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Phylogenetic Relationship of *Duttaphrynus melanostictus* From India and China as Revealed from the Study of 12S and 16S mtDNA Genes

Debojyoti Dutta and Sanjib Kr. Das
Department of Zoology, Acharya B.N. Seal College, CoochBehar, Govt. of West Bengal, PIN-736101, India

Corresponding Author: Debojyoti Dutta, Department of Zoology, Acharya B.N. Seal College, CoochBehar, Govt. of West Bengal, PIN-736101, India

ABSTRACT

In the present study, the phylogenetic relationship of *Duttaphrynus melanostictus* from West Bengal, India with other members of the Bufonidiae group was undertaken using partial mitochondrial DNA (mtDNA) genes. Mitochondria were isolated from the liver of *Duttaphrynus melanostictus* by a non-conventional method of membrane filtration. The technique allows trapping of mitochondria on cellulose acetate membrane followed by mtDNA isolation. 12S ribosomal RNA and 16S ribosomal RNA was sequenced with primers designed in our laboratory. mtDNA sequence from 18 different *Bufo* sp. found across the world were used for the phylogenetic analysis. Results were interpreted from the transition/transversion of nucleotides, genetic distance and maximum parsimony analysis. The findings indicates that *D. melanostictus* is very closely related to the *Bufo melanostictus* of China. The possible reasons of such close similarity between two distantly residing species (*D. melanostictus* of India and *Bufo melanostictus* of China) have been discussed.

Key words: mtDNA, phylogeny, *Duttaphrynus*, 12S rRNA and 16S rRNA

INTRODUCTION

Mitochondria are loaded with rare genetic information due to its unique pattern of maternal inheritance and thus make it an ideal tool for the study of genetic variation (Gyllensten *et al*., 1985) and phylogenetic relationships (Avise, 1986). The mitochondrial DNA (mtDNA) is a double stranded circular DNA, harbouring genes necessary for self replication and code for mitochondrial proteins that enable to yield energy rich ATP molecules. These genes have a clonal lineage of inheritance and their mutation rate is five to ten times faster than the nuclear genome. These features have given the mtDNA the uniqueness to be utilized by evolutionary biologist to predict the phylogenetic relationship of closely related species more accurately in evolutionary time scale.

Duttaphrynus melanostictus is a dominant toad of West Bengal, India which appears to have an intercontinental distribution. Apart from *D. melanostictus,* there are several other species that inhabit the Gangetic plains of West Bengal whose phylogenetic relationship is unclear. Several studies on the phylogenetic relationship of the family Bufonidae has been undertaken (Graybeal, 1997; Liu *et al*., 2000; Noonan and Gaucher, 2005; Frost *et al*., 2006; Pramuk, 2006) but always represented poorly by Indian specimens. Complete mtDNA sequence of *Bufo melanostictus* from China has been sequenced (GenBank ID: AY458592) (Zhang *et al*., 2005) but no such data of

D. melanostictus from West Bengal, India has ever been reported. Moreover, the relationship of the three most common toads, namely *Duttaphrynus melanostictus*, *B. stomaticus* and *D. himalayanus* found in West Bengal has remained elusive.

In the present investigation, we present an innovative method of cellulose acetate filter based method for separating mitochondria from the liver of *D. melanostictus* and subsequently isolated the mtDNA to sequence it 12S and 16S rRNA along with few tRNA genes and looked into the sequence similarity of the non protein coding regions with mtDNA of *Bufo melanostictus* from China and other members of the family Bufonidae from India and abroad to get a glimpse of their phylogenetic relationship. To distinguish our toad from *B. melanostictus* from China, we will refer our toad as *D. melanostictus* (I-WB).

MATERIALS AND METHODS

Animal model: *Duttaphrynus melanostictus* Schneider, 1799 (I-WB), that falls under the "least concerned" category of the IUCN red list 2007, was the animal model for the present study. The animals caught from the wild were sacrificed following the guidelines and approval the Animal Ethical Committee of the Institute.

