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Influence of Soil Salinity on Genetic Diversity and Phylogenetic Relationships in *Tetraena* Species: Insights from Electrical Conductivity Analysis, Inter-retrotransposon Amplified Polymorphism Markers, and DNA Barcoding

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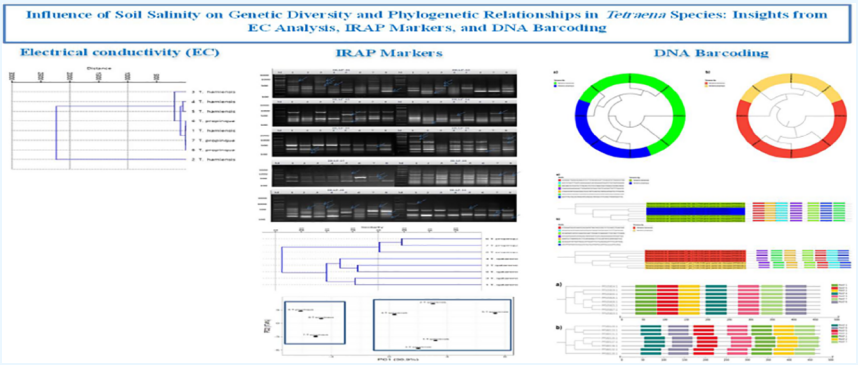

ABSTRACT: Soil salinity is a significant environmental stressor that impacts species distribution, plant development, and genetic diversity. Conservation and ecological management depend on an understanding of how *Tetraena* species respond to salinity. The genus *Tetraena*, which includes several species of succulent shrubs native to arid regions, is of significant interest for studying plant adaptation mechanisms. The study aims to evaluate the genetic diversity and ecological characteristics of eight groups of *Tetraena* species in Saudi Arabia using inter-retrotransposon amplified polymorphism (IRAP) markers, ycf5 and trnH gene sequences, as well as soil pH and electrical conductivity (EC). Soil pH indicated slightly alkaline conditions, while electrical conductivity (EC) ranged from 822 μS/cm in the *T. propinqua* population at Al Thumama Road (population 8) to 23,800 μS/cm in the *T. hamiensis* population at Al Jawhara-Dammam Road (population 2). The genetic relationships were determined by analyzing IRAP marker polymorphism, generated using 10 primers. Clustering through principal component analysis and biostatistical methods distinguished the populations of *T. propinqua* subsp. *Migahidii* (6, 7, and 8) from the populations of T. hamiensis var. qatarensis (1, 3), (4, 5), and (2). Ten primers had high polymorphism (60.5%) according to IRAP analysis between *T. hamiensis* and *T. propinqua*. The evolutionary trees of *T. propinqua* and *T. hamiensis* cluster together. Analysis of conserved motifs revealed common motifs that support the use of ycf5 and trnH as barcodes. The genetic diversity and population clustering of *T. hamiensis* and *T. propinqua* are influenced by environmental salinity and species-specific genetic adaptations. While *T. hamiensis* has more differentiation, maybe as a result of historical separation or localized adaptations, *T. propinqua* exhibits strong genetic similarities. These results demonstrate that common environmental stresses and species-specific characteristics are the main drivers of genetic diversity. Future studies should explore adaptive genetic mechanisms at the molecular level and assess the functional roles of salinity-responsive genes in support conservation efforts.

1. INTRODUCTION

Salinity in arable land is becoming an increasingly serious problem in many irrigated, dry, and semiarid places around the world, where low rainfall prevents salts from draining from the root zone, reducing crop output dramatically.[1] Saline soils are those with enough salt in the root zone to inhibit crop growth.[2] However, salt-induced damage varies according to species, variety, growth stage, climatic circum-

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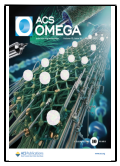

stances, and salt type, making it difficult to define saline soils precisely.[3] FAO[4] defines saline soil as having an electrical conductivity of the saturation extract (ECe) of 4 dSm to 1 or greater, with soils above 15 dSm to 1 considered extremely saline. As the population expands, competition for freshwater among the municipal, industrial, and agricultural sectors intensifies in many areas. As a result, freshwater allocation for agriculture has dropped.[5] This tendency is anticipated to continue and worsen, especially in less developed, arid nations that already face rapid population expansion and significant environmental challenges.[6]

