

Phylogenetic relationships of *Ansonia* from Southeast Asia inferred from mitochondrial DNA sequences: Systematic and biogeographic implications (Anura: Bufonidae)

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ABSTRACT

We investigated the phylogenetic relationships and estimated the history of species diversification and biogeography in the bufonid genus *Ansonia* from Southeast Asia, a unique organism with tadpoles adapted to life in strong currents chiefly in montane regions and also in lowland rainforests. We estimated phylogenetic relationships among 32 named and unnamed taxa using 2461 bp sequences of the mitochondrial 12S rRNA, tRNA^{val}, and 16S rRNA genes with equally-weighted parsimony, maximum likelihood, and Bayesian methods of inference. Monophyletic clades of Southeast Asian members of the genus *Ansonia* are well-supported, allowing for the interpretation of general biogeographic conclusions. The genus is divided into two major clades. One of these contains two reciprocally monophyletic subclades, one from the Malay Peninsula and Thailand and the other from Borneo. The other major clade primarily consists of Bornean taxa but also includes a monophyletic group of two Philippine species and a single peninsular Malaysian species. We estimated absolute divergence times using Bayesian methods with external calibration points to reconstruct the relative timing of faunal exchange between the major landmasses of Southeast Asia.

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1. Introduction

Oriental stream toads of the genus *Ansonia* Stoliczka, 1870 encompass 26 described species (Frost, 2009), but there remain at least five taxa still requiring formal descriptions (Matsui, unpublished data). They have a disjunct distribution, with two species in India and the remaining species in Southeast Asia (Matsui et al., 1998; Frost, 2009). In Southeast Asia, the genus is distributed

across the major landmasses of the Sunda Shelf (Voris, 2000), including the Malay Peninsula (Wood et al., 2008) as far north as central Thailand (Matsui et al., 2005b; Grismer, 2006a,b), Borneo (Inger, 1966; Malkmus et al., 2002), the Mindanao faunal region of the Philippines (Alcala and Brown, 1998), and a part of Sumatra (Iskandar and Mumpuni, 2004). This wide distribution, spanning the edge of the Asian mainland, continental land-bridge islands of the Sunda Shelf, and oceanic islands beyond the continental shelf makes *Ansonia* an ideal candidate for a phylogeny-based biogeographic study.

Despite this, an analysis of species-level relationships that would enable a biogeographic study is thus far lacking. Taking advantage of recently acquired taxon sampling and the fact that

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the monophyly of the *Ansonia* with respect to other bufonid genera is not contentious (Graybeal and Cannatella, 1995), we provide the first phylogenetic and biogeographic analysis of the genus. We estimate the phylogeny of Southeast Asian members of the genus *Ansonia* using mitochondrial gene sequences that have proven useful for elucidating species level relationships in many other groups (e.g., Tanaka et al., 1996; Wilkinson et al., 2002; Evans et al., 2003; Matsui et al., 2005a,c, 2006; Garda and Cannatella, 2007).

2. Materials and methods

2.1. Taxon sampling

We examined DNA sequences of 12S and 16S rRNA genes and the intervening tRNA gene for valine from 79 specimens of 32 species (including undescribed taxa) of the genus *Ansonia*, its putative allies, and a distant outgroup (*Dendrobates auratus* [Girard, 1855]

Table 1
Samples used for mtDNA analysis in this study together with the information on species identification, voucher, provenance, and GenBank accession numbers. RMB = Rafe M. Brown field collection numbers; specimens deposited in the National museum of the Philippines; ACD = Arvin C. Diesmos field collection numbers; specimens deposited in the National museum of the Philippines; BOR = BORNEENSIS collection of Institute for Tropical Biology and Conservation, University Malaysia Sabah; KUHE = Graduate School of Human and Environmental Studies, Kyoto University; SP = Sabah Parks; LSUHC = La Sierra University, Herpetological Collection; ZRC = Raffles Museum of Biodiversity Research, National University of Singapore. L = larva, UN = unnumbered.

Sample No.	Species	Locality	n	Data Bank Accession Nos.	Voucher
1	<i>Ansonia</i> sp. 1	Thailand, Kanchanaburi, Pilok	1	AB435249	KUHE 35066
2	<i>Ansonia kraensis</i>	Thailand, Ranong	3	AB435250–AB435252	KUHE 23517, 35814, 35817
3	<i>Ansonia inthanon</i>	Thailand, Doi Inthanon	1	AB435253	KUHE 19050
4	<i>Ansonia</i> sp. 2	Thailand, Pukhet	1	AB435254	KUHE 38071
5	<i>Ansonia siamensis</i>	Thailand, Khaochong	2	AB435255, AB435256	KUHE 23438, 23515
6	<i>Ansonia endauensis</i>	West Malaysia, Johor, Endau-Rompin	1	AB435257	LSUHC 7726
7	<i>Ansonia tiomanica</i>	West Malaysia, Pahang, Tioman Is.	2	AB435258, AB435259	LSUHC 2616, 4443
8	<i>Ansonia latirostra</i>	West Malaysia, Pahang, Sg. Lembing	2	AB435260, AB435261	LSUHC 4923, 4924
9	<i>Ansonia penangensis</i>	West Malaysia, Penang Is.	2	AB435262, AB435263	KUHE UNL (n = 2)
10	<i>Ansonia malayana</i>	West Malaysia, Larut	2	AB331712, AB435264	KUHE 15467, 15472
11	<i>Ansonia jeetskumarani</i>	West Malaysia, Pahang, Fraser's Hill	2	AB435265, AB435266	LSUHC 8049, 8050
12	<i>Ansonia platysoma</i>	East Malaysia, Sabah, Crocker, Mahua	2	AB435267, AB435268	BOR 12499, 12500
13	<i>Ansonia platysoma</i>	East Malaysia, Sabah, Kinabalu, Sayap	1	AB435269	BOR 23347
14	<i>Ansonia platysoma</i>	East Malaysia, Sabah, Kinabalu, Bundu Tuhan	2	AB435270, AB435271	KUHE L04B131, 132
15	<i>Ansonia platysoma</i>	East Malaysia, Sabah, Kinabalu, Poring	1	AB435272	ZRC 1.11917
16	<i>Ansonia</i> sp. 3	East Malaysia, Sarawak, Bario	4	AB435273–AB435276	KUHE 12380, 12433, 12434, 12448 BOR 22640, 22641, 22642
17	<i>Ansonia hamtschi</i>	East Malaysia, Sabah, Kinabalu, Silau Silau	3	AB331710, AB435277, AB435278	BOR 09247
18	<i>Ansonia spinulifer</i>	East Malaysia, Sabah, Tawau	1	AB435284	KUHE L05B005
19	<i>Ansonia spinulifer</i>	East Malaysia, Sabah, Maliau	1	AB435285	KUHE L04B138, 139, BOR 08433
20	<i>Ansonia spinulifer</i>	East Malaysia, Sabah, Crocker, Ulu Kimanis	3	AB435286–AB435288	KUHE 12065
21	<i>Ansonia spinulifer</i>	East Malaysia, Sarawak, Kuching	1	AB435289	LSUHC 4046, 4047
22	<i>Ansonia spinulifer</i>	East Malaysia, Sarawak, Sadong River	2	AB435290, AB435291	KUHE 17182
23	<i>Ansonia spinulifer</i>	East Malaysia, Sarawak, Gading	1	AB435292	BOR 08424, 08475
24	<i>Ansonia</i> sp. 4	East Malaysia, Sabah, Crocker, Ulu Kimanis	2	AB435279, AB435280	KUHE 12058, 17233, 17274
25	<i>Ansonia minuta</i>	East Malaysia, Sarawak, Kuching	3	AB435281–AB435283	BOR 12463
26	<i>Ansonia longidigita</i>	East Malaysia, Sabah, Crocker, Mahua	1	AB331711	BOR 12862
27	<i>Ansonia longidigita</i>	East Malaysia, Sabah, Crocker, Ulu Kimanis	1	AB435293	KUHE L04B133
28	<i>Ansonia longidigita</i>	East Malaysia, Sabah, Bundu Tuhan	1	AB435294	KUHE-12405
29	<i>Ansonia longidigita</i>	East Malaysia, Sarawak, Bario	1	AB435295	ZRC 1.11918
30	<i>Ansonia torrentis</i>	East Malaysia, Sarawak, Gn. Mulu NP	1	AB435296	BOR 22139
31	<i>Ansonia leptopus</i>	East Malaysia, Sabah, Tawau	1	AB435297	KUHE 17109
32	<i>Ansonia leptopus</i>	East Malaysia, Sarawak, Gading	1	AB435298	LSUHC 4991, 4992
33	<i>Ansonia latiffi</i>	West Malaysia, Pahang, Sg. Lembing	2	AB435299, AB435300	KUHE 17381, 17486
34	<i>Ansonia</i> sp. 5	East Malaysia, Sarawak, Lanjak Entimau	2	AB435301, AB435302	KUHE 17377, 17499, 17503
35	<i>Ansonia albomaculata</i>	East Malaysia, Sarawak, Lanjak Entimau	3	AB435303–AB435305	BOR 22928, KUHE L06B054
36	<i>Ansonia guibeii</i>	East Malaysia, Sabah, Kinabalu, Mesilau	2	AB435306, AB435307	BOR 22770, KUHE 17537
37	<i>Ansonia fuliginea</i>	East Malaysia, Sabah, Kinabalu, Pakka	2	AB331709, AB435308	ACD3543
38	<i>Ansonia muelleri</i>	Philippines, Mindanao	1	AB435309	RMB 639, 642
39	<i>Ansonia muelleri</i>	Philippines, Mindanao, Davao City	2	AB435310, AB435311	ACD 1617, 1640
40	<i>Ansonia muelleri</i>	Philippines, Mindanao, Davao, Mt. Apo	2	AB435312, AB435313	ACD 2631, 2702
41	<i>Ansonia muelleri</i>	Philippines, Mindanao, Mt. Hamigitan	2	AB435314, AB435315	ACD3600, 3601
42	<i>Ansonia mcgregori</i>	Philippines, Mindanao	2	AB435316, AB435317	SP 26033
43	<i>Sabahphrynus maculatus</i> (= <i>Ansonia anotis</i>)	East Malaysia, Sabah, Kinabalu	1	AB331708	BOR 08425
44	<i>Sabahphrynus</i> (= <i>Pedostibes</i>) <i>maculatus</i>	East Malaysia, Sabah, Crocker, Ulu Kimanis	1	AB331718	KUHE 35585
45	<i>Pelophylyne signata</i>	West Malaysia, Genting	1	AB331720	BOR 22088
46	<i>Pelostibes hosii</i>	East Malaysia, Sabah, Tawau	1	AB331717	BOR 08127
47	<i>Leptophryne borbonica</i>	East Malaysia, Sabah, Crocker, Ulu Kimanis	1	AB331716	KUHE 39029
48	<i>Bufo</i> (= <i>Duttaphrynus</i>) <i>melanostictus</i> <i>Dendrobates auratus</i>	West Malaysia, Penang Is.	1	AB435318 AY326030	

