



Unexpectedly high levels of lineage diversity in Sundaland puddle frogs (Dicroglossidae: *Occidozyga* Kuhl and van Hasselt, 1822)

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ABSTRACT

One of the most urgent contemporary tasks for taxonomists and evolutionary biologists is to estimate the number of species on earth. Recording alpha diversity is crucial for protecting biodiversity, especially in areas of elevated species richness, which coincide geographically with increased anthropogenic environmental pressures - the world's so-called biodiversity hotspots. Although the distribution of Puddle frogs of the genus *Occidozyga* in South and Southeast Asia includes five biodiversity hotspots, the available data on phylogeny, species diversity, and biogeography are surprisingly patchy. Samples analyzed in this study were collected throughout Southeast Asia, with a primary focus on Sundaland and the Philippines. A mitochondrial gene region comprising ~ 2000 bp of 12S and 16S rRNA with intervening tRNA Valine and three nuclear loci (*BDNF*, *NTF3*, *POMC*) were analyzed to obtain a robust, time-calibrated phylogenetic hypothesis. We found a surprisingly high level of genetic diversity within *Occidozyga*, based on uncorrected p-distance values corroborated by species delimitation analyses. This extensive genetic diversity revealed 29 evolutionary lineages, defined by the > 5% uncorrected p-distance criterion for the 16S rRNA gene, suggesting that species diversity in this clade of phenotypically homogeneous forms probably has been underestimated. The comparison with results of other anuran groups leads to the assumption that anuran species diversity could still be substantially underestimated in Southeast Asia in general. Many genetically divergent lineages of frogs are phenotypically similar, indicating a tendency towards extensive morphological conservatism. We present a biogeographic reconstruction of the colonization of Sundaland and nearby islands which, together with our temporal framework, suggests that lineage diversification centered on the landmasses of the northern Sunda Shelf. This remarkably genetically structured group of amphibians could represent an exceptional case for future studies of geographical structure and diversification in a widespread anuran clade spanning some of the most pronounced geographical barriers on the planet (e.g., Wallace's Line). Studies considering gene flow, morphology, ecological and bioacoustic data are needed to answer these questions and to test whether observed diversity of Puddle frog lineages warrants taxonomic recognition.

1. Introduction

Our knowledge regarding species diversity (Larsen et al., 2017; Mora et al., 2011; Myers et al., 2000) and underlying evolutionary

processes (de Queiroz 1998; Mayr 1947) continues to expand in many groups of organisms, including “well-studied” terrestrial vertebrates (Cozzuol et al., 2013; Geissmann et al., 2011; Nater et al., 2017; Welton et al., 2010). Over the past 15 years, an average of 155 species

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of anurans have been newly described each year (AmphibiaWeb, 2021). This increase derives particularly from new species discoveries from biodiversity hotspots, regions with high species richness and high levels of endemism (Orme et al., 2005), coinciding geographically with environmental degradation, and extinction risk (Marchese, 2015; Noss et al., 2015; Possingham and Wilson 2005). One such global set of biodiversity hotspots is the biogeographic region Sundaland, which comprises the Thai-Malay Peninsula, Borneo, Sumatra, Java, Bali, and smaller islands of the Sunda Shelf (Fig. 1), a tectonically relatively stable extension of Southeast Asia's continental shelf (Hall and Holloway, 1998; Myers et al., 2000). The contemporary shorelines of Sundaland, however, are geologically recent: Sumatra and Java started to rise towards the early Miocene (Gower et al., 2012; Hall, 2012; 2013; Meijaard and Groves, 2004), and Borneo has been separated from the continent for just under five million years (Hall, 2001; 2012; 2013). At present, the landmasses encompassed by the Sunda Shelf are separated by shallow seas with depths of ≤ 200 m (Sathiamurthy and Voris, 2006).

The northern border of Sundaland is marked by the Isthmus of Kra (Fig. 1), forming the link to the biodiversity hotspots of mainland Southeast Asia and Indochina (Sodhi et al., 2004). The western and southern borders of Sundaland are formed by deep water trenches (den Tex, 2011), whereas the eastern and north-eastern boundary corresponds to Huxley's modification of the Wallace Line (Huxley, 1868; Wallace, 1869). This biogeographic boundary separates the islands of Wallacea (much of eastern Indonesia) and the Philippine archipelago to

the northeast from the Asian ecozone including Sundaland (Brown et al., 2013; den Tex, 2011; Esselstyn et al., 2010; Hall and Holloway, 1998; Lohman et al., 2011). The island of Palawan, to the north of Borneo, has been viewed traditionally to be a biogeographical extension of Sundaland (Heaney 1986; Huxley 1868; Everett 1889; Leviton, 1963; Steere 1894). However, geologically, Palawan is a microcontinental plate that rifted from the margin of the Asian mainland, opening up the Western Philippine Sea and tectonically transporting Palawan and associated landmasses, as well as the Zamboanga Peninsula of Mindanao to their current locations (den Tex, 2011; Meijaard and Groves, 2004; Yumul et al., 2003; 2004; 2009; Padrones et al., 2017). The current extraordinarily high terrestrial faunal and floral species richness of Sundaland is considered, at least in part, a result of the lengthy, extraordinarily complex geological and climatological history of Southeast Asia (Darlington, 1957; den Tex, 2011; Esselstyn et al., 2010; Hall, 2002; Hall and Holloway, 1998; Heaney, 1986; Lohman et al., 2011; Brown et al., 2013). In the light of the accelerated habitat loss fuelling today's extinction crisis (Brooks et al., 2002; Pimm et al., 2014), reliable data on local genetic and species diversity, their geographical extent of distribution, and population abundance census data are essential to provide strong arguments for the conservation of biodiversity hotspots (e.g., Brooks et al., 2002; Forest et al., 2007; Funk et al., 2012; Mittermeier et al., 2011; Myers et al., 2000).

The rapid improvement of molecular phylogenetic methods, dramatically increased affordability of genetic data, and convenience of data collection equips taxonomists with effective tools to discover putative

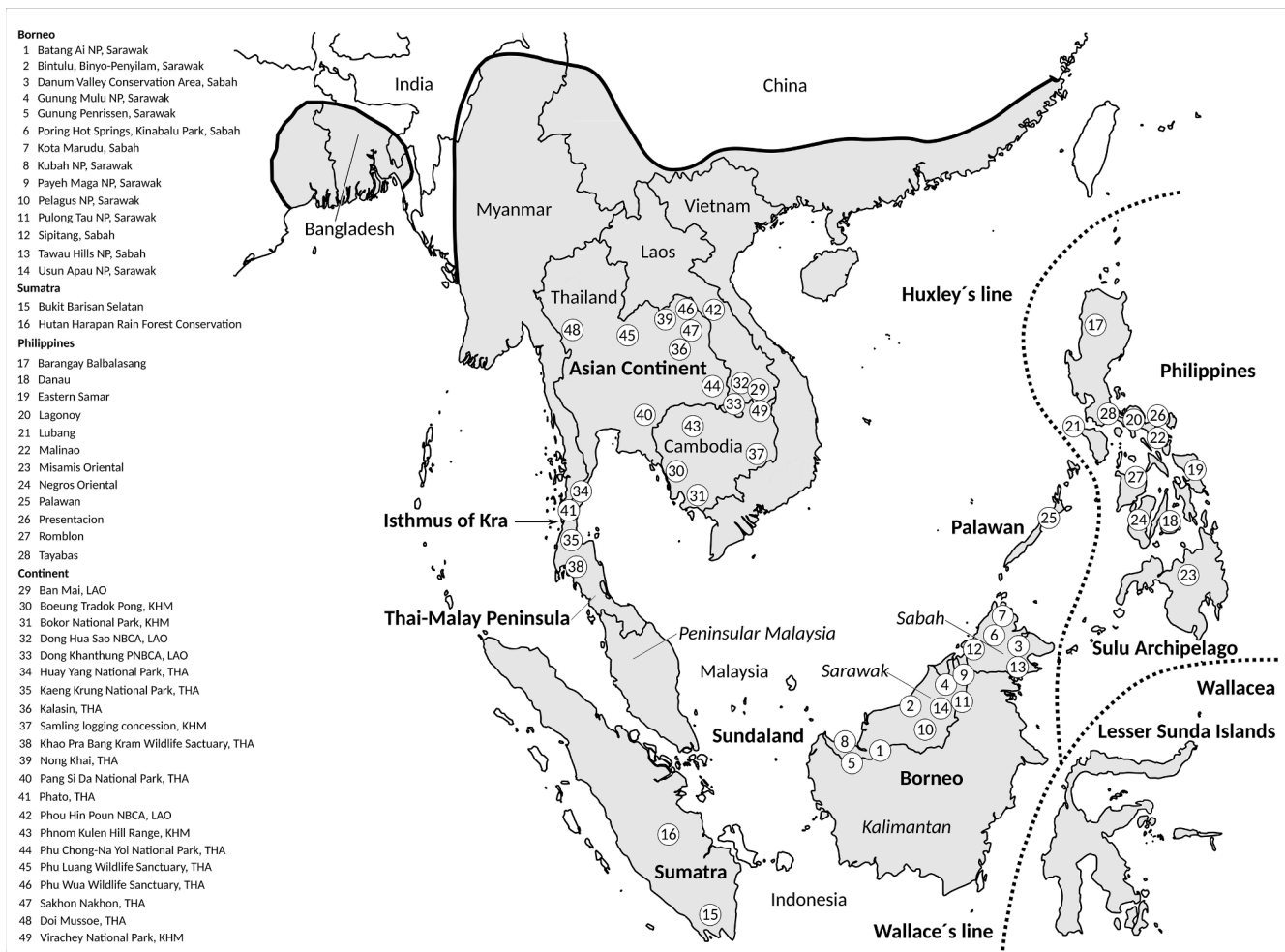


Fig. 1. Distribution area of *Occidozyga* in grey (Java and Lesser Sunda Islands not shown) and sampling localities of specimens used in this study. See Appendix for further details.

diversity and species boundaries (e.g., Barley et al., 2013; Chan et al., 2017; 2018; 2020; Hotaling et al., 2016; Vieites et al., 2009). Today's broad application of pluralistic, integrative taxonomy, including simultaneous inclusion of ecological information, behavioral data, phenotypic traits, and DNA sequence data, has promoted a burgeoning subdiscipline, accelerating identification of putative 'cryptic' species (Bickford et al., 2007; Padial et al., 2010; Stuart et al., 2006), defined as cases in which two or more at least superficially morphologically indistinguishable but genetically different species have been erroneously identified as a single, named/recognized taxon (Funk et al., 2012; Hanken, 1999; Jörger et al., 2012; Jörger and Schrödl, 2013; Matsui et al., 2016; McLeod, 2010; Pfenninger and Schwenk, 2007; Trontelj and Fiser, 2009; Vieites et al., 2009; Welton et al., 2013).

