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Additional information on the Giant Himalayan Horned Frog, *Megophrys periosa* Mahony, Kamei, Teeling & Biju, 2018 (Anura: Megophryidae)

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Abstract

In the last decade, there has been a major revision in the cryptic species complexes of the genus *Megophrys* Kuhl & Van Hasselt (and earlier *Xenophrys*), resulting in reports of many new species and new records from Arunachal Pradesh, India. Here we report additional information on the morphological variations of the recently described species *Megophrys periosa* Mahony, Kamei, Teeling, and Biju, 2018 (Anura: Megophryidae) from the surroundings of the type locality. The species was confirmed by generating the 16S rRNA sequences and comparing them with that of *M. robusta* Boulenger, 1908, a sympatric species with similar morphological characters. A preliminary phylogenetic tree has been made and discussed. Besides updating the altitudinal range for the species, a remark on the type locality is also made.

Keywords: Aruanchal Pradesh, Eastern Himalayas, Molecular Studies, North-East India

Introduction

The genus *Megophrys* Kuhl & Van Hasselt, 1822 is represented by 106 species all across the distributional ranges of the Tropical Asian region, including India, Bhutan, China, Sundas and the Philippines (Frost, 2020). At present, 17 species are reported from India (Dinesh *et al.*, 2020). Until the year 2012, most of the species were treated under the genus *Xenophrys* due to complexity in generic and species-level taxonomic identification (Dinesh *et al.*, 2012). With the advent of molecular tools and intensive field-based studies, and museum specimen examinations, taxonomic revisions as well as phylogenetic studies have been taken up in the *Megophrys* populations across India (Mahony *et al.*, 2013, 2018, 2020; Deuti *et al.*, 2017) and the neighbouring countries in recent years with the addition of 12 new species to science. *Megophrys periosa* was described by Mahony *et al.* (2018) from Arunachal Pradesh while resolving the taxonomic identity of one of the morphologically cryptic *Megophrys major* Species Group (MMSG) using molecular studies.

Previously, the genus *Megophrys* in Arunachal Pradesh had six species, namely *M. major* Boulenger, 1908, *M. robusta* Boulenger, 1908, *M. minor* Stejneger, 1926, *M. pachyproctus* Huang, 1981, *M. ancrae* Mahony, Teeling, and Biju, 2013 and *M. vegrandis* Mahony, Teeling, Biju, 2013 (Mahony *et al.*, 2013; Saikia and Sinha, 2018). Ohler *et al.* (2018) reported the presence of *M. major* Boulenger, 1908 from Arunachal Pradesh; however, Mahony *et al.* (2018) restricted *M. major* to Meghalaya, Manipur and Nagaland only with a possibility of it occurring in Western Myanmar.

As part of our ongoing studies on the Amphibian diversity in the Eastern Himalaya landscape, two large-sized *Megophrys* frogs were collected from the outskirts of Sessa Orchid Wildlife Sanctuary (SOWS) and Eaglenest Wildlife Sanctuary (EWS), both located in West Kameng district of Arunachal Pradesh. Due to morphological character crypticity prevailing in the genus, initial identification was limited up to the genus level as most of the morphological characters shared by the species *M. robusta* and *M. periosa* were similar.

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Mahony *et al.* (2018), while describing *M. periosa*, mentioned two morphological differences – absence of "*black dermal asperities on posterior abdomen*" and Finger I longer than Finger II – from *M. robusta*. However, Boulenger (1908) while describing *M. robusta* mentioned that Finger I is equal to or longer than II (i.e. II≤I) and the region of throat and breast spotted/marbled with brown. Due to this minor morphological character differences across the two species and with an overlapping range of distribution around Sessa in West Kameng district of Arunachal Pradesh (Mahony *et al.*, 2018) it becomes very difficult to accurately differentiate between these two species morphologically. Owing to the complexity mentioned, our collections were subjected to molecular studies (mt 16S rRNA) to confirm the identity of the species genetically.

Material and Methods

One male (V/A/NERC/1526) and one female (V/A/NERC/1527) specimens were collected respectively from the outskirts of EWS (27.0987 N; 92.5273 E; 1113 m asl) and SOWS (27.1045 N; 92.5268 E; 1429 m asl) on 31st August 2019 by Bikramjit Sinha. The collection locations are characterised by tropical moist evergreen and semi-evergreen forests interspersed with subtropical broad-leaved forests. Both the areas have thick vegetation with very little sunlight reaching the ground, evident from thick layers of moss on the surfaces. The amphibians were collected after dusk hours from moss covered logs and large boulders on the banks of the streams.