from GenBank; Table 1). Our sampling included 27 (22 described and five undescribed) species of *Ansonia*, one species each of *Sabaphryne* Matsui, Yambun, and Sudin, 2007, *Pedostibes* Günther, 1876, *Pelophryne* Barbour, 1938, *Leptophryne* Fitzinger, 1843, and *Bufo* Laurenti, 1768 (sensu lato) (Fig. 1 and Table 1). Our sampling also consists of a number of undescribed taxa. *Ansonia* sp. 1 and *Ansonia* sp. 2 are Thai species that resemble morphologically *A. inthanon* Matsui, Nabhitabhata, and Panha, 1998 and *A. kraensis* Matsui, Khonsue, and Nabhitabhata, 2005, respectively, in appearance. *Ansonia* sp. 2 has been reported as *A. kraensis* by Grismer (2006b), but differs in details of adult morphology. *Ansonia* sp. 3, *Ansonia* sp. 4, and *Ansonia* sp. 5 from Eastern Malaysia are morphologically similar to, but distinct from, *A. hanitschi* Inger, 1960, *A.*

minuta Inger, 1960, and *A. leptopus* (Günther, 1872), respectively. The recently described *A. jeetsukumarani* Wood, Grismer, Ahmad, and Senawi, 2008 and *A. latiffi* Wood, Grismer, Ahmad, and Senawi, 2008 from West Malaysia are morphologically similar to *A. malayana* Inger, 1960 and *A. leptopus*, respectively (Wood et al., 2008).

2.2. Preparation of DNA, PCR, and DNA sequencing

Tissues were obtained from either frozen or ethanol-preserved specimens. Total genomic DNA was extracted using standard phenol-chloroform extraction procedures (Hillis et al., 1996). We amplified targeted sequences through use of the polymerase chain reaction (PCR), using 10 primers combinations (Table 2) for 2.4 kb

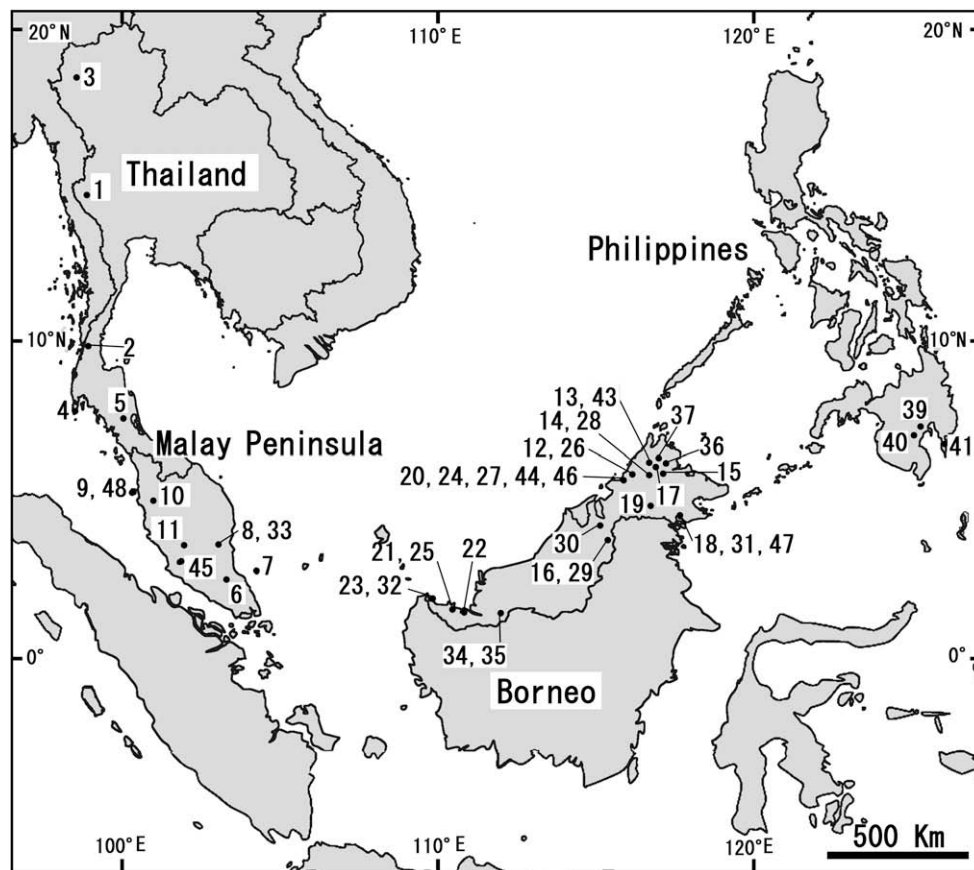


Fig. 1. Map of Southeast Asia showing sampling localities of *Ansonia* species included in this study. Locality numbers are included in Table 1.

Table 2

Primers used in this study. Sequence position indicates the starting position of the primer in the *Bufo* (= *Duttaphrynus*) *melanoticus* mitochondrial genome (Zhang et al., 2005) and is preceded by the amplified direction as indicated by heavy (H) or light (L) strand.

Name	Sequence position	Sequence 5'–3'	Reference
ThrLm	L93	AAARCATKGGTCTTGTAARCC	Modified from Shaffer and Mcknight (1996)
12S-3H	H795	CAAGTCCTTTGAGTTTAAAGCT	Made in this study
12SL1091	L698	AAACTGGGATTAGATACCCCACTAT	Modified from Kocher et al. (1989)
12StVal-H	H1546	AAGTAGCTCGCTTAGITTCGG	Made by T. Shimada
12S2	L1099	TACACACCCGCCGTACCCCTC	Kocher et al. (1989)
16Sa-H	H2140	ATGTTTTTGTAACAGCGCC	Palumbi et al. (1991)
16SL2021	L1619	CCTACCGAGCTTAGTRATAGCTGGTT	Tominaga et al. (2006)
16SH2715	H2363	AAGTCCATAGGGTCTCTCGTC	Tominaga et al. (2006)
16S1M	L2120	CCGACTGTTTACAAAAACAT	Fu (2000)
Hedges16H1	H2729	CTCCGGTCTGAACCTCAGATCACGTAGG	Hedges and Maxson (1993)

of 12S and 16S rRNA genes and the intervening tRNA gene for valine. Amplification was carried out in a 25- μ L reaction volume with Blend Taq (TOYOBO). The PCR cycle included an initial denaturation step of 5 min at 94 °C and 40 cycles of denaturation for 1 min at 94 °C, primer annealing for 1 min at 45–55 °C, and extension for 1–2 min at 72 °C. PCR products were purified using polyethylene glycol (PEG) purification procedures; these were used directly as templates for cycle sequencing reactions with fluorescent-dye-labeled terminators (ABI Prism Big Dye Terminators v.3.1 cycle sequencing kits). We cycle sequenced the amplified fragments using the same primers (Table 2). The sequencing reaction products were purified by ethanol precipitation following the manufacturer's protocol and then run on an automated ABI PRISM 3100 genetic analyzer. All samples were sequenced in both directions.

