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Cover: Nile Crocodile *Crocodylus niloticus* regulating body temperature on a warm day. Digital art on Procreate by © Aakanksha Komanduri.

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ARTICLE

Genetic polymorphism of Dhofar Toad *Firouzophrynus dhufarensis* (Parker, 1931) (Amphibia: Bufonidae) across central Saudi Arabia

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Abstract: This study investigated the genetic diversity of the Dhofar Toad *Firouzophrynus dhufarensis* in central Saudi Arabia, focusing on three populations: Al-Kharj, Al-Hariq, and Al-Aflaj. Using inter-simple sequence repeat (ISSR) markers, the analysis revealed notable variation in genetic polymorphism among these regions based on 30 individuals (10 per population) selected for genetic analysis from a total of 60 sampled specimens. Al-Kharj demonstrated comparatively higher levels of genetic diversity than the other populations, as reflected by polymorphism rates and diversity indices. In contrast, Al-Hariq and Al-Aflaj exhibited reduced polymorphism, suggesting that isolation may have been caused by habitat fragmentation and limited gene flow. Dendrogram analysis based on Nei's genetic distances indicated a closer relationship between Al-Hariq and Al-Aflaj, while Al-Kharj was more distinct. These findings underscore the conservation significance of Al-Kharj in maintaining amphibian genetic diversity in arid landscapes. Meanwhile, the genetic vulnerability of Al-Hariq and Al-Aflaj emphasizes the need of targeted habitat restoration and improved landscape connectivity. This research also demonstrates the utility of ISSR markers for preliminary genetic assessments in species lacking extensive genomic resources, reinforcing the need for broader geographic and genomic sampling. Future work should incorporate high-resolution markers and expand to populations in regions such as Oman to support transboundary conservation planning.

Keywords: Amphibian conservation, arid landscapes, gene flow, genetic diversity, habitat fragmentation, ISSR markers, landscape connectivity, phylogeography, population differentiation, population structure.

هدفت هذه الدراسة إلى استقصاء التنوع الوراثي لضفدع ظفار في وسط المملكة العربية السعودية، مع التركيز على ثلاث جماعات سكانية توزعت في الخرج والحريق والأفلاج. تم استخدام واسمات التكرارات البسيطة البينية ((ISSR ، حيث أظهرت التحليلات وجود تباين ملحوظ في مستوى التعدد الشكلي الوراثي بين هذه المناطق، وذلك استناداً إلى تحليل 30 عينة (10 عينات من كل جماعة سكانية) تم اختيارها للتحليل الوراثي من أصل 60 عينة جُمعت خلال الدراسة. أظهرت جماعة العينات المجموعة من الخرج مستويات أعلى نسبياً من التنوع الوراثي مقارنة بالعينات من الجماعات الأخرى، كما انعكس ذلك في نسب التعدد الشكلي ومؤشرات التنوع الوراثي. في المقابل، سجلت عينات جماعات مناطق الحريق والأفلاج مستويات أقل من التعدد الشكلي، مما يشير إلى احتمال تأثرها بالعزلة الناتجة عن تجزؤ الموائل الطبيعية ومحدودية تدفق الجينات بينها. كما بيّن تحليل شجرة القرابة الوراثية المعتمد على المسافات الوراثية وفقاً لمعامل ناي وجود علاقة وراثية أوثق بين عينات الجماعات من منطقة الحريق والأفلاج، في حين بدت جماعة الخرج أكثر تميزاً واختلافاً وراثياً وتؤكد هذه النتائج الأهمية المحافظة لجماعة الخرج باعتبارها مخزوناً مهماً للتنوع الوراثي للبرمائيات في البيئات الجافة. وفي الوقت ذاته، فإن الهشاشة الوراثية التي أظهرتها الجماعات التي تم الحصول عليها من مناطق الحريق والأفلاج تبرز الحاجة إلى تنفيذ إجراءات مستهدفة لاستعادة الموائل الطبيعية وتعزيز الترابط البيني بين المواقع المختلفة لتحسين تدفق الجينات كما تُظهر هذه الدراسة فعالية واسمات التكرارات البسيطة البينية كأداة مناسبة للتقييمات الوراثية الأولية للأنواع التي تفتقر إلى موارد الجينوم المتقدمة، مما يؤكد أهمية توسيع نطاق الدراسات المستقبلية لتشمل عينات جغرافية أوسع وتحليلات للجينوم أكثر شمولاً. ويوصى مستقبلاً باستخدام واسمات وراثية عالية الدقة وتوسيع نطاق الدراسة ليشمل جماعات سكانية من مناطق أخرى، مثل سلطنة عُمان، بما يدعم جهود

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Author contribution: All authors contributed equally to this work. Rawan Al-Shehri and Mohammed F. Albeshr are co-first authors.