Euthanised specimens were dissected to ascertain their respective sexes, and the liver sample was extracted and fixed in absolute alcohol (EMSURE®, Merck KGaA, Germany) for molecular studies. Initially, specimens were fixed in 10% formalin and preserved in 70% alcohol subsequently for further studies. Detailed morphometric measurements provided in Table 1, after Mahony *et al.* (2018). The following measurements were taken (in millimetres) with a Mitutoyo™ digital calliper: SVL (snout vent length), HW (head width), HL (head length), SL (snout length), SN (snout nostril distance), EN (eye nostril distance), EL (eye length), IUC (inter-orbital distance, UEW (upper eyelid width), IFE (distance between anterior eye corner), IBE (distance between the posterior eye corners), TYD (maximum tympanum diameter), TYE (tympanum eye distance), FAL (forearm length, from elbow to wrist), HAL (hand length, from wrist to tip of finger III), FIL (first finger length, from tip of finger I to the base where it joins finger II), FIIL (second finger length, from the tip of finger II to the base where it joins finger I), FIIIL (third finger length, from the tip of finger III to the base where it joins base of finger II), FIVL (fourth finger length, from the tip of finger IV to the base where it joins finger III), TL (thigh length), SHL (shank length), TFOL (tarsus and foot length), TFL (foot length), IMT (inner metatarsal tubercle length).

Table 1. Morphometric data of *M. periosa* specimens from Sessa. Measurements followed after Mahony *et al.* (2018) for comparison

Reg. No.	**V/A/NERC/1526**	**V/A/NERC/1527**
Sex	**Male**	**Female**
SVL	88.7	97.9
HW	34.6	42.4
HL	30.8	36.5
IFE	17.1	17.9
IBE	25.3	28.2
EL	9.4	9.9
TYD	4.2	4.6
TYE	8.6	9.5
SL	12.3	12.6
EN	4.7	5.6
SN	5.9	6.3
IUE	11.0	11.1
IN	10.8	11.4
UEW	7.9	9.0
FAL	19.2	20.9
HAL	24.3	24.8
FI	12.1	12.3
FIIL	10.3	10.5
FIIIL	16.1	16.2
FIVL	9.7	9.9
SHL	51.1	54.6
TL	49.3	54.6
TFOL	70.4	73.8
TFL	45.5	48.0
IMT	6.9	6.9

Methodology for the genetic analysis, including DNA extraction, PCR amplification of the 16S rRNA and the sequencing, were followed as described for the *Nanorana* frogs of northeast India by Saikia *et al.* (2020). With the 16S rRNA generated in the current studies for *Megophrys*, 16S rRNA sequences available for the species of *Megophrys* and *Xenophrys* at the GenBank were downloaded (Table 3) and aligned using MEGA 5.2 (Tamura *et al*., 2011) as in group and *Ophryophryne* was used as outgroup (Pyron and Weins, 2011). The final consensus tree was visualised using FigTree v1.4.0 for the Maximum Likelihood (ML) tree generated using RaxML (Silvestro and Michalak, 2012) under GTR+GAMMA+I model by running 1000 thorough bootstraps.

Results and Discussion

Morphological character crypticity in the genus *Megophrys* is challenging in the field conditions for the proper identification of the species and is an impediment for the species conservation efforts as the correct identification of taxon is crucial. Generation of mt DNA barcodes for such morphologically cryptic species is considered as an alternate tool for identification of the cryptic species which are in sympatry. In the present study, identity of *M. periosa* was confirmed only by generation of 16S mt DNA barcode and its phylogenetic tree. Wherein *M. robusta* and *M. periosa* exhibit morphological crypticity without any differentiable morphological characters but belongs to sister clades on the phylogenetic tree.

Our sequences for the *M. periosa* were forming monophyletic clade ('*periosa* clade') (Figure 1) with that of *M. periosa* of Mahony *et al.* (2018), confirming the identity of the species without any nucleotide base pair differences. One of the sequences, KY022309.1 of '*periosa* clade' in the GenBank is labelled as the nomen *Megophrys major* which needs to be corrected to *M. periosa* as these nomens can create sequence identity problems who rely on BLAST (Basic Local Alignment Search Tool) for the identification of the species. Phylogenetic relationships among the rest of the members were not showing robust boost strap values reflecting the under-sampling of taxon in the analysis (Figure 1). The nomen *M. major* is reflected in multiples clades of the tree (Figure 1) which needs voucher specimen based taxonomic studies as the samples are from different localities (Frost *et al*., 2006; Mahony *et al*., 2017, 2018) (Table 3).