Anurans constitute a large, globally distributed clade, which continues to increase rapidly in number of described species ($n = 7,148$ as of 23 October 2019; AmphibiaWeb, 2021; Frost, 2020). New species continue to be discovered, particularly from the tropical realm. Since 1985, the total number of recognized species has risen by over 60% (AmphibiaWeb, 2021). For instance, on the island of Borneo alone, 196 anuran species are known to date, although just eighteen years ago this number was only 141 (Frost, 2020; Inger and Voris, 2001). This dramatic increase in the number of recognized species may, in part, be a result of new discoveries from remote, previously inaccessible areas, but is also likely the result of application of integrative taxonomic approaches (Brown and Stuart, 2012; AmphibiaWeb, 2019). Furthermore, improved sampling efforts enabled the identification of cryptic species or even species complexes, such as in *Ansonia* (Hertwig et al., 2014; Matsui et al., 2010; Waser et al., 2017), *Chalcorana* (Inger et al., 2009; Stuart et al., 2006), *Duttaphrynus* (Wogan et al., 2016), *Fejervarya* (Kotaki et al., 2008, 2010), *Leptobrachium* (Brown et al., 2010; Hamidy et al., 2012; Hamidy and Matsui, 2014), *Leptobrachella* including the former genus *Leptotalax* (Chen et al., 2018; Dehling and Matsui, 2013; Eto et al., 2016; Eto and Matsui, 2016; Matsui et al., 2014b, 2014a), *Limnectes* (Evans et al., 2003; Matsui et al., 2016; Matsui and Nishikawa, 2014; McLeod et al., 2012; McLeod, 2010), *Meristogenys* (as *Amolops*, Matsui, 1986; Shimada et al., 2011), *Odorrana* (Bain et al., 2003; Stuart et al., 2006), *Philautus* (Dehling, 2010; Dehling et al., 2016), *Polypedates* (Brown et al., 2010), *Pulchrana* (Chan et al., 2020), *Sylvirana* (Sheridan and Stuart, 2018), *Staurois* (Matsui et al., 2007; Arifin et al., 2011), and *Sumaterana* (Arifin et al., 2018).

Within the Fork-tongued and Fanged Frogs of the family Dicroglossidae, the genera *Occidozyga* (Kuhl and van Hasselt, 1822) and *Ingerana* (Dubois, 1987) are allocated to the taxon Occidozyginae (Fei, Ye, and Huang, 1990) AmphibiaWeb, 2021; Frost, 2020), whereas the remaining genera of the dicroglossid frogs are amalgamated as Dicroglossinae (Anderson, 1871)(Chen et al., 2017; Frost et al., 2006; Pyron and Wiens, 2011; Chan and Brown, 2018). Although common, widespread, and recognized for a long time, the Puddle frogs of the genus *Occidozyga* constitute a prime example of a group of frogs for which knowledge of species diversity, phylogeny, and biogeography is conspicuously under-developed. These facts, plus their pattern of a wide geographical distribution from South and Southeast Asia to the western Pacific, render them an excellent candidate for a comprehensive, well-sampled phylogenetic and biogeographic study.

Species of *Occidozyga* are locally abundant in permanent or intermittent water bodies, including puddles and ephemeral ponds, lakes, rivers, swamps, marshes, ditches, or even seasonally flooded agricultural land, such as rice fields or irrigation systems (Inger et al., 2017). In comparison to other genera of the family Dicroglossidae, their tadpoles have a suite of derived characters, that constitute larval synapomorphies for *Occidozyga*, suggesting adaptations to an exclusively macro-carnivorous diet (Haas et al., 2014). The eleven currently recognized *Occidozyga* species are distributed throughout South and Southeast Asia, ranging from eastern India, Bangladesh and southern China to the Philippines and the Lesser Sunda Islands of Indonesia (Frost, 2020). The distribution of this genus overlaps at least partially

with five biodiversity hotspots: Indo-Burma, South-Central China, Sundaland, the Philippines, and Wallacea. Currently no species-level phylogenetic analysis of *Occidozyga* is available. Therefore, species delimitation remains incomplete and the distributions of these taxa are poorly understood. For example, *O. lima* (Gravenhorst, 1829) was formerly assumed to be a single, wide-spread species occurring from southern China to Java Island. However, the first study of population divergence revealed three unconfirmed candidate species, distinguished by $\geq 5\%$ genetic divergence in 16S ribosomal DNA sequences (Chan, 2013). The widespread species, *O. laevis* (Günther, 1858), was originally described from the Philippines, yet as currently defined, its known range ostensibly also includes Borneo, southern Thailand, and Peninsular Malaysia (Diesmos et al., 2015; Frost, 2020; Jaafar et al., 2012; 2009; Khonsue and Thirakhupt, 2001). *Occidozyga laevis* is often confused with *O. sumatrana* (Peters, 1877; originally described from Sumatra), yielding uncertainty regarding the species affiliation of many geographic populations from Sundaland, southern China and western India (Iskandar & Colijn, 2000; Frost, 2020). *Occidozyga martensii* (Peters, 1867) is distributed throughout Thailand, southern China, Vietnam, Laos, Myanmar, and north-western Malaysia, whereas *O. magnapustulosa* (Taylor and Elbel, 1958) is known only from its type locality in Thailand (Poyarkov et al., 2020). The remaining species seem to be endemic to one or a few islands of Sundaland and adjacent areas. *Occidozyga baluensis* (Boulenger, 1896), was originally described from Gunung Kinabalu on Borneo, but is known today to occur throughout Borneo (Inger et al., 2017; Sheridan et al., 2012). The three species *O. celebensis* (Smith, 1927), *O. semipalmata* (Smith, 1927) and *O. tompotika* (Iskandar, Arifin, and Rachmanasah, 2011) are endemic to Sulawesi, whereas *O. floresiana* (Mertens, 1927) is known only from Flores, and *O. diminutiva* (Taylor, 1922) is restricted to some southern islands of the Philippines.

Available data on ecology and intraspecific morphological variation for most species of *Occidozyga* are limited. Taxonomic uncertainty renders ecological research and statistical characterizations of morphological variability potentially ambiguous. The Bornean species, for example, can be categorized into two size classes based on the snout-vent length (SVL) of the females: *O. baluensis* is a medium-sized frog with SVL < 35 mm (Boulenger, 1896; Malkmus and Brühl, 2002), whereas some of the populations identified as *O. laevis* are relatively large, with SVL up to 48 mm (Inger et al., 2017). Moreover, *O. baluensis* possesses a cream-coloured ventral surface, with a contrasting pattern of numerous dark spots. This species prefers seepage areas in forests where adults and tadpoles are found in thin films of water (Haas et al., 2014; Inger et al., 2017). The remaining *O. laevis* or *O. sumatrana* populations from Borneo show a uniform coloration of the venter and throat, and are found in puddles, swamps, slow moving streams or at edges of forest ponds (Inger et al., 2017; pers. obs.).

In this study, we analyse the phylogenetic relationships within *Occidozyga* using mitochondrial (mtDNA) and nuclear sequence (nuDNA) data, derived from comprehensive geographical sampling, with a primary focus on Sundaland. Based on the resulting phylogenetic hypothesis, we evaluate whether current taxonomy reflects genetic diversity. We examine temporal and geographical distribution patterns of *Occidozyga* species using a time-calibrated phylogenetic hypothesis, in an attempt to identify the influence(s) of archipelagic radiations and dispersal events in the evolution of Puddle Frogs. Our results contribute to a better understanding of the underlying evolutionary and biogeographic processes that gave rise to the extraordinarily high anuran species richness in Southeast Asia. Finally, we anticipate that this work will soon be followed by genomic characterizations of gene flow, validation (or refutation) of species boundaries proposed here, and a starting point for subsequent studies of ecology, biogeography, and conservation of these enigmatic and overlooked amphibians.

2. Material and methods

2.1. Sampling and markers

Our taxonomic sampling consists of 151 samples from Sundaland, the Philippines and continental Southeast Asia of *Occidozyga* (Fig. 1, Appendix), except for those from Sulawesi, the Lesser Sunda islands, and from the Sulu Archipelago (*O. tompotika* Iskandar, Arifin, and Rachmanasah, 2011; *O. celebensis* Smith, 1927; *O. semipalmata* Smith, 1927; *O. floresiana* Mertens, 1927; *O. diminutiva* Taylor, 1922). Tissue samples were taken from adult specimen femoral muscles or liver, or from tadpole tail musculature; tissues were preserved in in RNALater buffer solution (Ambion/Applied Biosystems) or 90% pure ethanol. For out-group taxa, we included nine species of *Limnonectes* (Fitzinger, 1843), two *Fejervarya* (Bolkay, 1915) and one *Ingerana* (Dubois, 1981), based on previously published hypotheses of the phylogenetic relationships of diicroglossid frogs (Brown et al., 2015; Jetz and Pyron, 2018; Matsui et al., 2016; Pyron and Wiens, 2011; Wiens et al., 2009).