Isolation of mitochondria by membrane filtration: The liver was (0.8 to 1.2 g) removed aseptically and washed with chilled Lysis buffer-I (10 mM Tris-HCl; pH 7.8; 1 mM EDTA; pH 8.0; 0.32 M Sucrose) to rinse off the excess blood and then minced and homogenized in Dounce homogenizer. The homogenized tissue was subjected to centrifuge at 400 g for 15 min at 4°C. The supernatant was gently collected in a 2 mL syringe fitted with a filter paper holder containing a 0.45 μm Cellulose Acetate filter paper (Sartorius GmbH, W. Germany). The lysate was forced through the filter paper to trap the mitochondria in the cellulose acetate filter. The filter paper was then removed from the holder and was either processed immediately for mtDNA isolation or preserved at 4°C for mtDNA isolation later.

Isolation of mtDNA: Mitochondria trapped in filter paper was placed in 1 mL lysis buffer-II (10 mM Tris-HCl; pH = 8.0, 150 mM NaCl, 2 mM EDTA, 1% SDS, 100 μg Proteinase K), rocked gently for 5 min and then incubated at 55°C for 60 min. The membrane was gently removed and equal volume of buffered-saturated phenol was added to the sample and mixed gently by inversion. The mixture was then centrifuged at 13000 g for 5 min. The aqueous layer was collected and the process of phenol treatment was repeated twice. Finally, the aqueous layer was collected in a fresh tube. Two hundred microlitter of 5 M NaCl was then added to the sample and the mtDNA was precipitated with 2.5 volumes of absolute alcohol.

Primers and PCR parameters: Several primers were designed using the software Primer 3 from the mtDNA sequence available in the GenBank (Locus AY458592; Zhang *et al*., 2005, 2000). List of primers used to amplify and sequence 12S rRNA and 16S rRNA from *B. melanostictus* (I-WB) mtDNA is shown in Table 1. PCR was carried out following standard protocol in Primus 25 Peqlab with annealing temperature at 52°C. Sequencing of the amplified segments was done at Chromus Biotech, Bangalore. To remove any ambiguity in the sequenced bases, sequences were crossed checked with the elctrogpherogram of the complementary strand.

Sequences of other toad species included in the study to analyze the phylogenetic relationship of *D. melanostictus* (I-WB) are listed in Table 2.

Table 1: List of primers used to amplify 12S and 16S rRNA of *Duttaphrynus melanostictus*

Primer ID	Sequence 5`-3`	
Bm_01	Ggctccagtacctcttggtg	Lp
	Tcgtgtaggattgggctagg	Rp
Bm_01a	Acacccacaagggatctcag	Lp
	Tcgtgtaggattgggctagg	Rp
Bm_02N	Aatgggaagagatgggctac	Lp
	Acttgcctcttctgttgtgc	Rp
Bm_03N	Agcggatctaagtccaacct	Lp
	Gtggcgagtgctactttgtt	Rp
Bm_04N	Ctcgattccgatatgaccag	Lp
	Gccatgttgagagaatgagg	Rp
Bm_05	Accaaaaacatcgcctcttg	Lp
	Actccatccgcaataggttg	Rp
Bm_07	Ttttggcagttgctttcctc	Lp
	Aggaggcccttagctttcag	Rp
Bm_08	Cccaccaatctcatggaaac	Lp
	Ggtcaggaatagcgtggatg	Rp
Bm_09	Tccaaacaggcctcattctc	Lp
	Gaacgaagctcgctggatag	Rp

Table 2: List of Toads used for this study with their Gene Bank Accession number and number of nucleotides considered for analysis

Specimen name	Gene Bank Id	Nucleotides (bp)
Bufo melanostictus (I-WB)		5228
Bufo melanostictus(China)	AY458592	17374 bp (complete genome)
Bufo_andrewsi_(China)	FJ882808	3871
Bufo_brevirostris_(I-Wg-e)	FJ882786	3863
Bufo_bufo_(Euro)	FJ882806	3833
Bufo_divergens_(Malayasia)	FJ882802	3807
Bufo gargarizans (E-Asia-China)	DQ275350	17277 bp (complete genome)
Bufo_hololins_(I)	FJ882781	3842
Bufo himalayanus(China)	FJ882790	3861
Bufo_juxtasper_(Malayasia)	FJ882805	3818
Bufo_macrotis_(I-NE_SEA)	FJ882803	3867
Bufo_melanostictns (I-Wg-SL)	FJ882791	3864
Bufo_parietalis_(I-Wg-e)	FJ882784	3836
Bufo_scaber_(I-Wg_Sl)	FJ882785	3831
Duttaphrynus sp (I-Wg-SL)	FJ882792	3872
Bufo_stuarti_(I-NE_SEA)	FJ882788	3862
Bufo_verrncosissimus_(Russia_Iran)	FJ882807	3868
Bufo japonicus(Japan)	AB303363	17277 bp (complete genome)

