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Genetic diversity and phylogenetic structure of Marawh and Bidah pomegranate landraces from Al-Baha, Saudi Arabia, using ITS DNA barcoding

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Abstract

Pomegranate (*Punica granatum L.*) plays a vital cultural and economic role in the Al-Baha region of Saudi Arabia. Despite its significance, limited molecular data exist on the genetic structure of local landraces, particularly the distinct red and green fruit colour variants of the Marawh and Bidah cultivars. This study investigates the genetic diversity and phylogenetic relationships among these landraces using the nuclear internal transcribed spacer (ITS) DNA region. Maximum likelihood phylogenetic tree construction and network analysis (SplitsTree) were employed. Results reveal that red- and green-fruited landraces cluster into distinct clades, with red variants exhibiting reticulate patterns suggestive of introgression or incomplete lineage sorting. Genetic distance analysis confirmed a high similarity (~99.15%) between the green variants, despite their placement in separate clades. The findings provide crucial insights into the evolutionary history, cultivar authentication, and conservation strategies for pomegranate germplasm in Al-Baha. Future directions include genome-wide SNP analyses and expanded sampling to refine our understanding of these valuable genetic resources.

Keywords: *Punica granatum*; Phylogenetic analysis; Genetic diversity; Al-Baha; ITS DNA barcoding; SplitsTree; Pomegranate landraces.

1. Introduction

Pomegranate (*Punica granatum* L.) is an ancient fruit tree that plays a critical economic and cultural role in the Middle East and Mediterranean regions [1, 2]. In Saudi Arabia, especially in the Al-Baha region, pomegranate cultivation is a hallmark of local agriculture, with the Marawh and Bidah landraces being particularly valued for their distinctive fruit quality. Marawh is known for its two phenotypes, green and red, both of which are valued for their distinctive taste, balance of acidity and sweetness, and strong market appeal.

Several studies in Saudi Arabia have begun exploring the morphological and phytochemical traits of native pomegranate cultivars [3-5], but relatively few have applied molecular tools to assess genetic diversity and evolutionary structure [6, 7]. This lack of genetic insight hinders precise cultivar identification and conservation planning. In contrast, regional efforts in countries like Iran, India, and Turkey have successfully employed DNA markers such as RAPD, ISSR, and SSR to characterize population structure, uncover gene flow, and resolve cultivar identities [8-10]. These international benchmarks highlight the need for similar molecular frameworks to support pomegranate biodiversity management in Saudi Arabia.

Molecular tools such as DNA barcoding, especially using the internal transcribed spacer (ITS) region of nuclear ribosomal DNA, are powerful for assessing genetic diversity and population structure [11, 12]. These tools are crucial in contexts like Al-Baha, where cultivars with similar morphology might differ genetically due to historical propagation or local adaptation.

The objective of this study is to characterize the genetic diversity and evolutionary relationships of Marawh and Bidah landraces using ITS DNA barcoding, and to provide insights into cultivar structure, possible gene flow, and implications for conservation and breeding.

2. Materials and Methods

The ITS region was chosen for this study due to its high interspecific variability and proven efficacy in resolving species- and cultivar-level relationships within *Punica granatum* and other fruit-bearing taxa [11, 12]. Compared to plastid barcoding regions such as psbA-trnH or matK, ITS typically offers greater resolution in closely related genotypes and is especially effective in distinguishing between cultivated varieties with overlapping morphological traits [13, 14]. Additionally, the nuclear origin of ITS provides biparental inheritance patterns, allowing it to detect

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potential recombination or hybridization events that may not be evident from maternally inherited chloroplast markers [15, 16].

2.1. Sample collection

Leaf samples were collected from three randomly selected healthy trees for each of the four landraces: Marawh-Red, Marawh-Green, Bidah-Red, and Bidah-Green. Sampling was conducted on local farms in Bani Hasan, Al-Baha Province, Saudi Arabia (20°07′07″N, 41°22′12″E), during the peak growing season.

2.2. DNA extraction and amplification

Total genomic DNA was extracted from fresh leaf tissue using the NZY Plant/Fungi gDNA Isolation Kit, following the manufacturer's instructions. DNA concentrations were measured using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, UK).

