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RESEARCH ARTICLE

Molecular Phylogenetic analysis of *Bufo arabicus* (*Duttaphrynus arabicus*) in Saudi Arabia: according to mitochondrial DNA control region.

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Abstract

We investigated the genetic diversity and population structure of *Bufo arabicus* (*Duttaphrynus arabicus*)from the Arabian Peninsula in Saudi Arabia, according to mitochondrial DNA (D-Loop). Samples were collected from toes and prepared for mitochondrial DNA extraction and Polymerase chain reaction using the suitable primers. The products were sequenced using ABI Prism Big-dye. Phylogenetic analysis was carried out using DnaSP. V.5 version and maximum likelihood tree were constructed using MEGA 6. There were high polymorphic sites among the two species with high diversity of haplotypes. Nucleotide diversity of *Bufo arabicus* from Abha region was the highest population. Tajima's D test showed significant results from neutrality for all *Bufo arabicus* populations with $P< 0.05$. Fu's Fs was positive for the whole population and positive for the separated regional populations supported the allele deficiency due to recent population bottleneck or over-dominant selection without gene flow resulting in their endemism habitat as in Tihama region.

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1. Introduction:

Phylogeographic analysis provides a powerful tool for dissecting a species' evolutionary history and offering significant insight into the formation of present day biogeographic and systematic patterns (Avise, 2000). The long-term goal of systematics is to understand the historical relationships among organisms and construct a powerful tool to estimate robust species trees. Recently, the increased availability of molecular data and the parallel development of efficient analytical tools to extract the information have increased the accuracy of species tree estimation (Heled and Drummond, 2010).

Methodological advances related to estimation of the timing of population divergence, and in particular the incorporation in the analyses of the uncertainty associated with estimates of substitution rates and the temporal constraints imposed by the fossil record have greatly improved the understanding about the time scales involved in species formation in a wide variety of taxonomic groups (Aldenhoven et al., 2010; Arora et al., 2010; Blackburn et al., 2010).

The non-coding fragment, control region or displacement loop (D-Loop) of mitochondrial DNA, encompasses the sites of initiation of H strand replication and both H and L strand transcription. The D loop region is the most rapidly evolving part of the animal mitochondrial genome (Brown, 1984) has short sequence elements conserved among most vertebrates.

The Arabian toad, *Bufo arabicus* (*Duttaphrynus arabicus*. In: IUCN 2013) is by far the most commonly seen toad in the mountain wadis of the United States of Emirates (UAE) and Saudi Arabia. They are normally found in or near water and are active by day as well as by night, although they often seek shelter from direct sunlight in the heat of the day. Close inspection of mountain pools frequently reveals well-camouflaged toads, often clustered in damp or shady crannies. When other shelter is scarce, they may excavate small hollows in damp wadi gravel, resembling donkey hoof prints, perhaps to take advantage of a slightly cooler or more humid microclimate. The Arabian toad is absent, nevertheless, from the Ru'us al-Jibal in the north of the Hajar Mountains and also from Dhofar in southern Oman. This isolated distribution opens the possibility that the Arabian toad of the UAE and Oman will prove to be genetically distinct from the Arabian toad of western Arabia, although both are undoubtedly related to the *B. viridis* populations to the north. The genus *Bufo* contains more than 250 species, more than half the species of the whole family Bufonidae, and is distributed across Eurasia, Africa and the Americas (Duellmann and Sweet, 1999).

Bufo arabicus characterized by a rounded head and snout, along with small ear drums located behind the large, prominent eyes (Vine and Al-Abed, 1997). The female is also significantly larger than the male. The vocalization of the Arabian toad is a prolonged "krrraaaa", reminiscent of a creaking door hinges (Balletto and Cherchi, 1971). Cannibalism is also known to be widespread, with the larger adult toads consuming the smaller juveniles (Hellyer and Aspinall, 2005). *Bufo arabics* is an opportunistic, mesophilous species, encountered in almost all environments of the peninsula where there is some water availability. Its population in the Saudi Arabia may appear to be fluctuated significantly according to short-term environmental conditions, such as droughts. It was endemic to the Arabian Peninsula as well as it occurs in widely separated areas within northwest, central and south-west Saudi Arabia, and offshore Farasan Islands. It can also be found in west and south-central Yemen, north Oman and the United Arab Emirates. The Arabian toad has been recorded from sea-level to altitudes of 2,300 meters (Balletto and Cherchi, 1971).

