyla* species is clarified for the first time.

These phylogenetic results, along with molecular clock and ancestral area analyses, show the *Microhyla—Glyphoglossus* assemblage to have originated in Southeast Asia in the middle Eocene just after the first hypothesized land connections between the Indian Plate and the Asian mainland. While *Glyphoglossus* and *Microhyla* II remained within their ancestral ranges, *Microhyla* I expanded its distribution generally east to west, colonizing and diversifying through the Cenozoic. The Indian Subcontinent was colonized by members of five *Microhyla* species groups independently, starting with the end Oligocene—early Miocene that coincides with an onset of seasonally dry climates in South Asia. Body size evolution modeling suggests that four groups of *Microhyla* have independently achieved extreme miniaturization with adult body size below 15 mm. Three of the five smallest *Microhyla* species are obligate phytotelm-breeders and we argue that their peculiar reproductive biology may be a factor involved in miniaturization. Body size increases in *Microhyla—Glyphoglossus* seem to be associated with a burrowing adaptation to seasonally dry habitats. Species delimitation analyses suggest a vast underestimation of species richness and diversity in *Microhyla* and reveal 15–33 undescribed species. We revalidate *M. nepenthicola*, synonymize *M. pulverata* with *M. marmorata*, and provide insights on taxonomic statuses of a number of poorly known species. Further integrative studies, combining evidence from phylogeny, morphology, advertisement calls, and behavior will result in a better systematic understanding of this morphologically cryptic radiation of Asian frogs.

INTRODUCTION

The tropical areas of South and Southeast Asia include biogeographic regions recognized as global centers of biodiversity (*Myers et al., 2000*; *Bain et al., 2008*; *Stuart, 2008*; *De Bruyn et al., 2014*). Understanding processes that sculpted this diversity is hampered by a highly complex geological and climatic history of this region. Combining data on tectonics, paleoclimate, and phylogenetics has proved to be a powerful instrument for examining patterns of diversification within clades and understanding processes involved in the assembly of high biodiversity in regions like South and Southeast Asia (*De Bruyn et al., 2014*).

The tectonic collision between the Indian subcontinent (ISC) and the Eurasian landmass during the Early Cenozoic is widely recognized as a key event that caused significant geologic and climatic changes, such as the rise of the Himalaya, uplift of the Tibetan plateau, and a general drying of Central Asia (*Harrison et al., 1992*; *An et al., 2001*; *Guo et al., 2002*; *Molnar, 2005*; *Solovyeva et al., 2018*). This tectonic event also induced a major biotic interchange between the ISC and Eurasia and is widely regarded as a major driver of biotic diversification (*Wilkinson et al., 2002*; *Roelants, Jiang & Bossuyt, 2004*; *Li et al., 2013*; *Garg & Biju, 2019*). Numerous studies have demonstrated that floral and faunal elements reached and colonized tropical Asia from Gondwanaland via the

Figure 1 Distribution and species richness of Oriental tiny frogs of the genus *Microhyla*. Heatmap indicates approximate number of sympatrically co-occurring species (from 1 to 9); the highest species richness is observed in southern Vietnam and Malayan Peninsula. The individual species geographic range maps were adopted from the *AmphibiaWeb (2020)* database, and modified based on expert estimations in CorelDraw Graphics Suite X8. Grey/white dashed lines mark international borders on land/water, respectively. Base Map created using simplemappr.net. Photo shows *Microhyla heymonsi*—a widespread species occurring in Southeast and East Asia (by Nikolay A. Poyarkov).

Full-size 🖼 DOI: 10.7717/peerj.9411/fig-1

world's tiniest amphibians (*Das & Haas, 2010*; *Poyarkov et al., 2014*). In miniaturized groups of amphibians, a high proportion of cryptic diversity and rampant homoplasies are often recorded, obscuring our estimates of diversity and evolutionary relationships (*Hanken & Wake, 1993*; *Rovito et al., 2013*; *Parra-Olea et al., 2016*; *Rakotoarison et al., 2017*). Molecular phylogenetic analyses, optimally combined with behavioral and acoustic data, offer the best hope for clarifying diversity, species boundaries, and evolutionary relationships in many groups of Microhylidae, including the genus *Microhyla* (*Hasan et al., 2014a*; *Garg et al., 2019*; *Poyarkov et al., 2018a*, *2019*).

Despite significant progress in our understanding of *Microhyla* diversity in recent years, hypothesizing evolutionary origins of the genus remains a challenging task. *Microhyla* is the only Asian microhylid genus with a wide distribution over South, Southeast, and East Asia (see Fig. 1), making it an ideal model for studies on Asian biogeography. Phylogenetic relationships among members of the genus *Microhyla* have been discussed in several recent studies (*Matsui et al., 2011*; *Peloso et al., 2016*; *Tu et al., 2018*; *Nguyen et al., 2019*; *Biju et al., 2019*); however, they were generally based on limited sampling (<60% of recognized diversity). Monophyly of *Microhyla* was questioned by *Matsui et al. (2011)*, based on analysis of mitochondrial DNA (mtDNA) markers, but later corroborated by multi-locus phylogenetic analyses (*Peloso et al., 2016*; *Tu et al., 2018*). Only a few works have provided insights on biogeographic origin, patterns of distribution, and possible routes of colonization for the genus (*Vineeth et al., 2018*; *Garg et al., 2019*; *Poyarkov et al.,*

2019). Though the greatest species diversity of *Microhyla* is observed in Southeast Asia (up to nine sympatric species in Indochina, see Fig. 1), some studies suggested the possibility of an Indian origin for the genus and several species groups (*Garg et al., 2019*; *Garg & Biju, 2019*). That may be explained by biased taxonomic and geographic representation of *Microhyla* species in these works, primarily focused on South Asian taxa. Extensive taxon sampling of all known members of the genus *Microhyla*, including molecularly previously unstudied taxa from Southeast Asia, a more robustly resolved phylogeny, and sound divergence age estimates are important to understand historical distribution and diversification in this radiation of Asian frogs (*Garg et al., 2019*). Thus far, however, a comprehensive phylogenetic investigation with dating estimates within the genus *Microhyla* is lacking.

Herein, we identify unrecognized diversity and examine phylogenetic relationships among almost all described species of *Microhyla* based on extensive geographic and taxonomic sampling, including 48 of 50 nominal species (96% of recognized taxa); phylogenetic information for 12 species and a number of candidate new species is reported for the first time. We use the resulting phylogeny (based on both mtDNA and nuDNA markers) to test biogeographic hypotheses in space and time and provide a scenario for *Microhyla* diversification. Our study provides the first nearly complete phylogeny for the genus *Microhyla* and links the Indian Subcontinent with Sundaland, Indo-Burma, and East Asia, thereby allowing a better understanding of biogeographic history and diversification of the group. We also evaluate miniaturization and simulate body size evolution across different lineages of the genus, providing novel insights into morphological evolution in *Microhyla*.

MATERIALS AND METHODS

Taxon sampling

We used tissues from the herpetological collections of Zoological Museum of Moscow University (ZMMU; Moscow, Russia); Zoological Institute, Russian Academy of Sciences (ZISP; St. Petersburg, Russia); Vertebrate Zoology Department, Biological Faculty, Moscow State University (ZPMSU; Moscow, Russia); Amphibian Research Center, Hiroshima University (IABHU; Higashihiroshima, Japan); Danum Valley Conservation Area, Specimen collection (RMBR; Sabah, Malaysia); Department of Fisheries, Bangamata Sheikh Fazilatunnesa Mujib Science & Technology University (DFBSFMSTU; Jamalpur, Bangladesh); School of Agriculture and Natural Resources, University of Phayao (AUP; Phayao, Thailand); and the Institute of Biodiversity and Environmental Conservation, Universiti Malaysia Sarawak (UNIMAS; Sarawak, Malaysia) (information summarized in Table S1). Permissions to conduct fieldwork and collect specimens were granted by the Institutional Ethical Committee of Animal Experimentation of University of Phayao (permit number 610104022), the Institute of Animals for Scientific Purpose Development (IAD), Bangkok, Thailand (permit number U1-01205-2558), the Sarawak Forest Department and the Sarawak Forestry Corporation (permit number JHS/NCCD/600-7/2/107(Jld2)), the Department of Wildlife Conservation of Sri Lanka (permit number WL/3/2/1/14/12), the Forest Protection Departments of the Peoples' Committee of Gia Lai

Province (permit number #530/UBND-NC), the Department of Forestry, Ministry of Agriculture and Rural Development of Vietnam (permit numbers #142/SNgV-VP, #1539/TCLN-DDPH, #1700/UBND.VX and #308/SNgV-LS).

We analyzed 122 tissue samples representing 40 nominal species of *Microhyla*, 14 species have not been included in previous phylogenetic analyses. In our analysis, we also included GenBank sequences from 78 specimens of approximately 37 nominal *Microhyla* species, 29 other Microhylidae representatives, and five non-microhylid outgroups used for rooting the phylogenetic tree and divergence times estimation (Table S1). In total, we obtained molecular genetic data for 199 samples representing 48 nominal *Microhyla* species. Geographic location of sampled populations is presented in Fig. S1.
For alcohol-preserved voucher specimens stored in museum collections, we removed a small sub-sample of muscle, preserved it in 96% ethanol, and stored samples at −70 °C.

DNA extraction, amplification and sequencing

For molecular phylogenetic analyses, total genomic DNA was extracted from ethanol-preserved femoral muscle tissue using standard phenol-chloroform-proteinase K (final concentration 1 mg/ml) extraction procedures with consequent isopropanol precipitation (protocols followed *Russell & Sambrook, 2001*).

For mtDNA, we amplified sequences covering fragments of 12S rRNA, tRNAVal, and 16S rRNA mtDNA genes to obtain an up to 2478 bp-length continuous fragment of mtDNA. The 16S rRNA gene has been widely applied in biodiversity surveys in amphibians (*Vences et al., 2005*; *Vieites et al., 2009*) and 12S rRNA + 16S rRNA data have been used in several important studies on Microhylinae phylogeny (*Matsui et al., 2011*; *Peloso et al., 2016*). These fragments have also proven to be particularly useful in taxonomic studies of the genus *Microhyla* and closely-related taxa (*Hasan et al., 2012*, *2014a*, *2014b*; *Howlader et al., 2015*; *Matsui, 2011*; *Matsui et al., 2011*; *Matsui, Hamidy & Eto, 2013*; *Wijayathilaka et al., 2016*).

For nuDNA, we amplified a 729 bp-long fragment of brain-derived neurotrophic factor gene (*BDNF*). This marker was recently successfully applied in biodiversity and phylogenetic studies of Indian *Microhyla* species (see *Garg et al., 2019*; *Garg & Biju, 2019*; *Biju et al., 2019*). Primers used in PCR and sequencing were taken from the literature or designed by us and summarized in Table S2.

The PCR conditions for amplifying mtDNA fragments included an initial denaturation step of 5 min at 94 °C, and 40 cycles of denaturation for 1 min at 94 °C, primer annealing step for 1 min with TouchDown program from 65 °C reducing 1 °C every cycle to 55 °C, and extension step for 1 min at 72 °C, and the final extension step for 5 min at 72 °C. The PCR conditions for amplifying BDNF gene followed *Van der Meijden et al. (2007)* and included an initial denaturation step of 5 min at 94 °C followed with 32 cycles of denaturation for 1 min at 94 °C, primer annealing step for 1 min at 50 °C, and extension for 1 min at 72 °C, with final extension step for 5 min at 72 °C. PCRs were run on an Bio-Rad T100™ Thermal Cycler; sequence data collection and visualization were performed on an ABI 3730xl automated sequencer (Applied Biosystems, Foster City, CA, USA). PCR purification and cycle sequencing were done commercially

through Evrogen® (Moscow, Russia). All unique sequences were deposited in GenBank (see Table S1).

Phylogenetic analyses

In addition to newly obtained sequences, we also used 107 DNA sequences of Microhylidae from GenBank in our final alignments; sequences of *Rhacophorus schlegelii*, *Alytes dickhilleni*, *A. muletensis*, *Blommersia transmarina* and *B. wittei* were selected as outgroup taxa to help root our tree and were also used for time-tree calibration. Details on taxonomy, localities, GenBank accession numbers, and associated references for all examined specimens are summarized in Table S1.

Nucleotide sequences were initially aligned in MAFFT v6 (*Katoh et al., 2002*) with default parameters, subsequently checked by eye in BioEdit v7.0.5.2 (*Hall, 1999*), and adjusted as needed.

We reconstructed phylogenetic trees with two datasets:

(1) A mtDNA dataset joining 12S rRNA and 16S rRNA for all examined samples, used for assessment of species groups and estimation of cryptic diversity within *Microhyla* (230 sequences, including 199 sequences of *Microhyla*);

(2) A concatenated mtDNA + nuDNA dataset, joining long 12S rRNA—16S rRNA mtDNA fragment and *BDNF* gene sequences for 118 selected samples representing all major lineages within *Microhyla* (as revealed by initial analysis of mtDNA), used for obtaining a more robust phylogenetic hypothesis, time-tree estimation, and ancestral range reconstruction for *Microhyla*.

The optimum partitioning schemes for alignments were identified with PartitionFinder 2.1.1 (*Lanfear et al., 2012*) using the greedy search algorithm under an AIC criterion, and are presented in Table S3. Phylogenies were hypothesized via maximum likelihood (ML) and Bayesian Inference (BI). We used IQ-TREE (*Nguyen et al., 2015*) to reconstruct ML phylogenies. Confidence in tree topology for ML analysis was assessed by 1,000 bootstrap replications for ML analysis (ML BS). Bayesian inference (BI) was performed in MrBayes v3.1.2 (*Ronquist & Huelsenbeck, 2003*) with two simultaneous runs, each with four chains for 200 million generations. We checked that the effective sample sizes (ESS) were all above 200 by exploring likelihood plots using TRACER v1.6 (*Rambaut & Drummond, 2007*). The initial 10% of trees were discarded as burn-in. Confidence in tree topology was assessed by posterior probability for Bayesian analysis (BI PP) (*Huelsenbeck & Ronquist, 2001*). We a priori regarded tree nodes with ML BS values 75% or greater and BI PP values over 0.95 as sufficiently resolved (*Huelsenbeck & Hillis, 1993*; *Felsenstein, 2004*). For clarity, ML BS values between 75% and 50% (BI PP between 0.95 and 0.90) were regarded as tendencies and below 50% (BI PP below 0.90) were considered unresolved. The allele network for the *BDNF* gene was constructed using median-joining method in the PopArt v1.5 (*Leigh & Bryant, 2015*) with 95% connection limit.