Amplification of the ITS region was conducted using the primers ITS-S2F (5′-ATGCGATACTTGGTGTGAAT-3′) and ITS4rev (5′-TCCTCCGCTTATTGATATGC-3′). PCR conditions involved an initial denaturation at 94 °C for 3 minutes, followed by 35 cycles of 94 °C for 30 seconds, 58 °C for 40 seconds, and 72 °C for 50 seconds, with a final extension at 72 °C for 10 minutes using a Techne thermal cycler (GMI, USA).

2.3. Gel electrophoresis and sequencing

PCR products (~350 bp) were confirmed on 1.0% agarose gels and purified using the Expin PCR Purification Kit (Gene ALL, Korea). Sequencing was performed by Al Borg Laboratories (Saudi Arabia). Chromatograms were inspected for quality and trimmed manually.

2.4. Sequence analysis and phylogenetic inference

Sequences were aligned using MEGA12 software [17]. All sequences were deposited in GenBank Marawh-Green and Marawh-Red under the accession numbers PV810088 and PV810089, respectively and validated using BLASTn to confirm species identity. The best-fit model was identified as Kimura 2-parameter with invariant sites (K2P+I), and a maximum likelihood tree was generated with 1,000 bootstrap replicates.

2.5. Network analysis

To account for non-tree-like signals, a phylogenetic network was constructed using SplitsTree4 software [18], which visualizes potential reticulation and conflicting evolutionary signals, especially relevant in cultivated species with possible hybridization histories [11, 19].

2.6. Statistical Analysis

All phylogenetic and network analyses were supported by statistical evaluation. Sequence alignments and model selection (Kimura 2-parameter with invariant sites, K2P+I) were performed in MEGA12 [17]. Phylogenetic robustness was assessed using Maximum Likelihood inference with 1,000 bootstrap replicates. Reticulate relationships were further explored using SplitsTree4 [18], which computes split support values to visualize conflicting phylogenetic signals. Statistical significance in sequence identity confirmation was determined using BLASTn search scores and E-values against the NCBI database.

3. Results

Amplification of the locus was successfully performed in all samples from the two cultivars. The ITS2 sequences of Marawh-Green and Marawh-Red were deposited in GenBank under the accession numbers PV810088 and PV810089, respectively. To enable comparative analysis, a search of GenBank (NCBI) was conducted for *P. granatum* ITS2 sequences. Only sequences that were annotated with cultivar names and formed part of their validated dataset were included [6]. This approach ensured methodological consistency with published work and enhanced the reliability of cultivar-level phylogenetic interpretations.

The ITS sequences obtained from Marawh-Red and Marawh-Green exhibited high-quality chromatograms with clear base calling. ClustalW alignment revealed high sequence identity between Marawh-Red and Bidah-Red (>90%), along with several SNPs and a distinct 1-bp INDEL, offering resolution for distinguishing between the two red landraces. Similarly, Marawh-Green and Bidah-Green showed strong conservation (97% identity), with five small INDELs in the 5′ region and five SNPs, several of which were located near the 3′ end. These polymorphic sites serve as informative markers for differentiating the green cultivars [6, 20].

To place these cultivars in a broader genetic context, the ITS2 sequences of Marawh-Red and Marawh-Green were further analysed alongside selected *Punica granatum* accessions retrieved from GenBank using MEGA12. Multiple sequence alignment revealed several additional polymorphic sites, highlighting genetic variation across the dataset. Substitution model testing identified the Kimura 2-parameter model with a proportion of invariant sites (K2P+I) as the best fit based on the lowest Bayesian Information Criterion (BIC) score. Accordingly, evolutionary relationships were inferred using the Maximum Likelihood method under the K2P+I model, providing further insight into the phylogenetic placement of the two cultivars among global pomegranate accessions.