2.3. Sequence data

We initially acquired and edited sequence data for each sample using ABI PRISM Sequencing Analysis Software (V3.6.2). Alignment of data from all samples was initially performed using the ClustalW option in the BioEdit software (Hall, 1999). We obtained aligned sequences of 2461 bp (943 bp [12S], 74 bp [tRNA^{val}], and 1444 bp [16S]) that are deposited in GenBank (Accession Nos. AB435249–AB435318). We then performed tandem exploratory parsimony analyses following Moriarty and Cannatella (2004), in which (1) all nucleotide positions were included and (2) ambiguously aligned regions were excluded. We compared topology and parsimony bootstraps between the separate analyses and when these were not markedly different (not shown), we proceeded with subsequent analyses with ambiguous regions excluded. In total, 89 unalignable nucleotide positions were removed in final analyses for a total of 2372 aligned characters.

2.4. Phylogenetic analyses and divergence estimates

Phylogenetic relationships were estimated using maximum parsimony (MP), maximum likelihood (ML), and Bayesian methods (in BEAST, see below). We performed MP search using a heuristic search setting with random-addition sequences and TBR branch swapping in PAUP* 4.0b10 (Swofford, 2002). We determined the most appropriate model of sequence evolution using Akaike Information Criterion (AIC; Akaike, 1974) in Modeltest (v.3.7, Posada and Crandall, 1998) and selected the GTR+I+G model as the appropriate model for our data. We estimated phylogeny using ML under this model by selecting the best of 250 independent best-tree searches using GARLI v. 0.92 (Zwickl, 2006), each of which was run with 20,000 generations without likelihood improvement as a termination criteria. We assessed parsimony bootstrap support using non-parametric bootstrapping (Felsenstein, 1985) with 1000 pseudoreplicates in PAUP*, and 200 pseudoreplicates in GARLI to assess Likelihood bootstrap support. We regarded bootstrap values of 70% or greater for MP and ML, and Bayesian posterior probability of 0.95 as indicative of strong support (Huelsenbeck and Hillis, 1993; Hillis and Bull, 1993; Wilcox et al., 2002).

We extended our dataset by incorporating homologous bufonid sequences of the 12S/16S rRNA genes from Pramuk et al. (2007), with initial alignment using MUSCLE (Edgar, 2004) and subsequent corrective alignment by eye to reduce parsimony-informative characters. The final alignment had 162 taxa and 2850 characters before exclusion of ambiguous sites and 2462 characters after these sites were excluded. A time-calibrated phylogeny was estimated using BEAST (Drummond and Rambaut, 2007), which allows for the simultaneous Bayesian estimation of phylogeny and divergence times under non-autocorrelated relaxed

clock model. The BEAST analysis was run for 60 million generations under a GTR + G + I substitution model and uncorrelated log-normal “relaxed” clock rate model (Drummond et al., 2006). The MCMC chain was sampled every 1000 generations, for a total of 60,000 samples, and convergence to the stationary distribution was assessed through inspection of the likelihood and parameter sample plots in Tracer, and a burn-in of 10,000 samples was adopted. Large effective sample sizes (ESS) of all parameters across the post burn-in chain samples confirms that the MCMC chain was mixing well.

For calibration points, we used two of the calibrations provided by Pramuk et al. (2007): the Central/North American split (20 MYBP) as represented in the combined dataset by the age of the most-recent common ancestor of *Bufo* (= *Incilius*) *valliceps* Wiegmann, 1833 and *Bufo* (= *Anaxyrus*) *woodhousii* Girard, 1854, as well as the age of the *Bufo* (= *Rhinella*) *marinus* Linnaeus, 1758 clade (11 MY).

3. Results

3.1. Sequence and tree statistics

Sequence statistics for the three gene fragments and for the combined alignment, when all nucleotide positions were included, are provided in Table 3. Of 2461 nucleotides generated, 1141 were variable, and 931 were parsimony informative. The best substitution model selected by Modeltest was a general time-reversible model with a proportion of invariant sites and a gamma shape parameter estimated as 0.361 and 0.622, respectively (GTR + I + G; Rodriguez et al., 1990). We obtained 114 most-parsimonious trees with 4623 evolutionary steps, with a consistency index (CI) of 0.396 and a retention index (RI) of 0.787 (not shown). The likelihood value of the ML tree is $\ln L = -58,167.768$.

3.2. Phylogenetic relationships

Phylogenetic analyses employing three different optimality criteria yielded very slightly different topologies, and only the ML tree is presented in Fig. 2. Monophyly of bufonid taxa (*Bufo*, *Leptophryne*, *Pelophryne*, *Pedostibes*, *Sabahphryne*, and *Ansonia*) with respect to the outgroup *Dendrobates* was not supported, but monophyly of the genus *Ansonia* was always strongly supported.

Two major clades (Clades A and B in Fig. 2) of *Ansonia* were recovered with strong support, and with an uncorrected *p*-distance of 12.2%. The first of these (Clade A) consisted of two reciprocally monophyletic subclades: a mainland (Malay Peninsula and Thailand) clade (Subclade A1) with 11 species and a Bornean clade (Subclade A2) with six species (uncorrected *p*-distance between the two subclades = 11.1%). The other main monophyletic clade (Clade B) consists of one Malayan and two Philippine species nested within a predominantly Bornean (seven species) clade. Each of these clades/subclades was well supported in ML analyses (Fig. 2) and was also recovered, with strong support, in the MP and BEAST analyses.

Table 3

Alignment statistics for fragments of the 12S rRNA, tRNA^{val} and 16S rRNA (all nucleotide positions included); number of base pairs (bp), number of variable sites (vs), number of parsimony informative sites (pi), the transition-transversion ratio given for ingroups only (ti/tv).

	bp	vs	pi	ti/tv
12S rRNA	943	396	323	2.155
tRNA ^{val}	74	49	36	4.506
16S rRNA	1444	696	572	1.581
combined	2461	1141	931	1.814

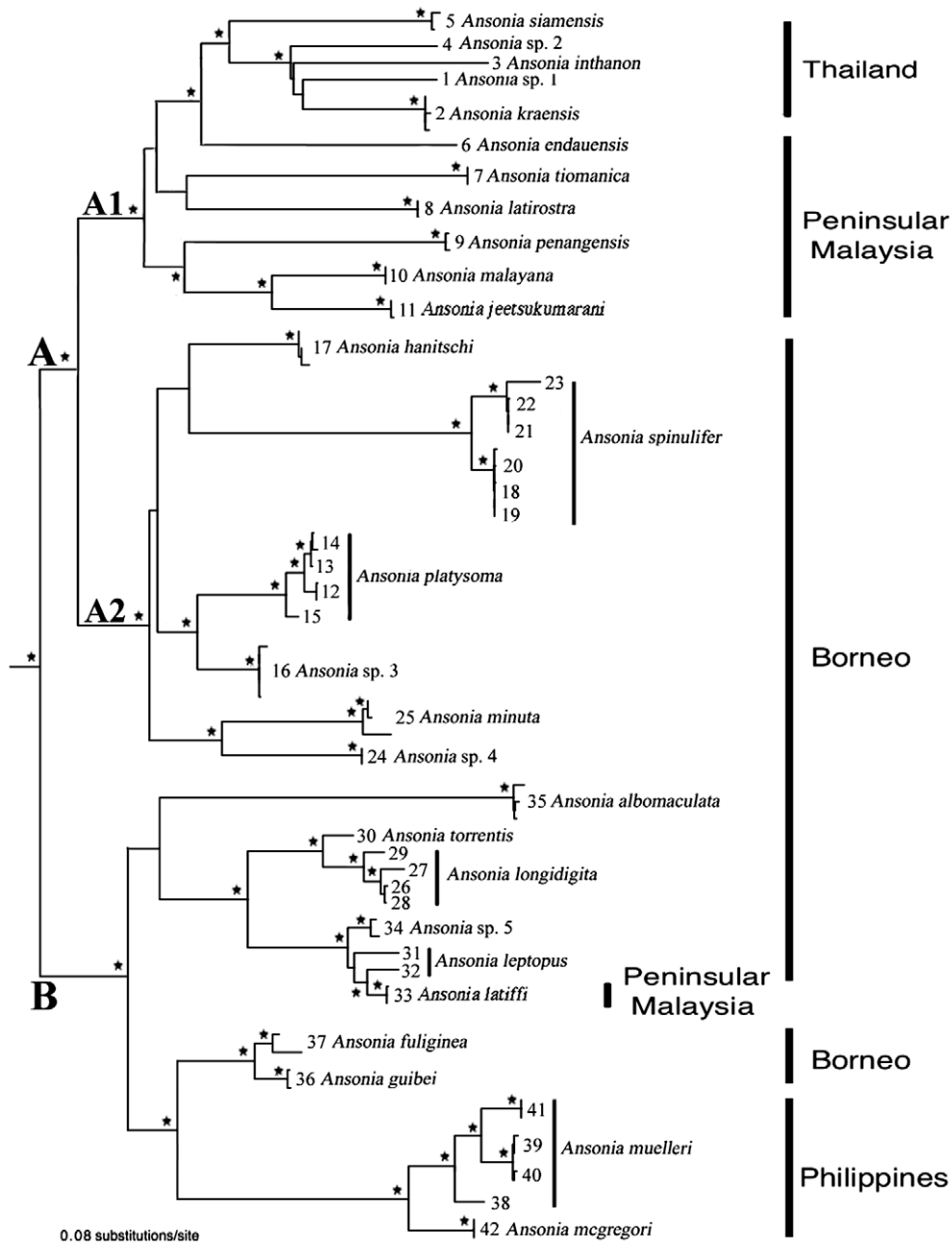


Fig. 2. Maximum likelihood phylogram from GARLI analysis of 12S rRNA, tRNA^{val} and 16S rRNA mitochondrial genes. Asterisks indicate nodes with significant Bayesian posterior probability (>0.95) and bootstrap supports for ML and MP (>70%) inferences. The number followed by species name corresponds to the locality number shown in Table 1 and Fig. 1.