Species delimitation

We examined putative species boundaries beyond those currently recognized by taxonomists based on two different species delimitation methods: The Automatic Barcode Gap Discovery (ABGD; *Puillandre et al., 2012*) and the Generalized Mixed Yule-Coalescent model (GMYC; *Pons et al., 2006*). These methods enable the delimitation of independently-evolving species based on genetic data (*Fujita et al., 2012*; *Dellicour & Flot, 2015*; *Eyer et al., 2017*) and do not require a priori hypotheses of putative species groupings, thereby limiting potential bias in species delimitation.

The ABGD method is a single-gene approach to statistical detection of barcode gaps in a pairwise genetic distance distribution (*Puillandre et al., 2012*). Barcode gaps, presumably occurring between intra- and interspecific distances, were used to partition the 16S rRNA dataset into species hypotheses (initial partition). Resulting inferences were then recursively applied to yield finer partitions (recursive partitions) until no further partitioning was possible. ABGD analysis was run on the 16S rRNA dataset through a web-based interface (https://bioinfo.mnhn.fr/abi/public/abgd/) using default parameters (10 steps of intraspecific divergence prior from Pmin = 0.001 to Pmax = 0.10, $X = 2$).

The GMYC method is also a single-gene approach to identifying species "boundaries" associated with shifts in branching rates between intra- and interspecies branching events on a time-calibrated ultrametric tree (*Pons et al., 2006*; *Fujisawa & Barraclough, 2013*). We used a Bayesian implementation of this method (bGMYC; *Reid & Carstens, 2012*), which was applied to the 16S rRNA data. We obtained the distribution of ultrametric phylogenetic trees of 16S rRNA haplotypes with BEAST v1.8.4 (*Drummond et al., 2012*), then used 100 random phylogenetic trees as an input for subsequent bGMYC analysis. We ran bGMYC for 50,000 generations with burn-in 40,000 and a thinning parameter of 100. We summarized results of bGMYC analyses in a matrix of pairwise co-assignment probabilities for each haplotype, shown as a heatmap (not presented).

In addition, both inter- and intraspecific uncorrected genetic *p*-distances were calculated using MEGA 6.1 (*Tamura et al., 2013*).

Divergence times estimation

Molecular divergence dating was performed in BEAST v1.8.4, including the concatenated mtDNA + nuDNA dataset. We used hierarchical likelihood ratio tests in PAML v4.7 (*Yang, 2007*) to test molecular clock assumptions separately for mtDNA and nuDNA markers. Based on PAML results, we then decided to use a strict clock for the nuDNA (BDNF) and an uncorrelated lognormal relaxed clock for mtDNA. We also used these models and partitioning schemes from the ML analysis. The Yule model was set as the tree prior and we assumed a constant population size and default priors for all other parameters. We conducted two runs of 100 million generations each in BEAST v1.8.4. We also assumed parameter convergence in Tracer and discarded the first 10% of generations as burn-in. We used TreeAnnotator v1.8.0 (in BEAST) to create our maximum credibility clades.

Since we could find no paleontological data for the Microhylinae, we relied on three recently estimated calibration priors for this subfamily obtained from recent large-scale

phylogenies of microhylids (*Kurabayashi et al., 2011*), and a fossil record of Gastrophryninae (*Sanchiz, 1998*; *Holman, 2003*; *De Sá et al., 2012*) as primary calibration points. We also applied two additional calibration points widely used in divergence time estimates of Anura: maximum age of the split between *Blommersia wittei* and *B. transmarina* from the Comoro islands at 15 MYA (*Vences et al., 2003*), and the minimum age of *Alytes muletensis*—*A. dickhilleni* split at 5 MYA (*Fromhage, Vences & Veith, 2004*). Calibration points and priors are summarized in Table S4.

Ancestral area reconstruction

To infer a biogeographic history of *Microhyla*, a model-testing approach was applied using the ML tree with Lagrange (*Ree et al., 2005*; *Ree & Smith, 2008*) in RASP v3.2 (*Yu et al., 2015*). Species occurrences were categorized according to nine biogeographic areas, modified from *Turner, Hovenkamp & Van Welzen (2001)*, *Wood et al. (2012)* and *Chen et al. (2018)*, reflecting patterns of endemism in *Microhyla* (see Fig. 2A): (A) Mainland East Asia; (B) Eastern Indochina; (C) Western Indochina; (D) Indian Subcontinent; (E) Malayan Peninsula; (F) Sumatra + Java + Bali; (G) Borneo and adjacent Philippine islands; (H) Sri Lanka; and (I) East Asian islands (Taiwan + the Ryukyus) (see Supplemental Information 1 for biogeographic area definitions and references). Maximum range-size was set to three areas, as no extant species occurs in more than three biogeographical regions. Matrices of modern distributions of taxa/ area are given in Table S5. We modeled discrete state transitions (for ranges) on branches as functions of time, enabling ML estimation of where ancestral linneages' geographic areas were at the time of cladogensis. A Lagrange analysis found ancestral area(s) at a node, split areas into two distinct lineages, and calculated probabilities of most likely geographic areas for the nodes (*Ree & Smith, 2008*). Analyses used two models (*Matzke, 2013*): Langrange Dispersal-Extinction-Cladogenesis (DEC; *Ree & Smith, 2008*), and the ML version of Statistical Dispersal-Vicariance Analysis (S-DIVA; *Ronquist, 1997*). We assessed model fit using the Akaike Information Criterion (AIC) and Akaike weights.

Given the tremendous geological complexity of the region through time, we applied the following time and dispersal constraints to the analyses: we set four periods, corresponding to the main stages of gradual ISC movements northwards, formation of the first land bridges between the ISC and Southeast Asia, and final accretion between the ISC and the Asian mainland based on data from recent geologic models (based on *Hall, 2012*; *Ding et al., 2017*; *Morley, 2018*; see Fig. 3 for schematic paleogeographic maps of South and Southeast Asia from early Paleocene to the Oligocene). The following time periods were designated: (1) 100–57 MYA corresponds to complete isolation of the ISC from Eurasia, (2) 57–50 MYA marks the first assumed land connections between India and modern-day Sumatra; (3) during 50–35 MYA the ISC likely continued counter-clockwise movement northwards, forming land bridges with modern-day Indo-Burma; and (4) 35–0 MYA corresponds to the firm collision and formation of a stable land connection between the ISC and Eurasia. Transition matrices between biogeographic regions for each time period are presented in Table S6.
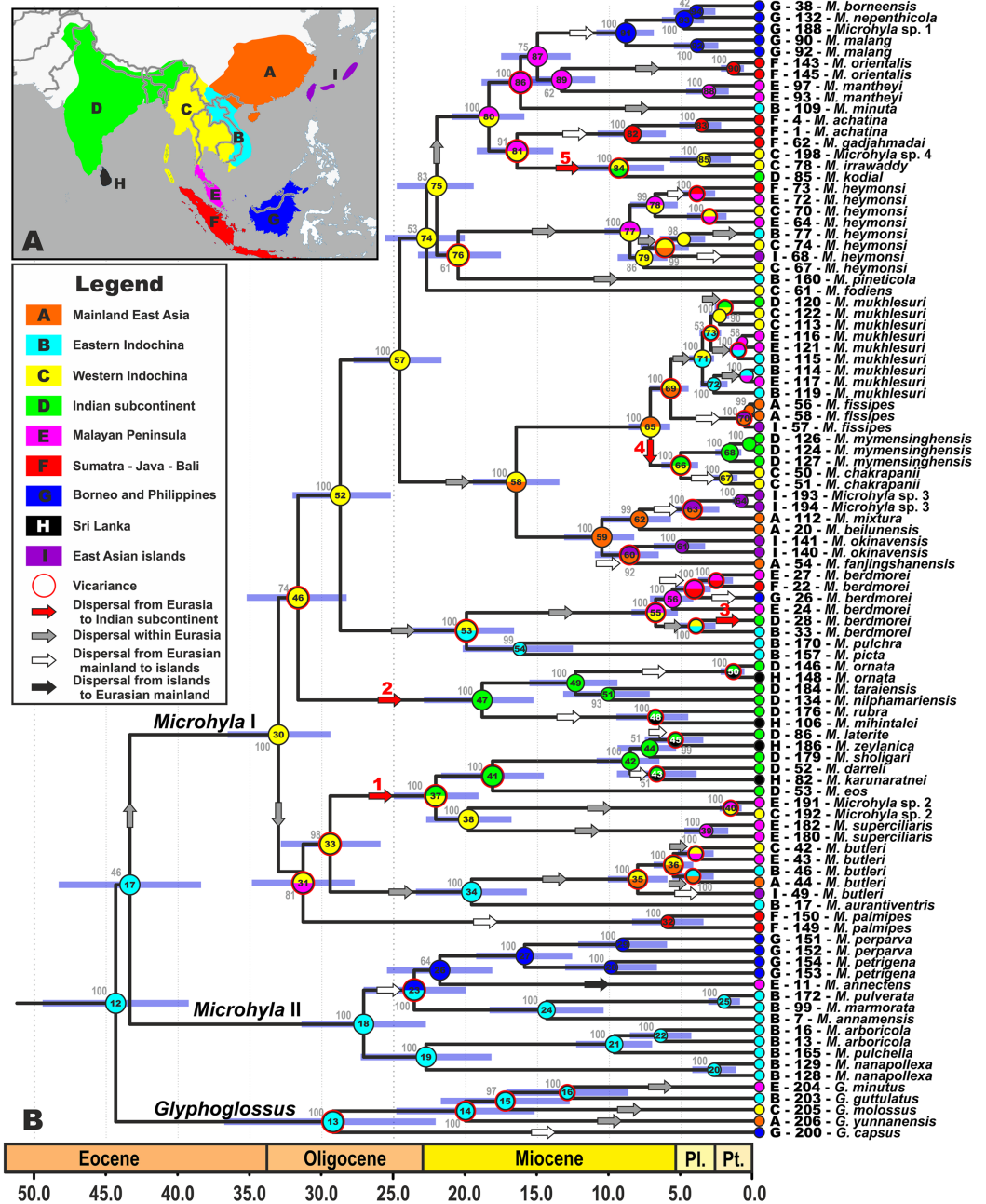
Figure 2 Biogeographic history of *Microhyla*. (A) Biogeographic regions used in the present study; (B) BEAST chronogram on the base of 3207 bp-long mtDNA + nuDNA dataset with the results of ancestral area reconstruction in RASP. For biogeographic areas definitions, species occurrence data and transition matrices see Supplemental Information 1, Tables S5 and S6. Information at tree tips corresponds to biogeographic area code (see Fig. 2A), sample number (summarized in Table S1), and species name, respectively. Node colors correspond to the respective biogeographic areas; values inside node icons correspond to node numbers (see Fig. S3 and Table S11 for divergence time estimates); values near nodes indicate marginal probabilities for ancestral ranges (S-DIVA analysis); icons illustrate vicariant and dispersal events (see Legend). Red arrows from 1 to 5 correspond to the dispersals to the Indian Subcontinent by *Microhyla* II lineages. Base Map created using simplemappr.net.

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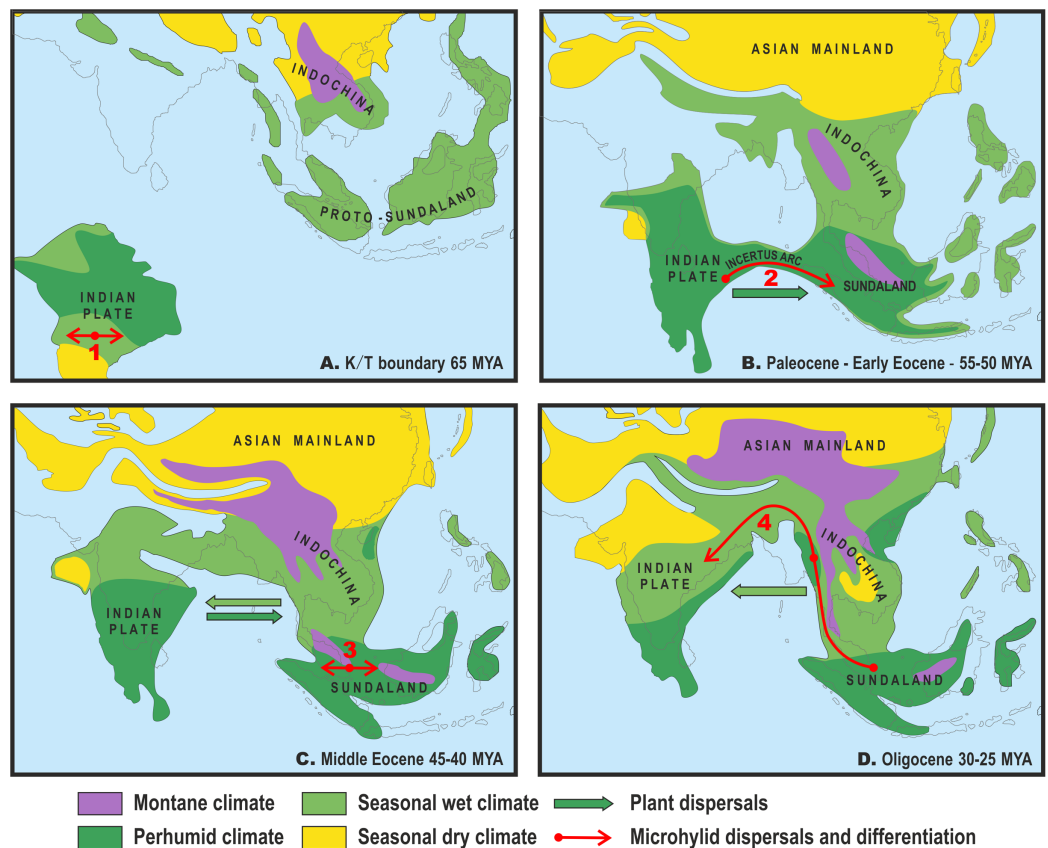
Figure 3 Paleogeography and climate of South and Southeast Asia, 60–25 Ma. Tectonic reconstructions modified from *Hall (2012)*; paleoclimate reconstructions based on *Morley (2018)*. Solid arrows indicate directions of plant dispersals (dark-green—perhumid floral elements, light-green—seasonal wet/seasonal dry elements) (from *Morley, 2018*); red arrows show probable areas of Microhylinae diversification and ways of their dispersal. (A) K/T boundary to early Paleocene: the isolated Indian subcontinent (ISC) is drifting northwards cradling perhumid tropical biota, Southeast Asia (SEA) has primarily seasonal wet or seasonal dry climate, no land connection between SEA and ISC, basal radiation of Microhylinae in the ISC; (B) Paleocene to early Eocene: the ISC and SEA are at the same latitude within same perhumid climate zone, first land connections between India and Sundaland via Incertus Arc, dispersal of Microhylinae from the ISC to SEA; (C) Middle Eocene: land connection between the ISC and mainland Southeast Asia (modern-day Myanmar), basal radiation of *Microhyla—Glyphoglossus* assemblage in SEA; (D) Oligocene: India drifts into northern high-pressure zone and seasonally dry climates predominate across the ISC and SEA, *Microhyla* II lineages colonize the ISC from SEA. Base Map created using https://www.simplemappr.net/. Full-size 🖼 DOI: 10.7717/peerj.9411/fig-3

Body size evolution

To assess body size evolution and miniaturization in *Microhyla*, we used weighted squared-change parsimony (*Maddison, 1991*) executed with Mesquite v3.31 (*Maddison & Maddison, 2017*). We compiled data on maximum snout-vent length (SVL) for both sexes for each *Microhyla* species reported in literature and/or determined from available voucher specimens (see Table S7); mensural data were taken with Mitutoyo dial caliper to the nearest 0.1 mm. SVL data for all *Microhyla* species are collated in Table S7.

