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Range extension of Ferguson's Toad *Duttaphrynus scaber* (Schneider) (Amphibia: Anura: Bufonidae) up to the northern most limit of Western Ghats, with its advertisement call analysis

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Abstract: *Duttaphrynus scaber* (Schneider) is known to occur in the southern Western Ghats, Eastern Ghats and northeastern India. However, there is no report of its occurrence from the northern Western Ghats from the states of Maharashtra and Gujarat. Here we report the occurrence of this species from the northernmost limit of the Western Ghats, a substantial range extension (approx. 550km) from the nearest known locality. We confirm our identification on the basis of morphology by comparing our specimens with a previously collected *Duttaphrynus scaber* specimen from Thrissur in Kerala State as well as on the molecular basis. Analysis based on the advertisement call of *Duttaphrynus scaber*, albeit preliminary, is provided for the first time for this species.

Keywords: Advertisement call analysis, *Bufo fergusonii, Duttaphrynus scaber,* range extension.

Duttaphrynus scaber (type locality 'Orientali India') was described by Schneider (1799). Boulenger (1892) described *Bufo fergusonii* (type locality 'on the Cavalry Parade ground' Thiruvananthapuram, Kerala) as a different species. However, after examining the lectotypes of *D. scaber* and comparing them with the holotype of *B. fergusonii*, Dubois & Ohler (1999) found these two species to be conspecific and hence they considered *B. fergusonii* as a junior subjective synonym of *D. scaber*.

Duttaphrynus scaber has been reported in India from

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Thriuvananthapuram in Kerala (Daniels 2005); Mysore (Daniel 1963), Lakkavalli State Forest (Krishnamurthy 1999) and Dharwad (Dutta 1997) in Karnataka; Chennai in Tamil Nadu (Rao 1915; Ravichandran 1996; Daniels 2005); Banjara Hills, Hyderabad (Donahue & Daniel 1966) in Andhra Pradesh and Sambalpur District (Dutta 1988) in Odisha. Recently the species has been recorded from the northeastern state of Manipur by Mathew & Sen (2009, 2010). Outside of India, the species has also been reported from Sri Lanka (Dutta & Manamendra-Arachchi 1996). Largely because of its wide distribution, IUCN Red List status of this species is Least Concern (Dutta & Manamendra-Arachchi 2004).

To date, *Duttaphrynus scaber* has not been reported from Maharashtra and Gujarat states, despite a number of amphibian surveys having been carried out (Padhye & Ghate 2002 and references therein; Naik & Vinod 1992, 1993a,b; Bhatta et al. 1999; Vyas 2000a,b, 2002, 2004a,b, 2007, 2008, 2012; Dinesh et al. 2009). There is a record of *Bufo fergusonii* (now *Duttaphrynus scaber*) in the list of amphibians from Shoolpaneshwar Wildlife Sanctuary, Gujarat (Sabnis & Amin 1992) but Vyas (2008; 2012) has raised the question about its validity. According to Vyas (2008), Naik & Vinod (1992, 1993a,b) never claimed any records of the species from Shoolpaneshwar Wildlife Sanctuary, which shows that the record of this toad species has been mistaken.

Therefore, we report this species from both the states with molecular evidence for the first time and describe its advertisement call.

Materials and Methods

We carried out amphibian surveys of Surgana Taluka in Nasik District, Maharashtra State and Ahwa Taluka, in Dang District, Gujarat State to assess the amphibian diversity of the northern most regions of the Western Ghats. Surveys were conducted from 1830-0230 hr on the 17 and 18 of July 2010. Specimens were identified in the field down to the species level and most were released. The presence of species was also reported on the basis of calls. Two specimens of *Duttaphrynus scaber* were collected from a road side puddle and brought back to the laboratory for confirmation. One of the two specimens was preserved in absolute ethanol and the other was preserved in 10% formalin and then transferred to 70% ethanol. These specimens were deposited at the museum of Zoology Research Laboratory, Abasaheb Garware College, Pune, under the accession numbers AGCZRL Amphibia 41 and AGCZRL Amphibia 42, respectively. Morphometry was performed using digital Vernier caliper (Areospace® China, least count 0.01mm).