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INTRODUCTION

The Dhofar Toad *Firouzophrynus dhufarensis* (Parker, 1931) is distributed across much of the Arabian Peninsula. It is currently classified as 'Least Concern' with a stable population (UAE National Red List Workshop 2022). Its range includes the western mountains near Mecca City and the central regions of Saudi Arabia, such as Ha'il and Riyadh Provinces. It also occurs in peripheral southern Arabia, including Yemen, Oman, and the United Arab Emirates (UAE), typically inhabiting wadis and areas with seasonal water sources (Cunningham & Feulner 2001; Cunningham & Wronski 2010; Soorae et al. 2010; Gardner 2013; Soorae et al. 2013; Alshammari & Ibrahim 2018). The species has also been reported by the IUCN as introduced in parts of the Riyadh region, although its native range boundaries within central Saudi Arabia remain uncertain (UAE National Red List Workshop 2022).

Within Saudi Arabia, *F. dhufarensis* inhabits a range of environments, from the southwestern provinces of Jazan, Asir, and Mecca to the more arid interior regions. It thrives in mountainous areas, valley streams, irrigated farms, and temporary wetlands (Balletto et al. 1985; Soorae et al. 2013; Al-Johany et al. 2014; Al-Qahtani & Al-Johany 2018). Observations from the Ibex Reserve and central provinces, including Al-Kharj, Al-Hariq, and Al-Aflaj, suggest its adaptability to natural and human-altered habitats (Alrefaei et al. 2022). Reports also confirm its presence in Wadi Abather, Al Madinah Province, and across sites with elevations ranging from 55–700 meters (Mashlawi & Masood 2024). While adapted to arid conditions, *F. dhufarensis* may be outcompeted by the Arabian Toad in more mesic environments (Soorae et al. 2013).

Despite its wide distribution, research on the species' genetic structure remains limited. Hafez et al. (2017) conducted a phylogeographic study across the Afro-Arabian regions using mitochondrial DNA (D-loop and 12S rRNA), which indicated low polymorphism and suggested either past population bottlenecks or balancing selection. Alrefaei et al. (2022) further explored the species' 16S rRNA in Riyadh populations, revealing high genetic similarity (99.35%) with Omani populations. These studies provide preliminary insights but highlight the need for further genetic investigation.

Accordingly, this study was designed as an exploratory assessment of genetic variation in *F. dhufarensis* across three populations in central Saudi Arabia. The specific objectives were to (i) quantify levels of genetic polymorphism using ISSR markers, (ii) evaluate whether measurable genetic differentiation exists among geographically proximate populations in an arid landscape, and (iii) provide baseline genetic information to inform future, more comprehensive studies. We hypothesized that populations would exhibit detectable genetic differentiation consistent with localized isolation in arid environments, while recognizing that testing underlying drivers of such patterns requires complementary genetic, ecological, and spatial data.

This study is intended as a preliminary assessment of population-level genetic variation in *F. dhufarensis* within central Saudi Arabia. Given the limited sample size, restricted geographic coverage, and the dominant nature of ISSR markers, the results should be interpreted as exploratory rather than definitive. Nevertheless, in the context of the Arabian Peninsula, where large-scale aridification, habitat fragmentation, and hydrological isolation have shaped amphibian distributions, such baseline genetic data remain valuable. By documenting spatial patterns of genetic polymorphism across arid landscapes, this study provides an initial framework for hypothesis generation and identifies priorities for future, higher-resolution genetic and phylogeographic investigations.

Study Area

This study was conducted in central Saudi Arabia, specifically in the regions of Al-Kharj, Al-Aflaj, and Al-Hariq, which are located south and south-east of Riyadh (Image 1); this description reflects their geographic position rather than a formal ecological or biogeographic division. These sites span approximately 22.15°–24.16° N and 46.50°–47.33° E. The region is characterized by an arid climate with extreme seasonal temperature variation, ranging from highs of 48°C in summer to lows of 3°C in winter. Annual rainfall is typically below 100 mm, while evaporation rates may exceed 2,000 mm. Elevations range from 320–650 m, contributing to local microclimatic and vegetative differences (Sayed & Masrahi 2023). The geology of the study area is predominantly composed of quaternary deposits, featuring significant karstic features, including wadis, sinkholes, and limestone formations. Soils are mainly sandy loam, supporting xerophytic vegetation. The Tuwaiq Mountains to the west create topographic heterogeneity, influencing hydrology and biodiversity. Traditional irrigation systems in Al-Aflaj have historically enabled agriculture in this otherwise arid landscape (Almalki et al. 2022).

Image 1. Sampling locations in Saudi Arabia.

MATERIALS AND METHODS

Sampling

Field sampling was carried out in January 2021. A total of 60 specimens of the Dhofar Toad were collected. Twenty specimens were initially obtained from each site. For genetic analysis, 30 individuals were randomly selected (10 per site) based on the quality of their samples. Specimens were preserved in 96% ethanol immediately upon collection to maintain DNA integrity, following the procedures of Zamani et al. (2011).

DNA Extraction

Genomic DNA was extracted from thigh muscle and skin tissues using a protocol adapted from Kumar et al. (2012). Approximately 5 mg of tissue was homogenized and incubated with 500 µL DNAzol reagent (Molecular Research Center, USA). Following centrifugation at 10,000 g for 2 minutes, the supernatant was transferred, and DNA was precipitated using 100% ethanol. After additional centrifugation and washing with 75% ethanol, the DNA pellet was air-dried and rehydrated in 50 µL nuclease-free water. DNA purity and concentration were assessed using a NanoDrop spectrophotometer, and integrity was confirmed by electrophoresis on a 1.5% SYBR green-stained agarose gel. All samples were diluted to a working concentration of 100 ng/µL.

