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AUTHOR(S):
Matsui, Masafumi; Yambun, Paul; Sudin, Ahmad

CITATION:

ISSUE DATE:
2007-11

URL:
http://hdl.handle.net/2433/85317

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Taxonomic Relationships of *Ansonia anotis* Inger, Tan, and Yambun, 2001 and *Pedostibes maculatus* (Mocquard, 1890), with a Description of a New Genus (Amphibia, Bufonidae)

Masafumi Matsui¹*, Paul Yambun² and Ahmad Sudin³

¹Graduate School of Human and Environmental Studies, Kyoto University, Sakyo, Kyoto 606-8501, Japan
²Research and Education Division, Sabah Parks, P.O. Box 10626, Kota Kinabalu 88806, Sabah, Malaysia
³Institute for Tropical Biology and Conservation, University Malaysia Sabah, Teluk Sepanggar, Locked Bag 2073, Kota Kinabalu 88999, Sabah, Malaysia

Examination of types and recently collected specimens revealed that *Ansonia anotis* Inger, Tan, and Yambun, 2001 and *Pedostibes maculatus* (Mocquard, 1890), both described from Kinabalu, Sabah, Malaysia, are hardly differentiated morphologically. Analyses of a total of 2,427 bp of the 12S rRNA, tRNAval, and 16S mitochondrial rRNA genes revealed that the two species are very close genetically. Thus *A. anotis* is regarded as conspecific and is synonymized with *P. maculatus*. Genetically, this species proved to form a lineage distinct from other bufonids from Southeast Asia, including species of *Ansonia* and *Pedostibes*. Because the species has also some unique morphological traits different from known bufonid genera, we propose to establish a new genus for *Nectophryne maculata* Mocquard, 1890.

**Key words:** Malaysia, molecular phylogeny, new genus, Sabah, synonymy

**INTRODUCTION**

Southeast Asia is one of the centers of amphibian diversification (Inger, 1999), and this holds for the toad family Bufonidae. On the island of Borneo, as many as six bufonid genera (*Bufo* Laurenti, 1768, *Ansonia* Stoliczka, 1870, *Leptophryne* Fitzinger, 1843, *Pedostibes* Günther, 1876, *Pelophryne* Barbour, 1938, and *Pseudobufo* Tschudi, 1838) have been recorded (Inger, 1966; Inger and Tan, 1996; Malkmus *et al*., 2002), and the number is even greater if we admit Frost *et al*.'s (2006) proposal to split *Bufo* into several distinct genera. This might promote uncovering the paraphyletic nature of the genus *Bufo* (e.g., Graybeal and Cannatella, 1995), but applying unfamiliar names for many taxa, without substantially providing their diagnostic characters, could result in further taxonomic confusion.

Some other Bornean genera are taxonomically more conservative than *Bufo* and occupy specific ecological niches (Inger, 1958). Of these, *Ansonia* is characterized by small to medium adult body size and a unique larva with a large oral sucker adapted for life in torrents. Both *Pelophryne* and *Pedostibes* are adapted for arboreal life, but *Pelophryne* is small in body size, and lays small numbers of large yolky eggs, while *Pedostibes* is moderate to large sized, and its eggs are numerous and small. Recent molecular studies indicate close relationships of *Pedostibes* with *Bufo asper* Gravenhorst, 1829 and *B. juxtasper* Inger, 1960 (Frost *et al*., 2006; our own observations, see Results).

Inger *et al.* (2001) described *Ansonia anotis* Inger, Tan, and Yambun, 2001 from Sayap, in the Kinabalu National Park of Sabah, Malaysian part of northern Borneo (Fig. 1). The species is characterized mainly by lack of a tympanum, large spatulate finger disks, and the unique morphology of the putative larva. However, because only several specimens have been obtained since its description, details of the species are unclear. Recently, on the joint expedition of UMS (University Malaysia Sabah) and JICA (Japan International Cooperation Agency) to the Crocker Range National Park (Fig. 1), Sabah, in 2002, four toads were collected. These specimens were very similar to *Pedostibes maculatus* (Mocquard, 1890), originally described as *Nectophryne*, and were identified as that species (Kueh *et al*., 2004). Like *A. anotis*, *P. maculatus* is characterized by lack of the tympanum and large finger disks (Boulenger, 1918; Inger, 1966), and these species could not be clearly distinguished from one another by any morphological traits described in the literature.