Thigh muscles of AGCZRL Amphibia 41 were used for the molecular analysis. The tissue was digested at 50^0C for two hours using the extraction buffer (0.1M NaCl, 0.05M Tris-HCl, 0.01M EDTA, 1%SDS) with 15µl Proteinase K (20mg/ml). DNA was then extracted using the conventional phenol:chloroform method (Sambrook et al. 1989). The partial fragment of each of the mitochondrial ribosomal RNA genes 12S (~400 bp) and 16S (~520 bp) were amplified using PCR. The 12S segment of mitochondrial DNA was amplified using universal primers L1091 (5'-AAACTGGGATTAGATACCCCA CTA-3') and H1478 (5'-GAGGGTGACGGGCGGTGTGT-3') as described by Kocher et al. (1989). While for 16S rDNA we used primers 16SA (5'-CGCCTGTTTATCAAAAACAT-3') and 16SB (5'-CCGGTCTGAACTCAGATCACGT -3') as described by Simon et al. (1994). PCR products were purified by using PEG-NaCl protocol (Sambrook et al., 1989). Products were sequenced on ABI 3730 DNA analyzer (Applied Biosystems, Hitachi), as per the manufacturer's instructions.

Sequences were manually checked for any ambiguity using BioEdit (Hall 1999) and were deposited in GenBank (http://www.ncbi.nlm.nih.gov/) under the accession number JQ898085 (12S rDNA) and JQ898086 (16S rDNA). Additional sequences of 12S and 16S rDNA for comparison were downloaded from GenBank. We retrieved 12S and 16S rDNA sequence for *Duttaphrynus scaber* (FJ882785), *Duttaphrynus melanostictus* (DQ283333), *Duttaphrynus stomaticus* (FJ882787), *Duttaphrynus parietalis* (FJ882784), *Duttaphrynus hololius* (FJ882781), *Duttaphrynus brevirostris* (FJ882786), *Pedostibes tuberculosus* (FJ882793), *Xanthophryne koynayensis* (FJ882782) and *Ansonia ornata* (FJ882797) for comparision. *Microhyla ornata* from family Microhylidae was used as an out-group and 12S (AB201176) and 16S (AY948728) sequence of the same were retrieved from GenBank. Sequences were aligned independently for each gene using MUSCLE (Edgar 2004). The individual gene sequences were trimmed using freeware DAMBE 5.2.77 (Xia & Xie 2001). Both the gene sequences were concatenated before performing the phylogenetic analysis. Phylogenetic and molecular evolutionary analyses were conducted using MEGA5 (Tamura et al. 2011). Best fit model for nucleotide substitution was selected based on minimum Akaike Information Criterion (AIC) value (Posada & Crandall 2001). We constructed phylogenetic trees based on maximum parsimony and maximum likelihood. Reliability of the phylogenetic tree was estimated using bootstrap values run for 1000 iterations.

Advertisement calls of two frogs were recorded with a mobile phone in AMR (Adaptive Multi-Rate) format at 8kHz sampling rate. Because the calls recorded on the mobile phone have limited sampling frequency we provide a very preliminary account of the advertisement call. Nevertheless, our analysis is important since there is no available information on the calls of this species. Calls were recorded at a distance of approximately 0.5m from the calling frogs. The toads were sitting on the sides of a temporary rain-water pool. The atmospheric temperature was 21.2^{0}C. Calls were analysed with Raven Pro 1.4 software (Bioacoustics Research Program 2003) and Praat sound analysis program version 5.2 (Boersma & Weenink 2009). Audio spectrograms were calculated with fast-Fourier transform (FFT) of 256 points, 50% overlap and 31.3 Hz grid-spacing, using Hanning windows. We tested the hypothesis that all different call parameters were same for both the individuals by performing unpaired t test.

