Resurrection of *Staurois parvus* from *S. tuberilinguis* from Borneo (Amphibia, Ranidae)

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Two forms of *Staurois* that are differentiated by body size occur parapatrically in the Crocker Range, Sabah, Borneo. Analyses of a total of 1,499 bp of the mitochondrial cytochrome b, 12S rRNA, and 16S rRNA genes revealed that the two forms could be completely split genetically. The two forms could be also clearly differentiated morphologically, not only by snout-vent length but also by the relative sizes of snout, eye, and finger disk. Comparisons of the two forms with all known species of the genus revealed the large and small forms to be *S. tuberilinguis* and *S. parvus*, respectively. The latter species has long been synonymized with the former, but we here consider them to represent different species.

Key words: biodiversity, cryptic species, molecular phylogeny, Southeast Asia, taxonomy

INTRODUCTION

In our survey of amphibians in Sabah, Borneo, we found frogs of the genus *Staurois* Cope, 1865, which is characterized by very large finger disks, each of which has a distinct transverse border proximally on the ventral surface, and complete webbing on all toes (Boulenger, 1918; Inger, 1966). These frogs were generally uniform in their habitus and color, but could be clearly differentiated as two forms by body size (small and large forms).

Staurois is a small genus occurring in the Philippines and Borneo and includes small- to moderate-sized torrent dwellers (*e.g.*, Inger, 1966; Malkmus *et al*., 2002). All recent authors (*e.g.*, Inger and Tan, 1996; Malkmus *et al*., 2002) have recognized three species, *S. latopalmatus* (Boulenger, 1887), *S. natator* (Günther, 1858), and *S. tuberilinguis* Boulenger, 1918, as the members of this genus from Borneo.

Preliminary examination of our two forms in conjunction with the key to the above three Bornean species (Inger, 1966; Malkmus *et al*., 2002) revealed that both could be tentatively identified as *S. tuberilinguis* because they lack webbed outer fingers and vomerine teeth, but possess a well-developed lingual papilla. This species includes *S. parvus* Inger and Haile, 1960, which is the smallest species of the genus ever described, as a synonym (Inger, 1966).

In order to clarify their relationships, we analyzed mitochondrial cytochrome b (cyt-b), 12S rRNA, and 16S rRNA gene sequences of the small form from the Crocker Range

and the large form from the Crocker Range and Sarawak, as well as of *S. natator* and *S. latopalmatus* for comparisons. We also made a morphological comparison of the two forms with available specimens, as well as with descriptions, of known species of the genus to determine their taxonomic status.

MATERIALS AND METHODS

For genetic comparisons, we studied a total of 15 specimens of "*S. tuberilinguis*": eight specimens of the small form from two localities in the Crocker Range, Sabah, and seven specimens of the large form from two localities in the Crocker Range and one locality in Sarawak (Appendix 1, Fig. 1). For comparisons, *S. natator* from Sabah and Sarawak, *S. latopalmatus* from Sabah, and another ranid, *Amolops marmoratus* (Blyth, 1855) from Thailand, were used. *Microhyla fissipes* Boulenger, 1884, a member of the ranoid family Microhylidae from Thailand, was chosen as an outgroup species.

DNA was extracted from small amounts of frozen or ethanol-preserved tissues using standard phenol-chloroform extraction procedures (Hillis *et al*., 1996). Amplification was done by the polymerase chain reaction (PCR), using primers L14841 (light chain; 5'-CCA TCC AAC ATC TCA GCA TGA TGA AA-3') / H15548 (heavy chain; 5'-AAT AGG AAG TAC CAC TCT GGT TTA AT-3') newly designed by Tomoko Tanaka-Ueno, and L14850 of Tanaka *et al*. (1994) / H15502 of Tanaka-Ueno *et al*. (1998) for cyt-b; primers 12SH of Cannatella *et al*. (1998) and H1548 of Matsui *et al*. (2005) for 12S; and primers L2188 of Matsui *et al*. (2005) and 16H1 of Hedges (1994) for 16S. The amplified fragments were sequenced with an automated DNA sequencer (ABI PRISM 3100) using the PCR primers and following the manufacturer's instructions. Newly obtained sequences were deposited in GenBank (accession numbers AB259717–259738).