ISSR-PCR Amplification

ISSR amplification was used to assess genetic diversity. Although ISSR markers are dominant and limited in resolving co-dominant variation, they are effective for detecting genome-wide polymorphism and are widely applied in preliminary population genetic studies, particularly in non-model and conservation-target species with limited genomic resources (Zietkiewicz et al. 1994; Bornet & Branchard 2001; Moradi et al. 2014). Because ISSRs do not allow direct estimation of allele frequencies or fine-scale gene flow, the approach employed here is intended to provide an initial assessment of genetic variability rather than a comprehensive reconstruction of population connectivity or evolutionary history. Nine primers (UBC 813, 814, 816, 817, 819, 821, 822, 825, and 828) were selected based on reproducibility and polymorphic potential (Moradi et al. 2014).

Each PCR reaction consisted of 1 µL of genomic DNA, 0.8 µL of primer, 10 µL of PCR master mix (Solarbio), and 8.2 µL of nuclease-free water (Promega) in a total volume of 20 µL. Amplification was performed using a ProFlex PCR system with an initial denaturation at 94°C for 3 minutes, followed by 35 cycles of 94°C for 30 seconds, annealing at 46–48°C for 30 seconds, and extension at 72°C for 1 minute. A hold at 4°C followed a final extension at 72°C for 2 minutes. PCR products were separated on 1.5% agarose gels in 0.5× TBE buffer. Gels were stained with SYBR Green, run at 100 V for 1 hour, and visualized under UV light. Molecular weight markers (100–5000 bp) were used to estimate band sizes, and gel images were captured using a BioDocAnalyze system.

To ensure reproducibility and minimize sensitivity to laboratory conditions, all ISSR-PCR reactions were conducted using standardized reagent concentrations, identical thermal cycling parameters, and the same PCR platform throughout the study. Amplifications were repeated independently for a subset of samples to confirm banding consistency, and only clear, reproducible bands observed across replicate reactions were scored. All gels were run under identical electrophoretic conditions and scored conservatively to reduce the inclusion of artefactual fragments. These standardization procedures were applied consistently across all primers and populations to ensure methodological reliability.

Data Analysis

Banding patterns were scored as binary data (1 = presence, 0 = absence). Fragment sizes were calculated using ONE-Dscan software (Scanalytics Inc., USA). Genetic diversity metrics, including percent polymorphic bands (PPB), Nei's genetic diversity (Nei 1987), and the Shannon diversity index (Shannon 1948) were calculated using POPGENE version 1.31 (Yeh & Yang 1999). Nei's unbiased genetic distances among populations were also computed. A dendrogram was constructed based on Nei's genetic distances using the unweighted pair group method with arithmetic mean (UPGMA) in MEGA version 11 (Tamura et al. 2021) to visualize genetic relationships among the three populations of *F. dhufarensis*. Genetic relationships among populations were inferred by constructing a dendrogram based on Nei's unbiased genetic distances using the unweighted pair group method with arithmetic mean (UPGMA).

RESULTS

The analysis of ISSR profiles revealed measurable genetic diversity among *F. dhufarensis* populations from Al-Aflaj, Al-Kharj, and Al-Hariq. Banding patterns generated by nine ISSR primers were scored as binary data (presence = 1, absence = 0) and summarized quantitatively as percent polymorphic bands (PPB), polymorphic loci per primer, and polymorphism information content (PIC) (Table 1 & 2). Primer performance varied among populations, with UBC 813, UBC 819, and UBC 828 consistently yielding the highest numbers of polymorphic loci and higher PIC values, indicating greater discriminatory power (Images 2 & 3).

Nine primers exhibited polymorphism levels that varied across populations. Al-Aflaj exhibited a polymorphism rate of 29% (30 polymorphic loci), Al-Kharj had the highest rate at 41% (24 loci), and Al-Hariq showed a lower polymorphism rate of 25.49% (31 loci). Primer-specific polymorphism ranged from 12% for UBC 817 to 50% for UBC 813 in Al-Aflaj; 15.3% (UBC 822) to 100% (UBC 813 and UBC 814) in Al-Kharj; and 18% (UBC 817 and UBC 819) to 44.44% (UBC 813) in Al-Hariq.

Al-Kharj exhibited the highest allelic richness and genetic variability across the nine ISSR primers, suggesting that this population may retain a broader representation of overall genetic diversity. In contrast, Al-Hariq and Al-Aflaj exhibited lower levels of polymorphism, potentially indicating reduced gene flow or historical isolation. Primer-specific amplification patterns also varied, with UBC 813 consistently generating the highest polymorphic rates in all populations. The dendrogram based on Nei's genetic distances confirmed a closer genetic relationship between Al-Aflaj and Al-Hariq (distance = 0.2). At the same time, Al-Kharj formed a separate cluster, consistent with its elevated intra-population diversity.