Sequent alignment analysis: Pair wise alignments of the mtDNA nucleotide sequence were computed with the aid of BLASTN 2.2.25 tool (Zhang *et al*., 2000) and multiple alignments with ClustalW. The sequences were checked visually before estimating the nucleotide statistics. Software program was designed in Office Excel-2007 to determine the transition and transversion from aligned sequences.

RESULTS

Transition/transversion of nucleotides: Phylogenetic analysis was conducted with the data set comprising of 3830 nucleotide sites which were aligned unambiguously. Of the 3830 sites,

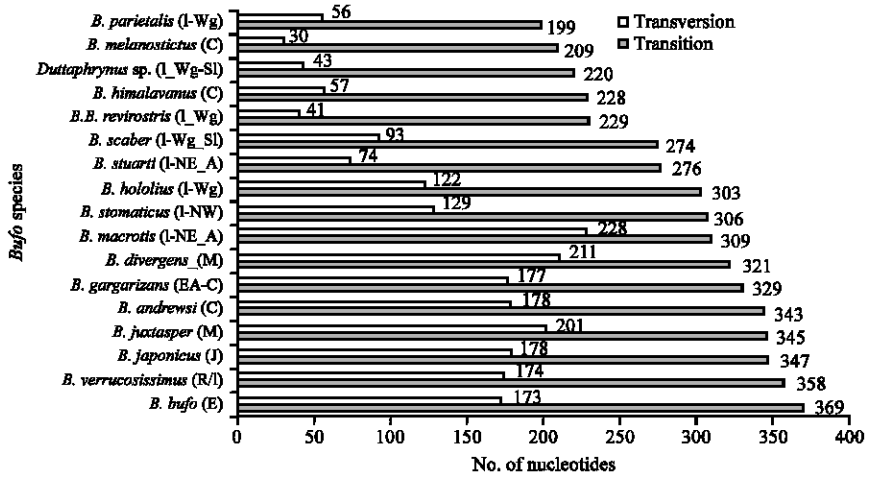

Fig. 1: Transition and Transversion in mtDNA nucleotide sequence observed in different *Bufo* sp. when compared with the same segment of mtDNA of *Duttaphrynus melanostictus* (I-WB)

2543 sites were conserved sequences, 1272 variable sites, 961 parsimony informative sites and 309 singleton sites. Transitions reflect the number of substitution purines (A G) or pyrimidine (C T) while number of transversions signifies the substitution of purines by a pyrimidine or vice versa. Transition-transversion data forms the basis of all the estimation for phylogenetic analysis though transitions are weighted more than the transversion during computation. Form the transition data, as shown in Fig. 1, it is apparent that least number of transitions occurred between *D. melanostictus* and *B. parietalis* (I-Wg-e) followed by *B. melanostictus* (China) *Duttaphrynus* sp., (I-Wg-SL) *B. himalayanus* (China), *B. brevirostris* (I-Wg-e). Although *B. parietalis* (I-Wg-e) shows minimum transitions from the mtDNA of *D. melanostictus* (I-WB), its number of transversions are significantly higher than *B. melanostictus* (China) and *Duttaphrynus* sp. (I-Wg-SL). Similarly, between *B. macrotis* (I-NE_A) and other species, *B. macrotis* show significantly more transversion than transitions and consequently placed more closely to *D. melanostictus* which is very unlikely. Indices drawn from the transition/transversion ratio alters the relationship of the considered species with *D. melanostictus* (WB-I) significantly (Fig. 2). *B. parietalis* (I-Wg-e) which was closest to *D. melanostictus* (WB-I) when only transition is considered comes to lie in the fifth position when both transition and transversion is considered. Similar alteration of relationship of *D. melanostictus* (WB-I) with other species is also noted in Fig. 2. Interestingly, transition/transversion ratio revealed that the value of quotient apparently increases with the relative closeness of the species.