RESULTS

Taxa, data, and sequence characteristics

Our aligned matrix of all mtDNA data comprised 2,478 bp, included 206 samples, representing 48 species of *Microhyla* (96% of the currently recognized species), five species (of nine currently recognized species) of the phylogenetically closely related genus *Glyphoglossus*, and 24 samples from outgroup taxa (see Table S1).

The concatenated mtDNA + nuDNA dataset comprised 3,207 bp, including 118 samples from 100 ingroup and 18 outgroup taxa. Summary information on fragment lengths and variability are collated in Table S8.

Phylogenetic relationships and species groups in *Microhyla*

Bayesian Inference and Maximum Likelihood analyses of the mtDNA-based genealogy for *Microhyla* and *Glyphoglossus* (Figs. 4 and 5; a simplified collapsed tree is shown in Fig. S2) resulted in a topology that was generally congruent with the phylogeny obtained from the concatenated mtDNA + nuDNA data, though the latter had higher support for most nodes (Fig. 6). The *BDNF* gene haplotype network resulted in the species clusters which were generally congruent with the phylogenetically and morphologically recognized groups of *Microhyla* and were separated from each other by at least four mutational steps (Fig. S3). Most of the examined *Microhyla* species showed sharing of *BDNF* haplotypes with exception of the species pairs *M. marmorata* + *M. pulverata*, *M. kodial* + *M. irrawaddy*, and *M. okinavensis* + *Microhyla* sp. 3 (Fig. S3). Overall, since the mtDNA + nuDNA phylogenetic tree was mostly better resolved and had greater support at more nodes than the mtDNA tree, we relied on the combined mtDNA + nuDNA topology for inferring phylogenetic relationships and biogeographic history of the genus *Microhyla*.

The BI- and ML-analyses of mtDNA data resulted in a majority of ingroup nodes receiving high values of both PP and BS support (Figs. 4 and 5). Observed topological patterns within the *Microhyla—Glyphoglossus* assemblage were generally congruent across analyses and agreed well with earlier phylogenies for the group (see "Discussion"), although with generally higher node support values in our study. All analyses unambiguously supported the monophyly of the *Microhyla—Glyphoglossus* assemblage; however, the basal node of this radiation was not sufficiently resolved in all analyses (Fig. 6; Fig. S2). The genus *Microhyla sensu lato* was subdivided into two major deeply divergent groups, that we identify here as *Microhyla* I and *Microhyla* II, while monophyly of the genus with respect to *Glyphoglossus* was not supported according to mtDNA data (see Fig. S2); a similar pattern was also reported in the mtDNA-based genealogy of *Matsui et al. (2011)*. Though basal divergence in the *Microhyla—Glyphoglossus* clade based on mtDNA + nuDNA data was also not strongly supported (Fig. 6), the topology suggesting monophyly of *Microhyla* I + *Microhyla* II agreed well with results of recent multilocus phylogenies for this group (*Peloso et al., 2016*; *Tu et al., 2018*).

All species of *Microhyla* I, *Microhyla* II, and *Glyphoglossus* clades were regularly grouped into one of nine well supported matrilines (Figs. 4 and 5A–5I); these same phylogenetic groupings were also revealed in the "total evidence" analysis (Fig. 6) in the

Figure 4 Updated mtDNA-genealogy of the *Microhyla—Glyphoglossus* assemblage (full tree, part 1). BI genealogy of *Microhyla* and *Glyphoglossus* samples examined in this study reconstructed from 2478 bp of mtDNA fragment. Values at nodes correspond to BI PP/ML BS, respectively; numbers at tips correspond to sample numbers summarized in Table S1. Colors and letters (A–I) correspond to species groups of the *Microhyla—Glyphoglossus* assemblage. Yellow and red bars present the results of species delimitation analyses from bGMYC and ABGD algorithms, respectively. Frog photos are given in one scale, scale bar corresponds to 10 mm, numbers near thumbnails correspond to species: (1) *Microhyla nepenthicola*; (2) *M. borneensis*; (3) *M. malang*; (4) *M. orientalis*; (5) *M. mantheyi*; (6) *M. minuta*; (7) *M. achatina*; (8) *M. irrawaddy*; (9) *M. heymonsi*; (10) *M. pineticola*; (11) *M. fodiens*; (12) *M. fissipes*; (13) *M. mukhlesuri*; (14) *M. chakrapanii*; (15) *M. okinavensis*; (16) *M. berdmorei* (Vietnam); (17) *M. berdmorei* (Malaysia); (18) *M. picta*; (19) *M. pulchra*; (20) *M. zeylanica*; (21) *M. sholigari*; (22) *M. karunaratnei*; (23) *Microhyla* sp. 2; (24) *M. ornata*; (25) *M. mihintalei*; (26) *M. butleri*; (27) *M. aurantiventris*; (28) *M. palmipes*; (29) *M. annamensis*; (30) *M. marmorata*; (31) *M. pulverata*; (32) *M. annectens*; (33) *M. petrigena*; (34) *M. perparva*; (35) *M. pulchella*; (36) *M. arboricola*; (37) *Glyphoglossus molossus*; (38) *G. guttulatus*. Photos by Nikolay A. Poyarkov, Indraneil Das, Vladislav A. Gorin, Parinya Pawangkhanant, Luan Thanh Nguyen, and Evgeniya N. Solovyeva. Full-size 🖼 DOI: 10.7717/peerj.9411/fig-4
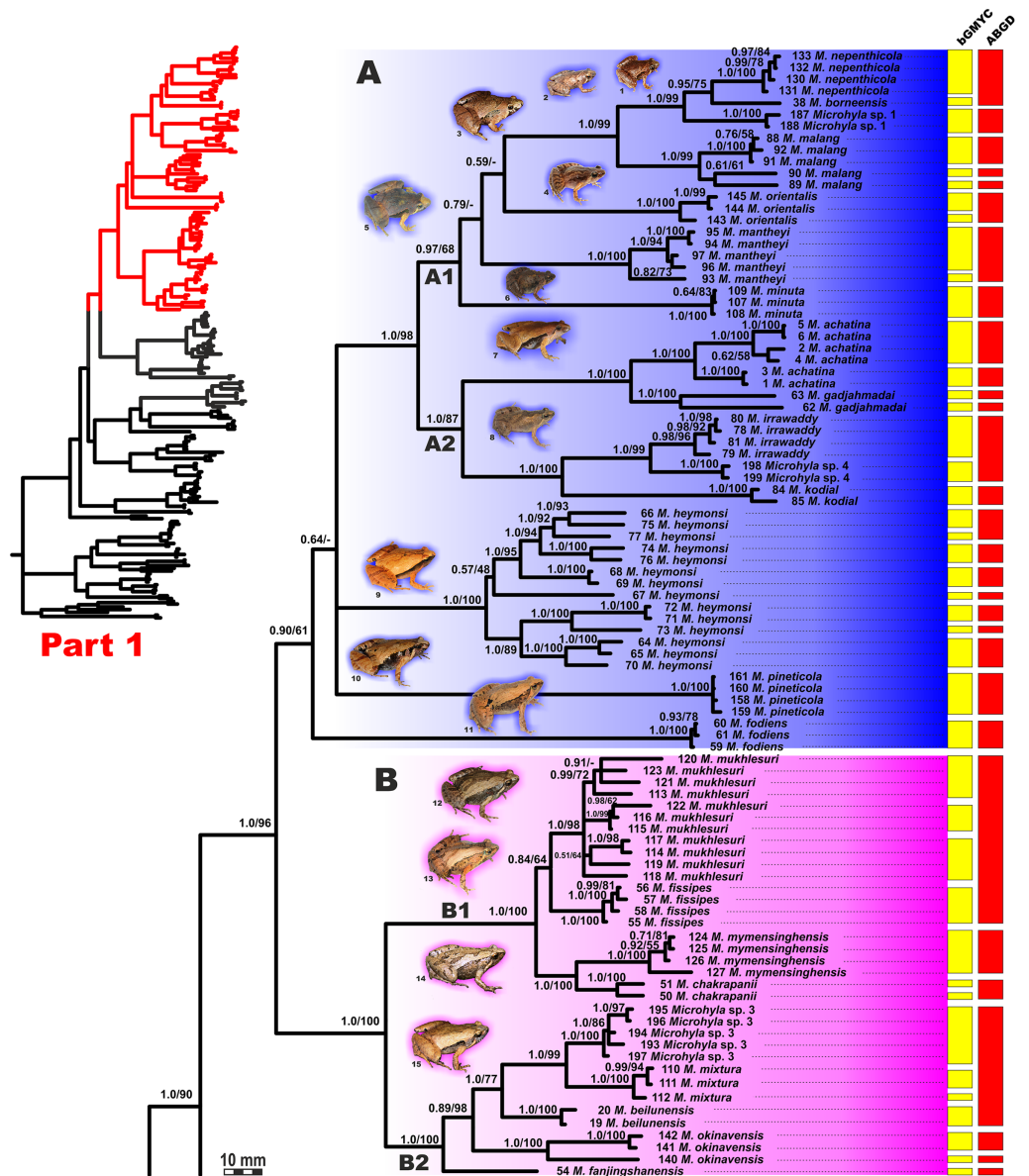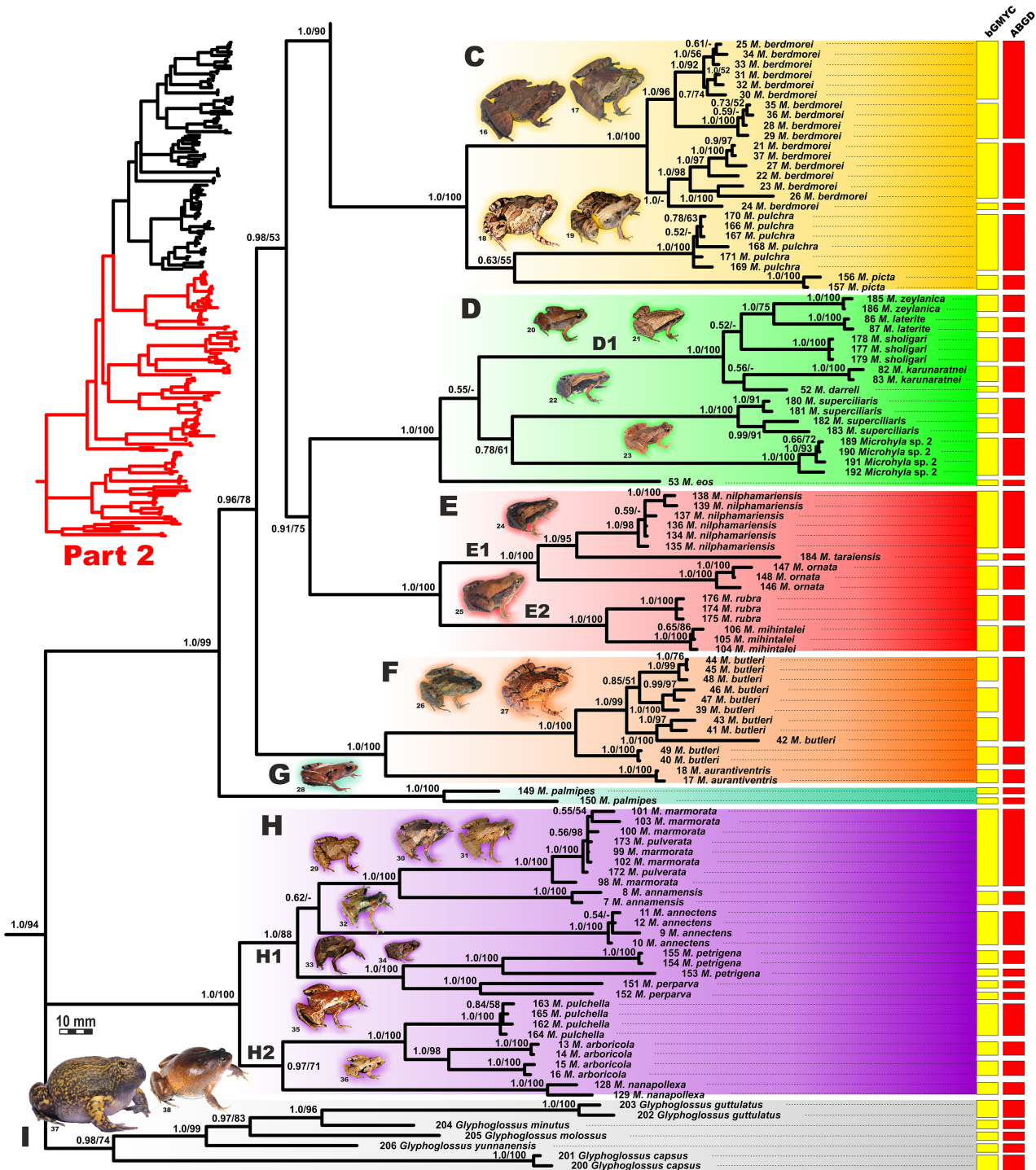
Figure 5 Updated mtDNA-genealogy of the *Microhyla—Glyphoglossus* assemblage (full tree, part 2). BI genealogy of *Microhyla* and *Glyphoglossus* samples examined in this study reconstructed from 2,478 bp of mtDNA fragment. Values at nodes correspond to BI PP/ML BS, respectively; numbers at tips correspond to sample numbers summarized in Table S1. For legend, see Fig. 4. Photos by Nikolay A. Poyarkov, Indraneil Das, Vladislav A. Gorin, Parinya Pawangkhanant, Luan Thanh Nguyen, and Evgeniya N. Solovyeva.