The genus *Tetraena*, belonging to the family *Zygophyllaceae*, comprises several species of succulent shrubs predominantly found in arid regions across Africa and Asia.[7] These plants are well adapted to extreme environmental conditions, making them of interest for their potential in understanding plant adaptation and resilience mechanisms.[8] *Tetraena* species are known for their morphological diversity, including variations in leaf shape, flower color, and growth habit.[9] The taxonomy of *Tetraena* has been a subject of debate, with earlier classifications grouping these species under the name *Zygophyllum*. However, recent molecular phylogenetic studies have supported the recognition of *Tetraena* as a distinct genus.[9−11] This change in classification led to a better understanding of the evolutionary relationships within the genus. The genus *Tetraena* is estimated to comprise around 40 species, distributed in Africa and Asia.[12−14] Two species, *T. propinqua* and *T. hamiensis*, are particularly noteworthy for their distributions and genetic diversity. *T. propinqua* is a low, perennial shrub with green, fleshy leaves and white flowers. Its brown fruits grow in sandy desert habitats across regions like Sinai, the Arabian Peninsula, Iran, Afghanistan, and Pakistan,[14] while *T. hamiensis* is found across Arabian Peninsula and Saudi Arabia.[9,15] Both species exhibit adaptations to arid environments, making them suitable candidates for studying plant resilience mechanisms. *Tetraena* species help to stabilize the sand, conserve soil moisture, and reduce erosion in arid and saline deserts.[16] They are also used as animal fodder and have medicinal properties for illnesses such as rheumatism, diabetes, asthma, and hypertension thanks to their phytochemical constituents.[17,18] *Tetraena* species have yielded a variety of chemicals such as terpenes, flavonoids, saponins, sterols, phenolics, essential oils, and esters.[19,20]

Diversity can be divided into four types: intra- and intercrop diversity, intra- and intercultivar diversity, intra- and intervarietal diversity, and intra- and interlandrace diversity.[21] Within cultivars, genetic diversity is divided into two kinds. The first is a monomorphic cultivar, which is uniform and homozygous and has similar physical and genetic characteristics. The second is a polymorphic cultivar, which is heterozygous and exhibits multiple phenotypes and genotypes.[21] Genetic diversity that is helpful for taxonomy and conservation measures is indicated by genetic markers like RAPD and chloroplast SSRs, which show high levels of polymorphism across species like *Z. coccineum* and *Z. album*.[11] Chloroplast genomics provides important insights into the phylogenetic relationships between *Zygophyllum* populations, particularly in China's LSC regions. This aids in species delimitation and understanding the evolutionary history, and it is critical for conservation under environmental stress.[22] The plastome is a distinguishing and identifying feature for the closely related *Tetraena* and *Zygophyllum* species.[9] SCoT assessed the genetic variations of 29 wild plants in Al Jubail, Saudi Arabia.[23] Geographic isolation and environmental stressors have caused significant genetic differentiation in *Zygophyllum* loci in the deserts of Northwest China. Researchers have examined genetic variation, ploidy levels, and adaptive genome structures.[10]

Numerous applications of molecular markers in plant molecular breeding and genomics have been documented.[24] Using particular or randomly designed oligonucleotide primers, the PCR technique allows specific DNA sequences to be practically amplified from genomic DNA sections. Nowadays, molecular markers are among the most helpful instruments for plant improvement research. Mostly polymorphic nucleic acids between individuals or populations make up these markers.[25] Molecular markers find and choose genotypes with desired qualities by analyzing molecular markers associated with target genes.[25] This strategy allows for a speedy and accurate selection of attributes. Genetic markers include morphological (visible features), cytological (chromosome variants), biochemical (based on proteins and isoenzymes, but with few observable sites), and molecular markers, which are frequently used because they directly represent DNA-level alterations.[26] RAPD is a quick, low-repeat molecular marker, while SSR is a widely used technology in crop genetic diversity research and molecular breeding.[27,28] SNP markers, caused by single nucleotide variations, offer high frequency, stable inheritance, and easy genotyping, making them useful for genetic studies of complex traits and diseases due to their wide distribution and high correlation with disease genes.[29]

Retrotransposons are distinct genetic elements resulting from ancient retrovirus insertions into plant genomes. Target segments on the genome between two loci where retrotransposons insert are amplified to create inter-retrotransposon amplified polymorphism (IRAP).[30,31] Furthermore, it can be used to find insertional polymorphism in retrotransposons. Their ability to replicate inside the genome has a substantial impact on genetic variation and genome size.[32] Several retrotransposon-based marker systems have been developed using genome sequence properties, the most prominent of which is IRAP.[33] LTR retrotransposon-associated IRAP is an effective method for finding insertion polymorphisms by amplifying DNA fragments from double retrotransposons. Because of its simplicity, IRAP is commonly used to detect genetic variation and investigate genetic diversity in plants.[34] The IRAP is thought to be a promising marker.[35] For example, IRAP markers have been discriminated against to estimate genetic diversity in the Phyllostachys bamboo genus.[36] Twelve Sakura genotypes underwent genetic analysis using the chosen IRAP markers.[37] The genetic relationships between 34 different species of *Lallemantia iberica* were using retrotransposon-microsatellite amplified polymorphism (REMAP) and IRAP.[30] The insertional polymorphism among 19 black pepper varieties was analyzed using seven LTR IRAP primers, in the single and combination forms.[38]

