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First genetic confirmation of *Theloderma stellatum* (Anura: Rhacophoridae) from Cambodia and notes on its distribution

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Cambodia, as a part of Indochina, harbours a highly diverse herpetofauna (Bain & Hurley 2011). During the last century, several surveys have provided insights into the taxonomy and distribution of Cambodia's amphibians and reptiles (Smith 1935, 1943, Bourret 1941, 1942, Saint Girons 1972). However, survey efforts have been geographically biased and especially recent studies concentrated on the foothills of the southern Annamite Mountains in the country's east (Long et al. 2000, Stuart et al. 2006, 2010, Rowley et al. 2010, Neang et al. 2011a, Geissler et al. 2012) and the Cardamom Mountains in the southwest (Daltry & Cheang 2000, Daltry & Wüster 2002, Long et al. 2002, Ohler et al. 2002, Swan & Daltry 2002, Daltry & Traeholt 2003, Stuart & Platt 2004, Stuart & Emmett 2006, Grismer et al. 2007a, 2007b, 2008a, 2008b, 2010, Wood et al. 2010, Neang et al. 2010, 2011a, 2011b, Murdoch et al. 2019). Recently, a first comprehensive checklist provided knowledge on the amphibians and reptiles of the Phnom Kulen National Park (PKNP) in northern Cambodia (Geissler et al. 2019), which is of particular interest as this region constitutes a biogeographic transition zone in between the Khorat Plateau in eastern Thailand, the Mekong River along the border to Laos, and the Annamite Mountains in the east (Bain & Hurley 2011, Geissler et al. 2015).

The mossy frogs of the genus *Theloderma* Tschudi, 1838 currently comprise 28 recognised species, including the subgenus *Stelladerma* Poyarkov et al., 2015 (Poyarkov et al. 2015, Frost et al. 2022). The latter includes three species, namely *T.* (*S.*) *horridum* (Boulenger, 1903), *T.* (*S.*) *stellatum* Taylor, 1962, and *T.* (*S.*) *vietnamense* Poyarkov et al., 2015. The two last taxa have been reported from Cambodia (Neang & Holden 2008, Poyarkov et al. 2015), but are morphologically very similar and hence difficult to distinguish based on morphology alone. However, most records and specimens have been assigned to one of those two taxa based on morphological characteristics only and genetic identification often has not been undertaken. Therefore, the distributional patterns of *T. stellatum* and *T. vietnamense*, particularly in Cambodia, remain unclear.

Geissler et al. (2019) recorded two specimens (one adult, one juvenile, ZFMK 92565-566) from Phnom Chor at PKNP, which were preliminarily assigned to *T.* cf. *stellatum* based on morphological characters only. However, the authors mention that the colour patterns of both *T. stellatum* and *T. vietnamense* is highly variable, even within the same individual. According to Poyarkov et al. (2015), *T. stellatum* features pinkish toe and finger pads, while those of *T. vietnamense* are rusty red. The specimens from PKNP, however, have rusty red pads and therefore might represent *T. vietnamense*. To clarify their specific identity, we analysed the PKNP specimens genetically and compared them to other *Theloderma* species, with focus on the subgenus *Stelladerma*.

Tissue samples were taken from museum specimens and processed at the Centre for Molecular Biodiversity Research at the ZFMK. We amplified for a fragment of the mitochondrial 16S rRNA using polymerase chain reaction (PCR). Used primers, PCR cycling conditions, and purification are described in Koch et al. (2013). Obtained sequences were checked with the original chromatograph data using PhyDE (http://www.phyde.de). Our data set was supplemented with sequences from GenBank and contained representatives from 29 species of *Theloderma*, including all three *Stelladerma* species as well as the members of the sister genus *Nyctixalus* Boulenger, 1882. Accession numbers and respective localities of specimens are provided in Appendix 1. Sequence alignment was done with MAFFT (Katoh et al. 2009), refined using the MUSCLE algorithm (Edgar 2004), and manually corrected

where necessary, resulting in 501 bp. The GTR+I+G model of nucleotide substitution was selected by the Akaike information criterion using ModelTest (Posada & Crandall 1998) as implemented in the R package 'phangorn' (Schliep 2011). Phylogenetic reconstruction by Bayesian inference (BI) was conducted in MrBayes 3.2.7 (Ronquist et al. 2012) using a random starting tree and four independent runs with a maximum of 10 million generations each, sampled every 1000. Runs were stopped when the average standard deviation of split frequencies had reached 0.01. Convergence of the Markov chains and ESS values were checked with Tracer v1.7 (Rambaut et al., 2014) and the initial 25% of the generations were discarded prior to building a consensus tree.