We compared the two species morphologically using available specimens, including three syntypes of *N. maculata*,...
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stored in the collection of Museum National d'Histoire Naturelle, Paris (MNHN), to determine their taxonomic relationships. Further, in order to clarify their phylogenetic relationships among Southeast Asian bufonids, we analyzed sequences of the 12S and 16S mitochondrial rRNA genes of the two species, as well as of representatives of related bufonids for comparisons.

MATERIALS AND METHODS

For morphological comparisons, we studied three specimens of *Ansonia anotis* (Sabah Parks, Kota Kinabalu [SP] 01762 (holotype) from Sungei (River) Wario, Sayap, Mt. Kinabalu, Sabah; SP 26033 and 26043 from Riou Hutan Simpan, Kiau, Mt. Kinabalu, Sabah), three syntypes of *N. maculata* from Kinabalu (MNHN 1899-266–268; syntypes), and two of four specimens of *P. maculatus* (Graduate School of Human and Environmental Studies, Kyoto University [KUHE] 38505 and 38506 from Ulu Kimanis of the Crocker National Park [Trail 5 of UMS 2002 expedition to the Crocker Range], Sabah).

Six body measurements were taken, following Matsui (1984): 1) snout-vent length (SVL), 2) head width (HW), 3) lower arm length (LAL), 4) thigh length (THIGH), 5) tibia length (TL), and 6) foot length (FL). All measurements were made to the nearest 0.1 mm with dial calipers. Dimensions were converted to a percentage ratio (R) in relation to SVL for comparisons, although small sample sizes examined prohibited statistical comparisons. We made slight dissections to examine tympanic structure and gonads, and prepared radiographs to examine gross osteology.

For genetic comparisons, we studied one specimen each of *A. anotis* and *P. maculatus* from among the specimens used for morphological comparisons. For comparisons, we used the following species (Table 1): *A. malayana* Inger, 1960; *A. hanitschi* Inger, 1960; *A. longidigita* Inger, 1960; *A. fuliginea* (Mocquard, 1890); *Pedostibes hosii* (Boulenger, 1892); *P. rugosus* Inger, 1958; *Pelophryne misera* (Mocquard, 1890); *P. brevipes* (Peters, 1867); *Leptophryne borbonica* (Tschudi, 1838); *Bufo juxtasper* (=*Phrynoidis juxtaspera* in Frost et al., 2006); *B. melanisticus* Schneider, 1799 (=**Duttaphrynus melanostictus** in Frost et al., 2006); *B. divergens* Peters, 1871 (=**Ingerophrynus divergens** in Frost et al., 2006); *Didynamipus sp. holothuriensis* Andersson, 1903 from GenBank (AY325991); and *Atelopus flavescens* Duméril and Bibron, 1841 from GenBank (DQ283259). We chose *Dendrobates auratus* (Girard, 1855) from GenBank (AY326030), a member of the supposed bufonid sister family, Dendrobatidae Cope, 1865 (Frost et al., 2006), as outgroup (Table 1).
We extracted DNA from small amounts of frozen or ethanol-preserved tissues using standard phenol-chloroform extraction procedures (Hillis et al., 1996). We conducted amplifications by the polymerase chain reaction (PCR) with the primers Thr Lrm (AAAR CATKGGCTTTGTTAARCC) modified from Shaffer and McKnight (1996) and Hedges 16H1 from Hedges and Maxson (1993) to obtain 2.4 kb of the 12S and 16S rRNA genes and the intervening tRNA gene for valine. We sequenced the amplified fragments in an automated DNA sequencer (ABI PRISM 3100) using the same primers and eight additional primers (sequences of primers available on request from the senior author), and following the manufacturer’s instructions. Sequences newly obtained will be deposited in GenBank (accession numbers AB331708–331721).

We obtained and eye-checked sequence data for each sample using ABI PRISM Sequencing Analysis Software (V3.6.2), and performed alignments of data from all samples by clustal option of the BioEdit software (Hall, 1999). After testing consistency among the genes by the use of incongruence length difference (ILD) tests with 1,000 randomized partitions (Farris et al., 1994) and confirming no significant heterogeneity, we combined the 12S, tRNA, and 16S rRNA gene for valine. We sequenced the amplified fragments in an automated DNA sequencer (ABI PRISM 3100) using the same primers and eight additional primers (sequences of primers available on request from the senior author), and following the manufacturer’s instructions. Sequences newly obtained will be deposited in GenBank (accession numbers AB331708–331721).