Sequence data for each sample were obtained and checked by eye using ABI PRISM Sequencing Analysis Software (V3.6.2). Alignments of data from all samples were performed with the clustal
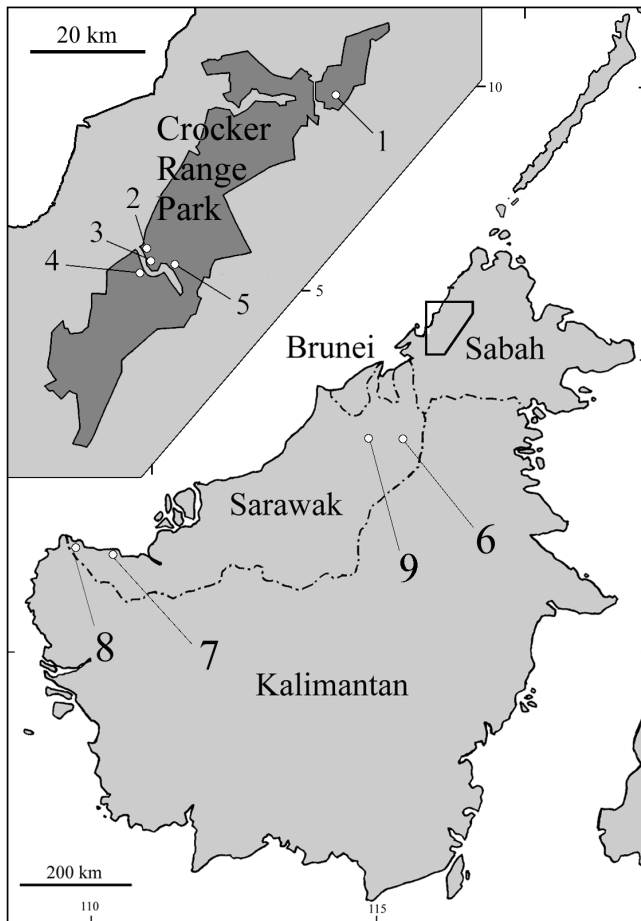
Fig. 1. Map of Borneo showing the localities where sampling of *Staurois* species was conducted. 1, Mahua; 2, Base camp; 3, Trail 4; 4, Trail 5; 5, Trail 11; 6, Bareo; 7, Serapi; 8, Sematan; 9, Mulu. Hatched area shows the Crocker Range (CR). See Appendix 1 for details.

option of BioEdit software (Hall, 1999). Initial ClustalX alignments of 12S and 16S were then inspected by eye and adjusted slightly. Prior to analysis, we tested for consistency among genes using incongruence length difference (ILD) tests with 1,000 randomized partitions (Farris *et al.*, 1994). No significant heterogeneity was observed among the cyt-b, 12S, and 16S genes. We therefore combined them into a single data set of 1,499 bp. Neighbor-joining (NJ) phylogenies based on Kimura two-parameter (K2p) distances (Kimura, 1980) and maximum parsimony (MP) phylogenies obtained by heuristic searches with TBR branch swapping were calculated in PAUP*4.0b10 (Swofford, 2002). The robustness of NJ and MP tree topologies was tested by bootstrap analyses (Felsenstein, 1985) with 1,000 replicates (Hedges, 1992).

For morphological comparisons, we studied a total of 89 specimens of "*S. tuberilinguis*" from Sabah and Sarawak (31 specimens of the small form and 58 specimens of the large form) and 16 specimens of *S. natator* from Sarawak (Appendix 1). Five body measurements were taken, following Matsui (1984): 1) snout-vent length (SVL), 2) head width (HW), 3) tibia length (TL), 4) snout length (SL), and 5) eye length (EL). All measurements were made to the nearest 0.1 mm with dial calipers under a binocular dissecting microscope. Dimensions were converted to a percentage ratio in relation to SVL, and statistical comparisons were made between the two forms for each sex. For these variables, Kruskal-Wallis tests with nonparametric multiple comparisons were performed to detect the presence

or absence of differences in frequency distributions. The significance level was set at 0.05.