Currently, little is known about this species' long-term population trend and their worldwide decline imposed the study of their risk factors. Global warming and pandemic fungal disease were the main risks to Arabian toad distribution (Vine and Al-Abed, 1997; Hellyer and Aspinall, 2005). Presently, the Arabian toad receives more protection by virtue of its presence in the Farasan Islands Protected Area, Saudi Arabia. Nevertheless, ecological study and monitoring of this species would be beneficial to determine its population size and trend, especially in light of ongoing urban expansion within its range, and the general decline of amphibians worldwide (Hellyer and Aspinall, 2005).

It can persist for extended periods by entering a dormant state, analogous to hibernation, known as an aestivation. Unbelievably, this species' aestivation periods are believed to last as long as three years. Outside of aestivation, the Arabian toad emerges rapidly from refuges in response to rain or even drizzle, and may form large congregations. Despite the fact that the skin of the Arabian toad, like many *Bufo* species, makes a noxious chemical, it does not seem to affect native predators, such as snakes and Brandt's hedgehog (Paraechinus hypomelas). One of the main practical applications of conservation genetics is the identification of Evolutionary Significant Units (ESUs) and Management Units (MUs) within a species and among populations. As defined by Moritz (1994), ESUs are geographically distinct populations which have evolved separately for a substantial period of time, being reciprocally monophyletic at mitochondrial DNA, and showing significant frequency differences of nuclear alleles.

Our study is the first to analyse the geographically comprehensive sampling of *Bufo arabicus* in KSA using the molecular markers from the mitochondrial genome (D-Loop, control region) and the adoption of a variety of analytical approaches including species tree estimation to produce a fully resolved topology which will set the basis for the clarification of the taxonomy, systematics, and evolutionary history of the group and will serve as a foundation for further studies on the process of species formation. As a first step towards understanding diversity within the species in Saudi Arabia D-Loop region fragment of mitochondrial genes was sequenced and combined with the only published sequence of *Bufo arabicus* in Yemen.

2. Materials and Methods

2.1. Study sites and sample collection

A total of 17 toads (*Bufo arabicus*) was sampled from 4 localities Tanomah, Wadi-Tarj, Tihama, and Abha. Streams and pools inhabited by toads were selected through field surveys. All individuals were sampled using toe-clipping technique (Sutherland, 1996a). Tissue samples were taken from toads caught by hand or using fishing nets in streams and pools. The animals were released into the pools close as possible to where they were found after the data were collected and the tissue sample taken. Tissues were preserved in 95% ethanol and stored at 4°C until their use for mtDNA extraction.

2.2. DNA extraction and primer selection

Total DNA was extracted from tissue samples using a standard phenol/chloroform method and Proteinase K with 10 mg/ml (Kirby, 1990). Two primers were selected from Goebel et al (1999) designed directly from the D-loop sequence as follows: Forward- Control B-H 5'-GTCCA TTGGA GGTTA AGATCTACCA-3'and Reverse: CytbA-L 5'-GAATY GGRGG WCAAC CAGTA GAAGA CCC-3'. Polymerase chain reaction (PCR) protocol was defined according to the T_m of the primers and the length of the expected PCR products (Hillis et al., 1997). PCR reactions were performed by a Perkin Elmer PCRthermal cycler. PCR products were checked by electrophoresis in

0.8% agarose in TBE buffer with ethidium bromide, and the bands visualized using ultraviolet illumination at 360 nm.

2.3. DNA sequencing

PCR products were cycle sequenced using the ABI Prism BigDyeTM Terminator. Cycle Sequencing Ready Reaction Kits (PE Biosystems) following the protocol suggested by the manufacturer. The sequence reaction receipt and the sequencing profile are as follows: DNA template (PCR product) 2 µl, dilution buffer 3 µl, ready reaction mix 2 µl, primer 1 µl, water to 20 µl; 30 cycles of denaturation at 96°C (10 s), annealing at 50°C (5s), extension at 60°C (4min).Sequencing reactions were precipitated by adding 2.5volumes of 95% ethanol and 1/10 volume of sodium acetate (pH 4.6), centrifuging for 10 min. At 4°C, removing the ethanol and repeating the procedure with 200 µl of 70% ethyl alcohol. This was done using an ABI Prism 377 Sequencer. Both the L and H strands of the amplified products were sequenced for all samples. Sequence electropherograms were edited using Chromas version 1.43.