Our male specimen (Figure 2a) is large (SVL 88.4 mm), which is as large as the largest known male of *M. periosa* [71.3-88.8 mm]. It has a pair of internal vocal slits on the floor of the mouth towards the rear side of the mandible. The female specimen (Figure 2b) is larger than the male (SVL 97.7 mm) and is only the second female of this species known till now after the allotype (112.0 mm). The specimen contains eggs (variable diameters of 2.8-3.1 mm) without pigmentation. There are no major morphometric variations observed *vis-à-vis* the type

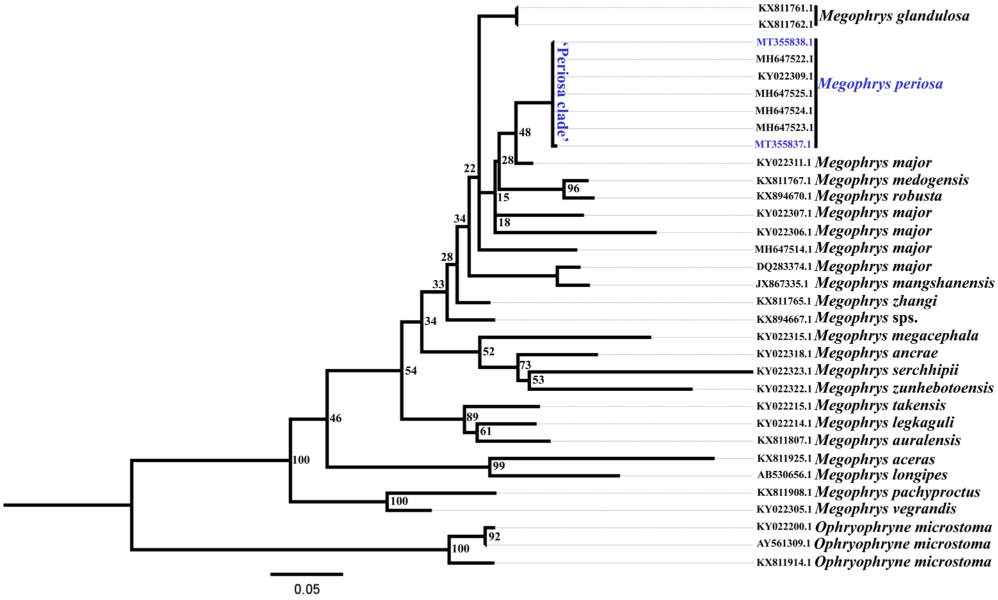

Figure 1. Maximum Likelihood (ML) tree for the species of *Megophrys* based on 521 bp of mt 16s DNA.

Figure 2. Field photographs of the specimens of *M. periosa* from Sessa, West Kameng, Arunachal Pradesh; **a** – V/A/NERC/1526 (♂) from EWS, and **b** – V/A/NERC/1527 (♀) from SOWS.

materials, except in V/A/NERC/1527 where SHL=TL. Only in paratypes (BHNS 6057 and BNHS 6061) it has been observed that SHL=TL; otherwise, SHL>TL in *M. periosa*.

Mahony *et al.* (2018), while describing this species, attributed the type locality as Pangin town in East Siang district of Arunachal Pradesh, while since 2015, Pangin town has been serving as the district headquarters of the newly created Siang District of the State. This error is probably due to the fact that the type series was collected in 2009, when Pangin was a part of East Siang District.

Currently, *M. periosa* is known from three locations in Arunachal Pradesh (Pangin, Along and Sessa), and provisionally from Manipur (Kangpat Khullen in Ukhrul district) and Kachin state of Myanmar. We are reporting this species additionally from the outskirts of SOWS and EWS in West Kameng district of Arunachal Pradesh, which is within 500 m aerial distance of the paratypes locality. Our report also extends the altitudinal range of occurrence for this species by more than 300 m from the previous range of 260 – 1110 m to the current range of 260-1429 m (Table 2).

The current study adds specimens to the museum collections of *M. periosa* to understand the morphological variations across the populations, and the sequences generated are expected to be used in the phylogeographic studies as the species is known to have a wide range of distribution across Arunachal Pradesh, Manipur and Myanmar.