Results and Discussion

During our field surveys we observed a total of three *D. scaber*. A single male *D. scaber* (not collected) was observed on 17 July 2010 on the banks of Ahwa Lake (20^{0}45'9"N & 73^{0}40'39"E, elevation 455m), Ahwa, District Dang, Gujarat at 2340hr. This male was calling in the open on the edge of the reservoir (Image 1). The two collected males were found calling in roadside puddles, which were flanked by paddy fields (20^{0}37'18"N & 73^{0}38'56"E, elevation 356m). These males were collected on 18 July 2010 at Supdahad, Ahwa Taluka, Dang District, Gujarat, at 0050hr. There was sporadic rain on the same day. Further, calling individuals of this species were observed near the Village Satkhamb, Taluka Surgana, District Nasik, Maharashtra (20^{0}35'31"N & 73^{0}34'47"E, elevation 385m). Judging from the number of calling males heard, the species appears to be locally abundant. The overall habitat was road surrounded by paddy fields and fragmented patches of deciduous forest (Image 2). Our new records of *D. scaber* represent a substantial northwestern range extension to the northernmost limits of the Western Ghats of India (Fig. 1).

Duttaphrynus scaber individuals were identified by the presence of distinct parietal ridges on head, numerous spiny warts on the entire body, a fawn coloured underside, the 1st and 2nd fingers equal in length, toes scarcely webbed, parotid glands rounded and tympanum less than half the diameter of eye (Daniel 1963; Dubois & Ohler 1999; Daniels 2005; Mathews & Sen 2010) (Image 3a–c). The specimens were also compared with a previously collected museum specimen from Thrissur, Kerala (Voucher No. AGCZRL Amphibia 98), which was identified by Dr. Annemarie Ohler (Museum National d'Histoire Naturelle, Paris). Morphometrics of the two specimens collected during the current survey and the specimen from Thrissur are given in Table 1.

Further identification of the specimens was confirmed by comparing the 12S and 16S mitochondrial rDNA partial sequences with the available Gene Bank sequence of *Duttaphrynus scaber* and other allied species. Percent divergence in partial sequences of 12S and 16S genes between the freshly collected specimens of *D. scaber* and GenBank sequences was 0.4%. As compared to other *Duttaphrynus* species considered in the study, *D. scaber* in our collection showed a minimum divergence of 5.3% and maximum of 8%. Modeltest performed in MEGA 5 revealed that the nucleotide substitution rates could be best described by General Time Reversal model with Gamma Distribution (AIC = 5247.40, lnL = -2595.61). Phylogenetic analysis based on maximum likelihood

Image 1. *Duttaphrynus scaber* male calling in the open on the banks of Ahwa Lake, Ahwa, Dang District, Gujarat.

Image 2. Habitat of *Duttaphrynus scaber* near Sup-Dahad Village, Ahwa Taluka, Dang District, Gujarat.