RESULTS

DNA sequences

We obtained sequences of 583 bp for cyt-b, 436–443 bp for 12S and 458–463 bp for 16S, and the aligned 12S (449 bp) and 16S (467 bp) data set combined with cyt-b yielded 1,499 nucleotide positions. Of these sites, 553 were variable and 355 were informative for parsimony analyses. We obtained a single most parsimonious tree with 923 evolutionary steps, a consistency index of 0.807, and a retention index of 0.873.

For cyt-b, the small and large forms of "*S. tuberilinguis*" from Sabah showed low within-population sequence divergence (K2p=0 to 0.012 and 0 to 0.014, respectively). The sequence divergence between populations from Sabah and Sarawak was also low (K2p=0 to 0.012) for the large form, contrasting with much higher divergence in *S. natator* (K2p=0.028–0.032). Sequence divergence was lower (K2p=0 to 0.002) for 16S than for cyt-b, both within the Sabah populations and between the Sabah and Sarawak populations of the small and large forms of "*S. tuberilinguis*". The Sabah and Sarawak populations of *S. natator* also showed low divergence (K2p=0.013). For 12S, no sequence divergence was observed either within the Sabah populations or between the Sabah and Sarawak populations of the small and large forms of "*S. tuberilinguis*", and *S. natator* showed only low divergence (K2p=0.002) between Sabah and Sarawak.

All phylogenetic analyses resulted in the same topology, and only the NJ tree from the combined data set is shown in Fig. 2. The following relationships were indicated by full bootstrap support (100%) in both the NJ and MP analyses: (i) monophyly of genus *Staurois* with respect to *Amolops* and *Microhyla*; (ii) monophyly of *S. natator* with respect to "*S. tuberilinguis*"; (iii) monophyly for each of the small and large forms of "*S. tuberilinguis*"; and (iv) a sister-group relationship of the small and large forms of "*S. tuberilinguis*". In addition, (v) monophyly of *S. natator* and "*S. tuberilinguis*", with respect to *S. latopalmatus*, was supported by a bootstrap value of 100% for the NJ analysis, but by 94% for the MP analysis.

Thus, the small and the large forms of "*S. tuberilinguis*" were clearly separated genetically, although the sequence divergences between them (K2p=0.096 for cyt-b, 0.018 for 12S, and 0.027 for 16S) were much smaller than the divergences between these forms and *S. natator* (0.248, 0.099, and 0.105, respectively, for the small form; 0.243, 0.097, and 0.109, respectively, for the large form) or *S. latopalmatus* (0.305, 0.121, and 0.129, respectively, for the small form; 0.303, 0.116, and 0.132, respectively, for the large form), and between the latter two species (0.287, 0.127, and 0.156, respectively).

Morphology

The two forms of "*S. tuberilinguis*" were morphologically very similar to each other, with the following characteristics in common. All specimens of the two forms had the first finger much shorter than the second, and consistently had a prominent lingual papilla but lacked vomerine teeth and an

outer metatarsal tubercle. They usually had a dark interorbital bar followed by a dark, V-shaped marking, and females had non-pigmented, creamy-colored ova.