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Table 2. Confirmed locality records for *Megophrys periosa* (AP=Arunachal Pradesh, MN=Manipur)

Location	Latitude	Longitude	Altitude (m)	Reference
Pangin, Siang Dist, AP	28.2094	94.9861	450	Mahony *et al.* (2018)
Sessa River, West Kameng, AP	27.1011	92.5275	1110	-do-
Rigo Vill., Along, West Siang, AP	28.1596	94.7886	260	-do-
Khangpat Khullen, Ukhrul, MN	24.6726	94.4722	820	-do-
Hepu Stream, Kachin, Myanmar	25.0903	96.4036	NA	-do-
Sessa River, SOWS, West Kameng, AP	27.1045	92.5268	1429	Present Report
Unnamed hill stream, Sessa, EWS, West Kameng, AP	27.0987	92.5273	1113	Present Report

Table 3. Details of 16S rRNA sequences downloaded from the GenBank and included in the generation of maximum Likelihood tree

Sl. No.	GenBank Accession No.	Species name as per GenBank	Locality	Publication details
1.	KX811761.1	*Megophrys glandulosa*	China	Chen *et al.* (2017)
2.	KX811762.1	*Megophrys glandulosa*	China	Chen *et al.* (2017)
3.	MT355838.1	*Megophrys periosa*	India: Arunachal Pradesh, Eaglenest Wildlife Sanctuary	Present study
4.	MH647522.1	*Megophrys periosa*	India: East Siang district, Arunachal Pradesh	Mahony *et al.* (2018)
5.	KY022309.1	*Megophrys major*	India: Arunachal Pradesh	Mahony *et al.* (2017)
6.	MH647525.1	*Megophrys periosa*	India: East Siang district, Arunachal Pradesh	Mahony *et al.* (2018)
7.	MH647524.1	*Megophrys periosa*	India: West Kameng district, Arunachal Pradesh	Mahony *et al.* (2018)
8.	MH647523.1	*Megophrys periosa*	India: West Kameng district, Arunachal Pradesh	Mahony *et al.* (2018)
9.	MT355837.1	*Megophrys periosa*	India: Arunachal Pradesh, Eaglenest Wildlife Sanctuary	Present study
10.	KY022311.1	*Megophrys major*	India: Arunachal Pradesh	Mahony *et al.* (2017)
11.	KX811767.1	*Megophrys medogensis*	China	Chen *et al.* (2017)
12.	KX894670.1	*Xenophrys robusta*	India	Deuti *et al.* (2017)
13.	KY022307.1	*Megophrys major*	India: Meghalaya	Mahony *et al.* (2017)
14.	KY022306.1	*Megophrys major*	India: Meghalaya	Mahony *et al.* (2017)
15.	MH647514.1	*Xenophrys major*	India: Kohima district, Nagaland	Mahony *et al.* (2018)
16.	DQ283374.1	*Xenophrys major*	Viet Nam: Vinh Phu	Frost *et al.* (2006)
17.	JX867335.1	*Xenophrys mangshanensis*	China	Unpublished
18.	KX811765.1	*Megophrys zhangi*	China	Chen *et al.* (2017)
19.	KX894667.1	*Xenophrys sp.*	India	Deuti *et al.* (2017)
20.	KY022315.1	*Xenophrys megacephala*	India: Meghalaya, East Khasi Hills district	Mahony *et al.* (2017)
21.	KY022318.1	*Xenophrys ancrae*	India: Arunachal Pradesh, Changlang	Mahony *et al.* (2018)
22.	KY022323.1	*Xenophrys serchhipii*	India: Tripura	Mahony *et al.* (2017)
23.	KY022322.1	*Xenophrys zunhebotoensis*	India: Nagaland	Mahony *et al.* (2017)
24.	KY022215.1	*Xenophrys takensis*	Thailand: Kampaeng, Khlong Lan National Park	Mahony *et al.* (2017)
25.	KY022214.1	*Xenophrys legkaguli*	Thailand: Mueang Sa Kaeo District, Pang Si Da National Park, Huay Kong Mou Now Stream	Mahony *et al.* (2017)
26.	KX811807.1	*Megophrys auralensis*	China	Chen *et al.* (2017)
27.	KX811925.1	*Xenophrys aceras*	China	Chen *et al.* (2017)
28.	AB530656.1	*Xenophrys longipes*	Malaysia: Genting highland	Hasan *et al.* (2014)
29.	KX811908.1	*Megophrys pachyproctus*	China	Chen *et al.* (2017)
30.	KY022305.1	*Xenophrys vegrandis*	India: Arunachal Pradesh, West Kameng, near Sessa village	Mahony *et al.* (2017)
31.	KY022200.1	*Ophryophryne microstoma*	China: Guangxi, Shiwan Dashang Nature Reserve	Mahony *et al.* (2017)
32.	AY561309.1	*Ophryophryne microstoma*	China	Yuchi *et al.* (2004)
33.	KX811914.1	*Ophryophryne microstoma*	China	Chen *et al.* (2017)

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