2.2. Laboratory protocols

Genomic and mitochondrial DNA was extracted using Wizard SV Genomic DNA Purification System (Promega AG, Switzerland) in accordance with the manufacturer's protocol. In case of crystallization of the buffer, we performed an additional washing step with nuclease-free water to remove excessive salt before extraction. Sequence data were generated from the mitochondrial 12S rRNA, tRNA-Val and 16S rRNA gene regions and three nuclear genes (brain-derived neurotrophic factor (*BDNF*), neurotrophin 3 (*NTF3*), proopiomelanocortin (*POMC*)), using forward and reverse primers (Tab. S1) for both PCR and cycle sequencing. For PCR reactions, we used 25 µl PCR reaction volumes containing 2 µl DNA, 12.5 µl GoTaq Hot Start Green Master Mix (Promega), 2 µl of each primer (10 µM), (for reaction with 16S and 16SD primers 1 µl (20 µM)) and 6.5 µl ddH₂O (for reaction with 16S and 16SD primers 8.5 µl ddH₂O). Cycling conditions for the three primer pairs to amplify the 12S, tRNA-Val and 16S rRNA gene regions were: denaturation at 94 °C for 2:00 min; 35 cycles at 95 °C for 0:30 min, 48.2 °C for 0:30 min and 72 °C for 1:00 min; then one final extension cycle at 72 °C for 5:00 min. Cycling conditions for *BDNF* were: denaturation at 94 °C for 2:00 min, 39 cycles at 94 °C for 0:20 min, 57 °C for 0:45 min, and 72 °C for 2:00 min, followed by one final extension at 72 °C for 5:00 min. For *NTF3* we used: denaturation at 94 °C for 3:00 min, 40 cycles at 95 °C for 0:30 min, 52.4 °C for 0:30 min, and 72 °C for 1:00 min, followed by one final extension at 72 °C for 7:00 min. Finally, for *POMC*, we followed: denaturation at 94 °C for 1:00 min, 35 cycles at 95 °C for 0:30 min, 56 °C for 0:30 min, and 60 °C for 1:12 min, followed by one final extension at 72 °C for 5:00 min. PCR products from the PCR with the primer pair 16S and 16SD were cleaned with the Wizard® SV Gel and PCR Clean-Up System (Promega) and no clean-up was required for the remaining markers. Sequencing was performed by LGC Genomics GmbH (Berlin, Germany).

2.3. Phylogenetic analyses

Sequences were checked for ambiguities, assembled, and aligned using Geneious Pro 10.2.6 (Kearse et al., 2012) (Biomatters Ltd., 2018) and the implemented MAFFT-plugin (Katoh and Standley, 2013). Our different markers were checked separately for contamination and laboratory errors. Uncorrected genetic p-distances were calculated in MEGA (Kumar et al., 2018). Finally, we concatenated our data and compiled three data partitions for subsequent phylogenetic analyses: mitochondrial (mtDNA = mitochondrial, consisting of concatenated sequences of 12S, tRNA-Val and 16S rRNA genes), nuclear (nuDNA = nuclear, consisting of concatenated *BDNF*, *NTF3*, *POMC* sequences), and combined (mtDNA + nuDNA = mitochondrial + nuclear, including all five gene regions).

The best-fit sequence substitution model of sequence evolution and partitioning scheme for each marker was determined with Partition-Finder 2.1.1 (Lanfear et al., 2016), based on the small sample-size corrected Akaike Information Criterion (AICc) and the 'models = GTR, GTR + I, GTR + I + G' for RAXML and 'models = all' for Bayesian setting with linked branch lengths. The Best-fit substitution models obtained for each gene are listed in Tab. S2a for ML and Tab. S2b for BI.

Maximum Likelihood (ML) analysis was performed in RAXML-NG (Kozlov et al., 2018) using a partitioned model, and implementing models selected in PartitionFinder. Branch lengths were linked (Duchêne et al., 2020) and 100 bootstrap replicates were run. RAXML-NG was then set to search for the best-scoring tree after performing bootstrapping. Bayesian inference (BI) was performed in MrBayes 3.2.6 (Ronquist et al., 2012). Four independent runs of Metropolis-coupled Markov chain Monte Carlo analyses were conducted, with each consisting of three heated and one cold chain. Analyses were run for 20 million generations with a sampling frequency of 100. Trace files were checked with Tracer v.1.7.1 (Rambaut et al., 2018) to assess convergence, and we conservatively discarded the first 25% of samples as burn-in (20,000,000 trees). Clades with a BI support ≥ 0.95 and $\geq 70\%$ in ML bootstrap values were considered robustly supported (Hillis and Bull, 1993; Ronquist et al., 2012). Modest support was assessed ≥ 0.75 in BI and $\geq 60\%$ in ML bootstraps value.

2.4. Divergence-time estimation

The ages of the divergences among lineages of *Occidozyga* were estimated using BEAST 2.5 (Bouckaert et al., 2019) and BEAUti 2.5 (Bouckaert et al., 2019). Our time-calibrated analysis was performed using the mitochondrial sequence data only, to avoid over-parameterisation and because no internal Diicroglossidae fossil calibrations are available, and divergence-time estimates from available literature for the split between Diicroglossinae and *Occidozyginae* differ significantly, ranging from 96.8 Ma to 59.9 Ma (Roelants et al., 2007; Wiens et al., 2009; Zhang et al., 2013). Thus, for our BEAST2 analysis, we used a range of mitochondrial divergence rates, (0.35–0.955% substitutions/my; Macey et al., 1998; 2001; Sanguila et al., 2011; Tan and Wake, 1995; Wang et al., 2008) as a crude general scalar. The standard deviation was set to be lower than one, which means that the standard deviation in branch rates was smaller than the mean rate. All analyses were conducted with a Yule model prior (uniform birth rate by default fixed to 1), a relaxed clock with log normal distribution with a rate for the clock of 0.0065, with a lower boundary of 0.0035 and an upper boundary of 0.00955. Uclmean was set to lognormal, M = 0.01, S = 0.9. uclstddev was set to exponential and defined to lie between 0 and 1. MCMC was set to 60 million generations and sampled every 1000 generations. We ran three separate runs, and log files of BEAST2 were checked for ESS values ≥ 200 in TRACER 1.7.1 (Rambaut et al., 2018). We then combined results in Log Combiner and visualized the tree in Figtree v1.4.2 (Rambaut, 2012).

2.5. Biogeographic analyses

The biogeographic range evolution history was reconstructed by testing six different models (DEC, DEC + J, DIVA, DIVA + J, BayArea, BayArea + J) and comparing them in a common maximum likelihood framework to find the best statistical fit using AIC in the R package BioGeoBEARS 0.2.1 (Matzke, 2013a; Matzke, 2014). The models allow to test alternative biogeographic hypotheses, such as dispersal, vicariance, and extinction. Five areas were defined that are covered by our ingroup sample: mainland Asia, the Philippines, Palawan, Borneo, and Sumatra. This coding scheme is a simplification of the complex paleogeographic history of Southeast Asia, because Borneo and the Thai-Malay Peninsula constituted the connected landmass of northern Sundaland until recently. The islands of Borneo and Sumatra were coded as separate areas (even though they are both part of Sundaland) to identify potential dispersal events between these islands and the other areas.

Maximum areas per species were set to two. Six models were compared: Dispersal-Extinction-Cladogenesis (DEC), Dispersal-Vicariance Analysis (DIVA), and BayArea, and each of these in combination with a parameter for long-distance dispersal “J”: DEC + J, DIVA + J, BayArea + J (Matzke, 2014; 2013b).

2.6. Sequence divergence

The software package MEGA (Kumar et al., 2018) was used to calculate the uncorrected pairwise divergences of 914 bp fragments of the 16S rRNA gene. Uncorrected p-distance values are not appropriate as the sole criterion for species delimitation, but are widely accepted as indicators of potential species boundaries and hidden species diversity in anurans (Fouquet et al., 2007; Matsui et al., 2016; McLeod, 2010; Vences et al., 2005b; 2005a; Vieites et al., 2009). In this study, lineages or clades with a genetic divergence in the 16S rRNA gene of > 5% were regarded as potentially distinct evolutionary lineages in the sense of Operational Taxonomic Units (OTUs) for possible future consideration/validation. OTUs supported by a consensus of supported clades that appeared in both mtDNA and nuDNA tree estimates are regarded as unconfirmed candidate species (UCS) (Jörger and Schrödl, 2013; Matsui et al., 2016; Vences et al., 2005b; 2005a; Vieites et al., 2009).