Full-size 🖼 DOI: 10.7717/peerj.9411/fig-5
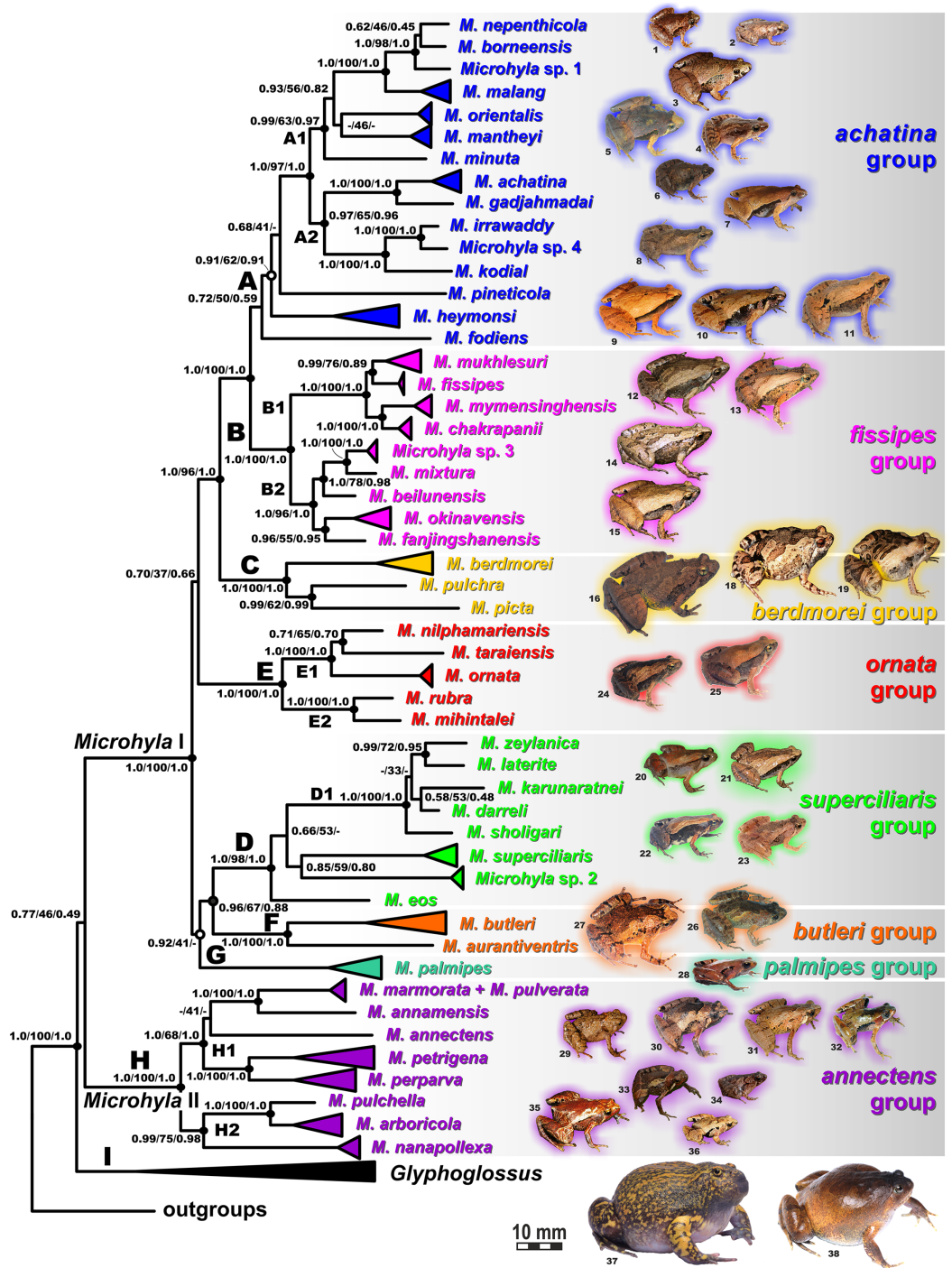
Figure 6 Maximum Likelihood tree for the "total evidence" analysis of the 3207 bp-long concatenated mtDNA + nuclear DNA dataset. Values at nodes correspond to BEAST PP/ML BS/BI PP, respectively; black and white circles correspond to well-supported (BI PP ≥ 0.95; ML BS ≥ 90) and moderately supported (0.95 > BI PP ≥ 0.90; 90 > ML BS ≥ 75) nodes, respectively; no circles indicate unsupported nodes. Colors and letters (A–I) marking the species groups in *Microhyla* species complex correspond to Figs. 4 and 5, but not to Fig. 2. Photos by Nikolay A. Poyarkov, Indraneil Das, Vladislav A. Gorin, Parinya Pawangkhanant, Luan Thanh Nguyen, and Evgeniya N. Solovyeva.

Full-size 🖼 DOI: 10.7717/peerj.9411/fig-6

BDNF haplotype network (Fig. S3) and in the most recent published phylogeny of the genus (*Biju et al., 2019*).

Microhyla I (subclade AI of *Matsui et al., 2011*) included seven major clades and 43 putative species of tiny to mid-sized terrestrial frogs (Figs. 4 and 5A–5G):

(A) Clade A corresponded to *M. achatina* species group and received only moderate levels of monophyly support in mtDNA-genealogy (0.90/61, hereafter node support values are given for BI PP/ML BS, respectively) (Fig. 4). Genealogical relationships within this group were poorly resolved. Phylogenetic positions of *M. fodiens* from central Myanmar, the *M. heymonsi* complex, and *M. pineticola* from Indochina were unresolved. Other species form a strongly supported monophyly (1.0/98), which is further subdivided into two subclades: (A1) comprising species from Sundaland (*M. borneensis*, *M. nepenthicola*, *Microhyla* sp. 1 from Sabah, *M. malang*, *M. orientalis*, *M. mantheyi*) and southern Vietnam (*M. minuta*) (0.97/68), and (A2) with species from Sundaland (*M. achatina*, *M. gadjahmadai*), Myanmar (*M. irrawaddy*, *Microhyla* sp. 4 from northern Myanmar), and southern India (*M. kodial*) (1.0/87). Monophyly of clade A was not supported in the "total evidence" analysis (0.72/50; Fig. 6), while the clade including all members of *M. achatina* species group except *M. fodiens* received moderate support (0.91/62; Fig. 6). The *M. achatina* species group occupied a more central position in the *BDNF* gene haplotype network (Fig. S3) and was generally poorly delineated, in agreement with results of *Garg et al. (2019)*.

(B) Clade B corresponded to *M. fissipes* species group (1.0/100) and consisted of two well-supported subclades (Fig. 4): (B1) included species from Indochina and southern mainland China and Taiwan (*M. fissipes*, *M. mukhlesuri*), and species from eastern India, Bangladesh and the Andaman Islands (*M. mymensinghensis*, *M. chakrapanii*) (1.0/100); (B2) encompassing species from mainland China (*M. mixtura*, *M. beilunensis*, *M. fanjingshanensis*) and the Ryukyus (*M. okinavensis*, *Microhyla* sp. 3 from Yaeyama Archipelago) (1.0/98). In the *BDNF* gene haplotype network, *M. okinavensis* and *Microhyla* sp. 3 (B2) were distantly placed from members of the *M. fissipes* species group with a minimum of 14 mutational steps (Fig. S3), also agreeing well with results of *Garg et al. (2019)*. The "total evidence" analysis suggested sister group relationships between *M. fanjingshanensis* and *M. okinavensis* (0.96/55; Fig. 6).

(C) Clade C included the *M. berdmorei* complex and *M. pulchra* from Indochina and southern China, as well as *M. picta* from southernmost Vietnam (1.0/100). Clade C was recovered as sister clade to a clade A + B (1.0/90 for mtDNA, and 1.0/96 for "total evidence" datasets, respectively; see Figs. 5 and 6); a similar topology of phylogenetic relationships was also reported by *Biju et al. (2019)*. In our *BDNF* gene haplotype network, *M. berdmorei* species complex is separated from *M. pulchra* + *M. picta* by at least eight mutational steps (Fig. S3).

(D) Clade D encompassed species from Sri Lanka (*M. zeylanica*, *M. karunaratnei*) and southern India (*M. laterite*, *M. sholigari*, *M. darreli*) (D1, see Fig. 5), but also included species from northeastern India (*M. eos*), western Thailand (*Microhyla* sp. 2 from Tenasserim) and Thai-Malay Peninsula (*M. superciliaris*). The *BDNF* haplotype

network suggests distant placement of *Microhyla* sp. 2, separated by at least seven mutational steps from other members of the *M. superciliaris* group (Fig. S3). The "total evidence" analysis strongly suggested sister group relationships between clades D and F (0.96/67; Fig. 6), in agreement with the phylogeny presented by *Biju et al. (2019)*.

(E) Clade E included species with distribution on the Indian Subcontinent and Sri Lanka, including *M. ornata* complex (E1, *M. ornata*, *M. nilphamariensis*, *M. taraiensis*) and *M. rubra* complex members (E2, *M. rubra*, *M. mihintalei*). In the *BDNF* gene haplotype networks, subclades E1 and E2 are separated from each other by at least three mutational steps (Fig. S3). MtDNA data suggested a tendency for joining the Clades D and E in a monophylum (0.91/75; Fig. 5), but it was not supported by the "total evidence" analysis, which instead placed Clade E as a sister group to the clade A + B + C, though with no support (0.70/37; Fig. 6). A similar topology was also proposed by *Biju et al. (2019)* but they also had insignificant node support (0.67/80). The monophyly of the clade joining matrilines A–E was moderately supported by mtDNA (Fig. 5), but not by the mtDNA+nuDNA dataset (Fig. 6).

(F) Clade F corresponds to *M. butleri* species group and joined the *M. butleri* complex from southern China and Southeast Asia with *M. aurantiventris* from central Vietnam (1.0/100). Clade F was strongly supported as a sister lineage with respect to Clade D based on the "total evidence" analysis (0.96/67) (Fig. 6) and the *BDNF* gene haplotype network (Fig. S3, separated by at least eight mutational steps). Monophyly of the clade joining D + E had moderate support in earlier phylogenies of the genus (0.91/81, see *Biju et al., 2019*).

(G) Clade G was represented by the *M. palmipes* species complex from Java and Sumatra; its phylogenetic position was poorly supported. In contrast to earlier data, suggesting sister relationships between *M. palmipes* and the group joining A + B + C + E clades (0.99/56; *Biju et al., 2019*), our "total evidence" analysis suggests sister group relationships of *M. palmipes* with respect to clade D + F but with moderate support (0.92/41; Fig. 6). The *BDNF* gene haplotype network also places *M. palmipes* closer to Clade D, separated by at least seven mutational steps (Fig. S3).

Microhyla II (subclade AIII of *Matsui et al. (2011)*) was represented by a single clade (H) and included nine nominal species of tiny to small-sized terrestrial or semi-arboreal frogs (Fig. 5H):

(H) Clade H corresponds to *M. annectens* species group and was further subdivided in two subclades: (H1) included species from Borneo (*M. perparva*, *M. petrigena*), the Thai-Malay Peninsula (*M. annectens*), and Annamite Mountains of Indochina (*M. annamensis*, *M. marmorata* and *M. pulverata*) (1.0/88); and (H2) joined species from central (*M. nanapollexa*) and southern parts of Annamite Mountains (*M. arboricola*, *M. pulchella*). *M. marmorata* was recovered paraphyletic, with respect to *M. pulverata* (1.0/100; Fig. 5). In the *BDNF* gene haplotype network, Clade H is separated from *Microhyla* I by at least 11 mutation steps; while H1 and H2 subclades are poorly delineated (Fig. S3).

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Finally, the large-sized fossorial frogs of the genus *Glyphoglossus* (subclade AII of *Matsui et al. (2011)*) was represented in our analysis by five of the nine recognized species; *G. capsus* from Borneo was recovered as a sister group to the clade that joined all other species from Indochina and Malay Peninsula (Fig. 5) (0.98/74). The most recent phylogenies of *Microhyla* did not include any species of *Glyphoglossus* (*Garg et al., 2019*; *Biju et al., 2019*). In our work, the genus *Glyphoglossus* is separated from *Microhyla* by at least eight mutational steps in the *BDNF* gene haplotype network (Fig. S3).

Species delimitation analyses

The BI matrilineal genealogy for the 48 nominal *Microhyla* species provided an initial assessment of species-level relationships (Figs. 4 and 5). Uncorrected genetic *p*-distances in 16S rRNA mtDNA gene within and among *Microhyla* species are given in Table S9. In addition to currently recognized taxa, our genealogy also depicted at least four lineages of *Microhyla* that likely represent unrecognized species (*Microhyla* spp. 1–4), and a number of deep lineages within species complexes (intraspecific genetic differences $p > 1.5\%$, for example, *M. malang*, *M. achatina*, *M. gadjahmadai*, *M. heymonsi*, *M. okinavensis*, *M. berdmorei*, *M. butleri*, *M. palmipes*, *M. petrigena*, *M. perparva* and *M. arboricola*; see Table S9), potentially constituting cryptic species diversity. Generally, interspecific divergences were $p > 3.0\%$, but in some cases, intraspecific divergences exceeded interspecific divergences (Table S9).