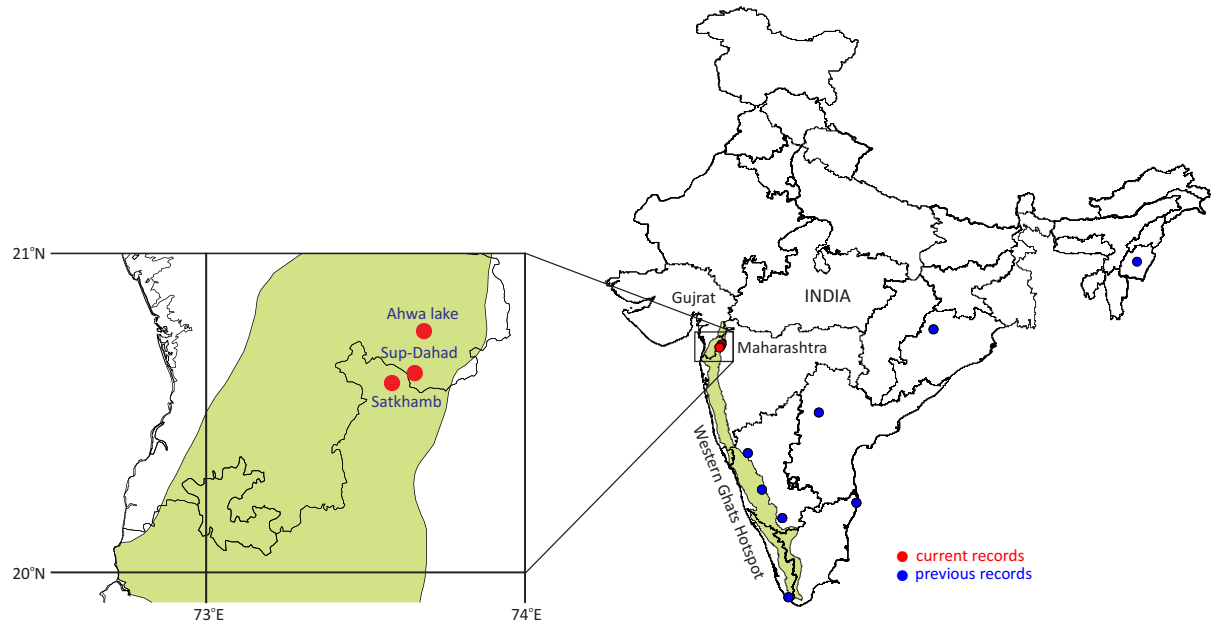

Figure 1. Distribution map of *Duttaphrynus scaber*.

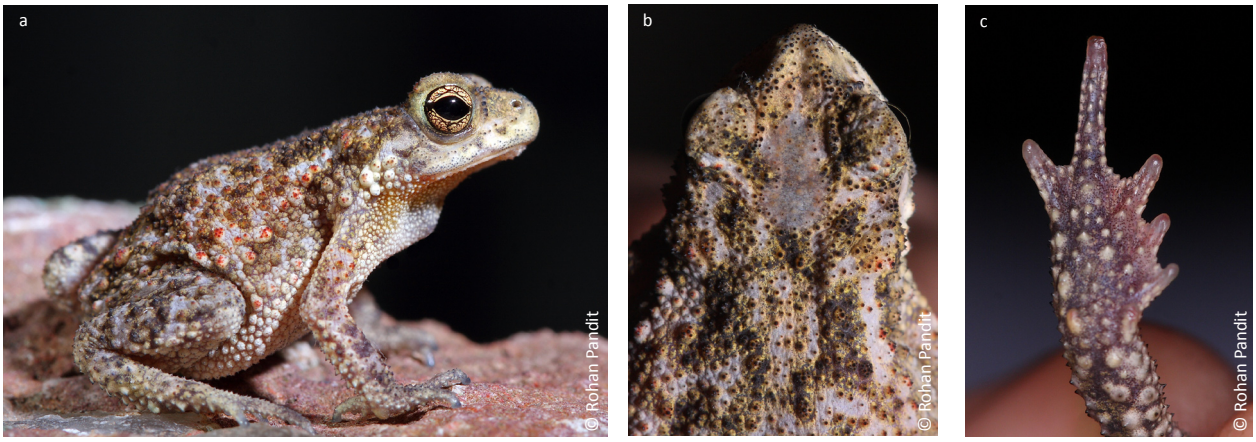

Image 3. *Duttaphrynus scaber* collected near Sup-Dahad Village, Ahwa Taluka, Dang District, Gujarat. a - Lateral view; b - dorsal view of the head region; c - close-up of the foot. Note the presence of distinct parietal ridges on head and numerous spiny warts, fawn coloured underside, toes scarcely webbed, parotid glands rounded and tympanum less than half the diameter of eye.