2.7. Species delimitation

Two different approaches of species delimitation were applied in order to compare the independently obtained results (Carstens et al., 2013). The general mixed Yule coalescent method (GMYC, Pons et al., 2006) uses maximum-likelihood statistics to find the transition points between inter- and intra-species branching rates. In this study we used a Bayesian implementation of the evolutionary model-based method, bGMYC (Reid and Carstens, 2012). The bGMYC approach outperforms existing GMYC implementations by accommodating different uncertainties in the model, e.g. the quality of the ultra-metric tree has less influence on the outcome (Esselstyn et al., 2012; Reid and Carstens, 2012; Talavera et al., 2013; Zhang et al., 2013). The input trees were generated by BEAST2 using the mtDNA partition. The outgroup was reduced to one terminal taxon. 60 million trees were sampled at a frequency of 300,000 and a burnin of 25% using LogCombiner to get a final subsample of 150 trees (bgmyc.multiphylo, mcmc = 50000, burnin = 15000, thinning = 500). The behaviour of the MCMC was checked visually according to the authors' recommendation (Reid and Carstens, 2012). The second species-delimitation approach applied was Poisson tree processes (PTP, Zhang et al., 2013). PTP does not require an ultra-metric tree but calculates the speciation rate based on the number of substitutions. In general, the method assumes higher substitution rates between species than within species. The mcmc-based mPTP was used, because the algorithm considers variable levels of intraspecific genetic diversity caused by a sampling bias or differences in the evolutionary history and is at least five times faster compared to the classic PTP method (Kapli et al., 2017). The RAxML tree based on the mtDNA partition with only one terminal taxon as outgroup was used as input (mcmc = 100 mio, mcmc_sample = 1 mio, mcmc_burnin = 5 mio).

3. Results

3.1. Partitions and substitution models

Our combined dataset was composed of 2066 bp of 12S-tRNA-Val-16S rRNA mtDNA, 1680 bp nuDNA (including 654 bp of *BDNF*, 588 bp of *NTF3* and 438 bp of *POMC*), and 3746 bp (mtDNA + nuDNA), respectively. PartitionFinder proposed the partition schemes and substitution models for the ML analyses (Tab. S2a) and BI (Tab. S2b).

3.2. Phylogenetic analyses and sequence divergence

Phylogenetic trees obtained with ML and BI analyses of the three data partitions (mtDNA + nuDNA, mtDNA, nuDNA) are congruent apart

from the lower resolution of nuDNA trees (Figs. 2 and 3). Occidozyginae is the sister clade to the subfamily Dicroglossinae (*Limnonectes* and *Fejervarya*). The monophyly of *Occidozyga* as currently recognized/defined was not supported, because the species *Limnonectes rhacodus* clustered with robust support within *Occidozyga* (Figs. 2 and 3). Within the remainder of the genus *Occidozyga*, unexpectedly high numbers of divergent evolutionary lineages were detected. Altogether, 29 divergent lineages were distinguished by sequence divergence of > 5% in our 16S rRNA gene tree and, further, by robust bootstrap support in analyses of this partition and the combined mtDNA + nuDNA dataset (Fig. 2, and Fig. 4). Even the nuDNA, when analyzed alone, recovered a majority of these same evolutionary lineages with moderate support (Fig. 3). As expected, branches in our nuDNA topology were considerably shorter, consistent with slower substitution rates of nuclear genes, and yielding generally lower resolution in comparison to the separate analyses of mtDNA and total evidence data set mtDNA + nuDNA (Fig. 2 and Fig. 3). The 29 evolutionary lineages (L1 to L29, see Fig. 2 and Fig. 4) were distributed across five, well-supported major clades, which we name: the *baluensis* group, the *laevis* group, the *sumatrana* group, the *rhacoda* group, and the *lima* group (Fig. 2 and Fig. 4). The *baluensis* group represented the sister clade to the remaining major clades, but relationships among these major clades were not robustly supported, particularly for the position of the *rhacoda* group.

The *baluensis* group exclusively comprises samples from Sabah and northern and central Sarawak (Fig. 2 and Fig. 4), allocated to four mitochondrial lineages (L1 to L4). L1 (Gunung Mulu NP) and L2 (Poring Hot Springs) are sister groups. L3 is distributed in central Sarawak (Bintulu, Pelagus), whereas L4 is widely distributed in western and eastern Sabah (Sipitang, Danum Valley CA, Tawau Hills Park) and northern Sarawak (Payeh Maga NP). The westernmost record of the widespread lineage L4 is only 170 km southeast of Poring Hot Springs, so that the distribution of L4 projects between the ranges of L2 and L1. Among these groups only L1, L2 and L3 are supported by nuclear data (Fig. 3).

The *laevis* group includes 11 distinct lineages (L5 to L15): L5 from Palawan, three from Borneo (L6 from Gunung Mulu NP and Sipitang, L7 from Danum Valley CA, L8 from Tawau Hills Park), and seven from the Philippines (L9 to L15) (Fig. 2 and Fig. 4). The lineages from the Philippine archipelago (L9 to L15), with the exception of L5 from Palawan, form a robustly supported clade, which is the sister group to L8 from Tawau Hills Park in eastern Sabah. Only L14 is not supported by nuclear data (Fig. 3).

The *sumatrana* group consists of three lineages from Sumatra and three from Borneo (L16 to L21), but Sumatra lineages do not form a clade, because L18 is more closely related to a clade from central and western Sarawak (consisting of L19 from Binyo-Penyilam 40 km east of Bintulu, L20 from Bintulu, L21 from Kubah NP) (Fig. 2 and Fig. 4). Only L21 is not supported by nuclear data (Fig. 3).

The *rhacoda* group consists of three lineages from Sarawak (L22 to L24) (Fig. 2 and Fig. 4). L22 includes the samples from Kubah NP and Gunung Penrissen assigned to *Limnonectes rhacodus* based on the presence of its diagnostic morphological characters (Inger et al 1996). The sister clade of L22 consists of the remaining two lineages L23 (a single specimen from the vicinity of the Batang Ai NP in central Sarawak), and L24 from northern and central Sarawak (Payeh Maga NP, Pulong Tau NP, Usun Apau NP).

Finally, the *lima* group includes all samples from the south-east Asian mainland available in this study (L25 to L29), which have been preliminarily identified as *O. lima*, or *O. martensii* (Fig. 2 and Fig. 4). L25 and L26 represent two evolutionary lineages regarded here as *O. lima*. The remaining three lineages (L27 to L29 from Thailand, Cambodia and Laos) cluster together and are referred to *O. martensii*. Only L26 is supported by nuclear data (Fig. 3).

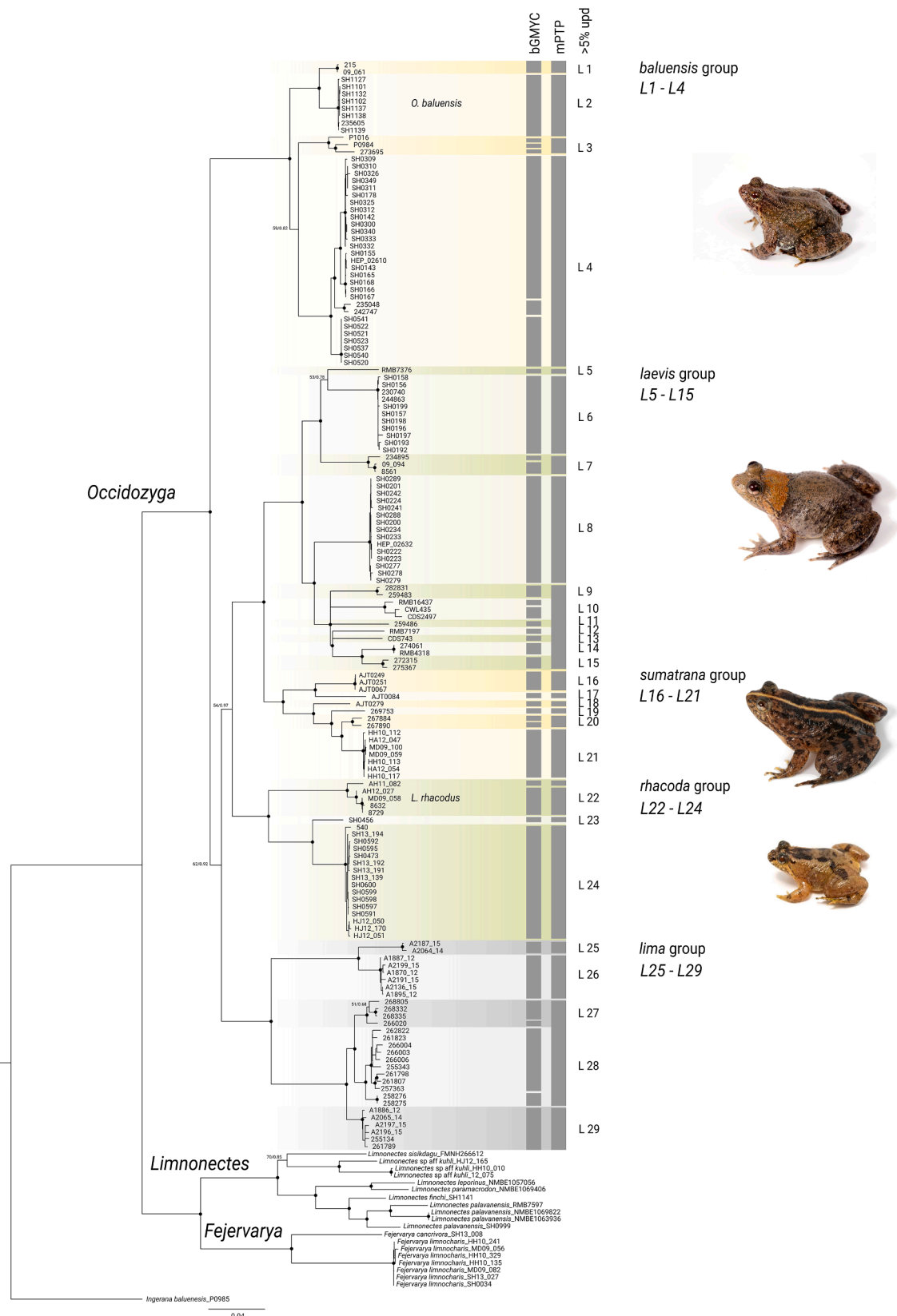


Fig. 2. Total evidence tree of combined data set (mtDNA+nuDNA) with ML support values on the left and BI probabilities on the right. Black circles indicate robustly supported nodes (ML $\geq 70\%$, BI ≥ 0.95). Unsupported nodes (< 50) are collapsed. Genetic diversity is analysed using three different methods, first by a $> 5\%$ p-distance for the 16S rRNA gene indicated by alternating yellow and green background coloration, furthermore by bGMYC and mPTP (both with a threshold of 0.95). Evolutionary lineages L1-L29 regarded here as unconfirmed candidate species are identified based on the $> 5\%$ distance criterion. The following lineages correspond to the nominal species *Limnonectes rhacodus* = *Occidozyga rhacoda* (L22), *O. baluensis* (L2), *O. laevis* (one of the lineages L9-L15), *O. lima* (L25+L26) and *O. martensii* (L27-L29). Terminal taxa are labelled with field numbers. For collection and genbank accession numbers see Appendix. Unit of the scale is substitution/site.