To assess the number of putative species-level lineages within the genus *Microhyla*, we implemented two alternative approaches to species delimitation via tree-based bGMYC and distance-based ABGD analyses. These methods show varying performance depending on sample and population sizes, speciation rates, and other parameters, with bGMYC showing a tendency to oversplit, while ABGD often overlumps putative species; however, when these methods agree, the resulting delimitation gains plausibility (*Dellicour & Flot, 2018*). The combined results of both bGMYC and ABGD analyses (Figs. 4 and 5; summarized in Table S10) resolved all described species of *Microhyla*, except for *M. pulverata*, which was indistinguishable from *M. marmorata*. This result is also corroborated by the mtDNA-genealogy (Fig. 5), the BDNF gene haplotype network (Fig. S3), and divergence data for 16S rRNA gene (Table S9). Both analyses suggest that species diversity within *Microhyla* is greatly underestimated: for 48 recognized *Microhyla* species included in our genealogy, the bGMYC analysis recovered 81, and ABGD recovered 63 species-level lineages (Table S10) (note that the two species missing from our analysis, *M. fusca* and *M. darevskii*, are not included in these totals). In most cases, analyses had congruent results, however in 14 species, bGMYC proposed more groups than were recovered by ABGD. Nonetheless, species delimitation analyses strongly indicate the presence of many unrecognized species-level lineages by further partitioning *M. heymonsi* (into 7–8 species), *M. bermodrei* (3–4 species), *M. malang* (3 species), *M. butleri* (2–4 species), and *M. gadjahmadai*, *M. okinavensis*, *M. palmipes*, *M. perparva*, *M. petrigena*, *M. achatina* and *M. arboricola* (each with 2 species) (Figs. 4 and 5; Table S10). For nine other taxa, the analyses gave incongruent results, with bGMYC splitting and ABGD lumping these disparate taxa (*M. beilunensis*, *M. chakrapanii*, *M. mantheyi*, *M. mixtura*,

M. mukhlesuri, *M. orientalis*, *M. superciliaris*, *Microhyla* sp. 3 and *Microhyla* sp. 4) (Table S10).

Divergence times estimation

The resulting BEAST chronogram (see Fig. S4; BEAST results for the ingroup are further detailed in Fig. 2) elucidates that the most recent common ancestor (MRCA) of *Microhyla* and *Glyphoglossus* originated between late Paleocene and early Eocene, ca. 50.8 Ma (44.1–57.0), and agrees with the analysis of *Feng et al. (2017)*, ca. 48.8 Ma (45.9–53.2), and is notably earlier than the estimate by *Garg & Biju (2019)* as 61.5 Ma (56.6–66.5). The group *Microhyla* + *Glyphoglossus* radiated within a relatively narrow time period in the middle Eocene ca. 43.8 Ma (38.7–49.1), slightly younger than estimates of *Garg & Biju (2019)*, who estimated this cladogenetic event at ca. 48.7 Ma (44.1–53.2). Diversification within the genus-level endemic radiations of *Microhyla* I, *Microhyla* II, and *Glyphoglossus* clades started in early to mid-Oligocene (from 35 to 25 Ma), generally agreeing with *Garg & Biju (2019)*. Estimated node-ages and the 95% highest posterior density (95% HPD) for the main nodes are summarized in detail in Table S11.

Historical biogeography

We were able to reconstruct biogeographic processes (vicariance, dispersal, and colonization routes) and ancestral areas (Fig. 2) for the genus *Microhyla* using the RASP biogeographic analysis. According to both the DEC and the S-DIVA model, the MRCA of *Microhyla* + *Glyphoglossus* (node 12, Fig. 2) most likely inhabited Eastern Indochina. Eastern Indochina was also reconstructed as an ancestral range for *Microhyla* II and *Glyphoglossus* lineages (nodes 18 and 12, respectively, Fig. 2), and *Microhyla* I likely originated in western Indochina (node 30, Fig. 2). *Microhyla* II expanded its range to Borneo and the Malay Peninsula, but *Microhyla* I dispersed more widely to all biogeographic regions within the modern range of the genus, including at least five independent dispersal events from western Indochina to the Indian Subcontinent (Fig. 2). Results of our analyses suggest numerous cases of dispersal from the Asian mainland to islands including Sundaland, but only a single case of reverse dispersal (from Borneo to the Malay Peninsula, see Fig. 2).

Body size evolution modeling

Reconstructions of maximum SVL ancestral states and their evolution in *Microhyla* and *Glyphoglossus* are shown in Fig. 7. Maximum adult SVL differed substantially for males and females, so data were analyzed separately for each sex. *Glyphoglossus* (adult male SVL 30.0–95.0 mm) are generally larger compared to *Microhyla* (adult male SVL 10.6–35.0 mm); the MRCA of the *Microhyla* + *Glyphoglossus* assemblage is reconstructed as a mid-sized frog (25.0–30.0 mm SVL for males, 30.0–35.0 mm for females). Most species of *Microhyla* were found to be smaller (male SVL roughly 11.5–25.0 mm) than their common ancestor, with five cases of miniaturization (male SVL 11.5–15.0 mm). However, both *Glyphoglossus* and *M. berdmorei* species groups of *Microhyla* I have subsequently increased their body size (up to 105 mm in *Glyphoglossus molossus*, up to 35 mm in *M. berdmorei*) (Fig. 7).
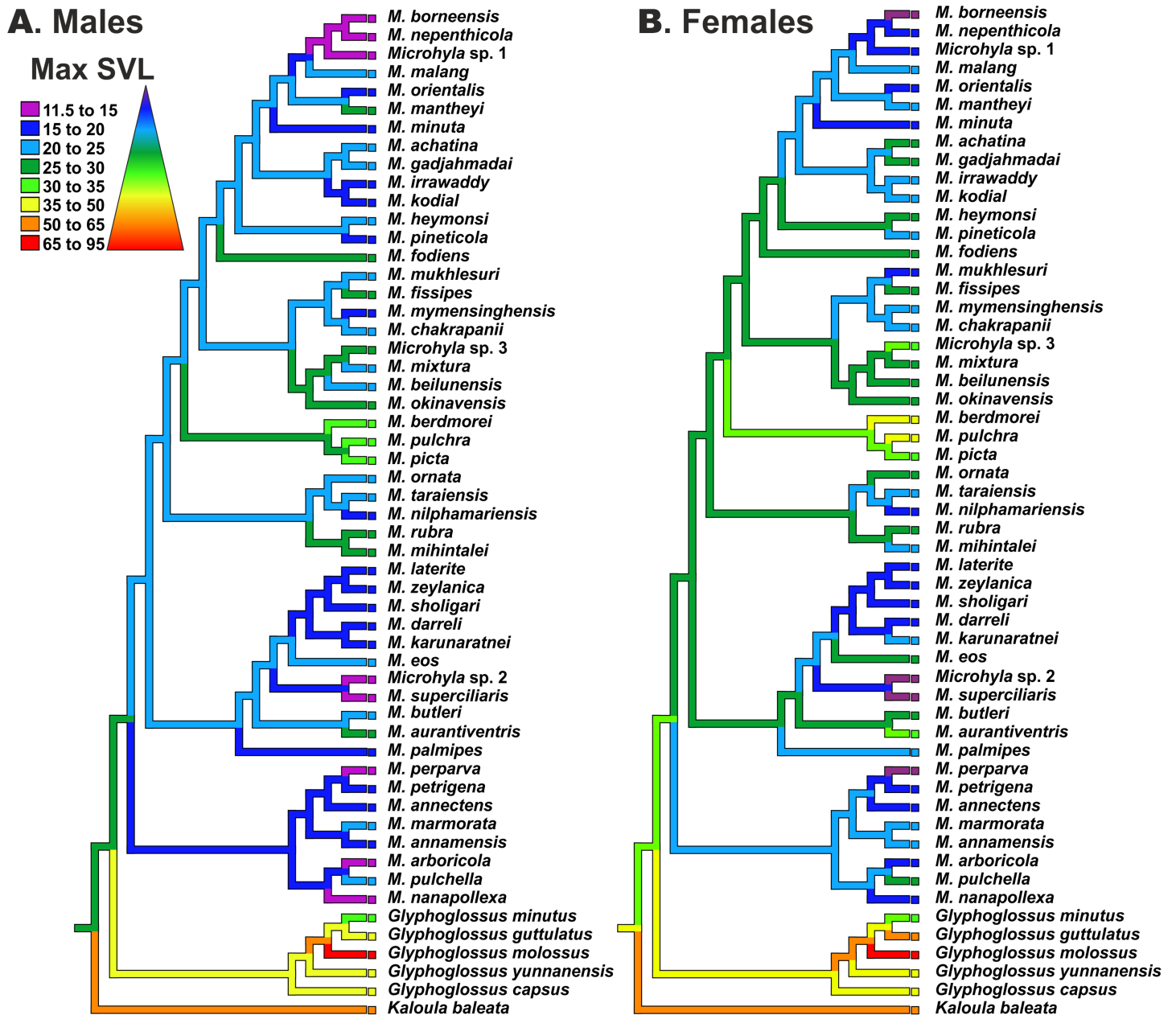
Figure 7 Body size evolution among members of the *Microhyla—Glyphoglossus* assemblage. See Table S7 for SVL data. Color of branches corresponds to average SVL in males (A) and females (B) (in mm). Full-size 🖼 DOI: 10.7717/peerj.9411/fig-7

DISCUSSION

Updated phylogenetic relationships of *Microhyla*

In phylogenetic systematics, extensive taxon sampling increases accuracy and support of evolutionary relationships (*Zwickl & Hillis, 2002*). Herein, we present an updated phylogenetic study of the genus *Microhyla*, with the most complete taxon sampling including 48 of the 50 currently recognized species. The absent taxa in our study are *M. fusca* and *M. darevskii*—two enigmatic species from central and southern Vietnam.

Microhyla fusca was described from a single specimen collected from southern Vietnam (*Andersson, 1942*); no additional specimens of this species were reported after its discovery despite numerous field survey efforts. *Microhyla darevskii* was described from a series of formalin-fixed specimens and morphologically resembles members of *M. berdmorei* species complex (*Poyarkov et al., 2014*); our repeated attempts to get DNA data from the type series of *M. darevskii* were not successful. Further studies of museum specimens and increased field survey efforts are required to clarify the taxonomic status and phylogenetic affinities of *M. fusca* and *M. darevskii*. We also did not sample *Microhyla maculifera*, a small-sized species described from Danum Valley in Sabah, Borneo (*Inger, 1989*); it might correspond to *Microhyla* sp. 1 in our analysis, collected from its type locality. Unfortunately, specimens of *Microhyla* sp. 1 included in our phylogenetic analyses were not available for morphological examination; we thus hesitate to identify this population as *M. maculifera* pending further morphological study.

The first phylogenetic study of Asian microhylids by *Matsui et al. (2011)* demonstrated paraphyly of *Microhyla* with respect to *Glyphoglossus* (at that time including *Calluella*). Subsequent multilocus phylogenetic studies (*Peloso et al., 2016*; *Tu et al., 2018*; *Garg & Biju, 2019*) strongly suggested sister group relationships between *Glyphoglossus* and *Microhyla sensu lato*, but still recognized the presence of two deeply divergent lineages within *Microhyla*. Our time-tree suggests that divergence between the two major clades of *Microhyla* I and *Microhyla* II happened soon after the basal split within the *Microhyla* + *Glyphoglossus* assemblage during the middle Eocene and similar divergence time estimates were obtained by a recent analysis by *Garg & Biju (2019)*. Robust phylogenies coupled with examination of external morphological and osteological characters are required to assess the evolutionary differences among the three subclades of the *Microhyla* + *Glyphoglossus* assemblage, likely warranting recognition as three distinct genera.

The first classifications of the genus *Microhyla* into species groups were based exclusively on morphological characters (*Parker, 1934*; *Dubois, 1987*; *Fei et al., 2005*) but have not been supported by more recent molecular data (*Matsui et al., 2011*; *Garg et al., 2019*). *Matsui et al. (2011)* assessed genealogical relationships among 20 *Microhyla* species and proposed recognition of five distinct species groups within the genus. Recently *Garg et al. (2019)* proposed a new scheme for grouping *Microhyla* species and recognized six species groups based on more extensive sampling of 33 species. Our phylogenetic hypothesis mostly agrees with these earlier proposed phylogenies of the genus (*Matsui et al., 2005*, *2011*; *Matsui, Hamidy & Eto, 2013*; *Matsui, 2011*; *Hasan et al., 2012*, *2014a*; *Howlader et al., 2015*; *Peloso et al., 2016*; *Wijayathilaka et al., 2016*; *Seshadri et al., 2016*; *Yuan et al., 2016*; *Khatiwada et al., 2017*; *Tu et al., 2018*; *Garg et al., 2019*; *Nguyen et al., 2019*; *Poyarkov et al., 2019*; *Biju et al., 2019*), and a much more extensive taxon sampling allows us to revise species groups more accurately in *Microhyla* (Fig. 6).

The *Microhyla achatina* group (Clade A, Figs. 4 and 6; part of group AId2 of *Matsui et al. (2011)*) includes species mostly from Southeast Asia (*M. achatina*, *M. gadjahmadai*, *M. heymonsi*, *M. pineticola*, *M. minuta*, *M. mantheyi*, *M. orientalis*, *M. malang*, *M. nepenthicola*, *M. borneensis*, *Microhyla* sp. 1), but also from Myanmar (*M. irrawaddy*, *M. fodiens*, *Microhyla* sp. 4) and southern India (*M. kodial*). This group includes tiny (male SVL 10.6 mm,

Das & Haas, 2010) to mid-sized (male SVL 29.1 mm, *Poyarkov et al., 2019*) frogs with lateral nostrils; dorsal skin shagreened to prominently granular skin; small disks on digits usually present and typically bearing terminal grooves; toe webbing rudimentary or absent; inner metatarsal tubercle rounded or oval-shaped, outer metatarsal tubercle rounded and small, or large, shovel-shaped (in *M. fodiens*, *Poyarkov et al., 2019*); usually with a mid-dorsal line or skinfold and a light streak extending from posterior corner of eye to axilla (*Garg et al., 2019*). The phylogenetic position of *M. minuta*, *M. pineticola*, and *M. borneensis sensu stricto* was assessed for the first time. Vietnamese *M. minuta* was recovered as a member of subgroup A1, joining species from Peninsular Malaysia (*M. mantheyi*), Java + Bali (*M. orientalis*), and Borneo (*M. malang*, *M. nepenthicola*, *M. borneensis*, *Microhyla* sp. 1). Our study also supports previously reported placement of south Indian *M. kodial* in one clade with Myanmar species *M. irrawaddy* and *M.* sp. 4 (*Poyarkov et al., 2019*); this clade is sister to the *M. achatina* + *M. gadjahmadai* clade from Java and Sumatra (subgroup A2). Unfortunately, phylogenetic positions of the *M. heymonsi* complex and *M. pineticola* remain unresolved. The morphologically different semi-fossorial *M. fodiens* from central Myanmar, previously identified as *M. rubra* (*Wogan et al., 2008*; *Peloso et al., 2016*), or *M.* cf. *berdmorei* (*Garg et al., 2019*), clearly belongs to this group and forms a highly divergent lineage (*Poyarkov et al., 2019*).