According to our genetic analyses, the specimen collected at PKNP is positioned within *T. stellatum* (Fig. 1), while all other genetically analysed specimens from Cambodia belong to *T. vietnamense*. Uncorrected p-distance of the PKNP sample to Thai *T. stellatum* is 1.9%, to *T. horridum* 6.8–7.6%, and to *T. vietnamense* 5.7–9.4%. The specimen collected by Stuart & Emmet (2006) from Areng Valley in the Cardamom Mountains of Cambodia (FMNH 267765), which was assumed to represent *T. stellatum* due to its geographic proximity to this species' type locality (Poyarkov et al. 2015), is confirmed to be *T. vietnamense*, as already noticed by Dever (2017). Poyarkov et al. (2015) map two records of "*T. stellatum*" in the Cardamom Mountains. However, this is due to a misinterpretation of the data provided by Neang & Holden (2008) and the Areng Valley specimen remains the only (vouchered) record from the Central Cardamoms (Fig. 1, locality 6). Since all analysed records from Mondolkiri Province in eastern Cambodia (Fig 1, localities 8–10) are *T. vietnamense*, the PKNP specimens are the first and only definite record of *T. stellatum* for Cambodia. The identity of the specimen from Oddar Meanchey Province (Fig. 1, locality 5) presented by Hartmann et al. (2013) remains uncertain as neither a specimen nor tissue sample were collected. While Geissler et al. (2019) were correct with their tentative determination of the PKNP specimens (Fig. 2), our findings indicate that the colour of toe and finger pads as constant colouration traits to distinguish between *T. stellatum* and *T. vietnamense* (Poyarkov et al. 2015) are too variable and therefore an identification based on these traits seems questionable. Furthermore, like in many colour traits, it is not applicable in preserved specimens due to fading (Fig. 2). In conclu-

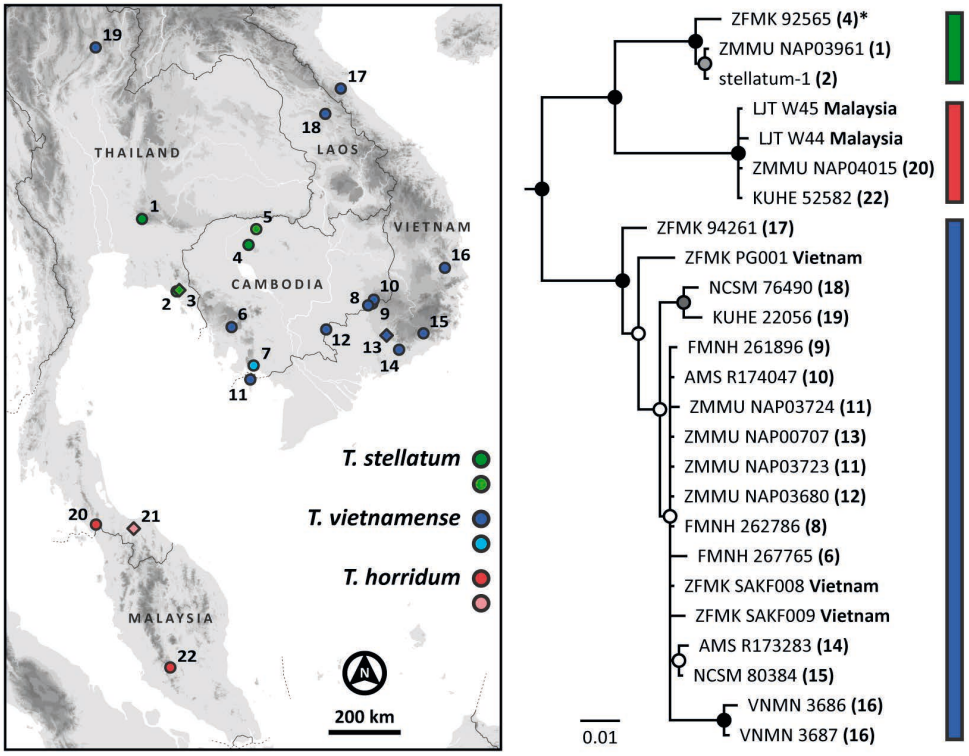

Figure 1. Geographic distribution and phylogenetic relationships of the subgenus *Stelladerma*, containing *Theloderma horridum* (red), *T. stellatum* (green), and *T. vietnamense* (blue). Note that all known records are shown for Cambodia, but only genetically verified localities and type localities are shown for other countries. Localities that could not be genetically verified are shown in lighter shades and type localities are indicated by diamond symbols. The Bayesian consensus tree is based on 501 bp of mitochondrial 16S rRNA with node support in terms of Bayesian posterior probabilities indicated by circles on nodes (nodes with BPP > 0.90 are white, BPP > 0.95 are grey, BPP > 0.99 are black, values < 0.90 are not marked). The PKNP sample is marked with an asterisk, numbers in parentheses refer to mapped localities (see also Table 1). Other *Theloderma* species and outgroups not shown for clarity.