2.4. Molecular diversity, neutrality and recombination tests

Sequences were aligned using Clustal X (Thompson et al. 1997) and COLLAPSE, version 1.2 (Posada, 1998) to reduce the final alignment of sequences to unique haplotypes. Number of haplotypes and values of mean haplotype (h) and nucleotide diversities (p) (Nei, 1987) for the mtDNA control region were estimated within the pool and within regions using DnaSP v5. 0 (Libradoand Rozas, 2009). Genetic distances within and between specieswere calculated with MEGA6 (Tamura et al., 2013). To test whether the data were consistent with neutral expectations which shows significant departure due to selection, expansion, or bottleneck, we also calculated Tajima's D statistic (Tajima, 1989) and Fu and Li's D and F statistics for each using DnaSP v5.0. (Fu and Li's, 1993).

We tested for the sudden expansion by comparing the observed mismatch distribution to the distribution generated by a population expansion model (Schneider and Excoffier 1999). We estimated effective population size per region using two theta (h) estimates (Watterson, 1975; Kimura, 1980; Tajima 1983). Pairwise distances between sites and regions were estimated using Fst and the corrected mean number of pairwise differences (Tajima, 1983). Testing for differences in haplotype frequency between regions was done using DnaSP version 5. 0 (Librado and Rozas, 2009) was used to calculate and show in graphic form the distributions of observed and expected pairwise nucleotide site differences, also called mismatch distributions, between all individuals within each group, and the respective expected values for growing populations. The model of sudden expansion describes an initial population at equilibrium, with the expected pairwise differences, $\theta 0$, ($\theta = 2Ne\mu$) and assumes rapid population growth, resulting in $\theta 1$ (Theta final). Tau, τ, is the time of the growth measured in units of substitutional time ($\tau = 2\mu t$; t is the time in generations, μ the substitution rate per locus and per generation). We estimated Tajima's D (1989) using DnaSP v5. 0 for each subpopulation.Tajima's D test of selective neutrality compares two θ estimators and its significance is evaluated by comparison of thetest statistic (D) with values randomly generated under"neutrality." Significant values indicate the population has deviated from neutrality, or that another demographic force has caused the deviation from expectation, such as population expansion (significant negative D), (Tajima, 1989).

2.5. Phylogenetic analyses

The best-fit model of evolution was estimated based on the Bayesian Information Criterion (BIC) as implemented in jModel test 1.0 (Guindon and Gascuel, 2003; Posada, 2008). Phylogenetic analyseswere based on Bayesian inference, maximum likelihood (ML) methods as implemented in MrBayesv3.1.2 (Huelsenbeck and Ronquist, 2001). The ML tree was then estimated (heuristic search with1000 replicates), and bootstrapping (500replicates) was used to assess support for internal nodes using MEGA 6.0 (Tamura et al., 2013).

3. Results

3.1. The anatomical characters:

Bufo arabicus haplotypes characterized by their middle size, couple of dark streaks extended from the nostrils to wrestle, their dorsal skin is granules by the presence of many flattened multispinose and multitubulate warts. Their limbs are slender and long with small hands. Skeletal features were determined according (Inger, 1972). The skull is middle sized, the brain case is comparatively high and the spoon-ethmoid dorsally exposed to a rather variable extent, quadrate-Jugal overlapping the maxilla in the posterior part of the target angle.

3.2. Molecular phylogenetic analysis

A total of 888 homologous base pairs of the control region of D-Loop sequences were obtained in all specimens with GenBank accession numbers from KJ 094415 to KJ094431. Sequence alignment was straightforward, no insertions or deletions were observed. A total of 14 mitochondrial haplotypes were identified among the 17 individuals. The best fitting evolutionary model for our data set found by Modeltest 3.6 was (HKY: Hasegawa-Kishino-Yano+ Gamma distribution) HKY+G model that had the best likelihood score (-lnL=-1601.616).The parsimony analysis generated fourteen most parsimonious trees. Trees generated from ML were the best product, including the total 17 samples together the only sequenced *Bufo arabicus* from Yemen (DQ 629592).

The estimated value of the shape parameter for the discrete Gamma Distribution is 0.0500. Substitution patterns and rates were estimated under the Hasegawa-Kishino-Yano (1985) model (+G) (Hasegawa et al., 1985). A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories, [+G]). The nucleotide frequencies are A = 31.77%, T/U = 33.60%, C = 24.06%, and G = 10.57%. The analysis involved 18 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 888 positions in the final data set. Evolutionary analyses were conducted in MEGA.6 (Tamura et al., 2013).

Our sequence results were aligned and compared together and to the unique *Bufo arabicus* sequence sample isolated from Yemen with accession number DQ 629592. Our isolates showed 56 variable sites, 11 parsimony positions, and 45 singlton site among them in front of DQ 629592. *Bufo arabicus* sequences from Saudi Arabia showed some distinguishable substitution profiles from that of Yemen, in the form of transitional substitution in 39 G/A except for ABH 50 (KJ 094431), 375 T/C prevailed in all sequences, Tihama and Abha shared the same transitional substitution at 423 C/T, all samples from Tanomah and Tihama showed the same transversional substitution haplotype at 455 A/T.