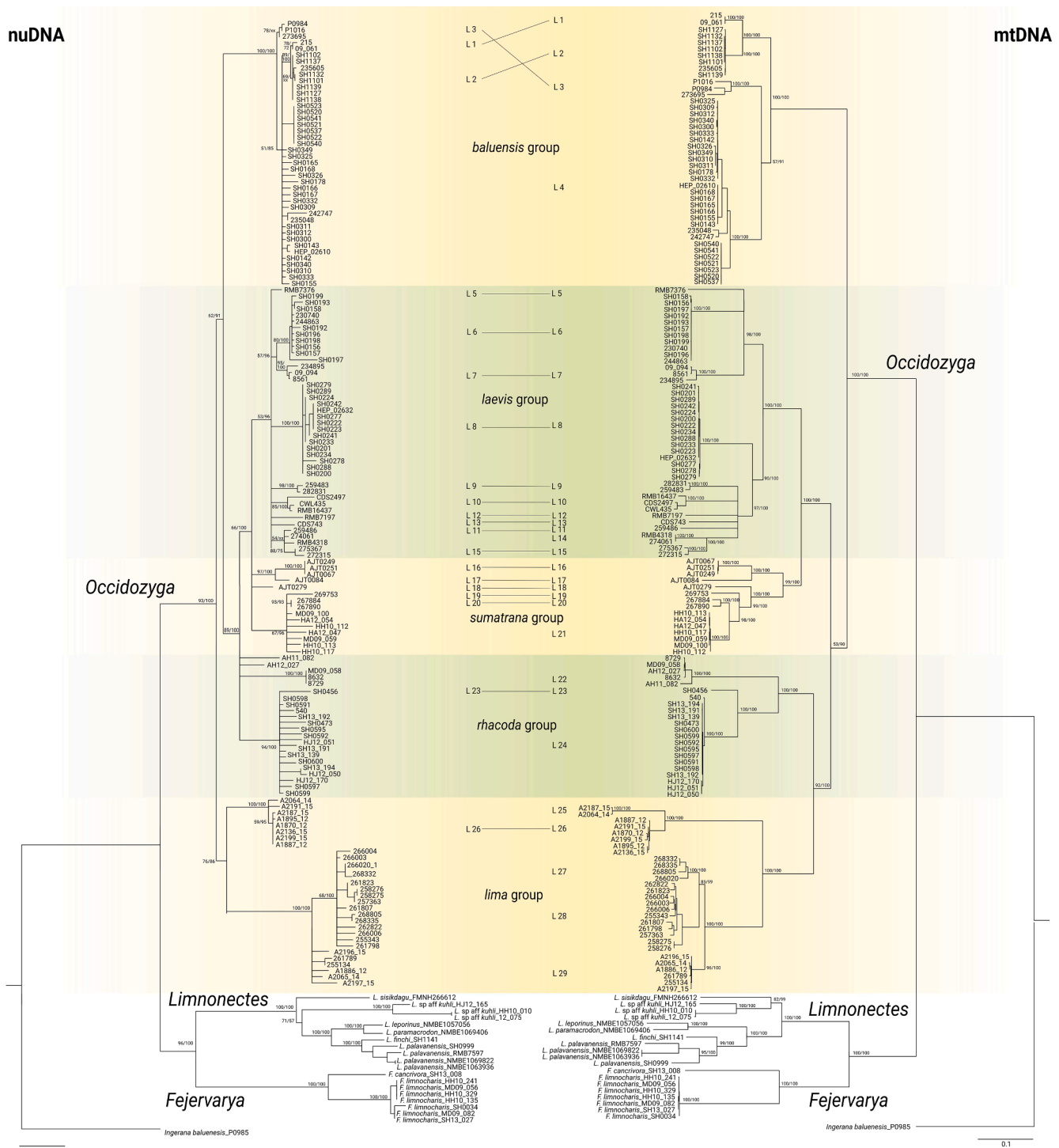


Fig. 3. Maximum-likelihood and Bayesian tree of both the nuclear data (nuDNA, on the left) and the mitochondrial data (mtDNA, on the right). Unsupported nodes (<50% bootstrap support) are collapsed. Support values on nodes are ML support left or above, BI probabilities right or below. Lineages are written out in the center of the figure only if they show at least moderate support (>50 ML, > 0.70 BI), which means lineages 4, 21, 22, 24, 25, 27, 28 and 29 are not supported in the nuclear trees. Unit of the scale is substitution/site.

3.3. Distribution

All lineages that cluster together in each of our five major clades are allopatrically distributed within the clade (Fig. 4). In most sampled localities, two lineages from different major clades are found sympatrically. Lineages of the *baluensis* (L1) and *laevis* groups (L7), for example, occur together in Gunung Mulu NP, Danum Valley CA (L4, L6), Tawau Hills Park (L4, L8), and Sipitang (L4, L7). Lineages of the *baluensis* (L3)

and *sumatrana* (L19, L20) groups likewise occur sympatrically in the Bintulu area, lineages of the *baluensis* (L4) and *rhacoda* (L24) groups co-occur in Payeh Maga NP, and finally, lineages of the *sumatrana* (L21) and *rhacoda* (L22) groups occur sympatrically in Kubah NP.

In all localities the co-occurring species of *Occidozyga* have clearly different habitat preferences and are not syntopically distributed. In the Payeh Maga NP, L24 was found in a muddy, slow moving stream running along an old logging road at 1000 m asl, while the vouchers of

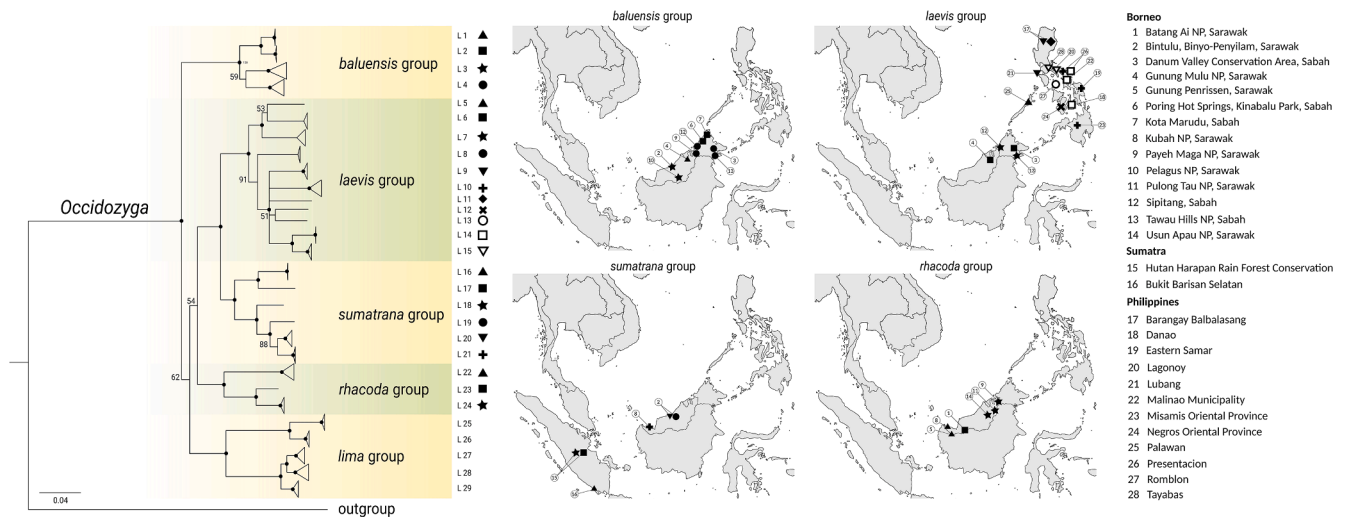


Fig. 4. Distribution of evolutionary lineages (>5% p-distance) of the four major groups of *Occidozyga* occurring on Sundaland and the Philippines. Phylogenetic relationships are illustrated using the simplified total evidence tree (see Fig. 2). Nodes within lineages and unsupported nodes are collapsed. Nodes marked by filled circles are robustly supported in ML and BI analyses. Unit of the scale is substitution/site. Symbols denote numbered lineages as shown at the tips of the tree. Circled numbers on the maps correspond to localities listed at right.

L4 were collected in a seepage area in the montane forest at 1100 m asl. In the Tawau Hills Park, L4 occurred in slow moving parts of a clear-water mountain stream at 1000 m asl, while L8 was in a marshy area at about 350 m asl. In the Danum Valley CA, both lineages occurred in lowland areas but in different microhabitats. The vouchers of L4 were collected in slow-running clear-water streams and adjacent seepage areas, while L7 was found in a shallow pond with muddy banks.

