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Phylogenetic relationships among different amphibian species captured from District Dera Ghazi Khan, Punjab, Pakistan

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SUMMARY

The present eight-month study was extended from March 2019 to October 2020 in District Dera Ghazi Khan, Punjab, Pakistan. During the survey, 25 amphibian specimens having 4 species, 3 genera, and 2 families were collected from Dera Ghazi Khan, Pakistan. Amphibian species captured include *Duttaphrynus stomaticus, Duttaphrynus melanostictus, Euphlyctis cyanophlyctis,* and *Hoplobatrachus tigerinus.* A total of 4 species were collected from DG Khan, whereas 2 toad species, *Duttaphrynus stomaticus* and *Duttaphrynus melanostictus*, were successfully amplified and sequenced. The successfully amplified sequenced genes obtain the Gene Bank accession numbers. After trimming ambiguous bases, the obtained 16SrRNA fragment of *Duttaphrynus stomaticus* was 538 bp, while the Cytb fragment of *Duttaphrynus melanostictus* was 639 bp. The 16SrRNA and Cytb fragments aligned with NCBI sequences comprised 493 and 450 bp, respectively. The mean intraspecific divergences of 16SrRNA and Cytb were 0.038 and 0.19, respectively.

Keywords: Phylogeny, Amphibians, Taxonomy, South Punjab

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INTRODUCTION

The Indo-Pakistan subcontinent has a unique geographical location in Asia. It was once a part of eastern Gondwana, the southern supercontinent that split into multiple plates in the distant past, including India, Africa, and South America. Plates travel through ancient oceans to reach their current position, according to plate tectonics theory. The country's reptile fauna is comprised of 195 species from 23 families. Pakistan is home to nine amphibian and thirteen reptile species that are indigenous to the country (Khan, 2004). As with other groups, these are a blend of Palearctic, Indo-Malayan, and Ethiopian forms (Khan, 2006).

Amphibians are cold-blooded vertebrates that live in both aquatic and terrestrial environments. In early life they spend their life in water to complete the process of metamorphosis and are unique among tetra pods due to their biphasic life cycle and permeable skin (Khan et al., 1999). The taxonomic position of the amphibians also makes this group evolutionarily very significant. The amphibians are most essential part of our ecosystem to check and balance the population of pests and insects and also act as the biological indicators of environmental changes. Herptiles not only play their roles in terrestrial ecosystem's food chain but also play their role

in aquatic ecosystems food chain (Ali et al., 2016). Amphibian eggs and tadpoles provide nutritious food to aquatic insects, birds and snakes. They form an important component of the wetland ecosystem (Khan et al., 1993).

In recent years, worldwide losses in herptiles populations have generated great alarm in the communities of scientists, and local accounts are vital instruments for advancing ecological understanding as well as management. Around 28% of the world's assessed reptiles and 30% of its assessed amphibians are threatened (Boone and Bridges, 2003; Gibbon et al., 2000; IUCN, 2009).

Individual identification, breeding pattern, degree of relatedness, and disturbance of genetic differences between them can all be collected using genetic variability within populations (Schierwater, 1994). Higher-level amphibian taxonomy and systematic understanding have evolved quickly in the last decade as a result of evolving and constantly improving molecular phylogenetic methods. The molecular markers are vital for estimating genetic diversity (Liu and Cordes, 2004). Despite the fact that herpetiles found in abundance, it is very tough to sample them quantitatively. In addition, keys of morphology are still considered authentic to identify species but molecular analysis should also take into known for precise taxa classification. Recent studies indicated that cryptic species identification through fragment sequencing of cytochrome b and mitochondrial 16S rDNA gene proven to be effective. In addition, expansions in molecular biology have led scientist to identify variations within and among different populations (Vences et al., 2004). Core objective of the study are to know the molecular identification of the amphibian species and to understand the phylogeny through phylogenetic relationships of different amphibian species of DG Khan.