The rates of different transitional substitutions were higher than tranversional substitutions in the whole population sequences. On one side, the highest transitional value was detected among A/G with 30.55% and the lowest was detected for G/A with 10.71%. On the other side, the highest transversional substitutions rate was among T/A and T/G with a rate value of 3.78% and the lowest was estimated within G/T and G/C with a rate value of 1.19%. The transition/transversion rate ratios are $k_1 = 8.549$ (purines) and $k_2 = 5.675$ (pyrimidines). The overall transition/transversion bias R was 3.055 where. The rate of variance model for some sites to be evolutionarily invariable was ([+I], 27.2102% sites). By separating the whole population from Saudi Arabia to regional populations, according the separation district, the population from Tanomah the overall transition/transversion bias R was 4.563; for Tihama was 3.005; for Abha 0.601.

3.3. Diversity indices

D-loop control region of mtDNA sequences were detected as haplotypes with high polymorphic sites. Haplotype diversity and nucleotide diversity were high in the analysis of the whole population with H=0.954 and $\pi = 0.0093$, respectively. The lowest nucleotide diversity ($\pi = 0.001502$) was detected among *Bufo arabicus* species from the Tihama region as it was the only subpopulation collected from low height lands of 40m, while the highest nucleotide diversity was ($\pi = 0.021$) among Abha population as it is the most height collected samples. Gene flow and genetic differentiation between populations detected the rate of evolution between populations with the Jukes and Cantor (1969), K (JC) using the total number of mutations. The results showed that the highest value of nucleotide divergence was between Tanamah and Abha population by 0.0138and the lowest value was between Tanoma and Tihama with a value of 0.00437.

3.4. Demographic and statistical analysis.

The results of Tajima's D test, Fu and Li's D*, Fu and Li's F*, and Fu's F are presented, including associated simulated p-values and are based on the fact that under the neutral model estimates of the number of segregating/polymorphic sites and of the average number of nucleotide differences. Tajima's D, Fu and Li's D* and Fu and Li's F* values were negative for all populations with significant values of deviation from neutrality. *Bufo arabicus* samples, indicating an excess of rare nucleotide site variants compared to the expectations under a neutral model of evolution.

Table 1. Statistical analysis of *Bufo arabicus* population in Saudi Arabia and its subpopulations.

Population (m)		H	S	Haplotype diversity	p_s	Θ	π	Tajima D	Fu and Li's D	Fu Li F	Fu's Fs
All samples	**17**	**14**	**55**	**0.95±0.0391**	**0.061937**	**0.018321**	**0.009340**	**-2.052 (*P<0.02)**	**- 2.795 (**P<0.02)**	**- 3.011 (**P<0.02)**	**-3.492**
Tanomah	**5**	**4**	**11**	**0.90±0.016**	**0.012387**	**0.005946**	**0.005405**	**-0.654302 (P >0.10)**	**- 0.654 (P >0.10)**	**-0.691 (P >0.10)**	**0.425**
Wadi Tarj	**2**	**2**	**5**	**1.000±0.500**	**0.00234**	**0.02312**	**0.0135**	**-**	**-**	**-**	**2.485**
Tihama	**6**	**3**	**4**	**0.600±2.151**	**0.004505**	**0.001973**	**0.001502**	**-1.295031 (P >0.10)**	**- 1.326 (P >0.10)**	**- 1.3962 (P >0.10)**	**0.297**
Abha	**4**	**4**	**38**	**1.000±0.177**	**0.042793**	**0.023342**	**0.021584**	**-0.781133 (P >0.10)**	**- 0.781 (P >0.10)**	**-0.825 (P >0.10)**	**1.068**