The two forms, however, did not overlap in body size in either sex, and could be differentiated by their SVL [males 19.7–23.6 mm (n=10) and females 25.7–31.1mm (n=14) in the small form, in contrast to 27.5–30.2 mm (n=19) and 32.8–36.7 mm (n=8) in the large form: Table 1]. In both forms, the sexes did not differ in HW or TL relative to SVL (Table 1). However, in both sexes, the small form had a sig-
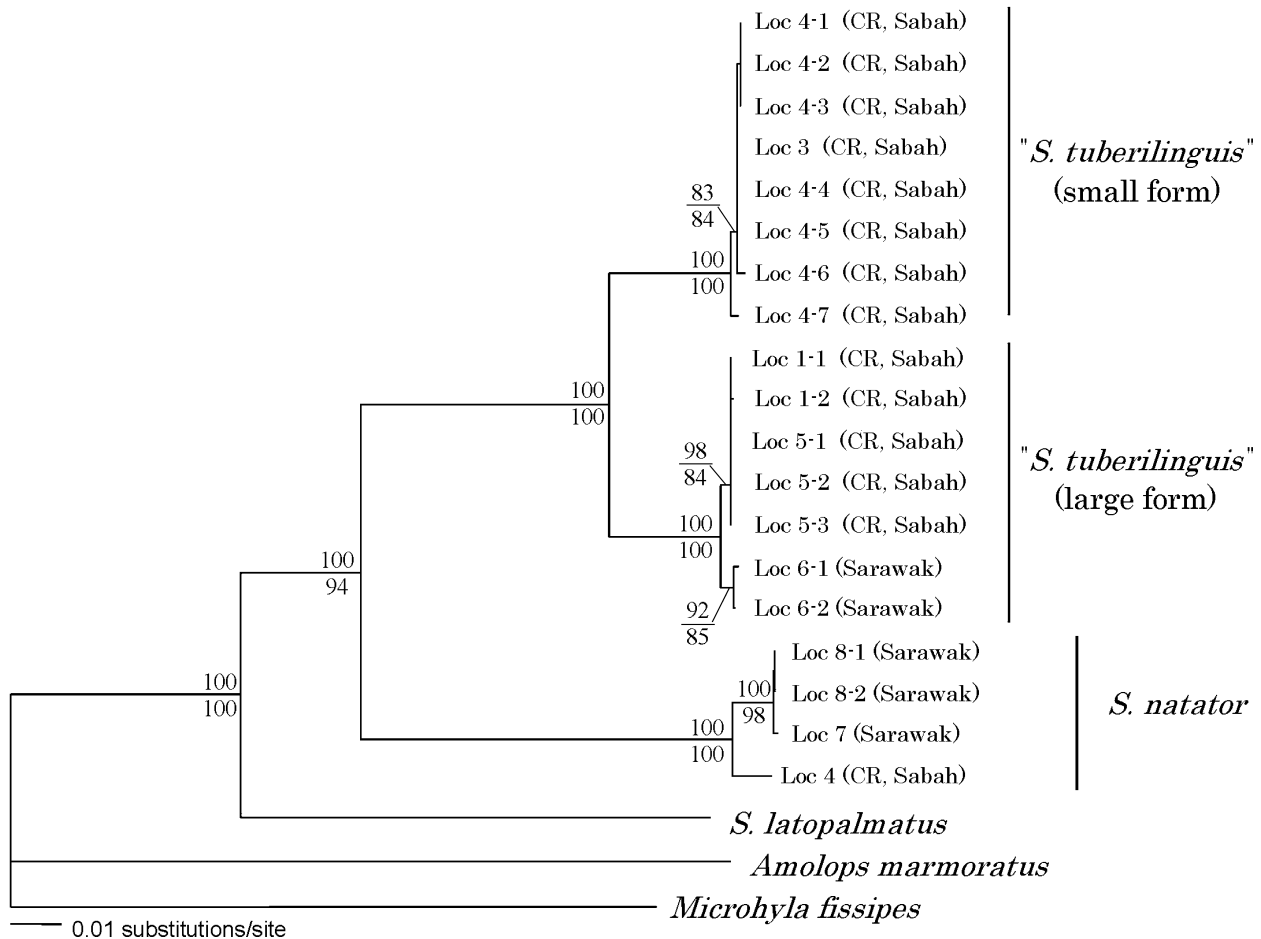

Fig. 2. Neighbor-joining tree based on 1,499 bp of the combined cyt-b, 12S rRNA, and 16S rRNA genes for species of *Staurois* and *Amolops*, with *Microhyla* as an outgroup. Bootstrap support is indicated for the NJ (1,000 replicates; above branches) and MP (1000 replicates: below branches) analyses. For abbreviations of sample localities, refer to Fig. 1.