One of the groups within the Subclade A1 contained *A. jeetsukumarani* from the Titi Wangsa Mountains, West Malaysia (Wood et al., 2008) and its sister species *A. malayana* from the adjacent Banjaran Bintang mountains (uncorrected *p*-distance = 5.3%). The lineage most closely related to these taxa, *A. penangensis* Stoliczka, 1870, is endemic to Penang Island lying immediately off the adjacent west coast (uncorrected *p*-distance = 9.0% from *A. malayana* plus *A. jeetsukumarani*). The remaining species of the Subclade A1 composed a poorly supported sister group containing the sister species *A. latirostra* Grismer, 2006 and the insular endemic from Tioman Island, *A. tiomanica* Hendrickson, 1966 (uncorrected *p*-distance = 10.4%), and its well supported sister clade consisting of a basal West Malaysian species, *A. endauensis* Grismer, 2006 from the southern tip of the Malay Peninsula, and supported group con-

taining all five Thai species (uncorrected *p*-distances from *A. endauensis* < 9.6%). Within the Thai species group, *A. siamensis* Kiew, 1985 from southern Thailand is the first diverging lineage (uncorrected *p*-distance from the other species = 8.6%), but the relationships among its well supported sister clade of the remaining species (uncorrected *p*-distances = 6.1–7.6%) were unresolved. The three samples of *A. kraensis* from the type locality of Ranong, Isthmus of Kra, grouped together but did not form a clade with *Ansonia* sp. 2 from the Phuket Island. Also in this group were *A. inthanon* from northwestern Thailand and *Ansonia* sp. 1 from Pilok, Kanchanaburi.

The Bornean group of the Subclade A2 was represented by four major lineages whose relationships were unresolved. The first of these is composed the supposed sister relationship between

A. minuta from western Sarawak and *Ansonia* sp. 4 from the Crocker Range (uncorrected *p*-distance = 6.6%). Another lineage containing only *A. spinulifer* (Mocquard, 1890) showed moderately deep separation (uncorrected *p*-distance < 2.8%) between populations from Sarawak and Sabah. A third lineage consisted only of *A. hanitschi* from Kinabalu, Sabah, and the fourth lineage was composed of the clade made of the sister species *A. platysoma* Inger, 1960 from the Crocker Range and Kinabalu and *Ansonia* sp. 3 from Bario, northern Sarawak (uncorrected *p*-distance = 4.4%).

The Clade B consisted of Bornean and Philippine taxa with one species, *A. latiffi* from central West Malaysia, nested among the Bornean species. Three major lineages were recognized in this clade, but their relationships to one another were unresolved. One of these major lineages contained the two Philippine sister species *A. muelleri* (Boulenger, 1887) and *A. mcgregori* (Taylor, 1922) (uncorrected *p*-distance = 4.3%) with the former showing moderate levels of divergence between population on Mindanao (uncorrected *p*-distances < 2.7%). The Philippine species formed a well-supported relationship with the sister species *A. fuliginea* (Mocquard, 1890) and *A. guibei* Inger, 1966 from Mt. Kinabalu, Sabah (uncorrected *p*-distance = 2.3%, and <9.0% between Philippine and Kinabalu species). *A. albomaculata* Inger, 1960 from Lanjak Entimau, Sarawak formed the second major lineage of Clade B and the third major lineage is a well-supported group composed of the remaining five species. This third supported lineage contains *A. longidigita* (Günther, 1872) from Sabah and Sarawak and *A. torrentis* Dring, 1984 from Mt. Mulu, Sarawak (uncorrected *p*-distance = 2.6%) forming one lineage, and a group containing *A. leptopus*, *A. latiffi*, and *Ansonia* sp. 5 forming the other (uncorrected *p*-distance between the lineages < 6.1%). The latter group (uncorrected *p*-distances between samples < 2.2%) was peculiar in that it is the only instance where a West Malaysian species, *A. latiffi*, was nested within a clade of Bornean species, and grouped in one clade with *A. leptopus* from Gading, Sarawak, making the latter species paraphyletic.

3.3. Divergence time estimation

Divergence time estimations reveal large overlap of 95% credibility interval on estimates of divergence times of many major clades of Southeast Asian *Ansonia* (Table 4), which precludes a confident conclusion with regards to the timing of colonization of the Sunda Shelf landmasses. However our data suggest that Southeast

Asian *Ansonia* diverged from remaining bufonids sampled in this analysis approximately 75 mya (95% Credibility Interval [CI] of 56.4–95.2 MYBP), with a Most Recent Common Ancestor [MRCA] of 66.4 MYBP (CI 49.5–84.0).

One major clade, Clade A, consisting of reciprocally monophyletic subclades from Malay Peninsula and Thailand (Subclade A1) and Borneo (Subclade A2) is approximately the same age as the other, predominantly Bornean Clade B (Table 4). The split between the Subclades A1 and A2 was estimated at 58.3 MYBP (CI 42.6–75.2), with the MCRA of the Malayan Subclade A1 estimated at 47.9 MYBP (CI 33.5–62.0) and that of the Bornean Subclade A2 estimated at 44.3 MYBP (CI 29.8–59.3).

Our data suggest at least two dispersal events within the predominantly Bornean Clade B: one to the Philippines and one to the Malay Peninsula. The Philippine taxa diverged from Bornean lineages 39.0 MYBP (CI 25.3–53.5), with a MRCA of 20.2 (9.8–31.8 MYBP). The single Malayan species within the predominantly Bornean clade dispersed with a split from Bornean lineages at 6.4 MYBP (CI 1.7–12.6) and an MRCA 0.4 MYBP (CI 0.0–1.2).

4. Discussion

4.1. Systematic implications

Southeast Asian bufonid genera were paraphyletic, seemingly consistent with recent studies emphasizing polyphyly even within the genus *Bufo* (sensu lato: e.g., Graybeal, 1997; Frost et al., 2006). Former *Ansonia anotis* Inger, Tan, and Yambun, 2001 was not grouped with other *Ansonia* taxa, but formed a well-supported clade with *Pedostibes maculatus* (Mocquard, 1890). These two species share the lack of a tympanum (Inger, 1966; Inger et al., 2001), and are not distinguishable from each other morphologically or ecologically. They have recently been considered conspecific, and were placed in a distinct genus, *Sabahphrynus* (Matsui et al., 2007).

As unambiguously supported in our results, monophyly of the genus *Ansonia* seems almost certain. All the known tadpoles in this genus possess a unique suite of synapomorphies associated with a large oral sucker for adhering to rocks in fast-flowing, high gradient streams (Inger, 1966; Matsui et al., 2005b). Possible tadpole of *Sabahphrynus* (= former *Ansonia*) *anotis* has a large oral sucker, too, but it additionally possesses a large abdominal sucker, which is absent in *Ansonia* larvae (Matsui et al., 2007).

This is the first to elucidate the phylogenetic relationships of this genus, allowing the study of several remaining unresolved taxonomic and biogeographic issues. We include all named taxa from Southeast Asia except for two species (*A. latidiscia* Inger, 1966 from Kalimantan and Sarawak, Borneo and *A. glandulosa* Iskandar and Mumpuni, 2004 from Sumatra). We also failed to obtain samples for the two Indian species; their generic assignment requires future confirmation before we can confidently conclude that they should be placed in the genus *Ansonia*.

Our analysis has proven useful for delimiting species boundaries and reaffirming existing species level taxonomy. Several allopatric populations were grouped into divergent, paraphyletic units, emphasizing the need for taxonomic reappraisal of those species. Our analysis also proved useful for identifying several cryptic candidate species. Several morphologically diagnosable putative undescribed species were supported as genetically distinct lineages, further bolstering up our confidence in recent and pending taxonomic studies (Wood et al., 2008; Matsui et al., unpublished data).

The genus *Ansonia* is clearly divisible into two distinct clades. The first of these (Clade A) contains 17 species and is made of a large monophyletic group of 11 species from Peninsular Malaysia and Thailand (Subclade A1) and its reciprocally monophyletic clade

Table 4
Estimated divergence times (MY) of main divergences of *Ansonia*.