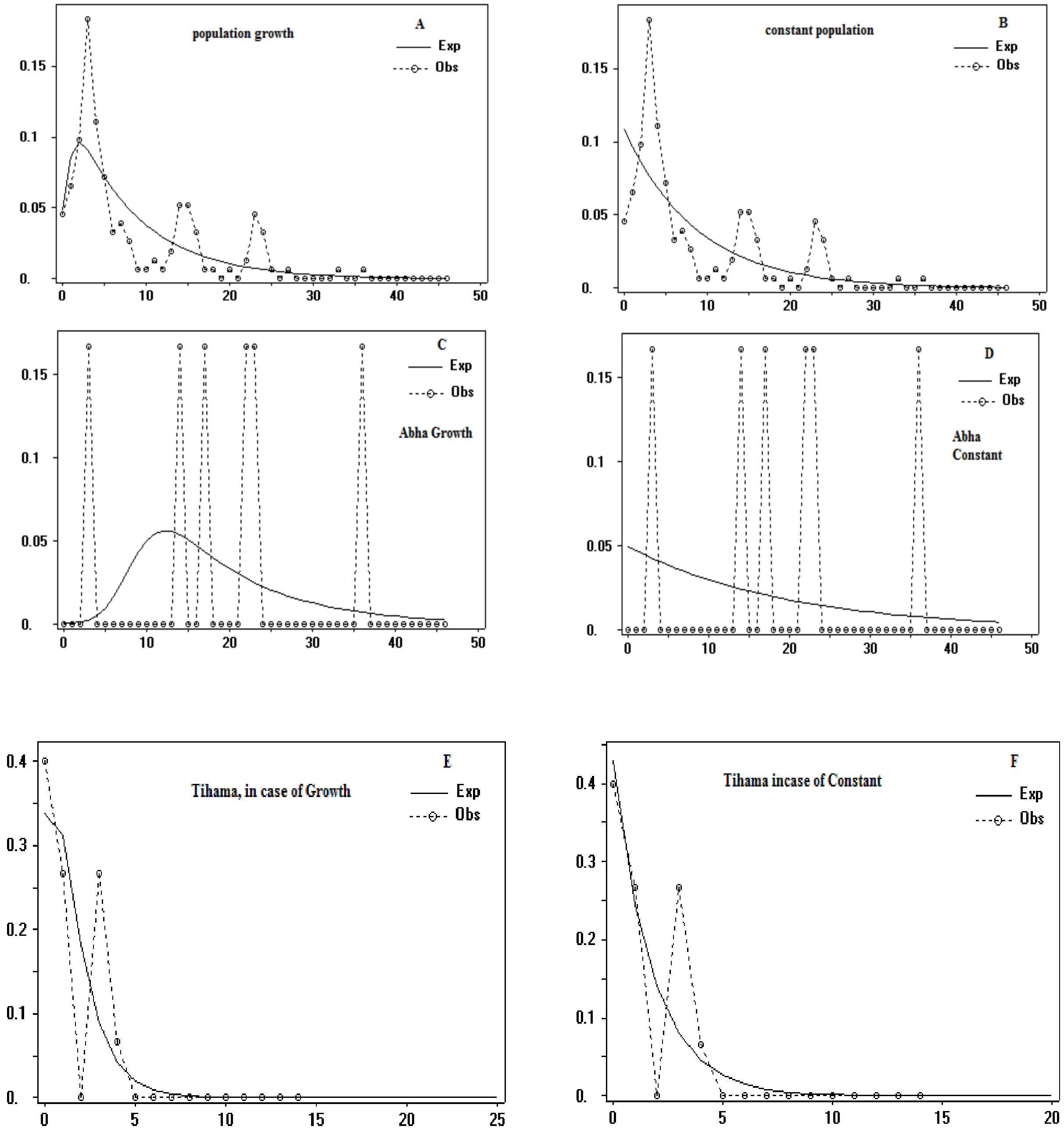
Table 1: All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed in any position. There were a total of 880 positions in the final data set.m = number of sequences, n = total number of sites, S = Number of segregating sites, $p_s = S/n$, $\Theta = p_s/a_1$, π = nucleotide diversity, and D is the Tajima test statistic, Fu and Li's D*, Fu Li F*, Fu's Fs are neurtrality results. 0.01<*P<0.05, is significant; P >0.10 is not significant.

Fig 1. Saudi Arabia map with selection sites localities.

Fig 2. *Bufo arabicus (Duttaphrynus arabicus*. In: IUCN 2013) species.

Fig 2. This species is generally restricted to sites of surface water in widely separated areas of the southern Arabian Peninsula. It is absent from eastern Yemen and southern and central Oman. It is present on the Farasan Islands, Saudi Arabia. It has been recorded from sea level to 2,300m. It is an opportunistic species that is present wherever there are water sources available.

Fig 3. Mismatch Pairwise no of differences within the whole isolated samples and within different subpopulations in case of constant and expansion modules.