The *Microhyla fissipes* group (Clade B, Figs. 4 and 6; part of group AId2 of *Matsui et al. (2011)*) joins species from East Asia (*M. fissipes*, *M. mixtura*, *M. beilunensis*, *M. okinavensis*, *M. fanjingshanensis* and *Microhyla* sp. 3), Indochina, and eastern India (*M. mukhlesuri*, *M. mymensinghensis*, *M. chakrapanii*). We also agree with *Garg et al. (2019)* in recognizing this species group as distinct from the *M. achatina* group, though monophyly of the latter is only moderately supported (Fig. 4). Members of this group are in general morphologically similar to *M. achatina* species group, but can be distinguished from the latter by finger tips lacking disks, toe tips rounded or bearing tiny disks lacking terminal grooves; by inner metatarsal tubercle elongated, outer metatarsal tubercle small, rounded; and by a dark band running from canthus rostralis posteriorly towards groin and posterior parts of belly (*Garg et al., 2019*). Our phylogeny recovered two well-supported subgroups within the *M. fissipes* group corresponding to Indochinese (B1) and East Asian (B2) taxa (Fig. 6) (*Yuan et al., 2016*), supported sister relationships of *M. chakrapanii* and *M. mymensinghensis* (*Garg et al., 2019*), and suggested sister group relationships of *M. fanjingshanensis* and *M. okinavensis* (Fig. 6).

The *Microhyla berdmorei* group (Clade C, Figs. 5 and 6; group AId1 of *Matsui et al. (2011)*) encompasses the largest *Microhyla* species and, according to our data, includes the wide-ranging *M. berdmorei* complex (SVL up to 45.6 mm; Southeast Asia), *M. pulchra* (SVL up to 36.5 mm; Indochina and southern China), and stout-bodied semi-fossorial *M. picta* from southern Vietnam (SVL up to 33.4 mm). The phylogenetic position of *M. picta* was investigated here for the first time, and was recovered as sister taxon to *M. pulchra*. Members of the *M. berdmorei* species group exhibit considerable morphological differentiation: smooth to sparsely granular skin on dorsum; finger tips rounded, toe tips rounded or bearing tiny disks with or without terminal grooves; toe webbing from rudimentary to complete reaching toe disks; inner metatarsal tubercle oval, outer

metatarsal tubercle from small to large, shovel-shaped (in *M. picta*); lacking mid-dorsal line or skinfold; with a light streak extending from posterior corner of eye to axilla; and a characteristic bright-yellow coloration of the groin and posterior parts of the belly. Based only on morphological characteristics, the unsampled *M. darevskii* (absent in our phylogeny), likely belongs to this species group.

The *Microhyla superciliaris* group (Clade D; part of group AIc of *Matsui et al. (2011)*) joins small-sized species from South and Southeast Asia, and includes a well-supported clade of southern Indian and Sri Lankan species (Figs. 5 and 6, D1, *M. zeylanica*, *M. laterite*, *M. sholigari*, *M. karunaratnei* and *M. darreli*; *M. zeylanica* group of *Garg et al. (2019)*), three species from northeastern India (*M. eos*), western Thailand (*Microhyla* sp. 2) and the Malay Peninsula (*M. superciliaris*). Morphologically members of this group are small-sized (male SVL under 21.5 mm), have dorsal orientation of nostrils; smooth to granular skin on dorsum; finger disks rounded or with weak disks lacking terminal grooves; toe disks having terminal grooves; inner metatarsal tubercle oval-shaped, outer metatarsal tubercle small and rounded; toe webbing reduced or well-developed; mid-dorsal skinfold generally present; with a light streak from posterior eye corner to axilla; and often with contrasting black and white blotches on belly. *Garg et al. (2019)* proposed recognizing *M. zeylanica* species group for taxa inhabiting peninsular India + Sri Lanka, but *Biju et al. (2019)* reported *M. eos* from northeastern India as a sister lineage of this clade, refraining from assigning this species to any species group. Herein, we propose recognizing the *M. superciliaris* species group for taxa inhabiting Southeast Asia, northeastern and southern India, and Sri Lanka.

The *Microhyla ornata* group (Clade E; group AIb of *Matsui et al. (2011)*) is comprised of species exclusively occurring in the Indian Subcontinent, subdivided into two groups, E1 (*M. ornata*, *M. nilphamariensis*, and *M. taraiensis*; corresponds to *M. ornata* group of *Garg et al. (2019)*), and E2 (*M. rubra*, *M. mihintalei*; corresponds to *M. rubra* group of *Garg et al. (2019)*) (Figs. 5 and 6). Morphologically *M. ornata* group includes small to mid-sized species with lateral nostrils; shagreened to granular dorsal skin; tips of digits lacking disks and terminal grooves; toe webbing rudimentary; inner and outer metatarsal tubercles present, latter may be enlarged; middorsal line or skinfold present; body flanks with dark band from nostrils to groin; and a light streak from posterior eye corner to axilla. Though *Garg et al. (2019)* proposed recognizing the more robust, semi-fossorial species *M. rubra* and *M. mihintalei* as a distinct *M. rubra* species group, we do not follow their scheme since phylogenetic relationships within Clade E are well resolved and subclades E1 and E2 are closely related (*Matsui et al., 2011*). In addition, adaptations to burrowing lifestyle are not unique for *M. rubra* but are found in other lineages of *Microhyla* as well, such as *M. fodiens* of Clade A, and *M. picta* of Clade C.

The *Microhyla butleri* group (Clade F; part of group AIc of *Matsui et al. (2011)*; see Figs. 5 and 6) includes the *M. butleri* complex from Southeast Asia and southern China and the closely related *M. aurantiventris* from central Vietnam. Morphologically members of this group show a dorsolateral nostril position; prominently granular dorsal skin; presence of weak disks on digits bearing terminal grooves; moderately developed webbing on toes; inner and outer metatarsal tubercles small; middorsal line or skinfold present;

characteristic "teddy-bear"-shaped dark marking on dorsum edged with light color; body flanks lacking dark band from nostrils to groin; and a light eye-axilla streak present (*Nguyen et al., 2019*).

The *Microhyla palmipes* group (Clade G; group AIa of *Matsui et al. (2011)*) presently includes a single species, *M. palmipes*, from Java, Sumatra, and adjacent offshore islands in Indonesia, and according to our data, likely represents a species complex (see below). Morphological data on *M. palmipes* are scarce; they are small-sized frogs (male SVL 16.0 mm) with lateral nostrils; shagreened skin on dorsum; weak disks on digits lacking terminal grooves; moderately developed webbing on toes; inner and outer metatarsal tubercles small; middorsal line absent; dark markings on flanks and a light eye-axilla streak present (*Bain & Nguyen, 2004*; *Poyarkov et al., 2014*).

Finally, the *M. annectens* group (Clade H of *Microhyla* II; group AIII of *Matsui et al. (2011)*; Figs. 5 and 6) encompasses tiny (male SVL 13.2 mm) to mid-sized (male SVL to 21.6 mm) frogs from Southeast Asia, and according to our phylogeny, is subdivided into two subgroups: H1 comprising species from Malayan Peninsula (*M. annectens*), Annamite Mountains in Vietnam (*M. annamensis*, *M. marmorata*, and *M. pulverata*), and Borneo (*M. petrigena* and *M. perparva*); and H2 including species from mountains of central and southern Vietnam (*M. arboricola*, *M. pulchella*, *M. nanapollexa*). *M. marmorata* was found to be paraphyletic with respect to *M. pulverata*. Morphologically, *M. annectens* group members are characterized by a relatively short body; lateral position of nostrils; sparsely granular to tubercular dorsal skin; complete toe webbing with well-developed, flattened, and slightly expanded disks on digits bearing terminal grooves; inner metatarsal tubercle present, and outer metatarsal tubercle present or absent. Further morphological studies are required to examine morphological differentiation among *M. annectens* group members. Phylogenetic positions of *M. annamensis*, *M. marmorata*, *M. pulverata*, *M. arboricola*, and *M. pulchella* are for the first time reported in the present study.

Our updated phylogeny reveals several lineages of *Microhyla* that likely represent undescribed species: *Microhyla* sp. 1 from Sabah, Malaysia (corresponds to *Microhyla* sp. 1 of *Matsui et al. (2011)*), *Microhyla* sp. 2 from western Thailand (previously not reported), *Microhyla* sp. 3 from Yaeyama Archipelago (previously referred to as *M. okinavensis*), and *Microhyla* sp. 4 from northern Myanmar (reported as *Microhyla* sp. A by *Mulcahy et al. (2018)*). Our study also recovered significant diversity within wide-ranging species complexes that might comprise undescribed cryptic species ($N = 31$). This suggests that the taxonomy of the genus *Microhyla* still remains largely incomplete.

Indian Collision and historical biogeography of *Microhyla*

The origin of Asian microhylids, including the subfamily Microhylinae, is connected with a break-up of Gondwana and the Indian Collision (*Van Bocxlaer et al., 2006*; *Van der Meijden et al., 2007*). Most likely, ancestors of Asian Microhylidae subfamilies diverged and diversified on the Indian Plate during its long isolation and northward drifting in the late Cretaceous and Paleocene (*Bossuyt & Milinkovitch, 2001*; *Kurabayashi et al., 2011*; *De Sá et al., 2012*) (see Fig. 3A). The basal divergence of the subfamily Microhylinae most likely took place on the Indian Plate prior to its first contact with Eurasia and the ISC is

regarded as the original source of Microhylinae diversity (*Garg & Biju, 2019*). However, Southeast Asia (not the ISC) presently harbors the largest number of Microhylinae lineages and species (*Frost, 2020*).

Several recent biogeographic studies suggested that collision of the ISC with the Asian mainland was a more complicated process than previously conceived, implicating early opportunities for faunal exchange between the ISC and present-day Southeast Asia (*Klaus et al., 2010*; *Li et al., 2013*; *Grismer et al., 2016*). The "Out of India" scenario, with early dispersal from the ISC to Sundaland via brief land connection in early Eocene, has also been proposed for the Microhylinae (*Garg & Biju, 2019*). Recent progress in tectonic plate modeling further corroborates the possibility for biotic exchange between the ISC and Sundaland via the Incertus Arc land bridge, starting 55–50 Ma (Fig. 3B), although exact timing and configuration of the landmasses remains under debate (*Hall, 2012*; *Ding et al., 2017*). Interestingly, paleoclimate reconstructions suggest that modern-day megathermal angiosperm-dominated tropical forests also originated in the ISC. They later dispersed from there and became established across Sundaland starting about 50 Ma (*Morley, 2018*) coinciding with the onset of a perhumid climate in Southeast Asia (Fig. 3B).

The present time tree analysis indicates that the ancestral radiation of *Microhyla* + *Glyphoglossus* into three main lineages (*Microhyla* I, *Microhyla* II and *Glyphoglossus*) happened during a short time frame in the middle Eocene (ca. 43.8 Ma), slightly later than previous estimates (48.7 Ma; *Garg & Biju, 2019*). Our biogeographic analysis strongly suggests that the *Microhyla* + *Glyphoglossus* MRCA, as well as the *Microhyla* I + *Microhyla* II ancestor, inhabited Eastern Indochina (Fig. 2), which was connected to Sundaland (Fig. 3C). Thus, our results support the Southeast Asian origin of the *Microhyla*—*Glyphoglossus* assemblage in contrast to the hypothesis by *Garg & Biju (2019)*, that suggested the dispersal of *Microhyla* from the ISC into Asia from the Oligocene to the Miocene.

The *Microhyla* II clade remained largely within its ancestral area with most members of the group inhabiting Eastern Indochina and a few species dispersing to Borneo and the Malay Peninsula (Fig. 2). Compared to other *Microhyla* species groups, members of the *Microhyla* II clade are generally small and associated with perhumid montane evergreen forests or tropical rainforests; they do not occur in lowland seasonally dry areas. In fact, in Indochina, their distribution is restricted to mountainous areas (*Parker, 1934*; *Poyarkov et al., 2014*).

On the contrary, members of the *Microhyla* I clade dispersed widely and achieved a pan-Oriental distribution (see Figs. 1 and 2). Members of this clade are diverse ecologically and morphologically. They vary in body size from the smallest to the largest *Microhyla* taxa, occupy habitats that include open seasonally dry savannahs (*Parker, 1934*), and several species within the *Microhyla* I clade evolved adaptations towards digging and estivation (*M. rubra*, *M. mihintalei*, *M. picta*, and *M. fodiens*; see *Poyarkov et al., 2019*). The MRCA of *Microhyla* I is hypothesized to inhabit western Indochina, the same region reconstructed as ancestral for all major internal nodes within *Microhyla* I, and for a

number of included lineages (*M. heymonsi*, *M. fissipes*, *M berdmorei*, and *M. superciliaris* species groups), respectively (Fig. 2).

Drifting of the ISC northwards led to a collision of the Indian plate with Eurasia from the Oligocene to the Miocene (*Aitchison & Ali, 2012*; *Hall, 2012*) (Fig. 3D). At the same time, the uplift of the Himalayas, coinciding with the middle Miocene thermal maximum, initiated the subsequent Miocene strengthening of the Indian monsoon and entailed the expansion of seasonally dry conditions across the northern parts of the Indian peninsula and Indochina. This resulted in the disappearance of closed tropical forests over much of the ISC (*Morley, 2018*) (Fig. 3D). Starting in the Oligocene, Indochina became the source of evergreen and seasonally dry floral elements that dispersed to the ISC with ongoing climate aridification (*Morley, 2000*). These conditions presumably facilitated colonization of the Indian Subcontinent by *Microhyla* I lineages.