Combination	Mean	CI
<i>Pelophryne signata</i> / <i>Ansonia</i>	74.9	(56.4–95.2)
<i>Ansonia</i> Clade A/Clade B	66.4	(49.5–84.0)
<i>Ansonia</i> Subclade A1/Subclade A2	58.3	(42.6–75.2)
Within <i>Ansonia</i> Clade B	51.5	(35.9–68.7)
Within <i>Ansonia</i> Subclade A1	47.9	(33.5–62.0)
Within <i>Ansonia</i> Subclade A2	44.3	(29.8–59.3)
<i>A. fuliginea</i> + <i>A. guibei</i> / <i>A. muelleri</i> + <i>A. mcgregori</i>	39.0	(25.3–53.5)
Thailand species/ <i>A. endauensis</i>	36.3	(23.7–49.8)
<i>A. siamensis</i> /other Thailand species	30.1	(19.0–43.2)
<i>A. longidigita</i> + <i>A. torrentis</i> / <i>A. leptopus</i> + <i>A. sp. 5</i> + <i>A. latiffi</i>	29.2	(16.6–45.5)
<i>A. minuta</i> / <i>A. sp. 4</i>	24.3	(10.6–39.2)
<i>A. platysoma</i> / <i>A. sp. 3</i>	23.2	(9.2–37.4)
<i>A. muelleri</i> / <i>A. mcgregori</i>	20.2	(9.8–31.8)
<i>A. malayana</i> / <i>A. jeetsukumarani</i>	19.8	(7.4–33.6)
<i>A. longidigita</i> / <i>A. torrentis</i>	14.6	(6.8–24.0)
<i>A. fuliginea</i> / <i>A. guibei</i>	13.0	(5.0–23.1)
Within <i>A. muelleri</i>	12.1	(5.4–20.0)
Within <i>A. spinulifer</i>	9.1	(3.3–16.6)
Within <i>A. longidigita</i>	7.2	(2.4–12.5)
<i>A. leptopus</i> (Sarawak)/ <i>A. latiffi</i>	6.4	(1.7–12.6)

of six species from Borneo (Subclade A2). Nearly one-fourth of the members of this clade species appear to be genetically distinct cryptic lineages, which may potentially warrant taxonomic recognition.

In the Subclade A1, *A. siamensis*, *A. inthanon*, and *A. kraensis* have been described from Thailand (Kiew, 1984; Matsui et al., 1998, 2005b), but suspected cryptic species, *Ansonia* sp. 1 (from Pilo, Kanchanaburi), and *Ansonia* sp. 2 (Puhket Island) are genetically distinct from these known taxa. It is conceivable that the monophyletic Thai group, whose internal relationships are unresolved, could constitute a single species. However the deep genetic divergences (uncorrected *p*-distances = 6.1–7.6%) between these terminals support our contention that each is a distinct evolutionary lineage. Future morphological studies will be necessary to determine whether *Ansonia* sp. 1 and *Ansonia* sp. 2 are morphologically diagnosable. Preliminary data (Matsui, unpublished data) suggest that *Ansonia* sp. 1 is phenotypically more similar to *A. inthanon* than it is to the other named taxa in this monophyletic clade (*A. siamensis* and *A. kraensis*). Together, these taxa from Thailand are most closely related to *A. endauensis* from near the tip of the Malay Peninsula (Grismer, 2006a), although they are genetically highly divergent (uncorrected *p*-distances > 9.6%). Other Peninsular Malaysian species, *A. malayana* (central Peninsula), *A. jeetsukumarani* (central Peninsula), and *A. penangensis* (Pinang Island), and *A. tiomanica* (Tioman Island) and *A. latirostra*, respectively, proved to be closely related, but with deep genetic divergences (uncorrected *p*-distances = 5.3–10.4%). The close relationship of *A. latirostra* and *A. tiomanica* appears to have first been postulated by Grandison (1972) in her referral of *A. latirostra* as “*Ansonia* ? *tiomanica*” (Grismer, 2006b).

The remaining six lineages in Clade A consist of a monophyletic group of Bornean taxa (Subclade A2). Surprisingly, *A. hanitschi* did not form a clade with its morphologically similar congener *A. platysoma*. *A. platysoma* is represented in our dataset by samples from Bundu Tuhan on Mt. Kinabalu (type locality), Sayap on Mt. Kinabalu, Poring, Mt. Kinabalu, and Crocker Range. Although these samples were not very divergent genetically (uncorrected *p*-distances < 1.4%), they varied in some morphological and acoustic characteristics, leading us to suspect that at least one cryptic species might be represented in these samples (Matsui, unpublished data). *Ansonia* sp. 3 from Bario, northern Sarawak, clustered with *A. platysoma* and superficially resembles with *A. hanitschi*. However, this population is readily distinguished morphologically and genetically (uncorrected *p*-distances between *A. platysoma* = 4.4%), and will eventually be recognized as another new species (Matsui, unpublished data).

Ansonia minuta is sister to *Ansonia* sp. 4. These two lineages cluster separately from *A. hanitschi* and *A. platysoma*, a relationship that is not surprising when considering the unique morphology of *A. minuta* (absence of nuptial pad in males and presence of distinct tarsal ridge: Inger, 1966). *Ansonia* sp. 4 from Crocker Range, Sabah, appears to represent another undescribed, morphologically distinct species (Matsui, unpublished data), with a high genetic divergence from *A. minuta* (uncorrected *p*-distance = 6.6%). One interesting species within the Bornean Subclade A2 is the genetically divergent *A. spinulifer* (uncorrected *p*-distances among samples < 2.8%). This species is larger than all other members of this clade (see above), and has long been synonymized with *A. leptopus* (Inger, 1960, 1966) or thought to be allied to *A. longidigita* (Inger and Dring, 1988). However, the strongly supported inclusion of *A. spinulifer* in this subclade conflicts with the proposed close relationship to *A. leptopus* and *A. longidigita*. Accordingly, the synonymisation of *A. spinulifer* to *A. leptopus* (Inger, 1960, 1966) or the association with *A. longidigita* (Inger and Dring, 1988) is untenable.

Interestingly, all peninsular and Thailand taxa of Subclade A1 have relatively small bodies (e.g., *A. endauensis*: male SVL = 17 mm,

female SVL = 29 mm [Wood et al., 2008]; *A. jeetsukumarani*: male SVL = 19–20 mm, female SVL = 25 mm [Wood et al., 2008]; *A. kraensis*: male SVL = 20–22 mm, female SVL = 24–28 mm [Matsui et al., 2005b]; *A. malayana*: male SVL = 20–22 mm, female SVL = 25–28 mm [Matsui et al., 2005b]; *A. latirostra*: male SVL = 22–24 mm, female SVL = 23–31 mm [Wood et al., 2008]; *A. inthanon*: male SVL = 23 mm, female SVL = 23–25 mm [Matsui et al., 1998]; *A. siamensis*: male SVL = 26–28 mm, female SVL = 32–35 mm [Matsui et al., 1998]; *A. tiomanica*: SVL = male SVL = 31 mm, female SVL = 38 mm [Wood et al., 2008]; *A. penangensis*: female SVL = 37 mm [Wood et al., 2008]). Bornean taxa (Subclade A2; see below) are also relatively small (e.g., *A. platysoma*: male SVL = 20–25 mm, female SVL = 20–26 mm [Wood et al., 2008]; *A. minuta* (male SVL = 21–24 mm, female SVL = 21–27 mm [Matsui, unpublished]; *A. hanitschi*: male SVL = 26–29 mm, female SVL = 29–32 mm [Matsui, 1979]), except for *A. spinulifer* (male SVL = 30–36 mm, female SVL = 40–44 mm [Matsui, unpublished]).

The remaining ten species of *Ansonia* fall into a well-supported monophyletic primarily Bornean Clade B. This clade is composed of seven Bornean species, two Philippine species, and one species from the Peninsular Malaysia (*A. latiffi*). Within this clade, the Bornean species *A. longidigita* and *A. torrentis* form a closely-related cluster, which is sister to of the cluster containing Bornean *A. leptopus* and *Ansonia* sp. 5 and *A. latiffi* from the Peninsula. Although biogeographically unexpected, this result is consistent with adult morphology; these species exhibit large body size (male SVL = 29–49 mm, female SVL = 51–64 mm) and *A. longidigita* and *A. leptopus* share a unique trait: the presumably derived long first finger (Inger, 1966). Exceptions to the morphological and life history homogeneity in this clade include the cases of *A. torrentis* which is not particularly large bodied (SVL = 31–33 mm: Dring, 1983), and *A. leptopus* which is uniquely reported to breed at the edges of rocky streams, contrasting all other *Ansonia* species that breed in strong currents (Inger, 1992; Inger and Voris, 2001).