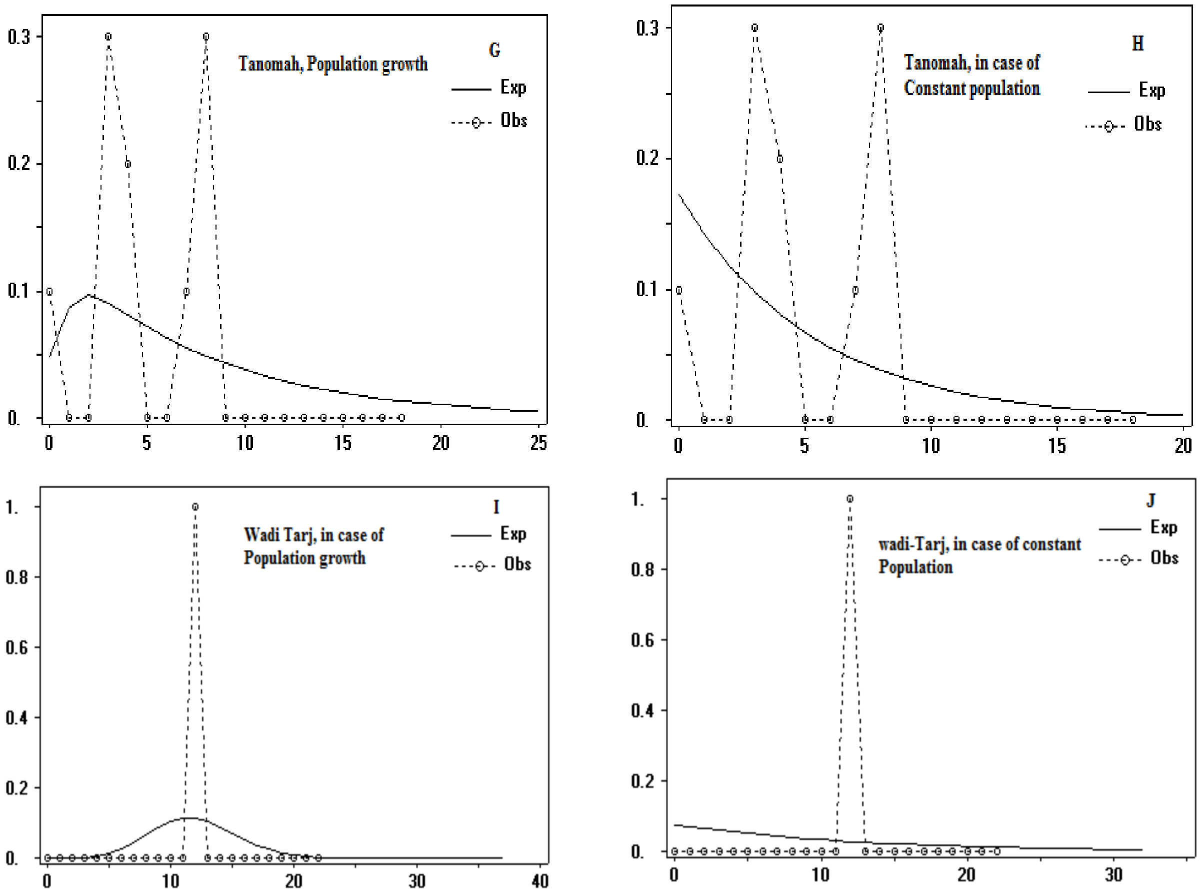


Fig 3. Mismatch distributions from 888 of the mtDNA control region of Bufo arabicus population in KSA and mentioned subpopulations.

The dotted line shows the frequency distribution of the observed pairwise differences. The solid show the frequency distribution of the expected pairwise differences under the sudden expansion model, performed in DnaSP v5. 0. Pairwise differences are counts of I, nucleotide differences; frequency is Fi. The model was applied to the whole population and within each group population in both situations in case of constant population size and population growth decline.

Fig 4. Maximum Likelihood tree (ML).

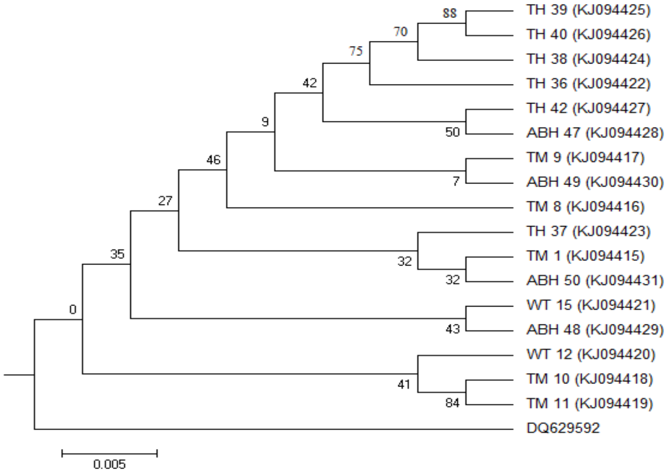


Fig.4. The evolutionary history was inferred by using the Maximum Likelihood method based on the HKY: Hasegawa-Kishino-Yano and Gamma distributed HKY+G model. The tree with the highest log likelihood (-1595.9994) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.0558). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 15.0756% sites). The analysis involved 18 nucleotide sequences. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed in any position. There were a total of 888 positions in the final data set. Evolutionary analyses were conducted in MEGA6.