MATERIALS AND METHODS

STUDY AREA

The research was carried out in Dera Ghazi Khan, Pakistan. The district Dera Ghazi Khan (30.0489° N, 73.6455° E) is situated in south of Punjab province and contain four tehsils namely TaunsaShrif, KotChutta, Barthi and Dera Ghazi Khan, Punjab, Pakistan. The district Dera Ghazi Khan is covered by Koh e Suleiman mountain ranges from north to south. In its west Barkhan district of Balochistan, Muzaffargarh district in east, Rajanpur in south and district of KPK Dera Ismail Khan in north are located. Indus River flows on its eastern side. Total 13,740 square kilometers area fall in the district D G Khan (Figure 1). The climate of the DG Khan is diverse due to high altitude of Koh e Suleman mountain ranges. Winter temperature varies from 10-20 centigrade but on few mountain places it drops to 1 centigrade. Summer is not too hot like other south Punjab districts it moderate between 30-40 centigrade. The topography of study area is semi arid make it not good for amphibians that’s why diversity of amphibians in DG Khan is low to moderate.

COLLECTION, PRESERVATION AND IDENTIFICATION OF SAMPLE

Amphibians’ samples were collected from selected sites of District Dera Ghazi Khan. Field surveys were done during early morning and before sun-set hours and specimens were collected using hand and drag. The data related to samples i.e.

habitat, date, time, species, sex, GPS coordinates and location were noted on Performa. All specimens were classified using taxonomic keys (Khan et al., 2006) and preserved in alcohol, carried to the laboratory for molecular analysis.

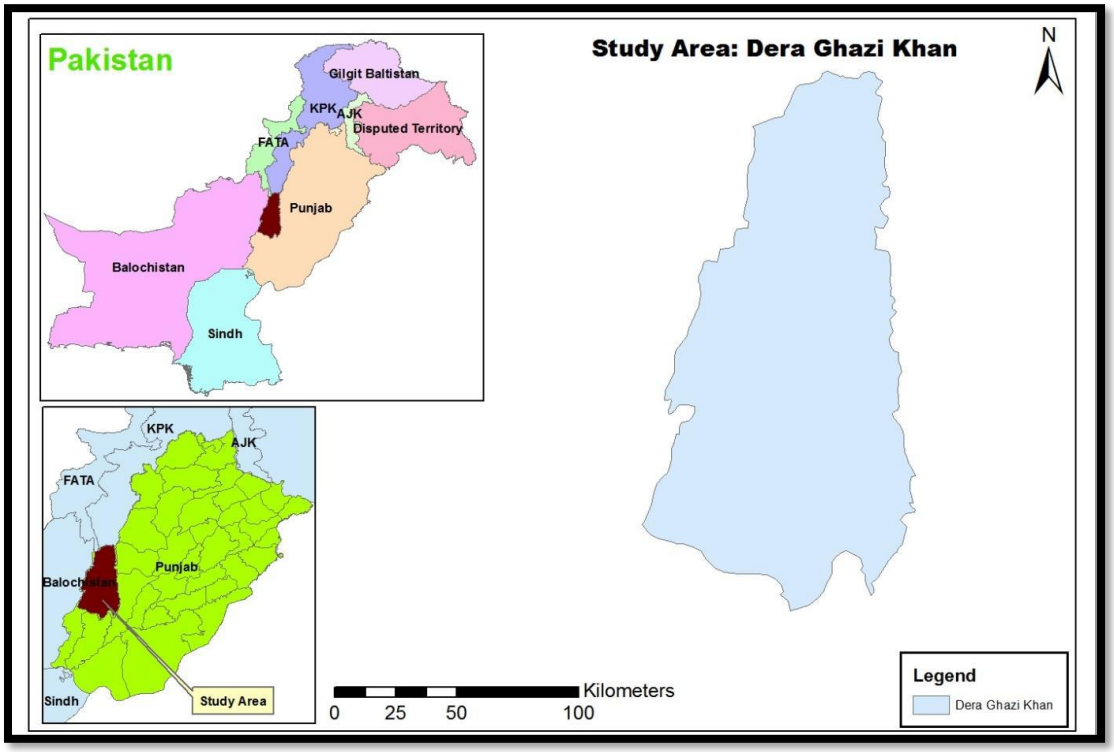

Figure 1: Map of the study area.