Our analyses show that *A. latiffi* is sister to *A. leptopus* from Sarawak, and is the only Peninsular species that was grouped in this primarily Bornean Clade B. The genetic distance of *A. latiffi* from *A. leptopus* from Sarawak (uncorrected *p*-distance = 1.5%) was considerably smaller than distances observed between taxa in the peninsular clade or the Bornean clade. Past treatments of this species have reported it as *A. leptopus* from the Malay Peninsula (Grandison, 1972; Berry, 1975; Inger and Voris, 2001). However, as noted by Dring (1979), these central peninsular populations are morphologically distinct from, and not conspecific with, Bornean *A. leptopus* (Wood et al., 2008). Similarly, *Ansonia* sp. 5 from Sarawak is morphologically distinct from *A. leptopus* and warrants distinct taxonomic recognition (Matsui, unpublished data), although the degree of its genetic divergence from *A. leptopus* and *A. latiffi* is not great (uncorrected *p*-distances < 2.2%).

The close relationship between *A. fuliginea* (SVL = 22–36 mm in males and 38–44 mm in females) and *A. guibei* (SVL = 32 mm in males and 34 mm in females) from Borneo is not surprising because these two species are restricted to higher elevations of Mt. Kinabalu, Sabah (around Pakka and Mesilau, respectively) and may represent *Ansonia* relic evolutionary lineages. However, they were not genetically divergent (uncorrected *p*-distance < 2.3%) in spite of their marked morphological differentiation (e.g., presence in *A. guibei* and absence in *A. fuliginea* of unique cloacal skin flap). Similarly, monophyly of Philippine species of *A. muelleri* (SVL = 32–39 mm in males and 43–50 mm in females) and *A. mcgregori* (SVL of 25–31 mm in males and 30–38 mm in females) was expected on the basis their distribution (Mindanao Island, Philippines) and conspicuous morphological synapomorphies (e.g., absence of visible tympanum). Although *A. mcgregori* was once synonymized with *A. muelleri* (Inger, 1954), our analysis revealed a moderate degree of genetic divergence (uncorrected *p*-distances < 2.7%) among

Philippine samples and supported the current taxonomic arrangement, and the recognition of two separate species (Inger, 1960; Alcalá and Brown, 1998). However, sufficient interpopulational divergence (uncorrected p -distances < 2.4%) observed in our result indicates that *A. muelleri* may warrant additional taxonomic partitioning.

In numerous taxa (*A. mcgregori* and *A. muelleri* [uncorrected p -distances < 2.7%]; *A. longidigita*, *A. torrentis* [uncorrected p -distances < 2.6%]; *A. leptopus*, *A. latiffi*, and *Ansonia* sp. 5 [uncorrected p -distances < 2.2%]; *A. guibei* and *A. fuliginea* [uncorrected p -distance = 2.3%]), the degree of genetic divergence did not always conform to taxonomic status as defined by morphological and ecological characters. Minor genetic distance between two taxonomically distinct species has been reported in a variety of other lineages of amphibians (e.g., *Hynobius* Tschudi, 1838 [Nishikawa et al., 2005], *Amolops* Cope, 1865 [Matsui et al., 2006], *Bufo* [Igawa et al., 2006]).

4.2. Evolutionary history

Geological history of Southeast Asian regions where *Ansonia* occurs has recently been reviewed (Hall, 1998; Voris, 2000; Sathiamurthy and Voris, 2006). Inger and Voris (2001) have shown that the Malay Peninsula and Borneo has a long history of connections, and that disjunction by the South China Sea occurred by about 5 MYBP in the early Pliocene. The two regions are estimated to have again connected much later for a short while, during the last glacial maximum (0.021 MYBP; Sathiamurthy and Voris, 2006). Brown and Alcalá (1994) noted the geological history of the Philippines, and inferred that Mindanao became aerial about 25–30 MYBP in the late Oligocene, but that land formation of Palawan is much later at about 6 MYBP in the late Miocene. *Ansonia* is diversified most markedly at Mt. Kinabalu, northern Borneo, and the uplift of granite pluton was estimated to have required 1–2 MY to form this mountain (Myers, 1978). Much more recently, during the glaciations of the Pleistocene and Holocene, sea level changes occurred several times in the region of the Malay Peninsula, Borneo, Philippines, and Sumatra, and faunal exchange occurred in this region (Brown and Alcalá, 1994; Sathiamurthy and Voris, 2006).

Compared with these geological data, the divergence times estimated in the present study were very old; 75 (CI 56.4–95.2) MYBP between Southeast Asian *Ansonia* and the remaining bufonids, 66.4 (CI 49.5–84.0) MYBP between two major clades, and 58.3 (CI 42.6–75.2) MYBP between the Bornean and Malayan subclades, all in the late Cretaceous. These estimates are much older than the time of divergence between two Southeast Asian bufonids, *Leptophryne* and *Pedostibes* (ca. <20 MYBP) estimated by Roelants et al. (2007).

With regards to much younger events, there seems to have been two routes directly connecting Philippines and Borneo. One in the eastern island arc, connected Mindanao, and another in the west, connecting Palawan (Brown and Alcalá, 1994; Brown and Guttman, 2002). *Ansonia* occurs only on Mindanao, and not on Palawan, where many anuran species common to Borneo occur. Thus, the common ancestor of *A. muelleri* and *A. mcgregori* is inferred to have dispersed once from Borneo through the eastern entryway before the formation of the western entryway to Palawan, and diverged in the Philippines. The alternative scenario that implies that the common ancestor differentiated into two lineages on Borneo and that each lineage separately dispersed to the Philippines is less likely. A dual invasion hypothesis was invoked to explain distribution of the *Rana signata* complex in both the western (Borneo–Palawan–Mindoro) and eastern (Borneo–Mindanao–Leyte–Samar–Luzon) island arcs (Brown and Guttman, 2002), but unlike these ranids that occur on many islands of Philippines, the two *Ansonia* species are restricted to one island of Mindanao.

Inger (1999) estimated the speciation of *A. muelleri* and *A. mcgregori* to have been promoted by environmental fluctuations in the Pleistocene. Our result, however, suggested that the two Philippine lineages diverged from each other 20.2 (9.8–31.8) MYBP in end of the Miocene, which is much older than Inger's (1999) estimate. Similarly, *A. latiffi*, a single Malayan species within the predominantly Bornean clade, is considered an invader via a Pleistocene land bridge, but was estimated to have been split from Bornean lineages much older at 6.4 (CI 1.7–12.6) MYBP in the end of the Miocene.

Macey et al. (1998), in studying evolution in Chinese species of the *Bufo bufo* group, used ND1, ND2 and intervening genes, and set the vicariance date of 10 MY between Asian and European species. This date is considered to correspond to increasing aridization of Central Asia by uplifting of the Transhimalaya and Tibetan Plateau, which blocked Indian monsoons. In estimating divergence times of *Ansonia* clades in this study, we combined our data with those of other bufonids given by Pramuk et al. (2007), and obtained divergence time of 32.9 MYBP between European *B. bufo* and Asian *B. andrewsi*. This is three times older than the vicariance time Macey et al. (1998) assumed for the *B. bufo* group, notwithstanding the fact that the region of mtDNA they studied is surely much faster in evolution than the one we used for the calibration times on average (Mueller, 2006).

Probably our very old estimates partly derived from the inappropriate calibration point chosen (no internal calibration point available within *Ansonia*), and partly from saturation of the mitochondrial gene sequences used, and the resultant greater compression of basal branches (Hugall et al., 2007), although we chose non protein-coding genes. The origin of *Ansonia* might be actually very old but our estimations seem too large, and minimum values of CI we obtained seem to be much more plausible at least for more recently diverged lineages. Our estimates should be seriously tested in future in the light of independent evidence, and at the same time, accumulation of detailed paleogeographic evidence is necessary.

Furthermore, future studies on the pattern of genetic differentiations in other stream dwelling genera uniquely diversified in Borneo, *Meristogenys* Yang, 1991 (Shimada et al., 2007), *Leptobranchella* Smith, 1925, and *Leptolalax* Dubois, 1980 (Inger and Voris, 2001), and comparisons of the tendencies observed with that found here in *Ansonia*, will permit deeper understanding of the evolutionary histories in other lineages.

4.3. Ecological relationships

As mentioned above, two different major clades, A and B, tend to differ in body size, and this phenomenon seems to be related to the prevailing sympatric distribution of two *Ansonia* species in many localities of Borneo. Most of Subclade A1 (the peninsular and Thailand) taxa are small-sized with the male SVL smaller than 30 mm. Species with SVL larger than 30 mm (females of *A. latirostra*, *A. tiomanica*, *A. siamensis*, and *A. penangensis*) never exceed 40 mm and can be classified as medium-sized. Species of the A2 (Bornean) Subclade are small-sized (SVL < 30 mm), and only female *A. hanitschi* is medium-sized. As noted above, *A. spinulifer* is exceptionally large-sized. Because *A. spinulifer* currently ranges widely in Borneo, and differentiation between Sabah and Sarawak populations seems to be not very old, this species has presumably secondarily attained its large body size.

Species of the Clade B (primarily Bornean–Philippines clade) are medium- to large-sized. In Borneo, syntopic distribution of two species is commonly observed, and species belonging to different major clades usually coinhabit in such cases. For example, *A. platysoma* or *Ansonia* sp. 4 and *A. longidigita* coinhabit the Crocker Range, Sabah, and similarly, *A. minuta* and *A. leptopus* co-occur in

Matang, Sarawak (Das et al., 2007). Most probably, differential body size between different clades facilitates sympatry. In some regions, exceptionally large-sized *A. spinulifer* is also sympatric with two species of different body size, but microhabitats tend to differ between the two large-bodied species.