4. Discussion

Our study was a geographically genetic analysis of the *Bufo arabicus* species in Saudi Arabia, according mitochondrial DNA sequences of rapidly evolving D-Loop region. Our main aim was to provide insights into the historical biogeography and investigate their matrilineal history in the different regions mentioned in the Saudi Arabia. It is of a surprise to find several amphibian species abundance in a harsh environment such as in the Arabian Peninsula. The work of Balletto et al. (1985) was considered as the more precise surveys dealing with the morphological and classification of Arabian anura. However, there are few researches on the distribution of frogs and toads in kingdom of Saudi Arabia and neighboring country such as Yemen (Hass,1957; Briggs, 1980).

Our results showed the presence of high haplotype and nucleotide diversities between and among the different populations of *Bufo arabicus* except in Tihama region subpopulation. According to the literatures, *Bufo arabicus* is the first toad to be classified from the region, but recent research on the amphibian chromosomes of the Arabian Peninsula has just started, and few papers have been published (Al-Shehri and Al-Saleh, 2008).Therefore, it is very important to study and protect such creatures because they are under grave threat not only due to general habitat alteration, but also to climate change, pollution and global warming. Its systematic relationships and speciation patterns within taxa of the *Bufo viridis* complex were with long debated issues.

The pairwise graphs in our population and some regional subdivisions indicated by multi-models for them in both cases of constant population and growth or decline referred to their regional distribution that they had undergone a sustained increase in population size, possibly because of a prior catastrophic decline in size or because species expanding its geographic range for the first time.

Furthermore, we investigated some statistical tests based on the distribution of pairwise differences between sequences within populations to test the selective neutrality of mutations detecting population growth or constant (Ramos-Onsins and Rozas, 2002) and Tajima’s D test (Tajima, 1989). We have got negative with highly significant results for the whole population analysis and non-significant values within sub-populations which may be due to the presence of a bias towards rare alleles.

The other tests are Fu and Li’sD*, Fu and Li’s F*, and Fu’sFs based on the neutral model prediction. Fu’s Fs test was negative for the whole studied population and positive for sub-populations with the highest value among Wadi-Tarj sub-population with 2.48. The positive results are evident for a deficiency of alleles which may be due to recent population bottleneck or from over-dominant selection, balancing selection, demographic processes such as population reduction, population subdivision, a recent bottleneck (Maruyama and Fuerst 1985), or migration which may yield positive values. So, Fu's simulations suggest (Fu and Li, 1993; Fu, 1997) that Fs is a more sensitive indicator of population expansion and genetic hitchhiking than Tajima's D. The population of Tanomah showed the highest transitional/transversional bias r (4.5) value, followed by Tihama (3.5), and finally Abha population (0.6) reflected their high diversity and divergence.

The analysis of the whole population showed high haplotype diversity (0.95±0.0391) and the low nucleotide diversity (0.009%) suggested that *Bufo arabicus* in the region of study may have undergone population expansion scenarios (Avise, 2000) but always in the Arabian peninsula only. The neutrality of mtDNA control region mutations was rejected on the basis of Tajima's D and Fu and Li's D tests. These two statistics are sensitive to factors such as bottlenecks and population expansions which tend to drive the values of Tajima's D and Fu and Li's D towards more-negative values (Tajima, 1996, Martel et al., 2004). Indeed, significant negative values of these two indices in this study indicated that *Bufo arabicus* in the Tihama population may have experienced population expansion regime in the past due to its geographical habitates. Moreover, it had a unimodel mismatched frequency distribution pattern based on the mtDNA sequence recorded well with the predicted distribution under a model of population expansion (Rogers and Harpending, 1992).