The Maximum Likelihood (ML) tree (Figure 1A) grouped Marawh-Red and Bidah-Red together with moderate bootstrap support (40%), supporting the findings from earlier SSR- and RAPD-based studies on phenotypic clustering and the pairwise genetic distance between them was (8%) [9, 21]. Conversely, Marawh-Green and Bidah-Green clustered within a separate clade, although with low resolution. Yet, the pairwise genetic distance between them was minimal (0.85%), indicating approximately 99.15% identity, an observation common in gene tree discordance reported in other fruit trees [22, 23]. SplitsTree4 network analysis further reinforced these findings: the red variants displayed clear reticulation, suggesting historical gene flow or hybrid ancestry, whereas the green cultivars formed a more tree-like, non-reticulate pattern (Figure 1B). This network complexity among red landraces aligns with earlier findings of higher genetic mixture in cultivated pomegranates revealed by SSR and AFLP markers [24-26].

Taken together, these results highlight contrasting evolutionary dynamics between red and green pomegranate accessions and demonstrate the power of combining ITS sequencing, phylogenetics, and network approaches in unraveling intra-specific diversity.

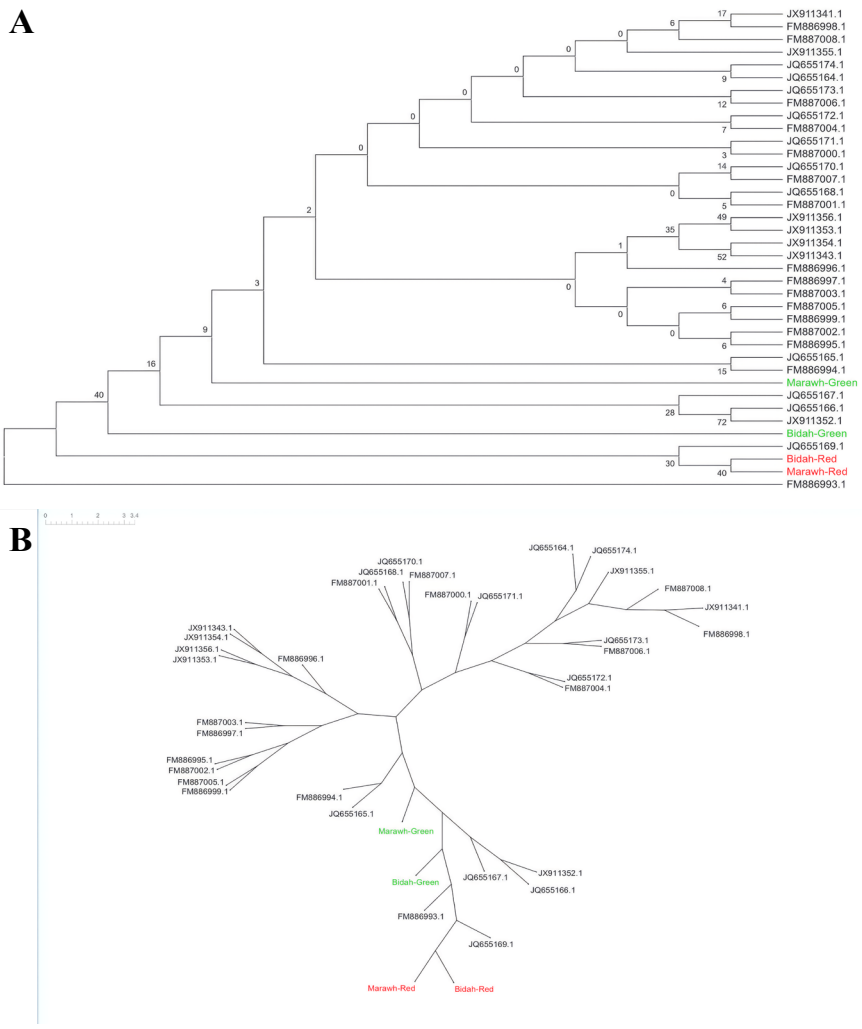

Fig. 1. Phylogenetic relationships among pomegranate landraces based on ITS sequences. A) Maximum Likelihood (ML) tree constructed under the Kimura 2-parameter + I model using 1000 bootstrap replicates in MEGA12. Bootstrap values are shown next to the branches. The tree reveals a distinct clustering of Marawh-Red and Bidah-Red as a sister clade, while Marawh-Green and Bidah-Green group within a broader conserved lineage. The sequence FM886993.1, representing a wild pomegranate cultivar, was included as an outgroup. **B)** SplitsTree4 network based on the same ITS dataset, illustrating reticulate relationships primarily among the red landraces.