Table 1. Morphometric variation in two forms of *Staurois* tentatively identified as *tuberilinguis* from the Crocker range, Sabah. SVL (means±SD, in mm) and medians of percentage ratios (R) of head width (HW) and tibia length (TL) to SVL, followed by ranges in parenthesis. For details of locality, refer to Appendix 1.

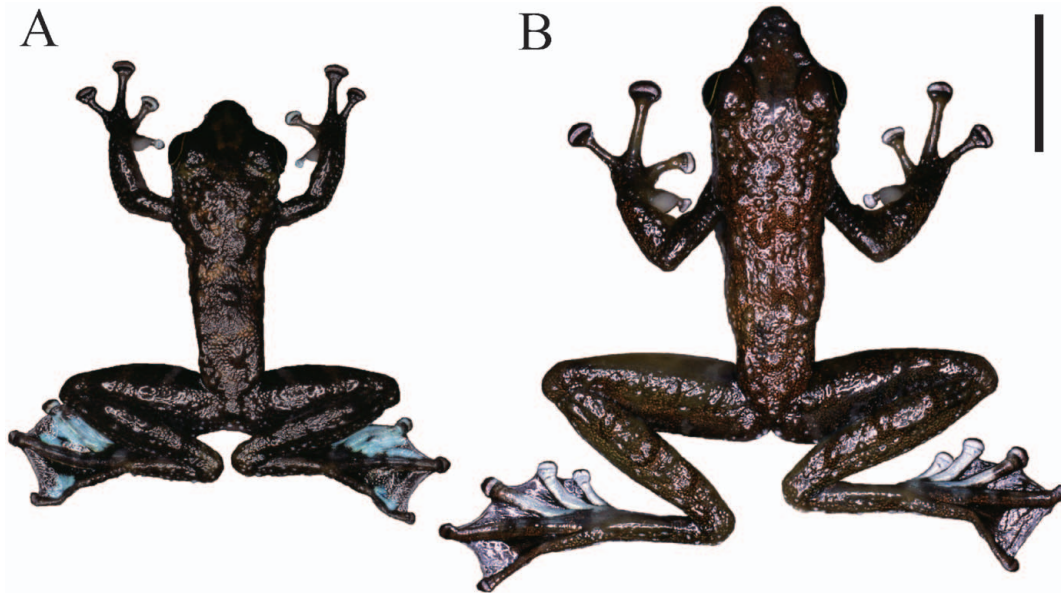| Locality		Males				Females		
		SVL	RHW	RTL		SVL	RHW	RTL
small from								
Trail 4	(n=3)	22.5±1.01	30.5	59.2	(n=6)	29.6±1.06	29.0	53.5
		(21.6–23.6)	(30.0–31.0)	(55.5–60.6)		(28.2–31.1)	(28.3–30.)2	(50.2–57.1)
Trail 5	(n=7)	21.2±0.68	30.6	58.9	(n=8)	27.8±1.39	29.5	57.9
		(19.7–21.6)	(29.0–31.3)	(57.5–59.9)		(25.7–30.0)	(28.5–30.7)	(53.3–58.5)
large form								
Trail 11	(n=14)	29.0±0.89	28.3	57.2	(n=5)	35.1±1.62	28.1	56.8
		(27.5–30.2)	(26.8–30.4)	(55.0–61.5)		(32.8–36.7)	(26.7–29.9)	(53.4–56.2)
Mahua	(n=5)	28.8±0.42	27.6	56.8	(n=3)	35.5±0.64	26.9	55.2
		(28.4–29.4)	(26.9–28.7)	(56.7–58.1)		(34.8–36.0)	(26.5–28.4)	(53.6–55.6)

Fig. 3. Dorsal views of males of the **(A)** small form (BORNEENSIS 23425) and **(B)** large form (BORNEENSIS 8635). Scale bar=10 mm.

nificantly wider head relative to SVL than the large form (Dunn's multiple comparison test, $P<0.05$). Similarly, the small form had a significantly longer tibia relative to SVL than the large form in males ($P<0.05$), though the two forms did not differ in females ($P>0.05$).