The average polymorphic information content (PIC) values also supported these findings, with Al-Aflaj showing the highest average PIC (0.772), followed by Al-Kharj (0.736), and Al-Hariq (0.716). Among all primers, UBC 813 yielded the highest PIC values across populations, establishing its value for future genetic assessments of *D. dhufarensis* (Figure 1).

The dendrogram revealed closer genetic proximity between Al-Aflaj and Al-Hariq (distance = 0.2), while Al-Kharj appeared more genetically distinct, suggesting population-specific divergence potentially driven by ecological or geographic isolation (Figures 2 & 3).

DISCUSSION

The ISSR-based genetic patterns observed among *Firouzophrynus dhufarensis* populations from Al-Kharj, Al-Hariq, and Al-Aflaj should be interpreted within the constraints of a preliminary study. While measurable differences in polymorphism were detected, the limited number of loci, dominance of ISSR markers, and restricted sampling design preclude strong inferences regarding historical demography or evolutionary processes. Nonetheless, the observed population-level differentiation is consistent with expectations for amphibian populations inhabiting arid and semi-arid regions of the Arabian Peninsula, where large-scale aridification, habitat fragmentation, and discontinuous surface water availability can promote isolation and reduced gene flow. These results therefore provide an initial indication of spatial genetic structuring that warrants further investigation using expanded sampling and higher-resolution genomic tools.

These patterns are consistent with ecological observations indicating that *F. dhufarensis* is physiologically tolerant of dry environments and can

Table 1. Genetic diversity parameters of *Firouzophrynus dhufarensis* populations based on ISSR markers.

Population	Total loci	Polymorphic loci	PPB (%)	Nei's genetic diversity (H)	Shannon index (I)
Al-Kharj	58	24	41.0	0.236	0.352
Al-Aflaj	104	30	29.0	0.191	0.281
Al-Hariq	122	31	25.49	0.178	0.263