DNA barcoding, proposed by Paul Hebert,[39] serves as a supplementary tool to morphological taxonomy for species identification and discovery. DNA barcoding is a molecular technique used for species identification and classification based on short, standardized DNA sequences.[40] The use of DNA barcoding has revolutionized species identification in plants, allowing for rapid and accurate species identification,

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particularly in complex plant groups like *Tetraena*. Several genes have been proposed as potential barcodes, including rbcL, matK, ITS, ycf5, and TrnH-psbA.[40−42] Lastly, environmental and ecological genomic research is a key field in which DNA barcoding is used.[40] TrnH-psbA is a highly variable noncoding plastid locus with an intergenic spacer that allows for strong species differentiation.[43,44] It can be amplified easily using universal primers, but alignment can be challenging due to its high insertion/deletion rates. Furthermore, the length of this region varies between families; in some cases, this region contains copies of rps19 in addition to a pseudogene between trnH and psbA. This poses an alignment challenge, even though high-quality bidirectional sequences can be obtained. To achieve sufficient resolution, the majority of researchers have suggested using trnH-psbA in conjunction with one or more loci.[43,45,46] However, research has demonstrated that it is a reliable indicator of flowering. To improve species discrimination, several genes, including ITS, ITS2, ycf5, and trnL.[47,48] *T. hamiensis* var. *qatarensis* and *T. simplex*, were reclassified from Zygophyllum to *Tetraena* using limited genetic evidence. To further study this categorization, researchers sequenced *T. hamiensis* and *T. simplex* and conducted extensive comparative genomics, phylogenetic analysis, and divergence time estimation. The full plastomes ranged from 106,720 to 106,446 bp, which is tiny when compared to typical angiosperm plastomes.[9]

This study's main goal is to investigate the ecological traits and genetic diversity of *Tetraena* species in Saudi Arabia, with an emphasis on the species boundaries, evolutionary links, and adaptation to desert conditions. Through the use of IRAP markers and ycf5 and trnH gene sequences, we want to evaluate the evolutionary history of various species and offer insights into their genetic variety. To learn more about the environmental conditions in which these species flourish, ecological characteristics including soil pH and electrical conductivity (EC) will also be evaluated. Clarifying species boundaries and advancing our knowledge of plant variety and evolutionary processes in arid environments are two benefits of combining genomic data with ecological considerations. In this study, we present novel findings on the genetic diversity and ecological adaptations of *Tetraena* species, providing new insights into their evolutionary history and environmental resilience in arid regions.

2. RESULTS

2.1. Soil Investigation. The soil pH across all sampled locations ranged from 7.8 to 8.49, indicating alkaline conditions. *Tetraena hamiensis* var. *qatarensis* exhibited a slightly broader pH range (7.9−8.49) for Al jawhara-Dammam road and Al Jubail−Riyadh road compared to *Tetraena propinqua* subsp. *migahidii* (7.8−7.91) for Riyadh-Al Qassim road and Al Thumama road. EC varied significantly among locations with *T. hamiensis* var. *qatarensis* growing in soils with EC values ranging from 888 to 23,800 μS/cm, suggesting its adaptability to both low and high salinity environments (Table 1). In contrast, *T. propinqua* subsp. *migahidii* was found in soils with lower and more stable EC values (822−899 μS/cm), indicating a preference for less saline conditions. These variations in soil parameters highlight the potential ecological adaptability of *Tetraena* species to different environmental conditions in Saudi Arabia.

Table 1. Analysis of Investigated Variations in pH and Electrical Conductivity (EC) across Eight *Tetraena* Species, each Collected from Distinct Habitats in Saudi Arabia

no.	samples	location	pH	EC μS/cm
1	Tetraena hamiensis var. *qatarensis*	Riyadh- Dammam road	8.1	888
2	*Tetraena hamiensis* var. *qatarensis*	Al jawhara-Dammam road	7.9	23,800
3	*Tetraena hamiensis* var. *qatarensis*	King abdullah bin abdulaziz road, Dammam	8.1	3120
4	*Tetraena hamiensis* var. *qatarensis*	Al Jubail industrial city road	8.23	2002
5	*Tetraena hamiensis* var. *qatarensis*	Al Jubail−Riyadh road	8.49	1503
6	*Tetraena propinqua* subsp. *migahidii*	Riyadh-Alkharj road	7.88	899
7	*Tetraena propinqua* subsp. *migahidii*	Riyadh-Al Qassim road	7.8	850
8	*Tetraena propinqua* subsp. *migahidii*	Al Thumama road (Al Narjas)	7.91	822