The two forms were also differentiated by several characteristics other than SVL. The snout was less slender and pointed in the small form than in the large form, as shown in Fig. 3. In the small form, the snout (SL=13.5–15.3% SVL, median=14.7% in males; 14.0–14.5% SVL, median=14.3% in females) was shorter than the eye (EL=16.5–18.8% SVL, median=17.7% in males and 15.7–16.7% SVL, median=15.8% in females), whereas in the large form, the snout (16.2–17.1% SVL, median=16.6% in males; 13.9–17.2% SVL, median=15.4% in females) tended to be longer than the eye (15.7–16.7% SVL, median=15.9% in males; 13.3–14.8% SVL, median=14.4% in females). Thus, the ratio of eye to snout in the small form (109.1–137.9%, median=120.6% in males; 109.3–114.6%, median=112.2 in females) was larger than in the large form (91.7–100.0%, median=96.8% in males; 83.9–98.1%, median=91.4% in females).

The two forms further differed in the relative size of the disk of the outer finger. In the small form, the width of the disk of the fourth finger was larger than the distance between its base and the junction of the third and fourth fingers, but the converse relationship was observed in the large form.

Staurois "*tuberilinguis*" collected from regions of Borneo other than the Crockers could be easily assigned to one of the two forms by SVL and consistently showed corresponding characters. In frogs from Bareo, Sarawak, 24 males and seven females had a mean ($\pm$SD) SVL of 28.2$\pm$0.7 (range= 26.8–29.7) mm and 35.8$\pm$1.3 (range=33.7–37.3) mm, respectively, and showed the characters of the large form. This morphological similarity of the Bareo population to the large form from the Crockers is consistent with the results of the DNA analysis presented above. In contrast, frogs from

Mulu, Sarawak, had a mean SVL of 21.5$\pm$0.5 (range=21.0–21.6) mm in four males and 29.1$\pm$0.9 (range=28.2–30.0) mm in three females, and possessed all the features of the small form.

DISCUSSION

From this study, it is certain that there are two genetically independent forms of "*S. tuberilinguis*" that can also be differentiated by body size and some other morphological characters. In order to confirm the identity of these two forms, it is pertinent to compare them with the descriptions of all species of the genus hitherto described: *S. latopalmatus*, *S. natator*, *S. guttatus* Cope, 1865, *S. nubilus* (Mocquard, 1890), *S. tuberilinguis*, and *S. parvus*.

Staurois latopalmatus from Borneo differs from the two forms, as well as all other species of the genus, in having the fingers fully webbed to the disks, as well as by a very short, "pug-like" snout (Boulenger, 1918; Inger, 1966; Malkmus *et al.*, 2002). *Staurois natator*, originally described from Philippines, is now considered to include *S. guttatus* from Borneo as a synonym (Inger, 1954, 1966; Malkmus *et al.*, 2002). Although Boulenger (1918) split *S. guttatus* from *S. natator* by the presence in the former and absence in the latter of vomerine teeth, this character is variable in *S. guttatus* (Inger, 1954, 1966; Malkmus *et al.*, 2002; our own observations). In contrast, the two forms of "*S. tuberilinguis*" consistently lack vomerine teeth. *Staurois natator,* including *S. guttatus,* clearly differs from our two forms in the absence of the lingual papilla (Boulenger, 1918; Inger, 1954, 1966; Malkmus *et al.*, 2002; our own observations). Another important difference of *S. guttatus* from our two forms is the color of the eggs; females of *S. guttatus* we observed (as Bornean *S. natator*) had entirely black ova, strongly contrasting to the pigmentless ova of the two forms of "*S. tuberilinguis*". In this regard, Boulenger (1918) noted the difference between the strongly pigmented eggs of *S. guttatus* and the unpigmented eggs of *S. natator*. This difference, later noted also by Inger

(1966), could be one reason for the future resurrection of *S. guttatus* from synonymy with *S. natator*.