DNA EXTRACTION AND GENETIC DIVERSITY

DNA extraction was done from tissues (salt extraction) by the following procedure. Turn on a water bath to 55ºC for blood and 70 ºC for tissue. Take a little bit of tissue (0.5 cm2) and cut it with a sterile scalpel blade (as finely as you can bear). Transfer the sample to a 1.5 ml micro centrifuge tube that has been labeled. 600 l of TNES buffer and 35 l of Proteinase-K (20 mg/ml) are added. Invert the tube many times to mix the sample. Incubate the tissue samples at 70oC overnight (or for 5-24 hours). If feasible, mix the samples by inverting the tubes on a regular basis (make sure the lids are clamped shut as they can sometimes break loose while warm). Shake the samples briskly for 20 seconds after adding 166.7 l of 6 M NaCl. Microfuge the samples at ambient temperature for 5-10 minutes at full speed (13000 rpm). Transfer the supernatant to a fresh 1.5 ml micro centrifuge tube that has been labeled. Add an equivalent volume of cold 100% ethanol (800 l) and gently mix by inverting the tube a few times. White DNA should precipitate out of solution. Centrifuge the sample at 4oC for 10-20 minutes at full speed (12-14,000 rpm).

Pour (or pipette) off the supernatant, being vigilant not to disturb the DNA pellet. 600 l of 100% ethanol is used to wash the DNA pellet. DNA pellet was washed with 600l of 70% ethanol (chilled at -20C for 24 hours) as described earlier. After removing the 70% ethanol, for a short time centrifuge the samples to ensure that all of the ethanol has settled to the bottom of the tube; pipette off the remaining ethanol.

Allow the sample to air dry for 20 minutes, depending on the temperature. Re-suspend the DNA in 100 l of sterile distilled water or "Tris-EDTA" as soon as the sample is dry.

PCR AMPLIFICATION

Polymerase chain reactions were developed with the use of primers. Each reaction was carried out in a 0.2 ml PCR tube with a 25 L reaction mixture. 6 L double distilled water, 1 L (25 mM) Primer F, 1 L (25 mM) Primer R, 12 L Master mix, and 5 L of DNA template were mixed to make this 25 L reaction mix. A negative control utilizing water (sterilized) as the template was also run in each reaction (Table 1).

Table 1: Reaction constituents Ratio for 25 µl PCR reaction mixture.

Reaction constituents	**Volume (µl)**
Double distilled water	6 µl
Primer F	1 µl
Primer R	1 µl
Master mix	12 µl
DNA template	5 µl
Total	**25 µl**

POLYMERASE CHAIN REACTION (PCR) CONDITIONS

The primers 16SA-L (5'-CGCCTGTTTATCAAAAACAT-3') and 16SB-H (5'-CCGGTCTGAACTCAGATCACGT-3') were used to amplify DNA. Thermal cycler with a 3 minute denaturing step at 94^{o}C which is followed by 40 cycles of denaturing for 30 sec at 94^{o}C, primer annealing for 30sec at 55^{o}C depending on the primer and elongation for 1 minute at 72^{o}C, with final 10 minutes elongation step at 72^{o}C and infinity at 4^{o}C (Vences et al., 2004).