Genetic distance: To determine a more genuine relationship of *Duttaphrynus melanostictus* (I-WB) with the other members of the Bufo group, nucleotide substitution pattern was determined with the aid of built in model selection program in MEGA 5.05 software. The Tamura-Nei's (TN93+G+I) model (Tamura and Nei, 1993) for nucleotide substitution pattern was selected as it produced the lowest BIC (Bayesian Information Criterion) scores. The genetic distance (Table 3) was estimated using TN93 nucleotide substitution model on the basis of both transition and transversion, with gaps and missing data completely deleted. There were 3767 positions that were considered in evolutionary comparisons (Tamura and Kumar, 2002). The rate variation among sites

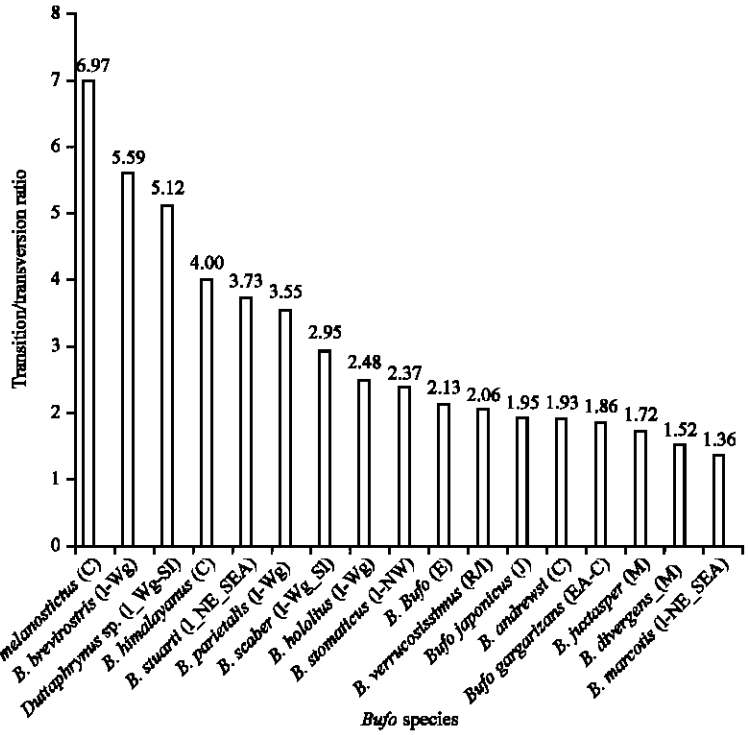

Fig. 2: Transition-transversion ratio of nucleotides of 18 different *Bufo* sp., Higher the value of the quotient, more closely the species is related to *Duttaphrynus melanostictus* (I-WB)

Table 3: Genetic distance of *Duttaphrynus melanostictus* (I-WB) with other Bufo members as revealed by Tamura-Nei's nucleotide substitution model having a gamma distribution parameter (0.1727), The difference in nucleotide composition 3767 positions were considered for analysis to calculate the distance which included both transition and transversion with gaps/missing data deleted

Species	Genetic distance *B. melanostictus* (I-WB)	Species	Genetic distance *B. melanostictus* (I-WB)
B. melanostictus (C)	0.095	*Bufo gargarizans* (EA-C)	0.345
B. parietalis (I-Wg)	0.102	*B. andrewsi* (C)	0.360
Duttaphrynus sp. (I_Wg-Sl)	0.115	*B. divergens*_(M)	0.367
B. brevirostris (I-Wg)	0.116	*Bufo japonicus* (J)	0.370
B. himalayanus (C)	0.126	*B. macrotis* (I-NE_A)	0.373
B. stuarti (I_NE_A)	0.178	*B. verrucosissimus* (R/I)	0.386
B. scaber (I-Wg_Sl)	0.200	*B. bufo* (E)	0.408
B. hololius (I-Wg)	0.266	*B. juxtasper* (M)	0.411
B. stomaticus (I_NW)	0.274		

was modeled with a gamma distribution (shape parameter = 0.1727). The distance data also revealed that *B. melanostictus* (China) *B. parietalis* (I-Wg-e) *Duttaphrynus* sp. (I-Wg-SL) *B. brevirostris* (I-Wg-e) *B. himalayanus* (China) are closely related species and slightly varies from the data obtained from the transition transversion ratio analysis.