Quaternary climatic oscillations, punctuated by the Pleistocene glaciations, caused massive changes to the distribution of species in the Palaearctic realm (Hewitt, 2000; Schmitt, 2007). Recent research on the micro evolution of East Asian species has focused largely on the locations of refuge during the Pleistocene glaciations and dispersal routes during the post-glacial period (Stöck et al., 2006; Zhang et al., 2008; Song et al., 2009; Ding et al., 2011). Much of the research has centered on tropical and subtropical species with geographic distributions that sometimes include the Palaearctic (Ding et al., 2011). Because Bufonids are a major part of the amphibian fauna of East Asia, they have been the subject of many investigations. The majority of previous studies has focused on anatomical characters. Inger (1972) recognized five species groups in East Asia and modern amphibians are believed to have originated in the early Triassic ~200 million years (my) ago. Fossil Xenopus was extensively represented in collections from Africa, representing the late Cretaceous (100my) to the early Pleistocene (10 May). The occurrence of Xenopus fossils in Yemen (Arabian Peninsula (Henrici and Baez, 2001; Bos and Sites, 2001) confirms the existence of the taxon in the late Oligocene (~25my) and reinforces the view of Tinsley et al (1996) that its distribution was once more widespread geographically than at present. So, our results of nucleotide divergence (KJC) postulated that *Bufo arabicus* population from Saudi Arabia may be diverged from *Bufo arabicus* species from Yemen and their migration started from this point in Abha to distribute in different migrational waves upward. The lowest nucleotide divergence value was between *Bufo arabicus* from Abha sub-population and *Bufo arabicus* from Yemen due to their close geographical distribution and sharing the same habitats of the Palearctic relicts in the high mountains with well watered biota.

In recent years there has been a growing interest in peripheral populations (Beebee, 2005; Eckert et al., 2008), which are often subjected to less favorable conditions and are more isolated than the incessant populations in the core. Some of these studies have focused on amphibian populations (Palo et al., 2003; Garner et al., 2004). Peripheral populations such as Tihama are more likely to be demographically impaired than core populations, especially due to its different climate change (Lesica and Allendorf, 1995).

Peripheral populations may or may not be shaped by different climatic and physical conditions, we hypothesized that the genetic structure of edge populations, relative to the core, may vary according to the difference in conditions. So, high taxonomic level endemism in the Arabian peninsula biodiversity hotspot has been typically attributed to the geological history of long-term isolation and severe environmental conditions which considered as a biogeographic factor that can dilute the composition of previously isolated biota. However, no molecular studies have focused on Arabian Peninsula dispersal as a possible source of endemism (Holness et al., 2010).

So, Additional analysis of more mtDNA populations for the fast and slowing mutated genes with nuclear DNA markers could give a more complete perspective on the neutral population structure of the population. Species with very small natural ranges may have limited dispersal capabilities, which may produced their restricted ranges (Slatkin, 1987; Kirkpatrick and Barton 1997). In these cases, gene flow between populations could be severely limited, potentially resulting in inbreeding depression or an inability to recolonize areas in case of local extinction (Marsh and Trenham 2001; Couvet, 2002; Kraaijeveld-Smit et al., 2005). Additionally, species with limited ranges may be more susceptible to environmental changes, which could occur on a small scale and affect the entire species (Lande, 1993; Arita et al., 1997; Simberloff, 1998).

As there is no studies exist on the intra specific phylogenetic structure and its association with geography over the native range of *Bufo arabicus* species in Saudi Arabia. An understanding of the biogeography of them throughout its range has very important implications for the conservation of the species. The estimated sequence divergence for *Bufo arabicus* D-loop may suggest an approximate time frame for the events which originating to detect the haplotype clades. However, further genetic studies using both mtDNA and nuclear markers (such as microsatellite loci), with greater sampling throughout wide distribution area are needed to confirm any hypothesis and highlights an important aspect of biodiversity, concentrations of endemic species, and forms an important component alongside other approaches in developing an integrated regional conservation assessment.

5. Conclusion

In conclusion, our study revealed a high genetic diversity within and between populations, but relatively little differentiation among them. On one side, there were close relationship between samples from Tihama sub-population which showed the same profile and their high bootstrap in the phylogenetic tree with confidence interval more than 70% on the clade node. On the other side, the observed patterns of genetic variation were most likely caused by a recent shared demographic history in the form of a reduced species area during the last glacial period with little gene flow and high bottleneck effect. So, this study provides a much needed framework for ongoing investigations of adaptive functional variation at the phenotypic and molecular level in the KSA region for the *Bufo arabicus*.

So, Based on these considerations, we are in need for further studies in the Arabian peninsula as this study is the first from the point of view of evolution and phylogeny with molecular basis and considered as a seed for studying their evolutionary aspects through old ages.

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Conflict of interest

All authors contributed equally and no potential conflict of interest including any financial, personal or other relationships with other people or organizations.

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