AGAROSE GEL ELECTROPHORESIS

The purity of the DNA and the validity of the PCR results were determined using agarose gel electrophoresis. The agarose gel (1%) was prepared in 0.5X TAE buffer, which was made from a 50X TAE stock solution made by mixing 28.6 ml glacial acetic acid, 0.5 molar EDTA and 121 g tris base in 500 ml of water. After mixing with DNA loading buffer containing 0.21% bromophenol blue, 0.21% xylene cyanol FF, 0.2M EDTA, and 50% glycerol, the DNA samples were loaded into the gel. The ethidium bromide stained DNA fragments visualized by UV fluorescence by gel documentation apparatus.

MOLECULAR ANALYSIS

The obtain sequences were submitted to GenBank and got the accession numbers and BLAST to download the closely related sequence of taxa. Mega X was used to alignment the sequence and construct Phylogenetic relationship.

RESULTS

The present eight months study was conducted from March 2019 to October, 2020 in District Dera Ghazi Khan, Punjab, Pakistan. During survey 25 amphibians specimens representing 4 species, 3 genera and 2 families were captured from study area.

Amphibian species captured from cultivated area include *Duttaphrynus stomaticus, Duttaphrynus melanostictus, Euphlyctis cyanophlyctis* and *Hoplobatrachus tigerinus.* Uncultivated area provides habitat to *Duttaphrynus stomaticus. Duttaphrynus stomaticus* and *Hoplobatrachus tigerinus* was recorded from cultivated areas. Canals, ponds and water channels provide habitat to *Duttaphrynus stomaticus, Euphlycti scyanophlyctis* and *Hoplobatrachus tigerinus*. Sandy patches extensively consist on sand and *Duttaphrynus stomaticus* is the only representative species of this habitat.

AMPLIFICATION AND SEQUENCE

A total of 4 species were included in this study whereas 2 toad species *Duttaphrynus stomaticus* and *Duttaphrynus melanostictus* were successful amplified and sequenced. In Table 2, summarizes the successful amplified species and the Gene Bank accession numbers. After trimming ambiguous bases, the obtained 16SrRNA fragment of *Duttaphrynus stomaticus* was 538 bp while Cytb fragments of *Duttaphrynus melanostictus* was 639bp. The 16SrNRA and Cytb fragments aligned with NCBI sequences comprised 493 and450bp respectively.

GENETIC DIVERSITY AND VARIATION

In Table 3, summarizes the Intraspecific and interspecific genetic identities of genus *Duttaphrynus* calculated using Kimura 2-parameter based on Cytb gene. The mean Intraspecific divergences of 16SrRNA and Cytb were 0.038 and 0.19 respectively show in Table 4. Figures 2 and 3, show Intraspecific and interspecific variation at different nucleotide positions.