3.4. Biogeography

In our reconstruction of historical biogeography (BioGeoBears; Matzke, 2013a) all six models were evaluated using the Akaike information criterion including accounting for the sample size (AICc; Burnham et al., 2011). Akaike weights were used to find the relative likelihood of the six models. The DEC + J model (Dispersal-Extinction Cladogenesis including a founder event (Ree, 2005; Ree and Smith, 2008)) was determined to be best-fitting due to the lowest AICc and highest AICc_{wt} (Tab. S3). The second-best model is DIVALIKE (a likelihood version of Dispersal-Vicariance Analysis (Ronquist, 1997)) without a founder event. DIVALIKE includes widespread vicariance. DEC on the other hand only includes vicariance in narrow ranges as well as in regional subsets of larger areas (see Matzke, 2013b for detailed model descriptions). Taking into account the palaeogeographic history of Sundaland a scenario without founder events seems less likely. The model does not reveal whether a founder event occurred via a land bridge or overseas dispersal. We use our temporal reconstructions and geological data to evaluate these alternative interpretations. The results of the biogeographic analyses (Fig. 5) for the deeper nodes are biased because most chosen outgroup taxa were from Borneo. We focus discussion of the outcomes of the ancestral area reconstruction on selected, more recent dispersal events from the defined area using the DEC + J model.

3.5. Divergence-time estimation

The resulting topology obtained with BEAST2 (Fig. 6) was essentially congruent with the RAXML and Bayesian trees. Only weakly supported nodes within the *O. baluensis* group and the phylogenetic relationships of the *O. rhacoda* group differed, because the *O. rhacoda* and *lima* groups were sister clades in the time calibrated tree (Fig. 6, Fig. S2). The estimated age of the genus *Occidozyga* was 31 million years (confidence

interval 19.5 to 51 my). The major clades corresponding to the five species groups separated from one another 25 to 14 Ma, while the evolutionary lineages within these groups split up between 12 and 4 Ma (Fig. 6, Fig. S3).

3.6. Species delimitation

The number of entities preliminarily distinguished by bGMYC was 51 with a threshold of 0.5 (50% of all trees separate the tips) and 40 with a threshold of 0.95 (95% of all trees separate the tips). The results of bGMYC divide some evolutionary lineages defined by the 5% p-distance criterion further (Fig. 2, Fig. S1). The number of entities identified in the mPTP analysis was 27 with a threshold of 0.5 and 24 with a threshold of 0.95. The mPTP analysis with a threshold of 0.95 does not separate L9 to L15 from the Philippines belonging to the *laevis*-group and L27 and L28 belonging to the *lima*-group (Fig. 2).

4. Discussion

4.1. Phylogeny and diversity

Understanding evolutionary processes that shaped the temporal and spatial distribution patterns of the current diversity requires credible identification and delimitation of the basal units of evolution (species) via a statistical, phylogenetic framework (Fujita et al., 2012; Funk et al., 2012; Hillis, 2019; Leaché et al., 2014; Luo et al., 2018; Rannala, 2015). However, the task of detecting the real species richness and its proper representation in taxonomy has been challenged by the increasingly frequent discovery of different levels of genetic differentiation and morphologically cryptic evolutionary lineages in many groups of organisms (Bickford et al., 2007; Chan et al., 2017; Funk et al., 2012; Singhal et al., 2018). This study on the widespread anuran genus *Occidozyga*, reveals a striking case of hidden genetic diversity in amphibians from Sundaland and the Philippines and demonstrates how current taxonomy probably does not reflect the degree of genetic divergence within this group. Using a well-established approach for delimitation of species boundaries in Southeast Asian amphibians based on sequencing of mitochondrial and/or nuclear marker genes (Chan et al., 2014a,b; Chan and Grismer, 2010; Matsui et al., 2016), we found a continuum of genetic structure ranging from probably recently differentiated populations to highly divergent evolutionary lineages (Fig. 6). These findings correspond to the concept, that the continuous

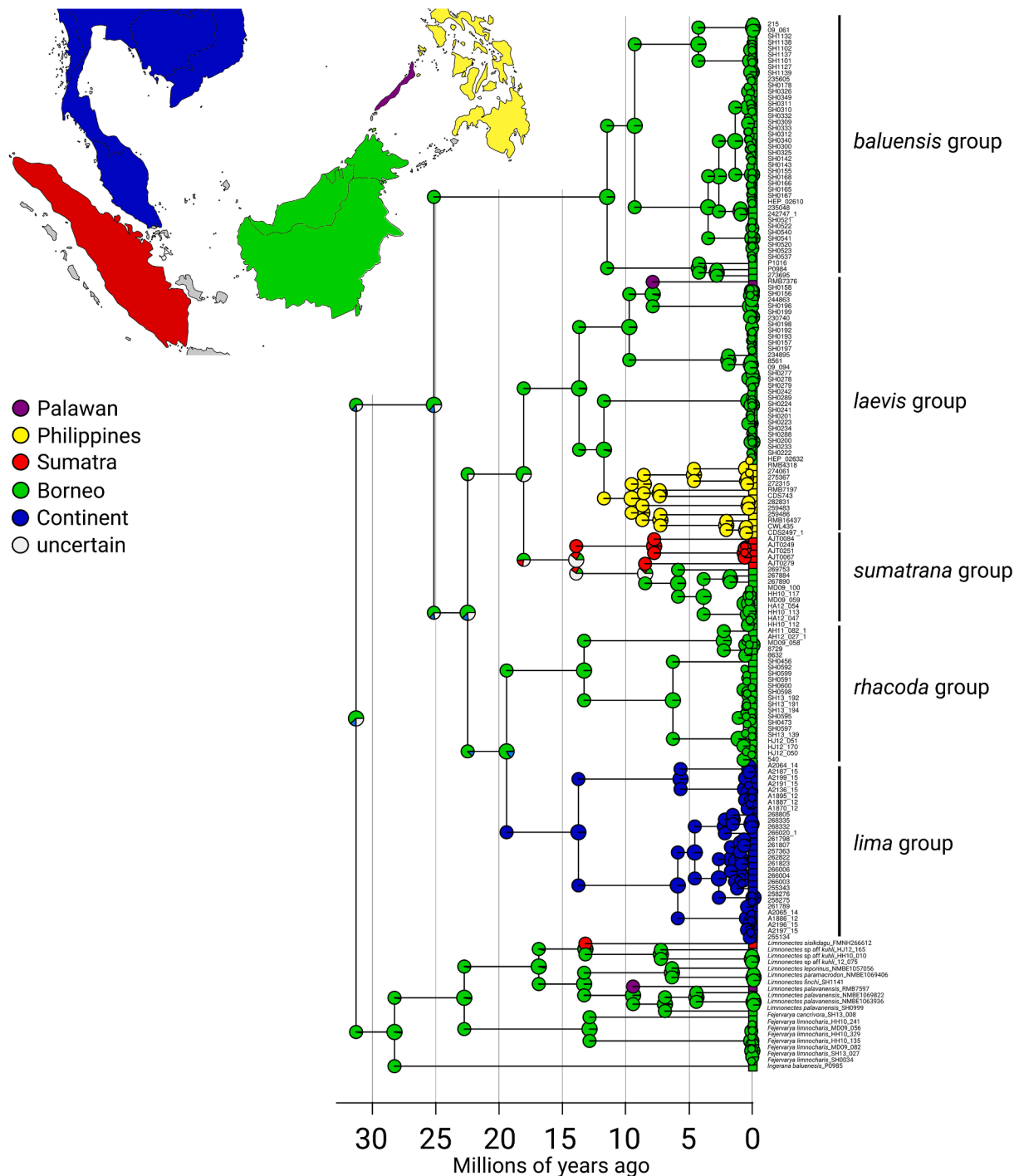
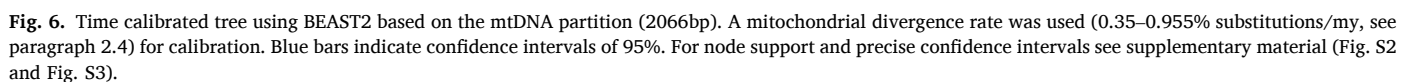


Fig. 5. Time calibrated tree using BioGeoBEARS with biogeographical reconstruction. Input-tree derived from BEAST2. Five areas were determined: Borneo (green), the Philippines (yellow), Palawan (purple), Sumatra (red) and the continent (blue). Grey partitions in the circles indicate the level of uncertainty at the respective node. Six models were tested: Dispersal-Extinction-Cladogenesis (DEC), DIVA (Dispersal-Vicariance Analysis), BayArea, and each of these in combination with a parameter for long-distance dispersal “J”. The best-fit model was “DEC+J”. According to the biogeographic history of Sundaland, *Occidozyga* originated in Borneo, followed by single colonizations of the Southeast Asian continent (ancestor of the *lima* group), Sumatra (ancestor of the *sumatrana* group, followed by return to Borneo by a common ancestor to lineages L19-L21), Palawan (lineage L5 of the *laevis* group), and Philippines (ancestor of lineages L9-L15 of the *laevis* group).

and complex process of speciation begins with populations becoming spatially isolated, which results in genetically diverged populations without reproductive isolation and ends with reproductive isolation between the evolved species, and that any current investigation can only be a snapshot of this process (Chan et al., 2017; 2020; Hendry et al., 2009).