Maximum parsimony analysis: The evolutionary history of *Duttaphrynus melanositctus* (I-WB) was inferred using the Maximum Parsimony method. The analysis of the mtDNA yielded

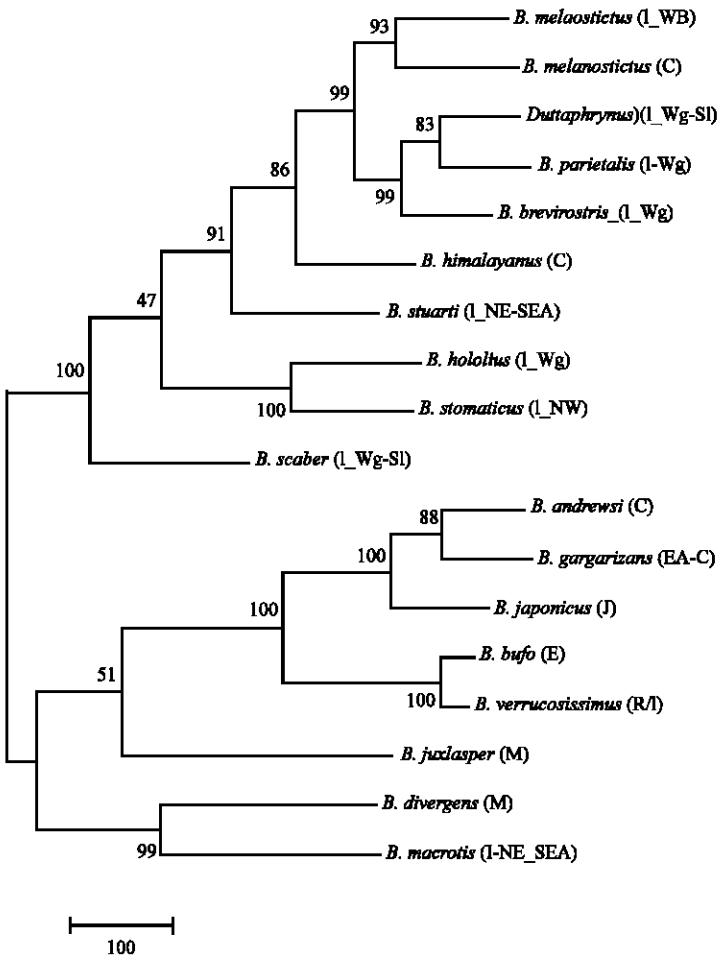

Fig. 3: Maximum Parsimony tree derived from 3080 mtDNA bp having 12S rRNA and 16S rRNA sequences from different *Bufo* species, Numbers at the nodes represents percentage of replicate trees in which the associated taxa clustered together in bootstrap test

2 parsimonious trees with a length of 3499 steps and having a consistency index 0.445463. The retention index and the composite index were 0.552779 and 0.275679 (0.246243), respectively. The figures shown next to the branches represents percentage of replicate trees in which the associated taxa clustered together in bootstrap test (500 replicates) (Felsenstein, 1985) which provides support for internal branches in the parsimony analysis. All sites were given equal weight in the parsimony analysis. Close-Neighbor-Interchange algorithm (Nei and Kumar, 2000) with search level 1, in which the initial trees were obtained with the random addition of sequences (10 replicates). The most parsimonious tree derived is shown below (Fig. 3). The tree is drawn to scale with branch lengths calculated using the average pathway method and are in the units of the number of changes over the whole sequence.