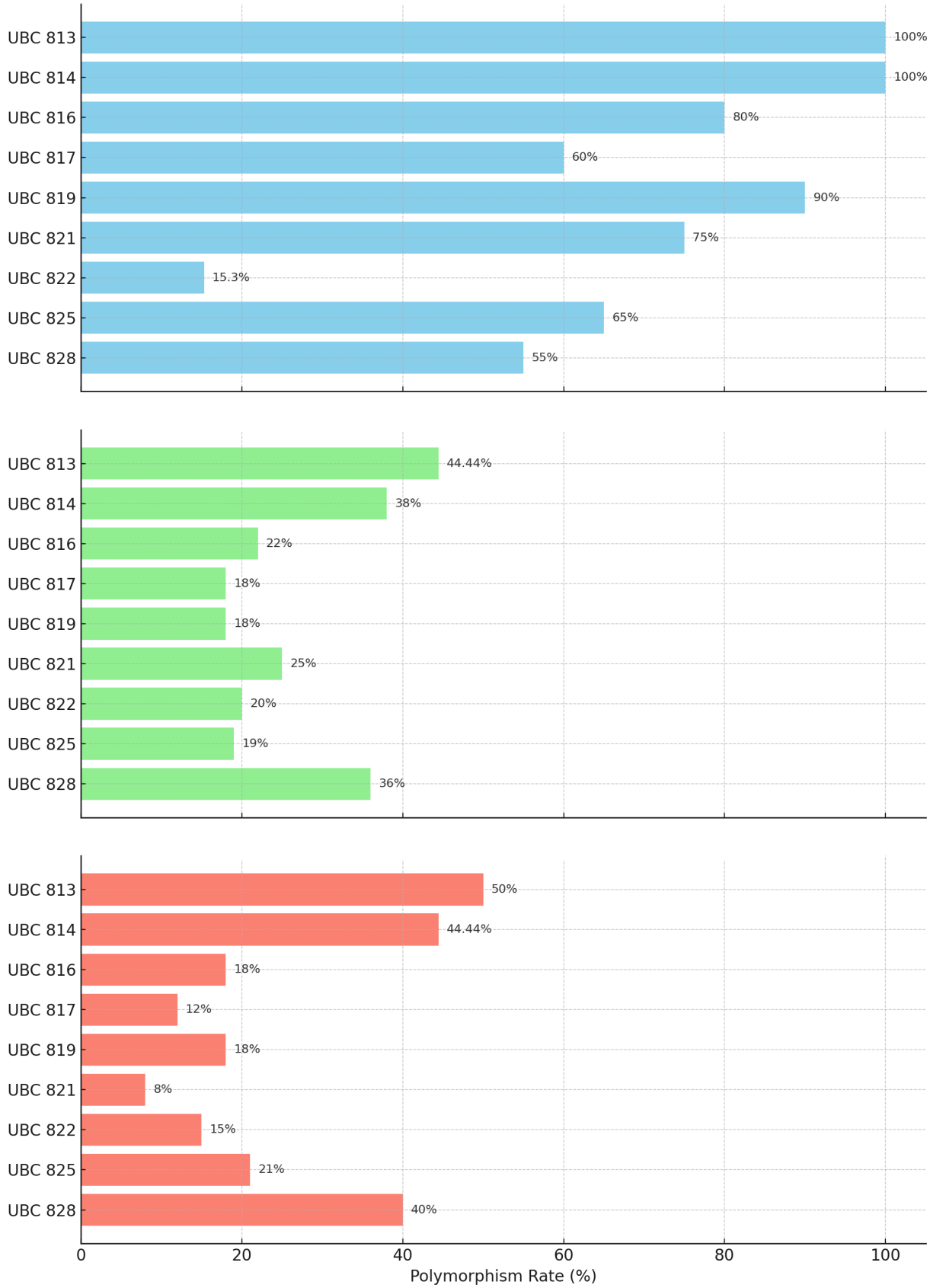

Figure 1. Percentage of polymorphic loci detected by nine ISSR primers in *Firouzophrynus dhufarensis* populations from A—Al-Kharj | (B—Al-Hariq | C—Al-Aflaj, illustrating intra-population genetic variability and differences in primer efficiency.

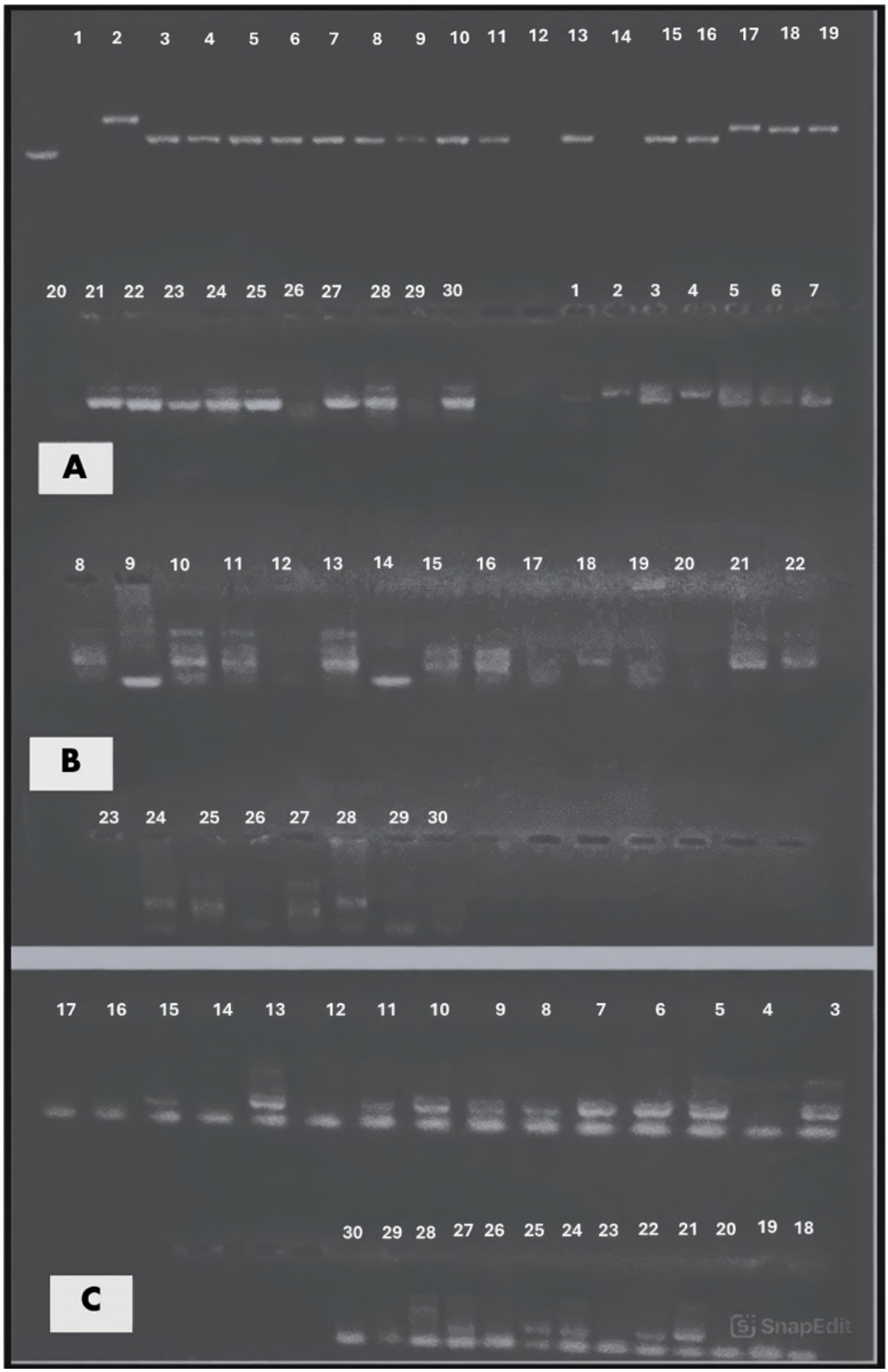

Image 2. Representative ISSR amplification profiles of *Firouzophrynus dhufarensis* from Al-Kharj, Al-Hariq, and Al-Aflaj populations generated using primers UBC 817–UBC 828. Distinct banding patterns illustrate variation in fragment presence and polymorphism among populations.