Our statistical species delimitation analyses uncovered a significantly higher number of distinct evolutionary lineages (OTUs, UCS) than currently recognized as valid species (Fig. 2, Fig. 3 and Fig. 4). The number of identified entities, however, depends on the species delimitation algorithm and the selected parameters ranging from 51 (bGMYC, threshold of 0.5) to 24 (mPTP, threshold of 0.95; Fig. 2, Fig. S1). In part,



these incongruent results can be explained by scattered geographic sampling from some areas and the fact that some lineages were represented by only a single specimen. In the following, we consider the 29 evolutionary lineages (OTUs) delineated by the operational criterion of > 5% uncorrected p-distance for the 16S rRNA gene, which has been widely-employed in amphibian systematics for putative species identification (Fouquet et al., 2007; Matsui et al., 2016; McLeod, 2010; Ron et al., 2006; Vences et al., 2005b; 2005a; Vieites et al., 2009). The results obtained with the mPTP species delimitation algorithm essentially agree with the boundaries defined by the 5% criterion, because only L9 to L15 from the Philippines and L27 and L28 belonging to the *lima*-group are not separated by mPTP (Fig. 2). Genetic isolation of lineages combined with geographic and paleogeographic information remains an important criterion for species status, and genetic divergence is evidence of at least temporarily interrupted gene flow in the past (Baker and Bradley, 2006).

Some portion of the evolutionary lineages retrieved in the analyses and distributed across all major clades of *Occidozyga* may represent cases of cryptic species—genetically divergent but morphologically indistinguishable species—suggesting that genetic divergence has not been accompanied by obvious phenotypic divergence. The frequently inferred phenomenon of phenotypically cryptic species diversity, resulting in a disparity between genetic diversity and taxonomic units, has been implicated in many metazoan taxa including amphibians (Austin, 1995; Bickford et al., 2007; Busack et al., 1985; Isaacson and Perry, 1977; Lavoue et al., 2011; Pfenninger and Schwenk, 2007; Sheridan and Stuart, 2018; Trontelj and Fiser, 2009). The existence of morphologically similar species could be explained by a strong selection on behavioural or physiological characters as the basis for adaptation. Species that rely on non-visual cues for mate choice, may, for example, develop new mate recognition signals of various modalities (e.g., behavioural, acoustic, or pheromonal) but may not necessarily undergo morphological divergence during speciation (Bickford et al., 2007; Narins, 1983; Barley et al., 2013).

A recent study showed, however, that gene flow and introgression among allopatrically diverged lineages after secondary contact can lead to an overestimation of cryptic species diversity. Hybrid populations were highly divergent from neighboring populations, but genetically unexpectedly similar to allopatric populations. The observed cryptic diversity might, therefore, also arise from highly admixed and structured metapopulation lineages (Chan et al., 2020, 2021). Because we consider these phenomena likely in co-distributed lineages situated on the same landscape (Sundaland), we emphasize that we consider our result of the unexpectedly high genetic diversity in *Occidozyga* only as a first step towards a deep investigation of the evolutionary processes that led to this pattern, and as a starting point for subsequent phylogenomic and taxonomic studies. Further integrative studies with a larger sample and additional lines of evidence including bioacoustic, ecological, and genomic data are thus required to distinguish between genetic structure associated with intraspecific geographic variation from processes of speciation (Sukumaran and Knowles, 2017).

4.2. Taxonomic identity

The nominal species *Occidozyga baluensis*, *O. laevis*, *O. lima*, *O. martensii* and *O. sumatrana* are each a member of one of the major clades that comprise multiple distinct evolutionary lineages (Fig. 2). This study was not conceived as a comprehensive taxonomic revision of *Occidozyga*, in part because it is based solely on analyses of molecular data. The unexpectedly high number of distinct evolutionary lineages, the brief nature of many original descriptions, and the imprecise information about the type localities in many of the original descriptions all constitute challenges for future taxonomic work. In particular, it will be difficult to assign lineages identified herein to available nominal taxa; we give tentative suggestions below.

Within the *O. baluensis* group, L2 most probably represents the nominal taxon *Occidozyga baluensis* (Fig. 2). The corresponding samples

were collected from Poring Hot Springs at the foothills of the Gunung Kinabalu massif, the type locality of this species (Boulenger, 1896). The absence of a supporting signal in the nuDNA data (Fig. 3) — except for L3 —, the relatively short branch length (Fig. 2) and the age estimates indicate that the lineages of the *O. baluensis* group diverged 12–4 million years ago (Fig. 6). The taxon *O. laevis* probably corresponds to one of the lineages L5 and L9 to L15 from the Philippine archipelago. The reliable match to a specific lineage, however, remains equivocal, because the type locality was merely provided as ‘Philippines’ (Frost, 2018; Günther, 1858). The assignment of the nominal taxon *O. sumatrana* to one of the lineages (L16 to L18) is uncertain, due to the unspecific type locality ‘Sumatra’ in the original description of this taxon (Frost, 2020; Peters, 1877). The *O. lima* group L27, comprising all samples from the south-east Asian mainland, could be conspecific with *O. martensii*, because these samples were collected in central Thailand, relatively close to Bangkok. The type locality of *O. martensii* was mentioned as ‘Bangkok (Siam)’ (Frost, 2020; Peters, 1867). The identity of *O. lima*, however, remains unclear because this taxon was described from ‘Java’, Indonesia (Frost, 2020; Gravenhorst, 1829). In all these cases, morphological comparisons of newly collected and genetically barcoded vouchers with the respective type materials of these taxa are needed as first steps toward a taxonomic revision of this genus. Furthermore, a revision should provide careful re-descriptions of these species and should specify clarified, or newly-defined type localities as references, which may then allow formal description of new species of *Occidozyga*.

4.3. Taxonomic amendments concerning *Limnectes rhacodus*

Within the *O. rhacoda* group, L22 is considered here as the nominal taxon *Limnectes rhacodus* (Fig. 2). This species was originally described as *Rana rhacoda* by Inger et al., 1996, who stressed its uncertain generic placement. Later, this species was assigned to the closely related genus *Limnectes* (Fitzinger, 1843), due to phenotypic similarity with members of that genus (Hoffmann, 2000). Although tissue samples from the type locality of *L. rhacodus* in Kalimantan were not available for this study, material collected from the Kubah NP (ZMH A11641) and Gunung Penrisen (ZMH A11483) in western Sarawak, close to the Indonesian border, was identified as *L. rhacodus*, based on external morphological characters in adult specimens. The unique combination of characters described by Inger et al., 1996 and lacking thereof in most members of this genus (i.e., snout vent length < 24 mm, tips of toes swollen, tympanum partially obscured by skin, dorsolateral fold interrupted, inverted V-shaped fold between shoulders absent, dorsal skin with numerous transverse wrinkles) allows for reliable identification of *L. rhacodus*. Pui et al. (2013) came to the same conclusions and stressed the similarities between *L. rhacoda* and species of *Occidozyga* in terms of external morphology, particularly skin structure, body proportions, and colour patterns. Furthermore, the genetically assigned tadpoles of L22 of *L. rhacodus* and *Occidozyga* (see Haas et al., 2014) share highly derived unique larval features (unpubl. data), reinforcing their unambiguous identification as members of *Occidozyga*. In conclusion, we transfer *Limnectes rhacodus* (*Rana rhacoda* Inger et al., 1996), to the genus *Occidozyga*; the name *Occidozyga rhacoda*, comb. nov. is proposed here. Accordingly, the revised distributional range of *O. rhacoda* now covers central Kalimantan, West Kalimantan and western Sarawak (Pui et al., 2013).

4.4. Spatio-temporal patterns of diversification

The spatio-temporal pattern of the diversification of *Occidozyga* was reconstructed on the basis of the timeline obtained from a relaxed molecular clock approach calibrated by mitochondrial substitution rates (Fig. 6) and of the available paleogeographic reconstructions of Sundaland and adjacent biogeographic regions irrespective of whether or not these evolutionary lineages are considered taxonomic units (Fig. 5). From the Eocene to Early Miocene, northern Sundaland was

one continuous, emergent but relatively low landmass, and was connected with mainland south-east Asia (Hall, 1998, 2012, 2013; Meijaard, 2004; Wilson and Moss, 1999). At the southern margin of Sundaland were volcanic arcs, whose volcanoes formed isolated islands (Hall, 2013). At the beginning of the Miocene or even at the end of the Oligocene, collision between Sundaland and Australia and, later, between Sundaland and the continental margin of South China led to mountain building of the central ranges of Borneo (Hall, 2012; Meijaard, 2004; Wilson and Moss, 1999).

In general, the evolution of species-rich clades in Southeast Asian anurans has been reported as the result of adaptive (genus *Kaloula*; Blackburn et al., 2013) or non-adaptive radiations (genus *Limnonectes*; Setiadi et al., 2011; review: Brown et al. 2013). Because our knowledge of the ecological and morphological differentiation of *Occidozyga* species is currently limited (see Introduction), which also applies for the rate of lineage proliferation, we cannot distinguish between alternative hypotheses as to whether the ancestors of the five major clades evolved as a result of adaptive or non-adaptive radiations (Brown et al. 2013; Gittenberger, 1991; Grant, 1986; Losos, 2004; Losos and Ricklefs, 2009; Pincheira-Donoso et al., 2015, 2013; Reaney et al., 2018; Rundell and Price, 2009; Schluter, 2000).