DISCUSSION

Unambiguously aligned 12S and 16S rRNA sequences of *D. melanostictus* (I-WB) with the non coding rRNA sequences of other *Bufo* species published by different authors formed the basis of the

current analysis. The primary aim was to investigate the phylogenetic relationship of the *D. melanostictus* found in West Bengal (India) with other *Bufo* species existing in India and its surrounding countries. The transition/transversion data (Fig. 1-2 and Table 3) provided a preliminary idea regarding the relationship of *B. melanostictus* (I-WB) with other *Bufo* species, but it was difficult to draw a phylogram convincingly and determine the evolutionary pattern from the data. The Maximum Parsimony (MP) analysis enabled to portray a phylogram (Fig. 3) that reveals the phylogenetic position of *D. melanostictus* (I-WB) within the *Bufo* group. MP tree distinctly positioned the 18 *Bufo* species under consideration into two clades based on the nucleotide composition of 12S and 16S rRNA of mtDNA. The first group comprised primarily of all Indian species except *B. melanostictus* (C) while the second group included *Bufo* species that are found outside the international boundary of India. The three species, *B. stomaticus* (I_NW), although found in West Bengal appears to be more closely related to *B. scaber* (I-Wg_Sl) and *B. hololius* (I-Wg), which are typically found in the Western Ghats of India. Most likely, the three species originated from a common ancestor and then migrated along the Western margin of India from North-West of India and got adopted recently in evolutionary time scale.

Surprisingly, the *D. melanostictus* (I-WB), which has pan India distribution appear to be very closely related to *B. melanostictus* from China rather than with *Duttaphrynus* sp. (I_Wg-Sl). The other species from China, *B. andrewsi* (C) got nested in the second clade, portray a considerable difference in nucleotide sequence. Neighbour joining and maximum likelihood analysis (not shown here) also indicated strongly that *D. melanostictus* (I-WB) and *B. melanostictus* (C) are indeed sister species though the two species have remained separated from each other by the Himalayas for considerable amount of time.

To explain such unexpected relationship of *D. melanostictus* (I-WB) and *B. melanostictus* (C), we have to understand the biogeographical distribution and adoptive radiation of the group Bufonidae. It has been estimated that the group Bufonidae originated some 55 million years (Myr) ago (Bocxlaer *et al*., 2009) and almost at the same time the Indian Sub-Continent, migrating through the Indian Ocean towards North to collided with Asia sometime in Late Cretaeceous 65 Myr (Klootwijk *et al*., 1992) to 40 Myr (Molnar and Tapponier, 1977) and established a link along the North Eastern region of Greater India (Chatterjee and Scotese, 1999). Since the initial collision of India with the Asian plate, India has moved an additional 2000 km to give rise to the spectacular Himalaya along its entire Northern edge. However, the rise of the Himalaya began 20 Myr (France *et al*., 1993) ago, much after the initial collision. Much of gain of height of the Himalaya took place during the last 15 Myr to form a barrier for dispersal of terrestrial vertebrates. From the above geographical background of the Indian subcontinent, it is evident that the Bufonids and other animals had enough opportunity to explore and radiate into India before the rise of the Himalayas. There are considerable palaeontological and geophysical evidence that the narrow bridge that formed along the north eastern margin with Asia allowed the influx of different animals into India. *Duttaphrynus* sp. (I_Wg-Sl), *B. parietalis* (I-Wg), *B. brevirostris* (I-Wg), *B. himalayanus* (C), *B. melanostictus* (I-WB) and *B. melanostictus* (C) had common ancestor just prior to the rise of the Himalayas. *Duttaphrynus* sp. (I_Wg-Sl), *B. parietalis* (I-Wg), *B. brevirostris* (I-Wg) migrated towards south India much before the Himalaya started gaining height and got adopted to more hot and humid climate of the Western Ghats and became endemic. The group of Bufonids that preferred to rise with the Himalaya got adapted to cold climatic conditions of the hills of Darjeeling, Sikkim and Southern parts of China underwent modifications gradually to suite

themselves to life at higher altitude and become *Duttaphrynus himalayanus*. The group that stayed behind to live in the Gangetic planes of West Bengal and the plains of China stared being called *Duttaphrynus melanostictus* and *B. melanostictus*, respectively.

CONCLUSION

In summary, the toads of India appear to have a monophyletic origin diversified in the late Oligocen-middle Miocene and the close similarity of *Bufo melanostictus* (I-WB) and *B. melanostictus* (C) is due their common origin and whatever difference noted in the mtDNA sequence of the two species is due to their separation and local environmental effects and separation induced due the formation of the Himalaya.

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