Two species endemic to Mt. Kinabalu, *A. fuliginea* and *A. guibei*, are medium-sized. Presence of few syntopic *Ansonia* species on the high mountain regions might have not affected their body size, and considering their relictual distribution, medium-sized body may be an ancestral character. Severe climatic conditions in high elevations may also have limited gigantism in these species. Furthermore, the size of *A. fuliginea* might be affected by syntopic, small-sized *Pelophryne misera* (Mocquard, 1890) (SVL = 16–23 mm; Inger, 1966; Malkmus et al., 2002).

Ansonia torrentis is similarly medium-sized, and this body size might be the result of miniaturization from a large-bodied or the retention of a primitive state. Interestingly, the two Indian species now assigned to *Ansonia* also are medium-sized (SVL = 27–31 mm in *A. ornata* Günther, 1876 [Matsui, unpublished data]; SVL = 36–41 mm in *A. rubigina* Pillai and Pattabiraman, 1981 [Pillai and Pattabiraman, 1981]). In contrast, *A. albomaculata* is small- to medium-sized exceptionally, and this body size might be resulted partly from interspecific relationships, because the species cohabit with larger sized *Ansonia* sp. 5 in Lanjak, Sarawak. Both the two species from Philippines are medium-sized, but they differ in body size, *A. mcgregori* being smaller than *A. muelleri*. Their body size might have been affected by resource allocation related to sympatric distribution (Inger, 1954). In order to test hypotheses posed here, accumulation of more data on the relationships between detailed distribution and body size, and application of pertinent analyses to them are necessary.

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References

- Akaike, H., 1974. A new look at the statistical model identification. *IEEE Trans. Automatic Control* 19, 716–723.
- Alcala, A.C., Brown, W.C., 1998. Philippine Amphibians, an Illustrated Field Guide. Bookmark, Makati City, Philippines.
- Berry, P.Y., 1975. The Amphibian Fauna of Peninsular Malaysia. Tropical Press, Kuala Lumpur.
- Brown, R.M., Guttman, S.I., 2002. Phylogenetic systematics of the *Rana signata* complex of Philippine and Bornean stream frogs: reconsideration of Huxley's modification of Wallace's Line at the Oriental-Australian faunal zone interface. *Biol. J. Linn. Soc.* 76, 393–461.
- Brown, W.C., Alcala, A.C., 1994. Philippine frogs of the family Rhacophoridae. *Proc. Calif. Acad. Sci.* 48, 185–220.
- Das, I., Jankowski, A., Makmor, M.I.B., Haas, A., 2007. Species diversity, elevational distribution and reproductive modes in an amphibian community at the Matang Range, Sarawak (Borneo). *Mitt. Hamb. Zool. Mus. Inst.* 104, 141–174.
- Dring, J.C.M., 1979. Amphibians and reptiles from northern Trengganu, Malaysia, with descriptions of two new geckos: *Cnemaspis* and *Cyrtodactylus*. *Bull. Brit. Mus. Nat. Hist. (Zool.)* 34, 181–241.
- Dring, J., 1983. Some new frogs from Sarawak. *Amphibia-Reptilia* 4, 103–115.
- Drummond, A.J., Ho, S.Y.W., Phillips, M.J., Rambaut, A., 2006. Relaxed phylogenetics and dating with confidence. *PLoS Biol.* 4, 699–710.
- Drummond, A.J., Rambaut, A., 2007. BEAST, Bayesian Evolutionary Analysis Sampling Trees, version 1.4.2. Available from: <<http://beast.bio.ed.ac.uk/>>.
- Edgar, R.C., 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 32, 1792–1797.
- Evans, B.J., Brown, R.M., Mc Guire, J.A., Supriatna, J., Andayani, N., Diesmos, A., Iskandar, D., Melnic, D.J., Cannatella, D.C., 2003. Phylogenetics of fanged frogs: testing biogeographical hypotheses at the interface of the Asian and Australian faunal zones. *Syst. Biol.* 52, 794–819.
- Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39, 783–791.
- Frost, D.R., 2009. Amphibian Species of the World: An Online Reference, version 5.3 (12 February, 2009). American Museum of Natural History, New York, USA. Electronic database accessible at: <<http://research.amnh.org/herpetology/amphibia/index.php>>.
- Frost, D.R., Grant, T., Faivovich, J.N., Bain, R.H., Haas, A., Haddad, C.F.B., De Sá, R.O., Channing, A., Wilkinson, M., Donnellan, S.C., Raxworthy, C.J., Campbell, J.A., Blotto, B.L., Moler, P., Drewes, R.C., Nussbaum, R.A., Lynch, J.D., Green, D.M., Wheeler, W.C., 2006. The amphibian tree of life. *Bull. Am. Mus. Nat. Hist.* 297, 1–370.
- Fu, J., 2000. Toward the phylogeny of family Lacertidae: why 4708 base pairs of mtDNA sequences cannot draw the picture. *Biol. J. Linn. Soc.* 71, 203–217.
- Garda, A.A., Cannatella, D.C., 2007. Phylogeny and biogeography of paradoxical frogs (Anura, Hylidae, Pseudae) inferred from 12S and 16S mitochondrial DNA. *Mol. Phylogenet. Evol.* 44, 104–114.
- Grandison, A.G.C., 1972. The Gunong Benom Expedition 1967. 5. Reptiles and amphibians of G. Benom with a description of a new species of *Macrocolamus*. *Bull. Brit. Mus. Nat. Hist. (Zool.)* 23, 45–101.
- Graybeal, A., 1997. Phylogenetic relationships of bufonid frogs and tests of alternate macroevolutionary hypotheses characterizing their radiation. *Zool. J. Linn. Soc.* 119, 297–338.
- Graybeal, A., Cannatella, D.C., 1995. A new taxon of Bufonidae from Peru, with descriptions of two new species and a review of the phylogenetic status of supraspecific bufonid taxa. *Herpetologica* 51, 105–131.
- Grismer, L.L., 2006a. A new species of *Ansonia* Stoliczka 1870 (Anura: Bufonidae) from a lowland rainforest in southern peninsular Malaysia. *Herpetologica* 62, 471–480.
- Grismer, L.L., 2006b. A new species of *Ansonia* Stoliczka 1872 (sic.) (Anura: Bufonidae) from central peninsular Malaysia and a revised taxonomy for *Ansonia* from Malay Peninsula. *Zootaxa* 1327, 1–21.
- Hall, R., 1998. The plate tectonics of Cenozoic SE Asia and the distribution of land and sea. In: Hall, R., Holloway, J.D. (Eds.), *Biogeography and Geological Evolution of SE Asia*. Backhuys Publishers, Leiden, pp. 99–131.
- Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* 41, 95–98.
- Hedges, S.B., Maxson, L.R., 1993. A molecular perspective on lissamphibian phylogeny. *Herpetol. Monogr.* 7, 27–42.
- Hillis, D.M., Bull, J.J., 1993. An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. *Syst. Biol.* 42, 182–192.
- Hillis, D.M., Mable, B.K., Larson, A., Davis, S.K., Zimmer, E.A., 1996. Nucleic acids IV: sequencing and cloning. In: Hillis, D.M., Moritz, C., Mable, B.K. (Eds.), *Molecular Systematics*. Sinauer Associates, Sunderland, MA, USA, pp. 321–378.
- Huelsenbeck, J.P., Hillis, D.M., 1993. Success of phylogenetic methods in the four-taxon case. *Syst. Biol.* 42, 247–264.
- Hugall, A.F., Foster, R., Lee, M.S.Y., 2007. Calibration choice, rate smoothing, and the pattern of tetrapod diversification according to the long nuclear gene RAG-1. *Syst. Biol.* 56, 543–563.
- Igawa, T., Kurabayashi, A., Nishioka, M., Sumida, M., 2006. Molecular phylogenetic relationship of toads distributed in the Far East and Europe inferred from the