Staurois nubilus from Palawan is also usually treated as a synonym of *S. natator* (*e.g.*, Taylor, 1921; Inger, 1954, 1966). One of the differences between the two species was reported to be the presence in *S. nubilus* and absence in *S. natator* of the lingual papilla (Boulenger, 1918), but presence of the papilla later proved to be limited to only some specimens of *S. nubilus* (Taylor, 1921; Inger, 1954). Another difference between the two species is in egg color (Boulenger, 1918), with feebly pigmented, pale brown ova in *S. nubilus* in contrast to unpigmented ova in *S. natator*. Inger (1954) listed other differences between the two species, while treating them as conspecific. Our two forms differ from *S. nubilus* in consistently having a lingual papilla and totally unpigmented ova.

Unlike all the above species, *S. tuberilinguis* from Borneo, including *S. parvus* as a synonym (Inger, 1966), agrees with our two forms completely in the absence of vomerine teeth and the presence of a lingual papilla and unpigmented ova. Boulenger (1918) erroneously stated that the first finger was shorter than the second in the original description of *S. tuberilinguis*, and this led Inger and Haile (1960) to describe *S. parvus*, which showed the converse relationship in finger lengths. Inger (1966) later discovered Boulenger's (1918) error and synonymized *S. parvus* with *S. tuberilinguis*. From the original description of *S. parvus* (Inger and Haile, 1960), it is clear that the most notable difference between the two species is in body size, *S. parvus* being much smaller than *S. tuberilinguis*.

As a morphometric value, Boulenger (1918) indicated only a SVL of 42 mm for a female syntype of *S. tuberilinguis*, and Inger (1966) presented an even larger value (43.4 mm) for that specimen. This is beyond the range of females of the large form we examined (32.7–37.5 mm). However, it is certain that the female reported by Boulenger (1918) is unusually large, because Inger (1966) gave the maximum size of the specimens he examined to be 38 mm, which is not different from our samples. Other than this body-size difference and the relationship of the length of the first and second fingers (see above), Boulenger's (1918) original description of *S. tuberilinguis* very well applies to our large form.

Similarly, the small form matches the description of *S. parvus* (Inger and Haile, 1960) in every respect, except for male tibia length and female head width, both relative to SVL. Inger and Haile (1960) described *S. parvus* based on a pair. The male and the female were reported to have a SVL of 22.9 and 29.0 mm, respectively. These values are well within the range of our small form (19.7–23.6 and 25.7–31.1 mm, respectively). When the measurements given by Inger and Haile (1960) (tibia length, head length, head width, and eye length) were converted to ratios to SVL, all were within the range of our samples of the small form, except for male TL and female HW. The TL of the male holotype of *S. parvus* was reported to be 52.8% SVL, a value smaller than for our males of both the small form (55.5–60.6%) and the large form (55.0–61.5%). In contrast, the HW of the female paratype of *S. parvus*, 27.2% SVL, is smaller than for our females of the small form (28.3–30.7%), but is within the range of the large form (26.5–29.9%). Not-

withstanding these slight morphometric differences, all the other characters, including a wide disk of the fourth finger, large eye, and short snout, agree between *S. parvus* and our small form.

In a very strict sense, the possibility that the two forms reported here represent undescribed species of *Staurois* is not precluded. However, it is at the moment most reasonable to consider that the large and small forms represent *S. tuberilinguis* and *S. parvus*, respectively. Both *S. tuberilinguis* and *S. parvus* can be differentiated from the other *Staurois* species by the combination of: consistent presence of a lingual papilla; possession of non-pigmented creamy ova; absence of webbed outer fingers; consistent absence of vomerine teeth; and usual absence of an outer metatarsal tubercle. *Staurois tuberilinguis*, with a SVL of 27–31 mm in males and 33–38 mm in females, is larger than *S. parvus*, with a SVL of 20–24 mm in males and 26–31 mm in females. *Staurois tuberilinguis* has a relatively narrower head and a more slender and pointed snout than *S. parvus*. In *S. tuberilinguis*, the snout tends to be longer than the eye, while in *S. parvus*, the snout is shorter than the eye, resulting in a ratio of eye to snout in *S. tuberilinguis* that is smaller than in *S. parvus*. In males, *S. tuberilinguis* has a relatively shorter tibia than *S. parvus*. Finally, the disk of the fourth finger is smaller than its distance from the palm in *S. tuberilinguis*, but the relationship is reversed in *S. parvus*.