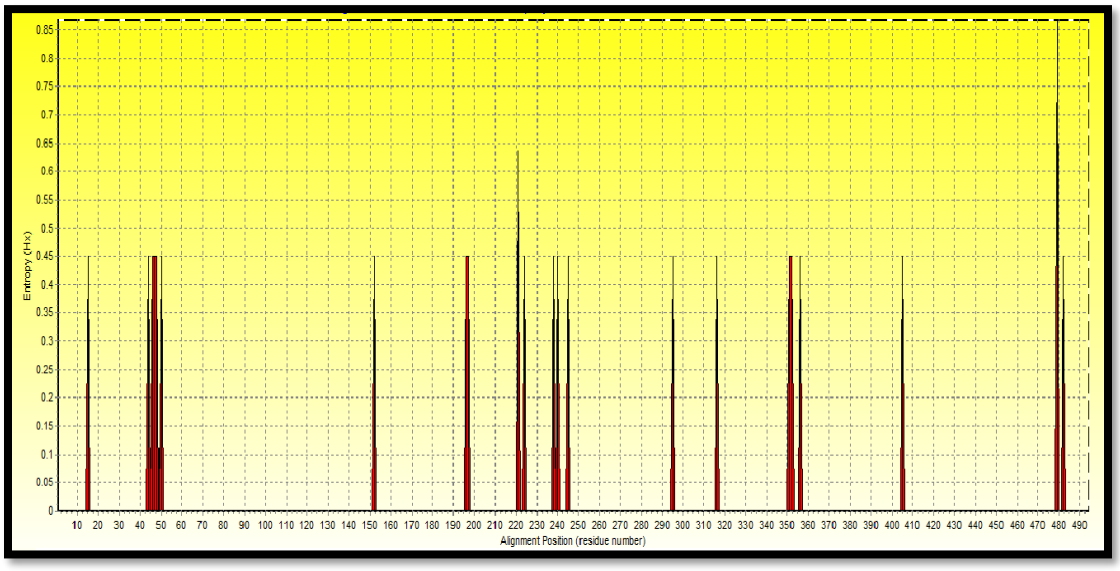

Figure 2: Intraspecific variation of 16S rRNA gene in *Duttaphrynus stomaticus* at different nucleotide positions.

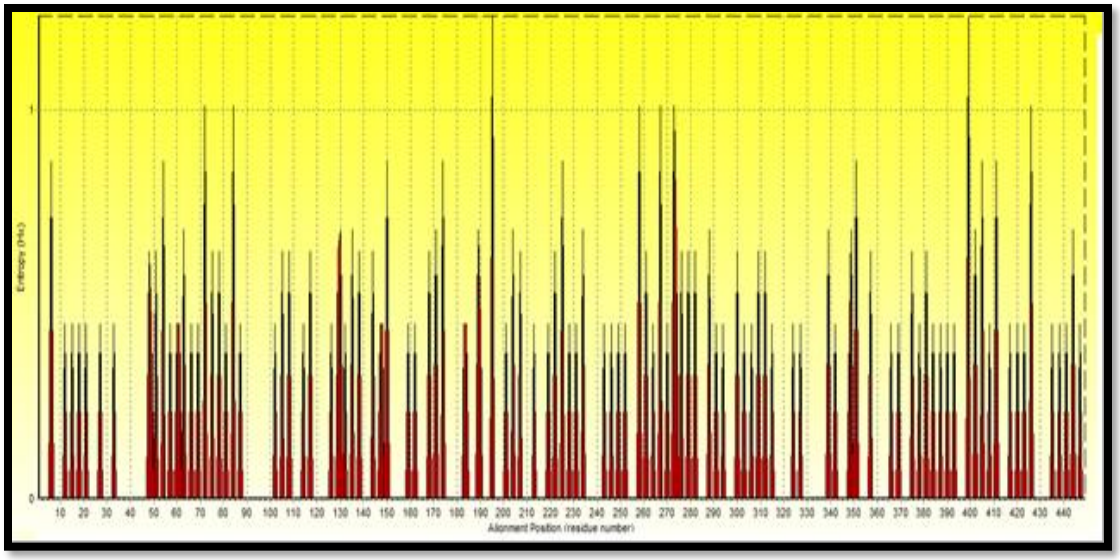

Figure 3: Intraspecific and interspecific variation of Cytb gene in *Duttaphrynus melanostics* at different nucleotide positions.

Phylogenetic analysis

During the study, 2 DNA sequences of *Duttaphrynus stomaticus* sand *Duttaphrynus melanostictus* were obtained. These DNA sequences have shown reliable and clear species identifications of almost all the species specimens. However, few specimens were morphologically identified as *Duttaphrynus stomaticus* but 16SrRNA sequence analysis identifies as *Duttaphrynus melanostictus*.

Although similarly comparable 16SrRNA sequences were frequently returned from public databases in blast searches, Cytb was less responsive. A few DNA barcoding investigations of Asian amphibians have recently been completed, and sequences for related amphibians are now available at NCBI. Figures 4 and 5, depict neighbor-joining trees of 16SrRNA and Cytb sequences based on Kimura 2-parameter distance.

Table 2: List of successful amplified species and the GenBank accession numbers of specimens used in this study.