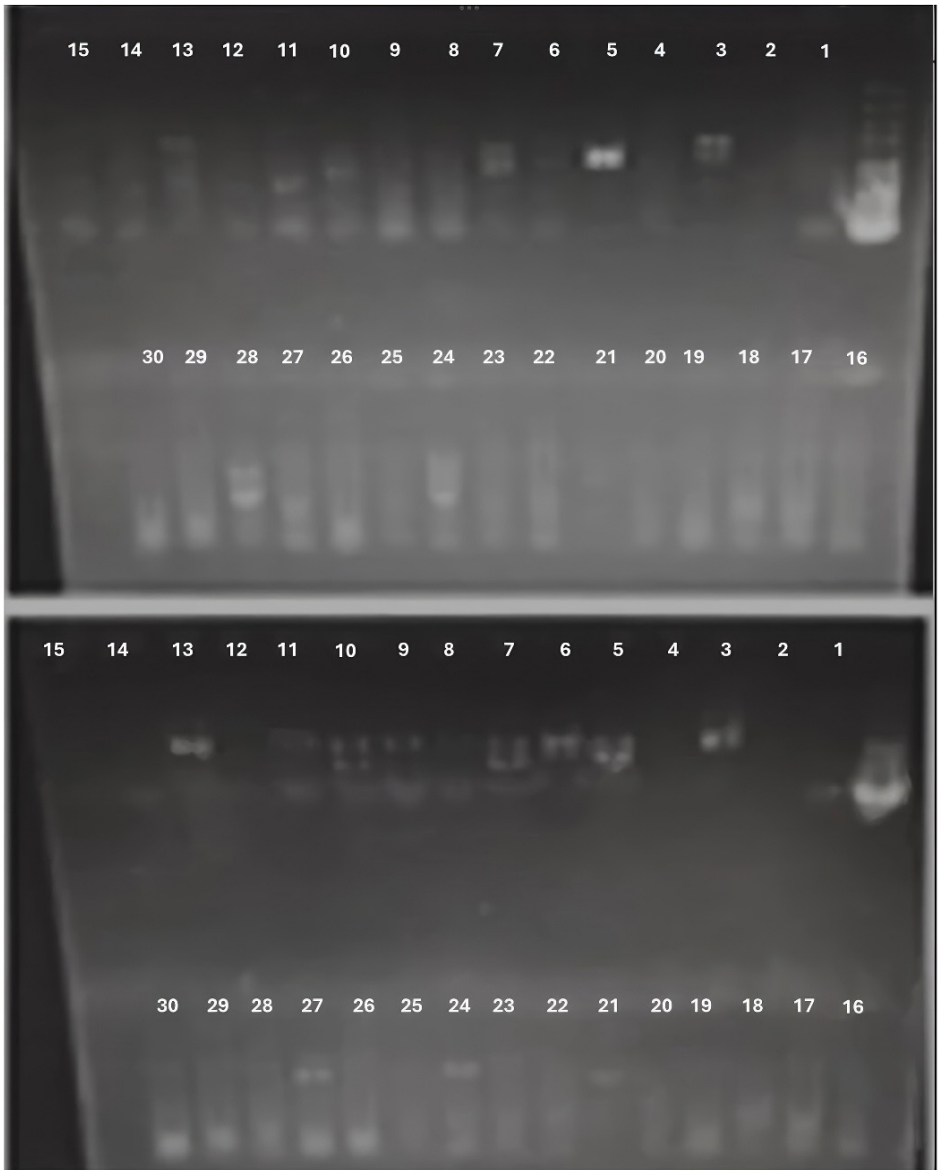

Image 3. ISSR banding profiles of *Firouzophrynus dhufarensis* individuals from Al-Aflaj, Al-Kharj, and Al-Hariq amplified using primers UBC 817 (A), UBC 819 (B), and UBC 825 (C), highlighting primer-specific polymorphism and inter-population genetic variation.

occupy a wide range of habitats, including mountains, wadis, and agricultural areas (Cunningham & Feulner 2001; Al-Johany et al. 2014). Similar trends have been reported for other Bufonidae species in arid landscapes, where habitat quality and landscape connectivity significantly influence genetic structure and long-term population viability (Zeisset & Beebee 2008; Alshammari & Ibrahim 2018; Alrefaei et al. 2022).

Primer performance further supports the genetic differences observed. UBC 813 and UBC 814 consistently yielded high polymorphism across populations, suggesting these primers may target variable genomic regions. In contrast, UBC 817 and UBC 821 produced lower polymorphism, possibly due to amplification of conserved sequences or reduced primer efficiency. These findings underscore the importance of primer selection and support the use of multi-primer ISSR strategies for assessing overall genetic diversity in species lacking extensive genomic resources, as ISSR markers are reproducible, highly polymorphic, and suitable for genome fingerprinting in non-model organisms (Zietkiewicz et al. 1994; Bornet & Branchard 2001).