The two forms are parapatrically distributed in the Crocker Range. On the western slope of this mountain range, *S. parvus* occurs at altitudes mostly from 750 to 820 m, whereas *S. tuberilinguis* has been collected only at 1,430 m. At lower elevations, the smaller *S. parvus* was found to co-occur with *S. natator*, which is even slightly larger than the larger *S. tuberilinguis*. This indicates the possibility of character displacement within a single species (*i.e.*, *S. tuberilinguis*), but the facts that the habitats that the two forms occupy are very close, and that the forms seem to be completely isolated genetically, precludes such a possibility. Furthermore, the two forms are not restricted to the Crockers, Sabah, but are also found widely in other parts of Sabah and Sarawak, and are clearly separated by body size and other morphological characteristics. These facts surely demonstrate their heterospecific nature. Thus, *S. parvus* should be treated as a distinct species and resurrected from synonymy with *S. tuberilinguis*.

From the low genetic divergences found between the two species, their speciation is considered to be a relatively recent event within Borneo. To elucidate the pattern of evolution of these two species, further studies are required that include investigaton of a more detailed pattern of distribution and behavioral characteristics such as acoustic ones.

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Appendix 1.   Specimens of *Staurois* examined in the present study. Voucher specimens are stored at the Institute for Tropical Biology and Conservation, University Malaysia Sabah (BORNEENSIS) and the Graduate School of Human and Environmental Studies, Kyoto University (KUHE).

Genetic analysis. *S. parvus*: BORNEENSIS 8802 [n=1, from Trail 4 of 2002 Ulu Kimanis Expedition by University Malaysia Sabah (UMS)]; BORNEENSIS 8405, 8406, 8417, 8448, 8450–8452 (n=7, from Trail 5 of Ulu Kimanis, Sabah). *S. tuberilinguis*: BORNEENSIS 8634–8636 (n=3, from Trail 11 of Ulu Kimanis, Sabah); BORNEENSIS 12489, 12527 (n=2 from Mahua, Tambunan, Sabah); KUHE 12389, 12441 (n=2, from Bareo, Sarawak). *S. natator*: BORNEENSIS 8449 (n=1 from Trail 5 of Ulu Kimanis, Sabah); KUHE unnumbered (n=1 from Serapi, Kuching, Sarawak); KUHE 17570, 17571 (n=2 from Sematan, Sarawak). *S. latopalmatus*: BORNEENSIS 8098 (n=1 from Base camp of Ulu Kimanis, Sabah).

Morphological analysis.--*S. parvus*: BORNEENSIS 8708, 8715, 8716, 8731, 8733, 8802, 8833–8835 (n=9, from Trail 4 of Ulu Kimanis, Sabah); BORNEENSIS 8417, 8448, 8450, 8451, 8455, 8465, 8694, 8695, 12893, 12895, 12896–12898, 12900, 12902 (n=15, from Trail 5 of Ulu Kimanis, Sabah); KUHE 10377, 10378, 10390, 10391, 10410, 10483, 10484 (n=7, from Mulu, Sarawak). *S. tuberilinguis*: BORNEENSIS 8484–8489, 8493, 8634–8636, 8668–8671, K9, K10, K17, K20, K21 (n=19, from Trail 11 of Ulu Kimanis, Sabah); BORNEENSIS 12489, 12527, 12528, 12530, 12610, 12612, 12682, 12683 (n=8 from Mahua, Tambunan, Sabah); KUHE 12199–12202, 12204, 12206–12211, 12232, 12234, 12235, 12237–12241, 12244–12247, 12352, 12353, 12357, 12359–12361, 12393, 12394 (n=31 from Bareo, Sarawak). *S. natator*: KUHE 17569–17575, 17615–17621, 17651, 17652 (n=16 from Sematan, Sarawak).