Genus	Family	Species	Label	Locality	Gene bank accession number	
					16S	Cytb
		Class Amphibia				
Duttaphrynus	*Bufonidae*	*Duttaphrynus stomaticus*	ZMUVAS26	29.5963N, 72.9269E	MN795537	
		Duttaphrynus melanostics	ZMUVAS25	29.7381N, 72.9458E		MN807947

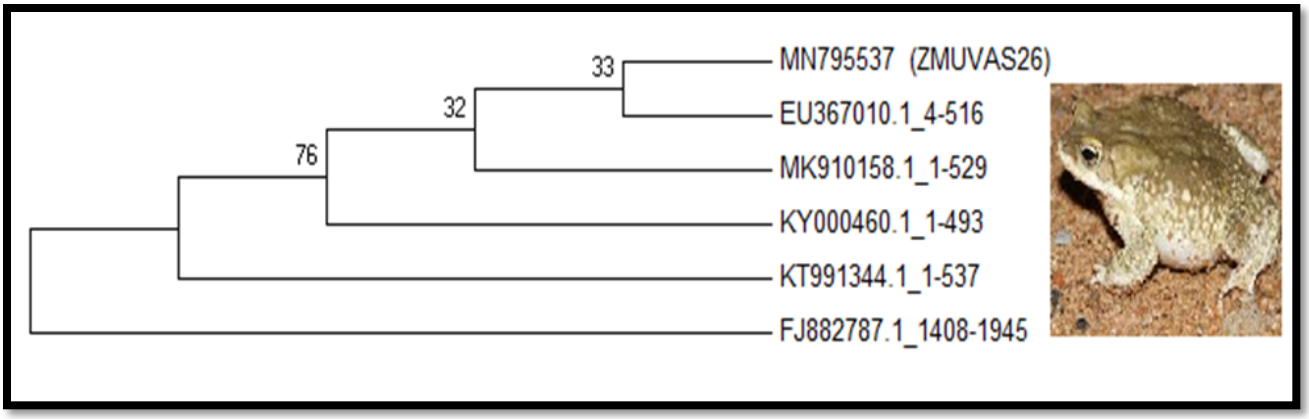

Figure 4: Neighbour-joining tree of *Duttaphrynus stomaticus* based of 16S rRNA sequence using Kimura 2-parameter distances. Bootstrap values are given above the nodes.

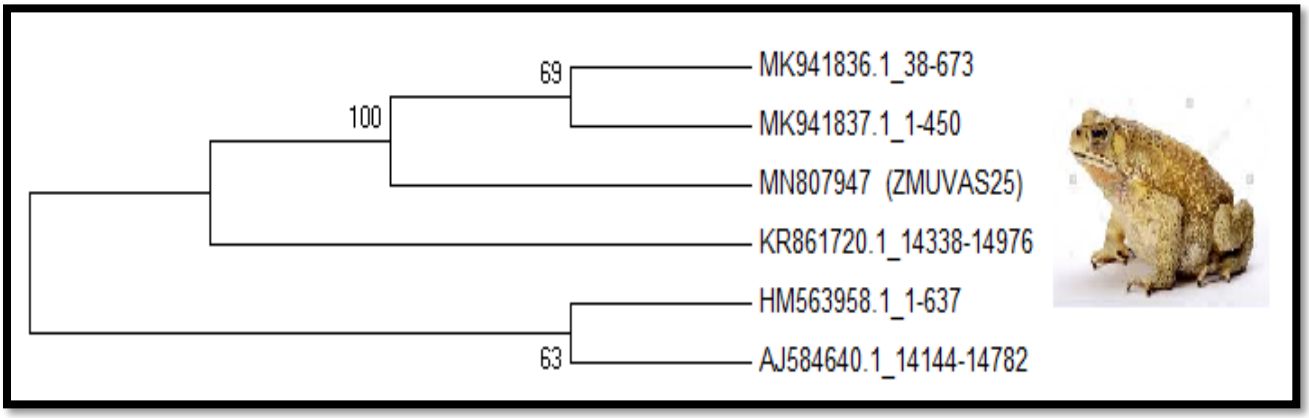

Figure 5: Neighbour-joining tree of *Duttaphrynus melanostics* based on Cytb sequence using Kimura 2-parameter distances. Boots trap values are given above the nodes.