The lower levels of genetic polymorphism observed in the Al-Hariq and Al-Aflaj populations should be interpreted cautiously. Reduced genetic diversity in amphibian populations is often associated with limited

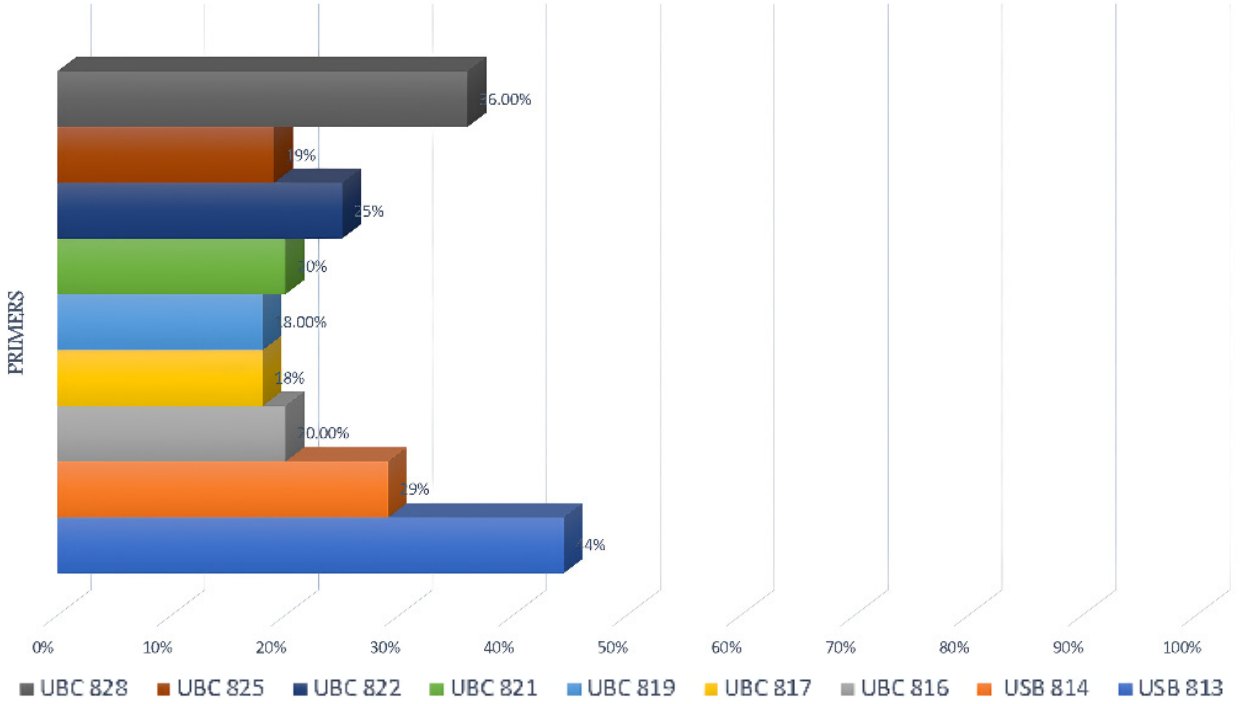

Figure 2. Primer-specific polymorphism rates in the Al-Hariq population of *Firouzophrynus dhufarensis*, expressed as the percentage of polymorphic loci across nine ISSR primers.

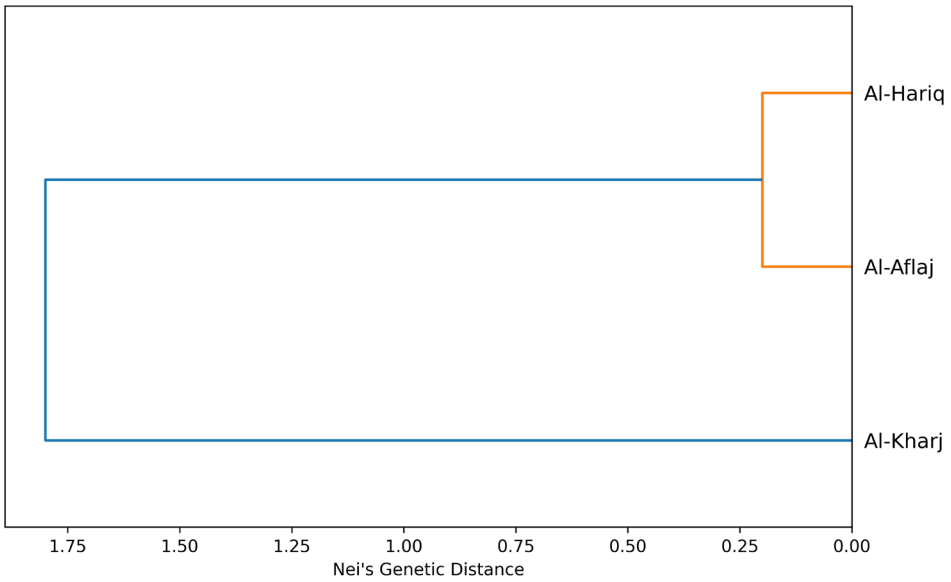

Figure 3. UPGMA dendrogram based on Nei's unbiased genetic distances showing genetic relationships among *Firouzophrynus dhufarensis* populations from Al-Kharj, Al-Hariq, and Al-Aflaj. The scale bar represents Nei's genetic distance, with shorter branch lengths indicating closer genetic similarity.