Most lineage proliferations within the major clades probably occurred by non-adaptive diversification caused by allopatric divergence in areas isolated by biogeographic boundaries with minimal or no ecological diversification, and niche conservatism without pronounced phenotypic changes (Debandi et al., 2012; Evans et al., 2003; Gittenberger, 1991; Kozak and Wiens, 2006; Reaney et al., 2018; Rundell and Price, 2009; Schluter, 2000). The complex geological and climatic history of southeast Asia presumably provided ample opportunity for the spatial separation of species into new lineages by vicariance or isolation following dispersal events (Brown et al., 2013, 2016). Sumatra, Java and the Lesser Sunda Islands result from volcanic activity of the Sunda Arc starting from about 45 Ma, when Australia began to move northwards relatively rapidly (Barber and Crow, 2005; Hall, 2009). The Barisan Mountains on the western side of Sumatra began to elevate from the end of the Oligocene, or later at the beginning of the Miocene (Barber et al., 2005; Hall, 2009). In the early Miocene, at about 20 Ma, Sumatra was separated from the remaining landmasses of Sundaland by the strait of Malacca, formed by a significant marine incursion onto the Sunda Shelf (Hall, 2013; 2012). This scenario corresponds with the dating of about 18 Ma for the separation of the *Occidozyga sumatrana* clade from the last common ancestor of the *O. laevis* group, followed by *in situ* diversification on Sumatra. The extent of emergent land of Sumatra at this time is controversial: either proto-Sumatra was a larger land mass (Hall, 2012; 2009; 1996; Hall and Holloway, 1998) or a chain of islands (Hall, 2012; Hall and Holloway, 1998; Meijaard and Groves, 2004). Subsequently, a geological structure, the Lampung High, provided a connection between the southern part of Sumatra and the landmasses of northern Sundaland during the early Late Miocene (Hall, 2013; Hall and Holloway, 1998; Meijaard and Groves, 2004). At this time, at about 11 Ma, lineages of the *O. sumatrana* clade may have used this land bridge as a dispersal corridor back to Borneo. The descendants of these migrants are distributed today in western and central Sarawak in northwestern Borneo (L19 to L21).

In the middle and late Miocene, a volcanic arc between Sabah and the Sulu islands could have permitted a connection between northern Borneo and the Philippines (Hall, 2013). The intermittently emergent volcanic islands of this arc could have served as stepping stones for the colonization of the Philippine archipelago by the common ancestor of the Philippine clade, as short overseas dispersals would suffice to cross narrow channels and successfully disperse across Huxley's modification of Wallace's line (Huxley, 1868; Brown and Guttman, 2002; Brown et al., 2013; Brown, 2016). The origin of the sister group L8 of the Philippine clade, Tawau Hills Park in southeast Sabah, and the dating of the split between L8 and the common ancestor of the Philippine clade at about 12 Ma are consistent with this paleogeographic scenario. In the

Pleistocene, the Philippine islands were less fragmented than today, and formed composite Pleistocene aggregate island complexes (PAICs; Brown et al., 2002; Brown and Diesmos, 2009) that were isolated from one another by deep-water channels (Evans et al., 2003; Heaney, 1985; 1986). A rapid *in situ* radiation of *Occidozyga* lineages in the Philippines presumably could have begun soon after a single colonization event from Borneo—as suggested by numerous short branches among the Philippine lineages L9 to L15. This rapid diversification could have been triggered by dispersal among, and subsequent speciation within, PAICs (Chan et al., 2021).

The island of Palawan is a microcontinental fragment from the South China margin, that drifted south as part of the Dangerous Grounds between in the Eocene and Miocene (Hall, 2013; 2012; Hall and Holloway, 1998; Holloway, 1982; Meijaard and Groves, 2004). This drift, caused by the subduction of the proto-Western Philippine sea, continued slowly during the Miocene until Palawan came close to the northern tip of Borneo at about 9 Ma, similar to its present-day position—but remained separated from Borneo by a shallow sea. The colonization from northern Borneo to Palawan may therefore have been possible at this time, assuming short distances for overseas dispersal. A terrestrial connection between Borneo and Palawan by an exposed narrow land bridge would have been possible only during the Late Quaternary (Middle Pleistocene) with sea-level fluctuations of at least 135 m below present-day levels (Heaney, 1986; McGuire and Alcalá, 2000; McGuire and Kiew, 2001; Brown et al. 2013; Robles et al., 2015).

Additional time-calibrated analyses, based on application of divergence dates between Occidozyginae and Limnonectinae as an external calibration point obtained from previous studies (96.8 Ma, Zhang et al., 2013; 74.5 Ma, Wiens et al., 2009; 63.8 Ma, Roelants et al., 2007), yielded significantly older estimates for the divergence events within *Occidozyga* between 80 and 35 Mya. A similar bias towards implausibly old clade ages was found in phylogenetic analyses of *Ansonia*, particularly by Matsui et al. (2010) and by Sanguila et al. (2011). The authors of the latter study argued that artefacts of the external calibration procedure – extreme rate variations in combination with other factors – could produce incorrect temporal calibrations (see also Graur and Martin, 2004). In comparison to the scenario presented above, the resulting alternative scenarios obtained with different calibrations are regarded less likely for the following reasons: First, between 49 and 30 Ma Sundaland was a continuous peninsula of the Asian continent fringed by volcanic arcs, and it was only much later in the early Miocene that Sumatra became a chain of volcanic islands (Hall, 2013; 2012; Meijaard and Groves, 2004). Second, the land bridge (Lampung High), between Sumatra and Borneo emerged in the late Miocene and provided a link for the dispersal back to Borneo. Sumatra was formed by the volcanic activity of the Sunda Arc only between 25 and 15 Ma (Hall, 2013; 2012; Hall and Holloway, 1998). Third, the most important argument against the older estimates of the diversification of *Occidozyga* is the paleogeography of Palawan. The Dangerous Grounds, which later became Palawan, rifted from the Asian mainland between the Eocene and Early Miocene, and reached its present position close to the northern shore of Borneo in the late Miocene (Fig. 9 in Hall, 2013; Yumul et al., 2005, 2009). This scenario corresponds well with our estimation of the divergence time of the Palawan lineage at about 9 Mya from the Bornean lineages of the *O. laevis* group by a short-distance overseas dispersal. It is still a matter of debate whether parts of Palawan were land-positive as it drifted south, or if Palawan was submerged for portions of its time as it drifted east (Hall, 1996; 2002; Meijaard and Groves, 2004). Holloway (1982) described a phase of uplift of land in the late Middle Miocene. Thus, an overseas dispersal from Sundaland to Palawan between 22 and 13 Ma is less plausible: not only because a much greater distance between the northern shoreline of Borneo and Palawan would have to have been traversed, but also because much of Palawan may even have been submerged at that time (Blackburn et al., 2010; Hall, 2013; 2012; Hall and Holloway, 1998; Yumul et al., 2005, 2009). In contrast to our results, the spatio-temporal pattern in *Barbourula* are more consistent with

the Palawan Raft hypothesis, according to which ancestors of the present species were isolated on Palawan, rafted from the Asian mainland southwards and colonized Borneo when Palawan reached its present position (Blackburn et al., 2010). However, our time-calibrated phylogeny of *Occidozyga* does not fit such isolation on Palawan for 40–9 mya. The Out-of-Palawan scenario described above was also evident for *Gekko* and *Sanguirana*, which dispersed northward at around the same time as demonstrated here for *Occidozyga*, or earlier, as the oceanic islands of the central and northern portions of the archipelago assembled into their current configuration, via the Mobile Philippine Belt (Brown et al., 2016; Chan and Brown, 2017; Siler et al., 2012).

4.5. Conclusion

Our preferred temporal framework based on mitochondrial substitution rates of the diversification of *Occidozyga* described in this study is generally consistent with the reconstruction of the paleogeography of Southeast Asia. The resulting scenario incorporates a complex spatio-temporal pattern of subsequent vicariance and dispersal events starting from today's Borneo caused by plate tectonics, orogeny, and climatic oscillations resulting in sea level fluctuations. De Bruyn et al. (2014) conducted a meta-analysis of phylogenetic studies in different groups of organisms from Southeast Asia and found a statistically higher level of diversity in Borneo and Indochina compared to Java and Sumatra. The long period of relative tectonic and climatic stability of northern Sundaland, including Borneo, for instance, was inferred by these authors as having paved the way for high levels of *in situ* diversification (de Bruyn et al., 2014). The uplift of mountain ranges, changes in the course of drainage systems and oscillating moderate climatic changes, probably altered the distribution of vegetation types like forests and savannah, separated populations temporarily and triggered subsequent allopatric speciation (Cannon et al., 2012; Heaney, 1991; van der Kaars and De Deckker, 2002). De Bruyn et al. (2014) argue that these highly diverse regions, Borneo and Indochina, have acted as sources of biodiversity for the remaining parts of Sundaland and the adjacent regions, which were colonized later in the Pliocene and Pleistocene. The evolutionary pattern detected in *Occidozyga* agrees markedly well with such an 'Out-of-Borneo' source of evolutionary lineages hypothesis, although diversification began some considerable time before the Pliocene and Pleistocene. In this study, we propose a scenario in which Borneo, as the largest part of northern Sundaland, was the geographic origin of the highest diversity of *Occidozyga* evolutionary lineages. From Borneo, the surrounding landmasses of the Sunda Shelf, Palawan, and the remaining Philippine archipelago were subsequently colonized by single dispersal events.

5. Acronyms of museum collections

Natural History Museum Bern (NMBE), Zoological Museum Hamburg (ZMH), Institute of Biodiversity and Environmental Conservation Kuching (IBEC, UNIMAS), Field Museum of Natural History Chicago (FMNH), Natural History Museum Erfurt (NME), Biodiversity Institute and National History Museum of Kansas (KU).

CRedit authorship contribution statement

Jana M. Flury: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization, Funding acquisition. **Alexander Haas:** Methodology, Validation, Investigation, Resources, Data curation, Writing - review & editing. **Rafe Brown:** Methodology, Validation, Investigation, Resources, Data curation, Writing - review & editing. **Indraneil Das:** Investigation, Writing - review & editing. **Pui Yong Min:** Investigation, Resources, Writing - review & editing. **Kueh Boon-Hee:** Investigation, Resources, Writing - review & editing. **Ulrich Scheidt:** Investigation, Resources, Writing - review & editing. **Djoko T. Iskandar:**

Investigation, Resources, Writing - review & editing. **Andre Jankowski:** Investigation, Resources, Writing - review & editing. **Stefan T. Hertwig:** Conceptualization, Methodology, Validation, Investigation, Resources, Data curation, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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