Figure 2. Dorsal and ventral views of preserved specimens of *Theloderma stellatum* from Phnom Kulen National Park, ZFMK 92565 (top) and ZFMK 92566 (bottom). The scale bar represents 1 cm.

sion, genetic barcoding is necessary to safely distinguish between *T. stellatum* and *T. vietnamense* and further field work is required to delineate the distribution of these two species including potential contact zones in Cambodia.

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Appendix 1

Samples, GenBank accessions and locality data used in this study.

Species	GenBank Acc. No. (16S rRNA)	Voucher	Country	#	Locality	Reference
Theloderma stellatum	KT461917	ZMMU NAP–03961	Thailand	1	Nakhon Nayok: Nang Rong	Poyarkov et al. (2015)
T. stellatum	KT461918	stellatum–1		2	Chanthaburi: Phliu NP	Poyarkov et al. (2015)
T. stellatum	–	FMNH 172249		3	Chanthaburi: Khao Sebab (type locality)	Taylor 1962
T. stellatum	MZ376719	ZFMK 92565	Cambodia	4	Siem Reap: Banteay Srei: Phnom Chor, Phnom Kulen NP	This study
T. cf. *stellatum*	–	–		5	Oddar Meanchey: Trapeang Prasat Dist.	Hartmann et al. 2013
T. vietnamense	KU561885	FMNH 267765		6	Koh Kong: Areng valley	Dever (2017)
T. cf. *vietnamense*	–	–		7	Kampot: Stueng Chral	Poyarkov et al. (2015)
T. vietnamense	KU561884	FMNH 262786		8	Mondolkiri: Keo Seima Dist.	Dever (2017)
T. vietnamense	DQ286981	FMNH 261896		9	Mondolkiri: Keo Seima Dist.	Stuart et al. (2006)
T. vietnamense	JN688171	AMS R174047		10	Mondolkiri Prov.	Rowley et al. (2011)
T. vietnamense	KT461888	ZMMU NAP–03724	Vietnam	11	Kien Giang: Phu Quoc NP	Poyarkov et al. (2015)
T. vietnamense	KT461919	ZMMU NAP–03723		11	Kien Giang: Phu Quoc NP	Poyarkov et al. (2015)
T. vietnamense	KT461921	ZMMU NAP–03680		12	Tay Ninh: Lo Go–Xa Mat NP	Poyarkov et al. (2015)
T. vietnamense	KT461889	ZMMU NAP–00707		13	Dong Nai: Cat Tien NP (type locality)	Poyarkov et al. (2015)
T. vietnamense	JN688170	AMS R173283		14	Binh Thuan Prov.	Rowley et al. (2011)
T. vietnamense	KU561887	NCSM 80384		15	Binh Thuan: Dai Ninh Hydropower Plant	Dever (2017)
T. vietnamense	KJ802922	VNMN 3686		16	Phu Yen: Krong Chai	Nguyen et al. (2014)
T. vietnamense	KJ802923	VNMN 3687		16	Phu Yen: Krong Chai	Nguyen et al. (2014)
T. vietnamense	MZ376720	ZFMK 94261		17	Quang Binh: Phong Nha–Ke Bang NP	This study
T. vietnamense	MZ376713	ZFMK-SAKF008		–		This study
T. vietnamense	MZ376714	ZFMK-SAKF009		–		This study
T. vietnamense	MZ376725	ZFMK PG001		–		This study
T. vietnamense	KU561886	NCSM 76490	Laos	18	Savannakhet: Tham Bing Cave	Dever (2017)
T. vietnamense	LC012862	KUHE 22056	Thailand	19	Phrae: Mae Yom NP	Nguyen et al. (2015)
T. horridum	KT461890	ZMMU NAP–04015		20	Satun: Tha Le Ban	Poyarkov et al. (2015)
T. horridum	–	BMNH		21	Pattani: Bukit Besar (type locality)	Boulenger 1903
T. horridum	LC012861	KUHE 52582	Malaysia	22	Negeri Sembilan: Kenaboi	Nguyen et al. (2015)
T. horridum	KC465842	LJT W45		–		Li et al. (2013)
T. horridum	KC465843	LJT W44		–		Li et al. (2013)