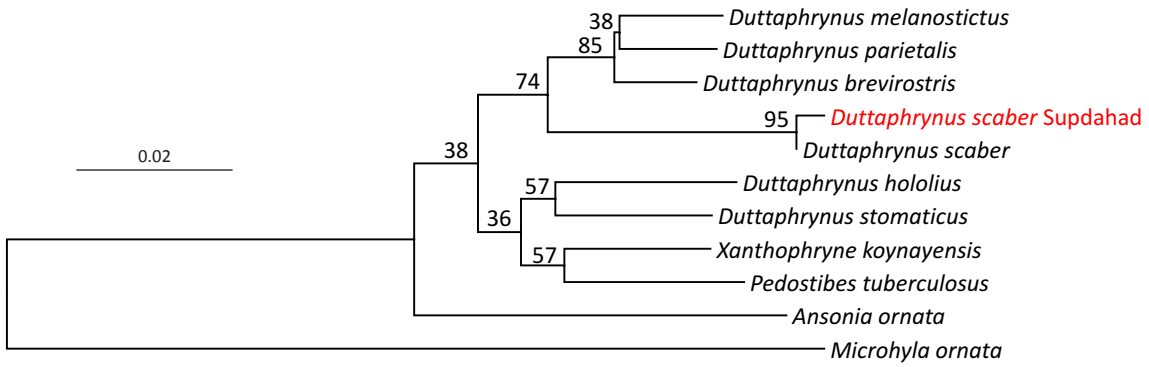

Figure 2. Bootstrap consensus trees using maximum likelihood method depicting the taxonomic status of specimen collected in the current study marked in red. The tree is drawn to scale, with bootstrap values as a percentage of 1000 iterations. There were a total of 821 positions in the final dataset of concatenated 12S and 16S rDNA partial gene sequences.

Table 1. Morphometrics (in mm) of specimens from Supdahad, Gujarat and a specimen from Thrissur, Kerala.

Characters	Voucher Number		
	*AGCZRL (male) Amphibia 41	AGCZRL (male) Amphibia 42	AGCZRL (female) Amphibia 98
Place of collection	Supdahad, Gujarat	Supdahad, Gujarat	Thrissur, Kerala
Snout to vent length	24.2	25.5	36.0
Head length	7.4	7.8	11.8
Head width	8.5	8.8	12.8
Inter narial distance	1.5	2.0	3.2
Snout length	0.9	1.1	1.7
Eye diameter	2.6	2.8	3.8
Inter orbital distance	2.7	2.9	3.4
Width of upper eyelid	1.8	2.3	3.1
Tympanum diameter (vertical)	1.0	1.1	2.6
Tympanum diameter (horizontal)	1.1	1.2	2.3
Eye-tympanum distance	0.8	0.9	0.3
Forelimb length	17.3	17.9	20.7
Hindlimb length	28.1	28.6	41.6
Femur	6.6	7.0	11.7
Tibia	8.4	8.5	11.6
Foot	13.1	13.7	18.3
Length of parotid gland	1.8	2.2	3.5
Width of parotid gland	1.8	2.2	3.3