RESULTS

Morphology

Specimens of *A. anotis* agreed well with the diagnosis given in the original description of Inger et al. (2001): "lacking a tympanum; snout projecting beyond lower jaw; first finger not reaching swollen tip of second; tips of outer fingers

### Table 1

<table>
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<tr>
<th>Species</th>
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<th>Accession number</th>
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<td>Dendrobates auratus</td>
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We also performed Bayesian analyses using the Markov chain Monte Carlo technique (MCMC) implemented in MrBayes 3.0b4 (Huelsenbeck and Ronquist, 2001). Hierarchal likelihood ratio test (hLRTs) using the program MODELTEST 3.06 (Posada and Crandall, 1998) selected the GTR+I+G model as the DNA substitution model that best fit our data. We initiated four independent analyses with a random starting tree that ran for 2.0 million generations. Because we found the log likelihood scores to stabilize after 50,000 generations, we conservatively discarded the first 1.0 million generations from each run as “burn-in”. We sampled one of every 100 generations from the remaining 1.0 million generations (10,001 trees across all four independent analyses) to calculate posterior probabilities (BPP) for each branch in the Bayesian tree, and considered BPP≥95% as significant support (Larget and Simon, 1999; Huelsenbeck et al., 2001).

### Table 2

<table>
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<tr>
<th>Species</th>
<th>Sp. Number</th>
<th>Sex</th>
<th>SVL</th>
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<th>RLAL</th>
<th>RTHIGH</th>
<th>RTL</th>
<th>RFL</th>
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<td>31.9</td>
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<td>–</td>
<td>–</td>
<td>62.4</td>
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<td>53.5</td>
<td>57.4</td>
<td>45.3</td>
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</table>

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We also performed Bayesian analyses using the Markov chain Monte Carlo technique (MCMC) implemented in MrBayes 3.0b4 (Huelsenbeck and Ronquist, 2001). Hierarchal likelihood ratio test (hLRTs) using the program MODELTEST 3.06 (Posada and Crandall, 1998) selected the GTR+I+G model as the DNA substitution model that best fit our data. We initiated four independent analyses with a random starting tree that ran for 2.0 million generations. Because we found the log likelihood scores to stabilize after 50,000 generations, we conservatively discarded the first 1.0 million generations from each run as "burn-in". We sampled one of every 100 generations from the remaining 1.0 million generations (10,001 trees across all four independent analyses) to calculate posterior probabilities (BPP) for each branch in the Bayesian tree, and considered BPP≥95% as significant support (Larget and Simon, 1999; Huelsenbeck et al., 2001).

RESULTS

Morphology

Specimens of *A. anotis* agreed well with the diagnosis given in the original description of Inger et al. (2001): "lacking a tympanum; snout projecting beyond lower jaw; first finger not reaching swollen tip of second; tips of outer fingers
expanded into distinct, spatulate discs; web extending beyond distal subarticular tubercles of third and fifth toes”. Similarly, by examining the types of *N. maculata*, we confirmed Mocquard’s (1890) original description except for color, which has faded seriously: “body slender, limbs very long; head short, snout truncate, length equal to eye; canthus rostralis angulated, vertical; nostril near tip of snout; interorbital as wide as upper eyelid; tympanum not distinct; limbs very thin, toes two-thirds webbed; fingers webbed only at base, flattened, tips wide and truncate, much stronger than toes; subarticular tubercles slightly visible, metatarsal tubercles very distinct, outer more protruded; tibio-tarsal articulation passes tip of snout; body covered by rough granulations, both dorsally and ventrally”.

**Fig. 2.** Dorsal (A–C) and ventral (D–F) views of (A, D) *Ansonia anotis* (holotype, SP 01762), (B, E) *Nectophryne maculata* (syntype, MNHN 1899-267), and (C, F) *Pedostibes maculatus* (KUHE 38506 [formerly BORNEENSIS 08682]). Scale bar=10 mm.