- nucleotide sequences of mitochondrial DNA genes. *Mol. Phylogenet. Evol.* 38, 250–260.
- Inger, R.F., 1954. Systematics and zoogeography of Philippine Amphibia. *Fieldiana Zool.* 33, 183–531.
- Inger, R.F., 1960. A review of the Oriental toads of the genus *Ansonia* Stoliczka. *Fieldiana Zool.* 39, 473–503.
- Inger, R.F., 1966. The systematics and zoogeography of the Amphibia of Borneo. *Fieldiana Zool.* 52, 1–402.
- Inger, R.F., 1992. Variation of apomorphic characters in stream-dwelling tadpoles of the bufonid genus *Ansonia* (Amphibia: Anura). *Zool. J. Linn. Soc.* 105, 225–237.
- Inger, R.F., 1999. Distribution of amphibians in southern Asia and adjacent islands. In: Duellman, W.E. (Ed.), *Patterns of Distribution of Amphibians: A Global Perspective*. Johns Hopkins Univ. Press, Baltimore, MD, USA, pp. 445–482.
- Inger, R.F., Dring, J., 1988. Taxonomic and ecological relations of Bornean stream toads allied to *Ansonia leptopus* (Günther) (Anura: Bufonidae). *Malay. Nat. J.* 41, 461–471.
- Inger, R.F., Voris, H.K., 2001. The biogeographical relations of the frogs and snakes of Sundaland. *J. Biogeogr.* 28, 863–891.
- Inger, R.F., Tan, F.L., Yambun, P., 2001. A new species of toad of the genus *Ansonia* (Anura: Bufonidae) from Borneo. *Raffles Bull. Zool.* 49, 35–37.
- Iskandar, D., Mumpuni, J., 2004. A new toad of the genus *Ansonia* (Amphibia, Anura, Bufonidae) from Sumatra, Indonesia. *Hamadryad* 28, 59–65.
- Kiew, B.H., 1984. A new species of toad, *Ansonia siamensis* (Bufonidae), from the Isthmus of Kra, Thailand. *Nat. Hist. Bull. SIAM Soc.* 32, 111–115.
- Kocher, T.D., Thomas, W.K., Meyer, A., Edwards, S.V., Paabo, S., Villablanca, F.X., Wilson, A.C., 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proc. Natl. Acad. Sci. USA* 86, 6196–6200.
- Macey, J.R., Schulte II, J.A., Larson, A., Fang, A.-L., Wang, Y.-Z., Tuniyev, B.S., Papenfuss, T.J., 1998. Phylogenetic relationships of toads in the *Bufo bufo* species group from the eastern escarpment of the Tibetan Plateau: a case of vicariance and dispersal. *Mol. Phylogenet. Evol.* 9, 80–87.
- Malkmus, R., Manthey, U., Vogel, G., Hoffman, P., Kosuch, J., 2002. *Amphibians and Reptiles of Mount Kinabalu* (North Borneo). ARG Gantner Verlag Kommanditgesellschaft, Ruggell.
- Matsui, M., 1979. Amphibians from Sabah I. Systematic and natural history notes. *Contrib. Biol. Lab. Kyoto Univ.* 25, 303–346.
- Matsui, M., Nabhitabhata, J., Panha, S., 1998. A new *Ansonia* from northern Thailand (Anura, Bufonidae). *Herpetologica* 54, 448–454.
- Matsui, M., Ito, H., Shimada, T., Ota, H., Saidapur, S.K., Khonsue, W., Tanaka-Ueno, T., Wu, G.-F., 2005a. Taxonomic relationships within the Pan-Oriental narrow-mouth toad *Microhyla ornata* as revealed by mtDNA analysis (Amphibia, Anura, Microhylidae). *Zool. Sci.* 22, 489–495.
- Matsui, M., Khonsue, W., Nabhitabhata, J., 2005b. A new *Ansonia* from Isthmus of Kra, Thailand (Amphibia, Anura, Bufonidae). *Zool. Sci.* 22, 809–814.
- Matsui, M., Shimada, T., Ota, H., Tanaka-Ueno, T., 2005c. Multiple invasions of the Ryukyu Archipelago by Oriental frogs of the subgenus *Odorrana* with phylogenetic reassessment of the related subgenera of the genus *Rana*. *Mol. Phylogenet. Evol.* 37, 733–742.
- Matsui, M., Shimada, T., Liu, W.Z., Maryati, M., Khonsue, W., Orlov, N., 2006. Phylogenetic relationships of Oriental torrent frogs in the genus *Amolops* and its allies (Amphibia, Anura, Ranidae). *Mol. Phylogenet. Evol.* 38, 659–666.
- Matsui, M., Yambun, P., Sudin, A., 2007. Taxonomic relationships of *Ansonia anotis* Inger, Tan, and Yambun, 2001 and *Pedostibes maculatus* (Mocquard, 1890), with a description of a new genus (Amphibia, Bufonidae). *Zool. Sci.* 24, 1159–1166.
- Moriarty, E.C., Cannatella, D.C., 2004. Phylogenetic relationships of North American chorus frogs (*Pseudacris*). *Mol. Phylogenet. Evol.* 30, 409–420.
- Mueller, R.L., 2006. Evolutionary rates, divergence dates, and the performance of mitochondrial genes in Bayesian phylogenetic analysis. *Syst. Biol.* 55, 289–300.
- Myers, L.C., 1978. Geomorphology. In: Luping, D.M., Wen, C., Dingley, R.E. (Eds.), *Kinabalu. Summit of Borneo*. Sabah Society, Kota Kinabalu, Malaysia, pp. 91–100.
- Nishikawa, K., Matsui, M., Tanabe, S., 2005. Biochemical phylogenetics and historical biogeography of *Hynobius boulengeri* and *H. stejnegeri* (Amphibia, Urodela) from the Kyushu region, Japan. *Herpetologica* 61, 54–62.
- Palumbi, S.R., Martin, A., Romano, S., McMillan, W.O., Stice, L., Grabowski, G., 1991. *The Simple Fool's Guide to PCR*, version 2.0. Privately Published. Compiled by Palumbi, S., Department of Zoology, University of Hawaii, Honolulu.
- Pillai, R.S., Pattabiraman, R., 1981. A new species of torrent toad (genus: *Ansonia*) from Silent Valley, S. India. *Proc. Ind. Acad. Sci. (Anim. Sci.)* 90, 203–208.
- Posada, D., Crandall, K.A., 1998. MODELTEST: testing the model of DNA substitution. *Bioinformatics* 14, 817–818.
- Pramuk, J.B., Robertson, T., Sites Jr., J.W., Noonan, B.P., 2007. Around the world in 10 million years: biogeography of the nearly cosmopolitan true toads (Anura: Bufonidae). *Global Ecol. Biogeogr.* 17, 72–83.
- Rodriguez, F., Oliver, J.F., Marin, A., Medina, J.R., 1990. The general stochastic model of nucleotide substitution. *J. Theoret. Biol.* 142, 485–501.
- Roelants, S., Gower, D.J., Wilkinson, M., Loader, S.P., Biju, S.D., Guillaume, K., Moriau, L., Bossuyet, F., 2007. Global patterns of diversification in the history of modern amphibians. *Proc. Natl. Acad. Sci. USA* 104, 887–892.
- Sathiamurthy, E., Voris, H.K., 2006. Maps of Holocene sea level transgression and submerged lakes on the Sunda Shelf. *Nat. Hist. J. Chulalongkorn Univ. Suppl.* 2, 1–43.
- Shaffer, H.B., McKnight, M.L., 1996. The polytypic species revisited: genetic differentiation and molecular phylogenetics of the tiger salamander *Ambystoma tigrinum* (Amphibia: Caudata) complex. *Evolution* 50, 417–433.
- Shimada, T., Matsui, M., Sudin, A., Maryati, M., 2007. Identity of larval *Meristogenys* from a single stream in Sabah, Malaysia (Amphibia: Ranidae). *Zool. J. Linn. Soc.* 151, 173–189.
- Swofford, D., 2002. PAUP*: Phylogenetic Analysis Using Parsimony (* and other methods), version 4. Sinauer Associates, Sunderland, MA.
- Tanaka, T., Matsui, M., Takenaka, O., 1996. Phylogenetic relationships of Japanese brown frogs (*Rana*: Ranidae) assessed by mitochondrial cytochrome b gene sequences. *Biochem. Syst. Ecol.* 24, 299–307.
- Tominaga, A., Matsui, M., Nishikawa, K., Tanabe, S., 2006. Phylogenetic relationships of *Hynobius naevius* (Amphibia: Caudata) as revealed by mitochondrial 12S and 16S rRNA genes. *Mol. Phylogenet. Evol.* 38, 677–684.
- Voris, H.K., 2000. Maps of Pleistocene sea levels in southeast Asia: shorelines, river systems and time durations. *J. Biogeogr.* 27, 1153–1167.
- Wilcox, T.P., Zwickl, D.J., Heath, T.A., Hillis, D.M., 2002. Phylogenetic relationships of the dwarf boas and a comparison of Bayesian and bootstrap measures of phylogenetic support. *Mol. Phylogenet. Evol.* 25, 361–371.
- Wilkinson, J.A., Drewes, R.C., Tatum, O.L., 2002. A molecular phylogenetic analysis of the family Rhacophoridae with an emphasis on the Asian and African genera. *Mol. Phylogenet. Evol.* 24, 265–273.
- Wood Jr., P.L., Grismer, L.L., Ahmad, N., Senawi, J., 2008. Two new species of torrent-dwelling toads *Ansonia Stoliczka*, 1870 (Anura, Bufonidae) from Peninsular Malaysia. *Herpetologica* 64, 321–340.
- Zhang, P., Zhou, H., Chen, Y.Q., Liu, Y.F., Qu, L.H., 2005. Mitogenomic perspectives on the origin and phylogeny of living amphibians. *Syst. Biol.* 54, 391–400.
- Zwickl, D.J., 2006. *Genetic Algorithm Approaches for the Phylogenetic Analysis of Large Biological Sequence Datasets Under the Maximum Likelihood Criterion*. Ph.D. dissertation. The University of Texas at Austin.