4. Discussion

This study reveals consistent differentiation between the red- and green-fruited pomegranate landraces in Al-Baha, mirroring trends reported from other Mediterranean and Iranian cultivars using molecular markers [19, 21]. For instance, Iranian studies employing SSR and chloroplast markers have revealed genetic structure and admixture among pomegranate genotypes, with some evidence of partial lineage sorting and gene flow [20, 22]. However, no consistent clustering based on fruit colour was observed in these studies.

Indian studies using SSR markers and ITS barcoding have shown low genetic distances between phenotypically distinct pomegranate cultivars. According to Patil et al. [9], most genetic variation existed within populations, likely due to gene flow and admixture. Similarly, [20] reported high intra-group polymorphism and conserved ITS regions, suggesting that environmental adaptation and historic cross-pollination have shaped genetic diversity more than visible traits. These findings highlight the limitations of relying solely on phenotypic traits for cultivar classification.

Despite high genetic similarity within the green group, their disrupted clustering may be attributed to incomplete lineage sorting or ancestral polymorphism, phenomena documented across various barcoding and cpDNA studies [11, 27]. These findings support the hypothesis that morphological traits such as fruit colour may not fully reflect underlying genetic relationships and may have emerged from localized selection pressures or epigenetic influences [28, 29].

The reticulate patterns observed among red variants support hypotheses of historic introgression, potentially driven by cultivation and seed exchange practices among farmers or cross-regional propagation routes connecting the Arabian Peninsula to Iran and the Levant [30, 31]. Such genetic blending has been shown to enhance diversity and adaptability in fruit trees [32, 33], which could explain the observed complexity in red cultivar networks.

Furthermore, the success of ITS barcoding in resolving intra- and inter-cultivar relationships confirms its utility for cultivar authentication, consistent with findings from India and other regions. This molecular resolution is essential for the protection of landrace identity and quality control in commercial propagation. In particular, studies such as [34] have demonstrated the effectiveness of molecular markers in distinguishing Egyptian pomegranate cultivars, supporting the broader application of nuclear

DNA markers for genetic fingerprinting and germplasm management.

The results align with genome-scale divergence patterns recently described in the *Punica* draft genome [7, 35], affirming that even non-coding regions like ITS can reflect underlying evolutionary structure. The Al-Baha landraces, with their contrasting fruit colours and genetic clustering, represent valuable genetic resources warranting conservation and deeper genomic analysis. Future studies may benefit from incorporating genome-wide SNP data and chloroplast markers to fully resolve lineage divergence and regional admixture patterns across the Arabian Peninsula.

This study represents a foundational step toward elucidating the genetic diversity and evolutionary structure of the Marawh and Bidah pomegranate landraces from Al-Baha, Saudi Arabia. By employing ITS DNA barcoding and robust phylogenetic tools, we demonstrated that these landraces separate distinctly along red and green fruit colour lines, with red varieties exhibiting signs of historical gene flow and green types displaying stronger lineage cohesion. These findings are in line with regional studies and highlight the utility of ITS in distinguishing closely related cultivars, even in morphologically conserved populations.

The molecular evidence supports the proposition that colour-based varietal identities in Al-Baha pomegranates are underpinned by measurable genetic divergence. Moreover, our results emphasize the importance of integrating molecular diagnostics into local breeding programs and germplasm conservation efforts to ensure the preservation of genetic resources unique to Saudi Arabia and to clarify patterns of diversity across the Arabian Peninsula.

Future research should incorporate larger sampling across more regions, additional nuclear and chloroplast markers, and high-throughput sequencing approaches such as genome-wide SNP profiling. Such efforts would provide a more comprehensive picture of pomegranate evolution in the Arabian Peninsula and inform strategies for sustainable cultivation, genetic improvement, and commercial authentication of Saudi landraces.

Conflict of interests
The author has no conflicts with any step of the article preparation.

Consent for publications
The author read and approved the final manuscript for publication.

Ethics approval and consent to participate
No human or animals were used in the present research.

Informed consent
The authors declare that no patients were used in this study.

Availability of data and material
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions
Abdulaziz Albogami: Conceived and designed the research; supervised the study; performed all laboratory procedures.

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