Our biogeographic analysis reveals at least five independent cases of *Microhyla* I dispersal from Western Indochina to the ISC (see Fig. 2). Two of these took place in Late Oligocene–Early Miocene: the *M. superciliaris* species group (29.2–22.5 Ma, Fig. 2, 1) and *M. ornata* species group (31.4–18.8 Ma, Fig. 2, 2). Both lineages underwent significant diversification in the ISC and reached as far south as Sri Lanka. Three other cases of the ISC colonization by *Microhyla* I include more recent dispersal events in Late Miocene— Pliocene by *M. berdmorei* (6.9–4.1 Ma, Fig. 2, 3) and *M. fissipes* species groups (*M. mymensinghensis*, 7.2–5.1 Ma, Fig. 2, 4) to northeastern India and Bangladesh, with the only case of dispersal to southern peninsular India being the species of *M. achatina* group in the Middle Miocene (*M. kodial*, 16.2–8.9 Ma, Fig. 2, 5). The confusing phylogenetic position of *M. kodial* within the Southeast Asian *M. achatina* species group originally inspired the hypothesis that this might be a result of a human-mediated dispersal and introduction (*Vineeth et al., 2018*). However, subsequent discovery of its sister species *M. irrawaddy* and *Microhyla* sp. 4 in central Myanmar have made the hypothesis of natural dispersal of the *M. achatina* species group members from Southeast Asia to the ISC more plausible. Interestingly, *M. irrawaddy* inhabits seasonally dry savannah areas, with minimal rainfall (*Poyarkov et al., 2019*). Hence, progressing aridification of the northern and central parts of the ISC starting in the late Miocene (*Deepak & Karanth, 2018*) could have created suitable habitats facilitating dispersal of *M. kodial* ancestors. Generally, western Indochina played an important role for the *Microhyla* I clade (Fig. 2) as this territory likely represents a "stepping stone" area connecting Southeast Asia and the ISC (Fig. 3D).

Diversification within *Microhyla* species group-level endemic radiations started in the Late Oligocene–Early Miocene and initiated multiple dispersals from Asian mainland to present-day islands and archipelagos (Fig. 2). These include multiple colonizations of Sundaland from both Indochina and the Malay Peninsula (by *M. annectens*, *M. palmipes*, *M. berdmorei* and *M. achatina* species groups; Fig. 2). This is not surprising, since these territories are believed to have been a single landmass throughout most of Cenozoic (*Cannon, Morley & Bush, 2009*; *Woodruff, 2010*; *Hall, 2012*). *Microhyla superciliaris* and *M. ornata* species groups experienced at least four independent dispersal events from southern India to Sri Lanka, corroborating results of recent studies, and suggesting a

complex history of dispersals between these regions (*Harikrishnan et al., 2012*; *Pyron et al., 2013*; *Agarwal et al., 2017*; *Karunarathna et al., 2019*). Our study confirms placement of *M. chakrapanii* from Andaman Islands inside the Southeast and East Asian *M. fissipes* species group as sister species of *M. mymensinghensis* (see *Garg et al., 2019*). This confirms the faunal similarity of the Andamans with Southeast Asia rather than with peninsular India (see *Das, 1994*, *1999*). Finally, members of *M. achatina*, *M. butleri*, and *M. fissipes* species groups have dispersed several times from the Asian mainland to East Asian islands: Taiwan (*M. fissipes*, *M. heymonsi*, and *M. butleri*), and two times independently colonized the Ryukyus (*M. okinavensis* and *Microhyla* sp. 3). These results also corroborate data that suggested faunal exchanges between Eurasian continent and East Asian islands (*Ota, 1998*; *You, Poyarkov & Lin, 2015*; *Yuan et al., 2016*; *Wang et al., 2017*; *Nguyen et al., 2020*) and require further study (*Lee et al., 2016*; *Tominaga et al., 2019*).

Interestingly, our phylogeny suggests numerous dispersal events from the Asian mainland to islands, with almost no dispersals back to the mainland (except for the *M. annectens* ancestor that is hypothesized to have dispersed from Borneo to Malay Peninsula, see Fig. 2). This also supports results of *De Bruyn et al. (2014)* who demonstrated that colonization events from younger Asian islands are comparatively rare, rather showing increased levels of immigration events as compared to Indochina or Borneo. Further studies are required to elucidate the role of islands in producing and preserving diversity in *Microhyla* frogs.

Implications for body size evolution in *Microhyla*

Miniaturization is a widespread and interesting morphological and ecological phenomenon in amphibians (*Hanken, 1985*; *Hanken & Wake, 1993*; *Rieppel, 1996*). It is common in several groups of frogs (*Clarke, 1996*; *Lehr & Coloma, 2008*) and reaches extremes in the Microhylidae (*Kraus, 2011*; *Rittmeyer et al., 2012*; *Oliver et al., 2017*; *Rakotoarison et al., 2017*; *Scherz et al., 2019*). The smallest Microhylinae and the smallest terrestrial vertebrate in Asia belong to *Microhyla* and include representatives of two different lineages within the genus: *Microhyla* I (*M. nepenthicola*, adult male size from 10.6 mm; see *Das & Haas, 2010*) and *Microhyla* II (*M. perparva*, males 10.5–11.9 mm, and *M. arboricola*, adult male size from 13.2 mm; see *Inger & Frogner, 1979*; *Poyarkov et al., 2014*). Osteological consequences of miniaturization in *Microhyla* are not well studied yet diminutive species in *Microhyla* II clade show partial (*M. arboricola*) or almost complete (*M. perparva*, *M. nanapollexa*) reduction of the first finger. Similar patterns have also been reported in other miniature microhylids (*Kraus, 2011*; *Rakotoarison et al., 2017*), however, patterns and drivers of body size evolution in *Microhyla* remain poorly understood.

In *Microhyla*, males generally tend to be smaller than females (see Table S7), yet our analyses revealed generally similar patterns of body size evolution in both sexes (Fig. 7). According to the most plausible scenario, the common ancestor of the *Microhyla* + *Glyphoglossus* assemblage was a mid-sized frog (male SVL 25–30 mm, female SVL 30–35 mm). Body size increased in the *Glyphoglossus* clade (up to 105 mm SVL), was slightly reduced in the *Microhyla* I clade, and significantly reduced in the *Microhyla* II

clade ancestors (see Fig. 7). *Microhyla* II members are all small-sized (<25 mm SVL in males) and three species of this group reach extreme miniaturization (<15 mm SVL in males; *M. arboricola*, *M. perparva*, *M. nanapollexa*). *Microhyla* I clade shows significant variation in body size: the *M. berdmorei* species group and, to a lesser extent, several species in other lineages (including *M. fodiens*, *M. rubra*, *M. mihintalei*, *M. aurantiventris*) demonstrate increased body sizes, while members of two lineages within *M. achatina* (*M. nepenthicola* + *M. borneensis* + *Microhyla* sp. 1) and *M. superciliaris* species groups (*M. superciliaris* + *Microhyla* sp. 2) are diminutive (Fig. 7).

Our analyses suggest that adult body size has independently changed several times in the evolution of the *Microhyla* + *Glyphoglossus* assemblage. At least two lineages show an increase in body size, while four other lineages demonstrate extreme miniaturization (Fig. 7). Significant increases in body size in these frogs seem to be connected with a fossorial life style. For example, all members of *Glyphoglossus* as well as some large *Microhyla* (*M. picta*, *M. fodiens*, *M. rubra* and *M. mihintalei*) have stout body habitus and are excellent burrowers (*Poyarkov et al., 2019*). Most of these species inhabit open seasonally dry habitats at low elevations and burrowing is an important strategy for estivation during dry periods. Increased body size might not only facilitate digging, but is also advantageous due to lower surface-area to volume ratios, hence leading to less evaporative water loss during estivation (*Tracy, Christian & Tracy, 2010*).

Evolutionary causes for extreme miniaturization in frogs remain under debate (*Scherz et al., 2019*), but are probably connected with life history strategies, such as exploiting new food resources (*Lehr & Coloma, 2008*) or adaptation to specific microhabitats such as leaf-litter or moss (*Kraus, 2011*). *Scherz et al. (2019)* noted that one miniaturized Malagasy microhylid species is arboreal and breeds in water-filled leaf-axil phytotelmata, while all others are terrestrial. Interestingly, at least the three smallest known *Microhyla* species are also obligatory phytotelm-breeders and reproduce in water-filled pitcher-plants (*M. nepenthicola*, see *Das & Haas, 2010*; and *M. borneensis*, see *Parker, 1928*) or water-filled tree hollows (*M. arboricola*, see *Poyarkov et al., 2014*; *Vassilieva et al., 2017*). Among all other *Microhyla* species, *M. arboricola* is the only known arboreal species with a unique reproductive mode: developing tadpoles of this species are obligately oophagous (*Vassilieva et al., 2017*). *Microhyla arboricola* has a reduced clutch size (16 ± 8 eggs), compared to other *Microhyla* species (usually over 400 eggs per clutch) (*Vassilieva et al., 2017*). Egg size appears to be one of the main constraints for miniaturization in animals (*Polilov, 2015*). On one hand, reduced clutch size might favor the choice of phyototelmata for reproduction due to the absence or low density of predators in such habitats; on the other hand, diminutive body size might be advantageous for phytotelmic frogs because it allows them to exploit smaller phytotelmata than are available to larger frogs (*Scherz et al., 2019*). Breeding biology of *M. petrigena* and *M. nanapollexa* is not yet reported. However, our field observations suggest that the latter species also reproduces in tree hollows. Further studies might shed light on evolutionary interdependencies between phytotelm-breeding and extreme miniaturization in *Microhyla*.

Taxonomic implications and cryptic diversity in *Microhyla*

Diminutive frogs are recognized as a source of astonishingly high undescribed cryptic diversity at different taxonomic levels due to incomplete phylogenetic information and widespread homoplasies in morphology (*Hanken & Wake, 1993*; *Rittmeyer et al., 2012*; *Scherz et al., 2019*). Until recently most miniature frog groups also attracted little attention by taxonomists (*Rakotoarison et al., 2017*). This is also true for the genus *Microhyla* as the only available systematic study addressing the genus by *Matsui et al. (2011)* provided important insights on phylogeny and taxonomy of these frogs, but did not provide other insights. In accordance with results of *Matsui et al. (2011)*, our phylogeny indicates that morphology-based classification schemes of *Parker (1934)*, *Dubois (1987)*, and *Fei et al. (2005)* do not reflect actual phylogenetic relationships among *Microhyla* species, most likely due to high frequency of homoplasies both in adult and larval morphology. Further thorough morphological and osteological studies along with a robust phylogeny are required to diagnose supraspecific-level taxa within the *Microhyla + Glyphoglossus* assemblage.

Since the sampling used in *Matsui et al. (2011)* was incomplete, and the number of recognized *Microhyla* species has since increased almost two-fold, many questions of *Microhyla* taxonomy remain unaddressed. In the present article, we used an updated and almost complete phylogeny of the genus along with species delimitation methods to resolve several long-standing areas of confusion in the genus *Microhyla*. Our analyses suggest that despite recent progress in *Microhyla* taxonomy, the current number of recognized *Microhyla* species is still greatly underestimated. Based on species delimitation analyses, 15 (ABGD estimate) to 33 lineages (bGMYC estimate) probably reflect new species requiring formal description (Table S10). However, due to possible overlap in levels of intra- and interspecific divergence, species delineation in *Microhyla* based on genetic differentiation alone is problematic (*Garg et al., 2019*). An integrative approach including morphology and acoustics must be applied for further progress in *Microhyla* taxonomy (*Rakotoarison et al., 2017*).

Below we give a brief summary of taxonomic implications of our results. The smallest member of the genus, *M. nepenthicola*, was described by *Das & Haas (2010)* from Kubah, Sarawak, but was synonymized with *M. borneensis* by *Matsui (2011)* based on morphological examination of the *M. borneensis* holotype from Kidi (sic for Bidi), Sarawak, Borneo (*Parker, 1928*). *Matsui (2011)* did not include in his molecular analysis materials from the type locality of *M. borneensis*; however, his taxonomy was widely accepted (*Frost, 2020*). We analyzed topotypic *M. borneensis* specimen from the Bidi region (Fig. 4), specifically, from the Deded Krian National Park, near Bau, western Sarawak, and show it be a sister species of *M. nepenthicola*, and sufficiently divergent from the latter in 16S rRNA sequences ($p = 4.6\%$, Table S9) to constitute a separate species. The bGMYC analysis also supports distinctiveness of *M. nepenthicola* from *M. borneensis* (Fig. 4). The name-bearing population from the former locality (*M. nepenthicola*) is found on sandstone massifs of western Sarawak, and is diagnosable in showing a pale brown dorsum with darker subtriangular pattern on scapular region, its adjacent areas lacking

dark variegation; and flanks with an elongated dark area. On the other hand, the latter population (*M. borneensis*), restricted to the limestone hills of the interior, shows a gray-brown dorsum, areas outside dark subtriangular pattern with dark gray variegation; and flanks with isolated dark blotches. Therefore, we propose to revalidate the species *M. nepenticola Das & Haas, 2010*. Our data further suggest that *M. borneensis*, *M. nepenthicola*, and *Microhyla* sp. 1 from Sabah form a clade of morphologically similar and closely related taxa. Further studies are required to fully clarify morphological differences between *M. nepenthicola* and *Microhyla* sp. 1.

Significant genetic differentiation is revealed within several species of the *M. achatina* species group suggesting presence of cryptic diversity. Examples include *M. malang* (3 putative species: populations from Sarawak, Sabah and Kalimantan); *M. orientalis* (2 putative species: populations from Bali and Java; our study confirms the occurrence of *M. orientalis* in Java); *M. mantheyi* (2 putative species within Malayan Peninsula); *M. achatina* (2 putative species within Java); and *M. gadjahmadai* (2 putative species within Sumatra) (see Fig. 4). Genetically, the most diverse cryptic species complex in *Microhyla* is the *M. heymonsi* complex. Earlier studies already recognized the presence of several deeply divergent intraspecific lineages within *M. heymonsi* (*Garg et al., 2019*). Our new analyses revealed 7–8 highly divergent ($p > 3.0\%$) lineages from China and northern Vietnam, Thailand and Laos, Thailand and south Vietnam, Taiwan, Myanmar, peninsular Malaysia, and Sumatra (Fig. 4). The taxonomic status of these lineages has yet to be assessed. Our study also confirms the clear distinctiveness of an undescribed species *Microhyla* sp. 4 from northern Myanmar, and the full species status of *M. minuta*, *M. pineticola*, *M. irrawaddy*, and *M. fodiens*, respectively.