* used for DNA analysis

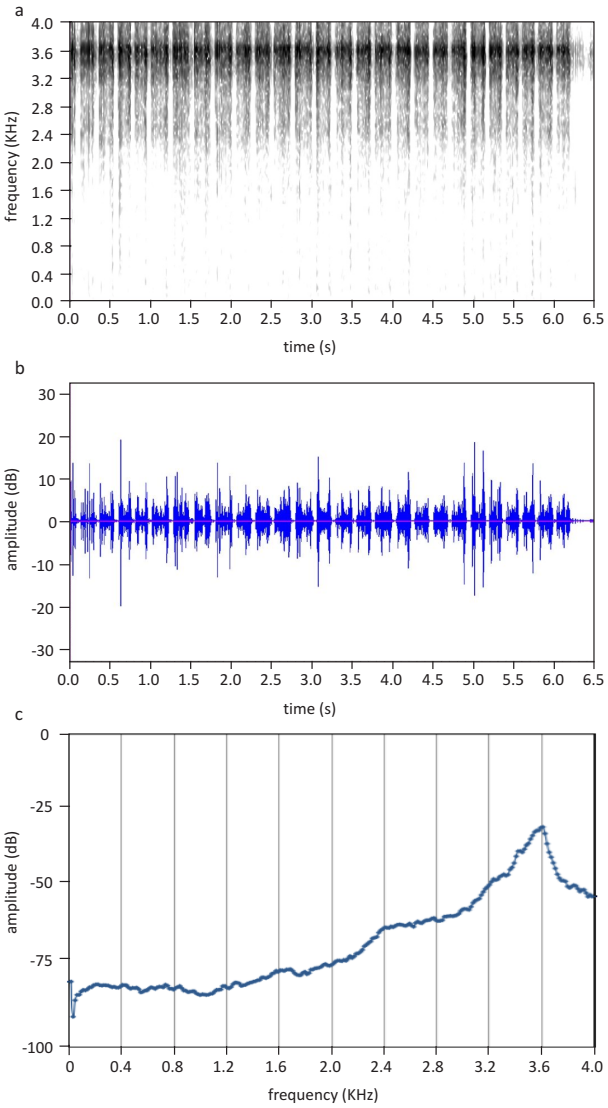

Figure 3. Advertisement call analysis of *Duttaphrynus scaber* (Call-2). (a) The spectrogram of advertisement call, (b) the waveform of advertisement call and (c) power spectrum depicting the dominant frequency. Note that frequency was spread over a band in the region of 3.0 to 4.0 KHz, the dominant frequency with highest amplitude is 3.5 to 3.7 KHz and pattern with rapid amplitude modulation within each note.

method suggests further conspecificity with a known *Duttaphrynus scaber* specimen from Western Ghats for which the sequences are available in GenBank (Fig. 2).

The advertisement call of *Duttaphrynus scaber* is a sustained call that is mildly harsh and scratchy in tone (Audio 1). It has a regular beat pattern giving the feel of a small saw quickly going back and forth with a brief interlude at each end. The call file is provided as supplementary data. The frequency of the call is spread over a band mostly in the region of 2.5–4.5 kHz (Fig. 3a). The dominant frequency was 3.6 kHz (Table 2, Fig. 3c). Though the sampling freq was 8 kHz the software used for analysis could render the upper bound unto 4 kHz that is evident from the fig. 6a. Due to the limitations of the sampling frequency the energy levels were cut off at 4 kHz however it can be predicted that the frequency range could reach 4.5 kHz which is evident from the Fig. 3a. Each note thus had an average duration of 155.8 (sd = 24.50) ms, the note interval was about 85.6 (sd = 10.60) ms and number of notes per minute was 251 (Table 2). The waveform (Fig. 3b) shows the beat pattern of the call and the rapid amplitude modulation within each note highlights the scratchy sound each note makes. Comparison of the two calls from two different males revealed that there was no significant difference in higher frequency (t = -0.562, df = 40, P = 0.577), dominant frequency (t = -1.959, df = 40, P = 0.057) and call duration (t = 1.295, df = 40, P = 0.203) of the two calls but there was significant difference in the lower frequency (t = -3.683, df = 40, P = 0.001) and inter-note duration (t = 2.495, df = 38, P = 0.017). This indicates that

Table 2. Call parameters for two individuals of *Duttaphrynus scaber*. Mean values are accompanied with standard deviations in parenthesis.

Parameter	Call 1	Call 2
Number of notes	16	26
Note rate per minute	234.30	267.84
Note duration (ms)	162.0 (31.39)	152.00 (18.80)
Inter-note duration (ms)	90.7 (9.88)	82.56 (9.99)
Lower frequency (KHz)	3.32 (0.05)	3.37 (0.04)
Higher frequency (KHz)	3.67 (0.03)	3.67 (0.02)
Dominant frequency (KHz)	3.55 (0.03)	3.57 (0.04)

except for the lower frequency and inter-note duration the call pattern for two individuals was not significantly different. Even though the recorded calls had limitations in terms of sampling frequency, the preliminary analysis provided here is important as, to our knowledge, there is no other information about the calls of this species.

Our report of *Duttaophrynus scaber* based on both morphological and genetic data extends the geographical distribution of the species up to the northern most limit of the Western Ghats of India. From the previous reports, this species was known from southern Western Ghats up to Karnataka, Eastern Ghats up to Odisha and Manipur from northeastern India. However its presence in northern Western Ghats indicates that the species may be distributed throughout most of peninsular India. Shoolpaneshwar Wildlife Sanctuary, the place from where Sabnis & Amin (1992) reported the species, is far away from the location indicated by the current locality records. Further, the specimens collected by them (if any) should be confirmed by DNA analysis. This emphasizes the need for detailed surveys for the presence of this species in Satpura ranges as well as plains of Maharashtra State.

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