DISCUSSION

Amphibians are predominantly distributed internationally with the exception of a few isolated islands and columns (Ali et al., 2016). Pakistan covers an area of 796,096 square kilometers between longitude 60 ° 52, 75 to 22 ° E, and latitude 24 ° to 37 ° N, and the semi-arid lands of Pakistan make it a poor country of amphibians compared to India and Southeast Asia. Pakistani amphibians belong to the three families of *Bufonidae*, *Microhylidae* and *Ranidae*. Over all 6285 species of amphibians in world including 24 species out these 9 are endemic to Pakistan (Khan, 2006). The amphibian has major impact on distribution and population density of many bird species, mammals and other animals. In addition, amphibians are important in conservation because many species are endangered by different human activities viz., construction of buildings, deforestation, fires, use of fertilizers, and use of pesticides, deep tilling and global warming which destroy or change in their habitats (Helmer and Scholte, 1985). Many anthropogenic hazards causing problems for these taxa and their survival is under threat to extinction of many species (Ali et al., 2016). Almost 30% amphibians of the world (1895 out of 6285) are threatened (IUCN 2009).

Our findings of current study show molecular analysis based on 16S-RNA gene sequence confirmed the identification of two different populations of

amphibians viz, common toad (*Duttaphrynus stomaticus*), Asia toad (*Duttaphrynus melanostictus*), skittering frog (*Euphlyctis* c*yanophlyctis*) and bullfrog (*Hoplobatrachus tigerinus*) from district Dera Ghazi Khan, Punjab, Pakistan. The results suggested the existence of four distinct molecular operational taxonomic units of *Duttaphrynus stomaticus*, *Duttaphrynus melanostictus, Euphlyctis cyanophlyctis* and *Hoplobatrachus tigerinus* from study area (Dubey et al., 2009).

Traditionally, Sanger sequencing is used in molecular identification of herpetofauna. However, DNA barcoding of herpetofauna for species identification should be preceding using standardized markers and protocols should be proceeding for practical reasons.

CONCLUSION AND RECOMMENDATIONS

Identification of amphibians on morphological basis is still considered authentic. However, there are some cryptic species which are difficult to identify. Therefore, for accurate identification it is recommended that molecular analysis should be adopted.

Table 3: Intraspecific and interspecific genetic identities of genus *Duttaphrynus* calculated using Kimura 2-parameter based on Cytb gene.

Clade		***Duttaphrynus melanostics*** **MN807947 (ZMUVAS25)**	***Duttaphrynus melanostics*** **MK941836.1**	***Duttaphrynus stomaticus*** **MK941837.1**	***Bufote s raddei*** **KR861720.1**	***Bufo mazatlanensis*** **HM563958.1**	***Bufo melanostictus*** **AJ584640.1**
Duttaphrynus melanostics	MN807947 (ZMUVAS25)	ID					
Duttaphrynus melanostics	MK941836.1	0.997	ID				
Duttaphrynus stomaticus	MK941837.1	0.997	1	ID			
Bufotes raddei	KR861720.1	0.866	0.868	0.868	ID		
Bufo mazatlanensis	HM563958.1	0.877	0.877	0.877	0.846	ID	
Bufo melanostictus	AJ584640.1	0.844	0.846	0.846	0.832	0.855	ID

Table 4: Comparison of 16S rRNA and Cytb genetic divergence based on K2P.

Species	16S rRNA	Cytb
Duttaphrynus stomaticus	0.038	----
Duttaphrynus melanostics	---	0.19

Note: K2P is Kimura 2-parameter.

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Authors' contributions: Latif has designed this project, collected data and written this article; while all authors have critically analyzed this article and approved as final.