The soil salinity analysis, based on EC measurements, demonstrated a strong correlation with genetic clustering among the studied populations (Figure 1). *T. hamiensis* from

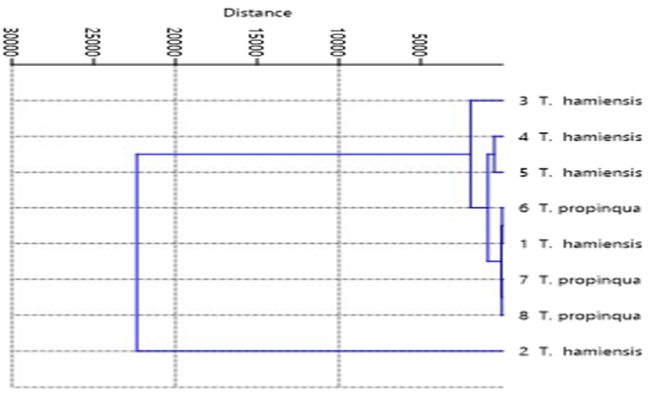

Figure 1. A distance pair group (UPGMA) tree illustrating the relationships among the eight populations (five *T. hamiensis* and three *T. propinqua*) based on the analysis of electrical conductivity (EC).

population 2 exhibited distinct salinity characteristics, aligned with its placement in a separate genetic branch. Similarly, *T. hamiensis* from population 3 displayed unique salinity conditions, consistent with its isolated position within the main cluster. In contrast, *T. hamiensis* from population 1 and *T. propinqua* from populations 6, 7, and 8 clustered together, likely due to shared salinity levels. Additionally, *T. hamiensis* from populations 4 and 5 grouped closely, reflecting similar salinity conditions and strong genetic relatedness. These results suggest that soil salinity is a key environmental factor influencing genetic diversity and population clustering in *Tetraena* species.

2.2. Molecular Characterization as Revealed by IRAP Markers. A total of 10 IRAP primers were used to assess genetic diversity in *Tetraena* species, generating 134 bands, of which 82 were polymorphic, resulting in an average polymorphism percentage of 60.5% (Table 2). Figure 2 shows photographs of IRAP fingerprinting. The number of total bands (TNB) per primer ranged from 7 (IRAP-2202) to

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Table 2. Name and Sequence of 10 IRAP Primers, Total Number of Bands (TNB), Monomorphic Bands (MB), Polymorphic Bands (PB), and Percentage of Polymorphism (P %), Mean of Band Frequency (F), and Polymorphic Information Content (PIC)

no.	P. name	sequence (5′→3′)	TNB	MB	PB	P%	*F*	PIC
1	IRAP-2175	TTAGACCCGGAACCGCCGTG	15	3	12	80	0.49	0.373
2	IRAP-2198	ATCCTTCGCGTAGATCAAGCGCCA	14	5	9	64	0.51	0.358
3	IRAP-2197	GAAGTACCGATTTACTTCCGTGTA	13	6	7	54	0.64	0.356
4	IRAP-2200	ATGTGACAGTCGACTAACCAC	12	4	8	67	0.55	0.372
5	IRAP-2202	TGGCGCTTGATCTACGCGAAGGA	7	4	3	46	0.75	0.305
6	IRAP-2204	AACTTGATCCAGATCATCTCC	13	5	8	62	0.53	0.374
7	IRAP-4334	CCATGGCGAGCAGATGTGCT	16	8	8	50	0.63	0.361
8	IRAP-4370	ATGCCGTATTCTCAGCATCC	15	7	8	53	0.64	0.371
9	IRAP-4351	CAGGCAAGAATGAGCGTCTC	17	5	12	71	0.54	0.374
10	IRAP-4340	ATGGTTGTCGAAACTCCAGC	12	5	7	58	0.65	0.353
	total		134	52	82			
	mean		13.4	5.2	8.2	60.5	0.69	0.359