Within the *M. fissipes* species group, the bGMYC analysis indicated the presence of three cryptic species-level lineages within *M. mukhlesuri*, although the ABGD analysis lumped *M. mukhlesuri* with *M. fissipes* (Fig. 4). Significant genetic differentiation in mtDNA-markers was reported for *M. mukhlesuri* by *Yuan et al. (2016)*, but they were not corroborated by nuclear DNA. Further integrative studies are required to assess variation within *M. mukhlesuri*. Deep divergence was revealed between populations of *M. okinavensis* from Okinawa and Amami islands ($p = 4.8\%$); populations from Yaeyama Archipelago formerly treated as *M. okinavensis* grouped with *M. mixtura* and most likely represent an undescribed species, *Microhyla* sp. 3 (*Tominaga et al., 2019*; *Hasan et al., 2014a*). Shallow divergence was also found among populations of *M. chakrapanii* from different islands of the Andaman Archipelago (Fig. 4).

Substantial genetic divergences also uncovered cryptic species lineages within the *M. berdmorei* species complex (Fig. 5) (*Hasan et al., 2012*). The bGMYC analysis suggested presence of four distinct groups from Malayan Peninsula, Malaysia + Sumatra + Borneo, Indochina, and Bangladesh. Populations from northern Thailand previously described as *M. fowleri* are grouped within the Indochinese lineage of *M. berdmorei*, suggesting synonymy of the former (see *Matsui et al., 2011*). Further studies, including examination of topotype material for *M. berdmorei* (Myanmar) and *M. darevskii* (central Vietnam), are needed to estimate taxonomic statuses of these newly revealed lineages and extent of their distribution. Within the *M. superciliaris* group, our study confirmed occurrence of

M. superciliaris in southern Thailand (Songkhla), however the deep divergence of this population from the *M. superciliaris* population in Malaysia ($p = 1.6\%$) suggests that it is necessary to reevaluate the taxonomy of Thai populations (Fig. 5). We also report an undescribed species *Microhyla* sp. 2, occurring in western Thailand where genetic variation among examined populations (Suratthani and Phetchaburi) also suggests presence of two cryptic lineages.

For the *M. butleri* species group, our work confirms deep divergence and full species status of the recently described *M. aurantiventris* (*Nguyen et al., 2019*), but also reveals additional undescribed lineages within *M. butleri* that we treat here as a species complex. From two (ABGD) to four (bGMYC) cryptic lineages were recovered within this complex, with the most divergent lineages being distributed in Taiwan + mainland China versus the rest of the species range in Indochina and the Malay Peninsula (Fig. 5). We included only two *M. palmipes* samples in our analysis that were notably divergent in 16S rRNA sequences ($p = 3.6\%$) and originated from Bali and Sumatra; likely they both represent distinct species.

For the *M. annectens* species group, our species delimitation analyses reveal a number of cryptic and undescribed lineages. Our study confirms genetic distinctiveness of recently described *M. arboricola* and *M. pulchella* (*Poyarkov et al., 2014*), as well as of *M. annamensis*, for which genetic information was not previously available. We also added to our analysis a number of populations of *M. marmorata* throughout the species' range and for the first time, including specimens of *M. pulverata* (collected from ca. 10 km north of the type locality in Gia Lai Province, central Vietnam). Both species were described by *Bain & Nguyen (2004)* based on morphological evidence and the main characters considered to be diagnostic for these species were belly coloration (marbled in *M. marmorata* versus dusty in *M. pulverata*) and skin texture. Our genetic data reveal almost no genetic differentiation between samples of *M. marmorata* and the topotypic *M. pulverata* ($p = 0.4\%$, see Table S9), the latter are nested within the *M. marmorata* radiation and do not form a clade (Fig. 5). Moreover, our observations showed that belly coloration is highly variable within *M. marmorata*, especially in the southern part of the species range, and cannot be used as a reliable diagnostic character. Due to the lack of genetic and morphological differentiation, we hereby formally treat *Microhyla pulverata Bain & Nguyen (2004)* as a subjective junior synonym of *Microhyla marmorata Bain & Nguyen (2004)*. Some other species of the *M. annectens* species group show deep intraspecific divergences in 16S rRNA sequences, such as *M. arboricola* ($p = 2.6\%$ between populations from Dak Lak and Khanh Hoa provinces of Vietnam), *M. petrigena* ($p = 3.7\%$ between populations from Sarawak and Sabah), and *M. perparva* ($p = 5.1\%$ between populations from Sarawak and Indonesian Kalimantan) (Fig. 5). It is likely that these lineages represent distinct species, including several new taxa awaiting formal description.

CONCLUSIONS

Herein, we provide an updated phylogenetic hypothesis for the genus *Microhyla*. An exhaustive taxonomic sampling for this group is challenging due to the high number of

narrow-ranged or point-endemic species across South and Southeast Asia. In the present study, however, we examined mtDNA and nuDNA markers for 48 of 50 recognized *Microhyla* species (96%), including 12 nominal species and several undescribed candidate species that have not been examined phylogenetically before our work, thus providing the most comprehensive taxonomic sampling for *Microhyla* to date. Our data further highlight the importance of broad phylogenetic sampling and ground-level field research to gather an accurate picture of global biodiversity, phylogenetic relationships, and evolutionary patterns in cryptic groups such as microhylid frogs.

We recognize nine species groups within the *Microhyla—Glyphoglossus* assemblage (*M. achatina*, *M. fissipes*, *M. berdmorei*, *M. superciliaris*, *M. ornata*, *M. butleri*, *M. palmipes*, *M. annectens* species groups and *Glyphoglossus*), divided into three clades of probable genus-level differentiation: *Microhyla* I, *Microhyla* II and *Glyphoglossus*. Further integrative research combining phylogenetic and morphological lines of evidence is required to fully diagnose these recognized groups and test our taxonomic arrangement. The basal radiation of the *Microhyla—Glyphoglossus* assemblage is dated to the middle Eocene and likely took place in Southeast Asia. Following drifting of the Indian Plate northwards and formation of firm land bridges between the subcontinent and Asian mainland in Oligocene, ancestors of *Microhyla* colonized India several times from Southeast Asia and later diversified there. Our analysis also suggests that such dispersal occurred independently in five different species groups of the *Microhyla* II clade. Our results further corroborate the growing set of evidence for early-Eocene land connections between the Indian Subcontinent and Southeast Asia. Progressing aridification since the late Oligocene—Miocene likely facilitated dispersal of Southeast Asian biotic elements to India including the ancestral lineage-genus *Microhyla*. Our study further highlights the importance of Indochina not only as a cradle of autochthonous amphibian diversity and a key evolutionary hotspot for the herpetofauna (*Bain & Hurley, 2011*; *Geissler et al., 2015*; *De Bruyn et al., 2014*), but also as a stepping stone facilitating dispersal between Sundaland, the Indian Subcontinent, and East Asia (*Woodruff, 2010*; *Chen et al., 2017*; *Suwannapoom et al., 2018*; *Poyarkov et al., 2018b*). Further phylogenetic studies across different faunal groups with Indo-Southeast Asian affinities are required to clarify impact of complex paleogeography and paleoclimate history on formation of extant biodiversity in Asia.

Comprising the smallest tetrapods in Asia, frogs in the genus *Microhyla* represent a potential model group to study evolutionary drivers and constraints of vertebrate miniaturization. Our study suggests that four groups of *Microhyla* independently achieved extreme miniaturization with adult body sizes <15 mm. Evolution of body size in *Microhyla—Glyphoglossus* assemblage seems to be driven by natural history: the largest body sizes are observed in burrowing species adapted to estivation during the dry season, while three of the five smallest known *Microhyla* species occur only in perhumid montane forests and are phytotelm-breeders. Further research is required on how reproductive ecology in phytotelmata, often leading to reduction of clutch size, facilitates extreme miniaturization in *Microhyla*.

The present work clearly indicates a vast underestimation of diversity and species richness of *Microhyla*. We revalidate *M. nepenthicola* as a valid species, synonymize *M. pulverata* with *M. marmorata*, confirm species-level differentiation for a number of taxa not included in earlier phylogenies, and reveal a large number of cryptic lineages representing putative undescribed species. Alternative approaches to species delimitation suggest that at least 15–33 lineages of *Microhyla* likely correspond to species-level differentiation. Further integrative studies combining genetic, morphological, and acoustic parameters are essential for a better understanding of evolutionary relationships and taxonomy within this morphologically cryptic and diverse radiation of Asian frogs.

ACKNOWLEDGEMENTS

ADDITIONAL INFORMATION AND DECLARATIONS

Funding

Competing Interests

Nikolay A. Poyarkov is an Academic Editor for PeerJ. Suranjan Karunarathna is employed by Nature Explorations and Education Team (Sri Lanka). Luan Thanh Nguyen is employed by Asian Turtle Program—Indo-Myanmar Conservation (Vietnam). Other authors have declared that no competing interests exist.

Author Contributions

- Vladislav A. Gorin conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Evgeniya N. Solovyeva conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Mahmudul Hasan conceived and designed the experiments, performed the experiments, analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.
- Hisanori Okamiya conceived and designed the experiments, performed the experiments, analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.
- D.M.S. Suranjan Karunarathna analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.
- Parinya Pawangkhanant analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Anslem de Silva analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.
- Watinee Juthong analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.
- Konstantin D. Milto analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Luan Thanh Nguyen analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.
- Chatmongkon Suwannapoom performed the experiments, analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.

- Alexander Haas conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- David P. Bickford analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Indraneil Das conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Nikolay A. Poyarkov conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.

Field Study Permissions

The following information was supplied relating to field study approvals (i.e., approving body and any reference numbers):

No field studies were carried out specifically for this work; tissue samples and specimens stored in museum collections were used in this study. However, some specimens stored in the mentioned collections were collected by the coauthors of this manuscript or with their participation during numerous field trips in a time frame over 15 years. Specific permits from Malaysia, Sri Lanka, Thailand and Vietnam are as follows:

- The Sarawak Forest Department and the Sarawak Forestry Corporation granted a fieldwork permit to Indraneil Das (permit number (13)JHS/NCCD/600-7/2/107(Jld2));
- The Sarawak Forest Department and the Sarawak Forestry Corporation granted permission for fieldwork under permit number NPW.907.4.2–26, NPW.907.4.2–43; NPW.907.4–35; NPW.907.4–36; NPW.907.4.2–8 and NPW.907.4.2(II)–73; NCCD.907.4.4 Jld.7–39, issued to Indraneil Das (Malaysia), and export permits (04635, 07094–97 and 07484);
- The Department of Wildlife Conservation of Sri Lanka granted fieldwork permission (permit number WL/3/2/1/14/12 issued to Anselm de Silva, 30 April 2008);
- The Institute of Animals for Scientific Purpose Development (IAD), Bangkok, Thailand granted permission for fieldwork under permit number U1-01205-2558, issued to Chatmongkon Suwannapoom;
- The Institute of Animals for Scientific Purpose Development (IAD), Bangkok, Thailand granted permission for fieldwork under permit number UP-AE59-01-04-0022, issued to Chatmongkon Suwannapoom (Thailand);
- The Institutional Ethical Committee of Animal Experimentation of the University of Phayao approved field collections under certificate number 610104022 issued to Chatmongkon Suwannapoom;
- The Department of Forestry, Ministry of Agriculture and Rural Development of Vietnam granted permission for fieldwork under permit number #170/TCLN–BTTN (issued 7 February 2013), #831/TCLN–BTTN (issued 5 June 2013), #400/TCLN-BTTN

(issued 26 March 2014), #547/TCLN-BTTN (issued 21 April 2016), #432/TCLN-BTTN (issued 30 March 2017), issued to JRVTRTC, Nikolay A. Poyarkov (Vietnam);

- The Department of Forestry, Ministry of Agriculture and Rural Development of Vietnam approved additional fieldwork (permit numbers #142/SNgV-VP (Gia Lai Province, issued 4 May 2016), #1539/TCLN-DDPH (issued 19 September 2018), #1700/UBND.VX (Nghe An Province, issued 22 March 2018) and #308/SNgV-LS (Quang Nam Province, issued 01 April 2019) given to Nikolay A. Poyarkov), Thanh Hoa Province (#3532/UBND-THKH, issued 27 March 2019); Lam Dong Province (#5832/UBND-LN, issued 22 October 2012); Dak Lak Province (#1567/UBND-TH, issued 06 April 2011; #388/SNgV-LS, issued 24 April 2019; #995/SNN-CCKL, issued 12 April 2019); Lao Cai Province (#1148/UBND-TNMT, issued 26 March 2019);

- In Cat Tien NP, fieldwork was conducted in accordance with Agreement #37/HD on the scientific cooperation between Cat Tien NP and JRVTTC;

- In Bu Gia Map NP, fieldwork was conducted in accordance with Agreement #137/HD NCKH of 23 June 2010 on the scientific cooperation between Bu Gia Map NP and JRVTTC;

- Forest Protection Departments of the Peoples' Committee of Gia Lai Province granted permission for fieldwork to Nikolay A. Poyarkov (permit number #530/UBND-NC (issued 20 March 2018)).

DNA Deposition

The following information was supplied regarding the deposition of DNA sequences:

The sequences described here are available at GenBank: MN534393 to MN534449; MN534450 to MN534550; MN534551 to MN534657; MN534658 to MN534765; MK208926 to MK208938; MH286426 to MH286427.

Data Availability

The following information was supplied regarding data availability:

The raw data has been supplied as voucher specimens and tissue samples stored in the following herpetological collections:

(1) Zoological Museum of Moscow University (ZMMU; Moscow, Russia);

(2) Zoological Institute, Russian Academy of Sciences (ZISP; St. Petersburg, Russia);

(3) Vertebrate Zoology Department, Biological Faculty, Moscow State University (ZPMSU; Moscow, Russia);

(4) Amphibian Research Center, Hiroshima University (IABHU; Higashihiroshima, Japan);

(5) Danum Valley Conservation Area, Specimen collection (RMBR; Sabah, Malaysia);

(6) Department of Fisheries, Bangamata Sheikh Fazilatunnesa Mujib Science & Technology University (DFBSFMSTU; Jamalpur, Bangladesh);

(7) School of Agriculture and Natural Resources, University of Phayao (AUP; Phayao, Thailand);

(8) Institute of Biodiversity and Environmental Conservation, Universiti Malaysia Sarawak (UNIMAS; Sarawak, Malaysia).

(9) For the full list of voucher specimens examined, see Table S1.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.9411#supplemental-information.

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