**Fig. 3.** Views of living (A) *Ansonia anotis* (SP 26033 from Riou Hutan Simpan, Kliau, Mt. Kinabalu) and (B) *Pedostibes maculatus* (KUHE 38505 [formerly BORNEENSIS 08681], from Trail 5 of Ulu Kimanis, Crocker Range).
The holotype of *A. anotis* is a female with SVL 52.6 mm, which is slightly larger than that given in the original description (52.1 mm: Inger et al., 2001). The other two specimens considered as males are 39.0 and 35.5 mm in SVL (Table 2). Of three syntypes of *N. maculata*, two females are 51.5 and 45.4 mm in SVL, and an individual of unknown sex is 37.5 mm in SVL. Of the *P. maculatus* collected from the Crocker, the largest one is the female 48.6 mm in SVL; the male is 30.4 mm in SVL. The remaining two specimens of unknown sex are 31.9 and 39.7 mm in SVL. Thus, *A. anotis, N. maculata*, and *P. maculatus* do not differ from each other in SVL in either sex, although the maturity of males is not clear. These species also had similar relative size in head (median of RHW=29.0, 28.5, and 27.9%, respectively), and hindlimb characters (medians: RTHIGH=51.9, 52.4, and 55.1%; RTL=59.2, 56.6, and 61.4%; RFL=43.7, 43.7, and 45.5%, respectively; Table 2).

As described by Inger et al. (2001), the holotype of *A. anotis* in preservative is slight gray to brown on dorsal and lateral surfaces, scattered with many small, roundish dark spots, and pale brown on ventral surfaces. Limbs are barred with dark bands (Fig. 2). In the two males, the ground color of the dorsum was light green in life (Fig. 3). Mocquard’s (1890) original description for *N. maculata*, “Dorsum grey brown scattered with small irregular black spots, isolated or confluent; transverse bands on limbs; ventral surface a little lighter grey brown”, was barely evident because the three specimens are all faded (Fig. 2). Color of *P. maculatus* both in preservative and in life (Fig. 2, 3) was almost identical to that of *A. anotis*.

In addition, *A. anotis* was nearly identical with *N. maculata* and *P. maculatus* in morphological characteristics other than the morphometric and color traits. The following characteristics were found in common with them: lacking bony crests on head, parotoid gland, tympanum, columnella, and eustachian tube; snout projecting beyond lower jaw; hand very large, first finger much shorter than second; tips of outer fingers expanded into distinct, spatulate discs; web extending beyond distal subarticular tubercles of third and fifth toes. Males lack vocal sac opening, nuptial pad, and mandibular spines.

**DNA sequences**

We obtained sequences of 888–908 bp for 12S, 69–73 bp for tRNAval, and 1,372–1,403 bp for 16S, and the aligned 12S (926 bp), tRNAval (73 bp), and 16S (1,428 bp) data set yielded 2,427 nucleotide positions. Of these sites, 1,077 were variable and 722 were informative for parsimony analyses. *Ansonia anotis* and *P. maculatus* differed only slightly genetically. The sequence divergences between them (K2p=0.015 in 12S, 0.014 in tRNAval, and 0.032 in 16S) were much smaller than divergences between *A. anotis* and four other *Ansonia* species (minimum K2p=0.111, 0.075, and 0.156, respectively) or between *P. maculatus* and other two *Pedostibes* species (minimum K2p=0.098, 0.143, and 0.145, respectively).

We obtained six most parsimonious trees with 311 evolutionary steps, with a consistency index of 0.521 and a retention index of 0.335. All phylogenetic analyses resulted in the similar topologies (only the Bayesian tree from the combined data set is shown in Fig. 4). The following relationships were indicated, with high support, by all three methods employed: (1) monophyly of bufonid species other than *Atelopus* with respect to *Dendrobates* (BS=97% in MP, etc.)
BS=99% in NJ, BPP=100%); (2) monophyly of four species of Ansonia other than A. anotis (99%, 100%, 100%, respectively); (3) sister-group relationship of two species of Pelophryne (100% in all); (4) sister-group relationship of A. anotis and P. maculatus (BS=100% in all). In addition, the results of the present study clearly indicate that A. anotis and P. maculatus are morphologically, genetically, and ecologically hardly distinguishable, and that the former should be synonymized with the latter, pending the question of the male nuptial pad. Slight genetic differentiation found between the Kinabalu and Crocker samples indicates the presence of some degree of isolation between the localities. A similar, but larger, degree of genetic differentiation between these two regions has been detected in a stream-living ranid, Meristogenys kinabaluensis (Inger, 1966) (Shimada, unpublished data).