dispersal, isolation, or small effective population sizes in fragmented or arid landscapes (Frankham 2005; Storfer et al. 2010; Haddad et al. 2015). The present study does not directly test these processes, and no spatial, environmental, or landscape genetic analyses were conducted. Therefore, causal links between genetic variation and habitat fragmentation or environmental heterogeneity cannot be confirmed and should be regarded as hypotheses requiring further investigation.

Recent landscape-scale studies from arid regions

Table 2. Primer-wise ISSR polymorphism and polymorphic information content (PIC) across populations.

Primer	Polymorphism range (%)	PIC (Al-Kharj)	PIC (Al-Hariq)	PIC (Al-Aflaj)
UBC 813	44.44–100	0.812	0.798	0.821
UBC 814	38–100	0.781	0.754	0.768
UBC 816	22–35	0.701	0.689	0.712
UBC 817	12–18	0.665	0.648	0.671
UBC 819	18–42	0.743	0.721	0.758
UBC 821	20–30	0.692	0.676	0.701
UBC 822	15–28	0.684	0.667	0.695
UBC 825	25–40	0.728	0.709	0.736
UBC 828	32–45	0.756	0.732	0.771

of the Arabian Peninsula and adjacent deserts further demonstrate that population connectivity in dryland systems is often shaped by complex interactions among historical aridification, topography, hydrology, and species-specific dispersal capacity. For example, Pola et al. (2024) showed that even broadly distributed desert taxa can exhibit pronounced genetic structuring across environmentally heterogeneous arid landscapes, with connectivity often constrained by discontinuous habitats rather than simple geographic distance. Although such studies typically rely on higher-resolution genomic or spatially explicit approaches, their findings provide an important regional framework for interpreting preliminary genetic patterns observed in arid-zone amphibians such as *F. dhufarensis*. Within this context, the present results should be viewed as an initial indication of localized population differentiation rather than evidence of range-wide isolation or connectivity.

From a conservation perspective, preserving genetic diversity across the species' range is critical, as genetic variation is a key determinant of population resilience in the face of environmental change and stochastic events (Frankham 2005). Al-Kharj may serve as a priority site due to its elevated genetic variability. At the same time, Al-Hariq and Al-Aflaj may benefit from habitat restoration and connectivity-enhancing measures such as ecological corridors. Establishing such linkages could help mitigate the effects of genetic drift, reduce inbreeding, and improve population resilience, especially in fragmented or isolated habitats where landscape-level barriers hinder gene flow (Botstein et al. 1980; Storfer et al. 2010; Haddad et al. 2015; Mashlawi & Masood 2024).

The geographically narrow sampling design represents an important limitation of this study. While the three sampled populations provide insight into local-scale genetic variation within central Saudi Arabia, they do not capture the full extent of genetic diversity across the species' core range in southern Arabia, particularly Oman and Yemen, where *F. dhufarensis* is considered native. As a result, the present data cannot be used to infer species-wide connectivity, historical dispersal routes, or range-wide isolation patterns. Future studies incorporating populations from Oman, Yemen, and southwestern Saudi Arabia are essential to place the central Saudi populations within a broader phylogeographic framework and to robustly evaluate patterns of connectivity, isolation, and potential post-aridification divergence across the Arabian Peninsula.

A key limitation of this study is the exclusive reliance on ISSR markers, which, despite their reproducibility and utility for preliminary assessments, provide lower resolution than co-dominant or sequence-based markers. As dominant markers, ISSRs do not allow direct estimation of heterozygosity, contemporary gene flow, or historical connectivity among populations. Future research should therefore integrate more informative molecular tools, such as microsatellites, mitochondrial DNA sequences, and genome-wide SNP approaches, alongside broader geographic sampling. Such integrative analyses would substantially improve inference regarding population origins, dispersal dynamics, and the evolutionary processes shaping genetic structure across the Arabian Peninsula.

This study provides the first ISSR-based assessment of genetic diversity in central Saudi Arabian populations of the Dhofar Toad. The results reveal clear population-level differences in genetic polymorphism, with Al-Kharj exhibiting comparatively higher genetic diversity, while Al-Hariq and Al-Aflaj show reduced variability, likely reflecting isolation and limited gene flow. These findings highlight the conservation importance of maintaining habitat connectivity and protecting genetically diverse populations in arid landscapes. Although ISSR markers offer valuable baseline insights, future studies employing higher-resolution genomic markers and broader geographic sampling are needed to clarify population origins, connectivity, and conservation units across the species' Arabian range. While the present study is intentionally limited in scope, integrating higher-resolution genetic markers together with morphological, ecological, and spatial data will be essential in future research to rigorously evaluate population connectivity, adaptive variation, and conservation units across the species' Arabian range.

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