Taxonomic history of Pedostibes maculatus

Mocquard (1890) without any reason placed maculata in Nectophryne Buchholz and Peters in Peters, 1875, but Barbour (1938) later moved the species to Pelophryne with some consideration. Barbour thought that several bufonid species from Asia, then included in Nectophryne, are closely related and differ from African members. He divided the Asian species into two genera: Pedostibes Günther, 1876 (type species P. tuberculosus Günther, 1876 from Malabar, India) and his new genus Pelophryne Barbour, 1938. The diagnostic characteristics Barbour (1938) listed for Pelophryne include usually seven, and perhaps occasionally six, presacral vertebrae, fusion of the coccyx (=uro-style) to the sacrum, and presence of coccygeal expansions. Barbour (1938) placed N. maculata in Pelophryne, although he noted that because of the hidden tympanum, this species might turn out not to be congeneric with any species of Pelophryne. Barbour (1938) probably believed N. maculata to have the coccyx expanded dorsally and fused to the sacrum, and seven presacral vertebrae, like other members of Pelophryne.

Inger (1966) examined the syntypes of N. maculata and clarified Barbour’s (1938) mistakes. He ascertained that the species has a coccyx that is not equally expanded dorsally but fused to the sacrum, and eight presacral vertebrae. These traits were confirmed by our examination of radiographs. Inger (1966) also noted that two of the three syntypes (MHN 89.266–267) have numerous small ova (more than 50 per ovary, also confirmed in the present study), unlike members of Pelophryne, with a small number of large eggs, but like Pedostibes. Inger (1966) thus moved Pelophryne maculata to Pedostibes, even though the species lacks a parotoid gland, unlike its congeners. All subsequent authors (e.g., Inger and Tan, 1996; Malkmus et al., 2002; Matsui, 2006) have followed this classification.

Notwithstanding close morphological similarities with P. maculatus in metamorphs, Inger et al. (2001) described A. anotis as a new species of Ansonia, whose tadpoles are famous for their unique conformation of the sucker-type oral disc (Inger, 1960, 1966). Inger (1992) reported from Sabah a unique bufonid tadpole, which he assigned to the genus Ansonia and called the Ansonia “sucker”. It has a large oral disc only slightly narrower than the widest part of the body, unlike other Ansonia larvae (Inger, 1966), but at the same time lacks an upper jaw sheath, unlike congenic species. What is more unique is that it characterized has a sharply defined abdominal sucker immediately behind the oral disc, similar to that found in the torrent-dwelling ranids Meristogenys Yang, 1991 and Hua Yang, 1991 (not sensu Frost et al., 2006), but which has never been seen in Bornean bufoinds.

Because Inger et al. (2001) found no species of Ansonia other than the juvenile paratype of A. anotis, and no form of larval Ansonia other than the Ansonia “sucker” (1992) at Puruol, one of the known habitats of A. anotis in the Crocker Range, they associated these (metamorph and larva) as conspecific. In most Ansonia species, the tympanum is exposed, but it is hidden under the skin in A. mcgregori (Taylor, 1922) and A. muelleri (Boulenger, 1887) from the Philippines. Inger et al. (2001) considered the complete absence of tympanum in A. anotis to be a more extreme condition, within a generic morphcline, than in the two Philippine species. Also, the larval upper jaw sheath in Ansonia varies from large to very small, as if leading to the absence in A. anotis. Furthermore, postdental portion of the scotorial lip also varies from small to large, and the abdominal sucker found in A. anotis can be viewed as an extension of such a morpholine. Thus, Inger et al. (2001) concluded that both the metamorph and the larva represent extremes of morphological specialization among Ansonia species, although they admitted the possibility that A. anotis warrants distinct generic status.

Designation of lectotype of Nectophryne maculata and new generic allocation

To prevent future taxonomic confusion, we select
Sabahphrynus new genus

Diagnosis
A bufonid genus lacking tympanum annulus, columella, and eustachian tube; head without bony crests; no parotoid gland; tips of outer fingers expanded into distinct, spatulate discs; distal phalanges T-shaped; subarticular tubercles normal, though weak; toe webbing moderately developed; male without vocal sac opening or mandibular spines; ova numerous, small and unpigmented; coccyx neither expanded dorsally nor fused to the sacrum, movable; eight presacral vertebrae; quadrate-jugal complete: pectoral girdle arciferal. If correctly identified, the tadpole is also unique in the possession of expanded mouthparts and abdominal sucker, and absence of upper jaw sheath.

Comparisons
Among the above diagnostic characters, absence of a tympanum is the most easily confirmed visually. An invisible tympanum is not rare among various bufonid lineages; in many cases, the tympanum is actually present but hidden under the skin (e.g., in some Bufo [sensu lato], some Ansonia, Nannophrynus Günther, 1870, and probably Bufoioides Pillai and Yazdani, 1973). Actual absence of a tympanum and columella is more limited to some lineages mostly from Africa (Altiphrynoides Dubois, 1987; Capensibufo Grandison, 1980; Didynamipus Andersson, 1903; Laurentophrynus Tihen, 1960; Mortonsophrynus Tihen, 1960; Nimbaphrynoides part of Nectophrynoides Noble, 1926; Nimbaphrynoides Dubois, 1987; Werneria Poche, 1903; and Wolterstorffina Mertens, 1939) (Grandison, 1978, 1981) and Middle to South America (some Atelopus; Crepidophrynus Cope, 1889; Dendrophryniscus Jiménez de la Espada, 1871; Melanophryniscus Gallardo, 1961; Oreoophryne Boulenger, 1895; Osornophrynus Ruiz-Carranza and Hernández-Camacho, 1976; and some Rhamphophrynus Trueb, 1971) (Savage and Kluge, 1961; Cannatella, 1986).

The new genus is differentiated from most of these by its larger body size and by widely expanded, T-shaped phalangeal tips (vs. slightly modified, T-shaped or knobbed phalangeal tips in Altiphrynoides, Nectophrynoides, and Werneria) (Grandison, 1978); by eight presacral vertebrae (vs. up to seven in Didynamipus, Laurentophrynus, Mertensophrynus, some Nectophrynus, Nimbaphrynoides, Oreophrynella, Osornophrynus, and most of Rhamphophrynus) (Trueb, 1971; Grandison, 1978; Cannatella, 1986); by the absence of sacro-coccygeal fusion (vs. fused sacrum and coccyx in Wolterstorffina) (Grandison, 1978); and by the arciferal pectoral girdle (vs. arciferal-firmisternal in some Atelopus, Dendrophryniscus, Didynamipus, and Melanophryniscus) (Trueb, 1971; Cannatella, 1986). It also differs from Capensibufo by the absence of parotoid gland, presence of toe webs, and a large clutch of small eggs (vs. presence of parotoid gland, absence of toe webbing, and a small clutch of large eggs) (Grandison, 1981), and from Crepidophrynus by normal phalangeal formulae (vs. phalanges reduced) (Savage and Kluge, 1961).

Eymology
The taxon is named for the state of Sabah, Malaysia from where the unique species has been recorded. The suffix -phrynus is from the Greek, phrynos, meaning a toad.

Type species
Nectophrynus maculata Mocquard, 1890.

ACKNOWLEDGMENTS
We are grateful to the following for their encouragement and/or permission to conduct research, or for field companionship: S. Anuar, L. Apin, K. Araya, A.-A. Hamid, Y. Hashimoto, T. Hikida, I. Jaafar, A. H. Kassim, J. K. B. Kueh, T. Kusano, D. Labang, K. Nishikawa, H. Ota, L.-H. Seng, T. Shimada, T. Tachi, N.-S. Wong, and H.-S. Yong. We also thank A. Ohler and A. Dubois (MNHNP), N. Jamir and M. B. Lakim (SP), and M. Maryati (UMS) for allowing the senior author to examine specimens under their care. A. Tominaga conducted the DNA analysis, K. Nishikawa kindly prepared radiographs, and T. Shimada examined specimens in SP and also provided literature. The Economic-Planning Unit (former Socio-Economic Research Unit) of Malaysia, Sabah Parks, and the State Government of Sarawak kindly permitted us to conduct the project, and UMS, JICA, SP, Tawau Hills National Park, and the Forest Department, Sarawak kindly provided all the facilities for conducting research. Field trips and examination of museum specimens by the senior author were made possible by Grants under The Mombusho JSPS Scientific Research Programs (Nos. 01040151, 02041051, 03041044, 10041166, 15370038).

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(Received March 14, 2007 / Accepted July 24, 2007)