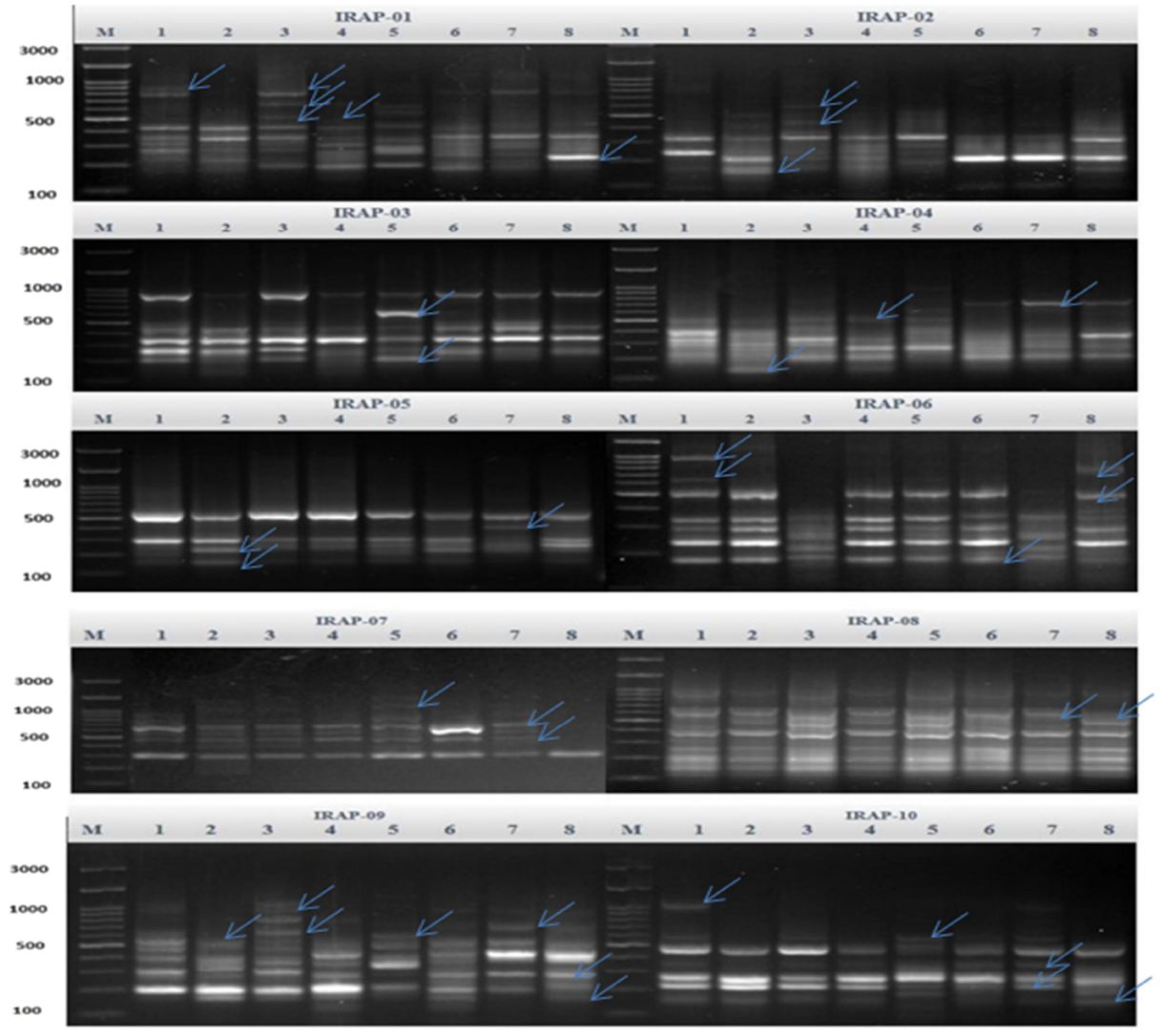

Figure 2. Ten primers of inter-retrotransposon amplified polymorphism (IRAP) profiles were utilized to screen five *T. hamiensis* and three *T. propinqua* populations on a gel concentration of 1.5%, M: DNA ladder.

17 (IRAP-4351), with a mean of 13.4 bands per primer. The number of polymorphic bands (PB) varied from 3 (IRAP-2202) to 12 (IRAP-2175 and IRAP-4351), with a mean of 8.2. The polymorphism percentage (P%) ranged from 46% (IRAP-2202) to 80% (IRAP-2175), indicating moderate to high genetic variation among the studied samples (Table 2). The mean polymorphic information content (PIC) value was 0.359, suggesting a moderate level of marker informativeness. Additionally, the highest PIC value (0.374) was observed in IRAP-2175, IRAP-2204, and IRAP-4351, indicating their

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effectiveness in detecting genetic variation. These results highlight the efficiency of IRAP markers in assessing the genetic diversity within *Tetraena* species.

The calculated genetic diversity parameters for the *Tetraena* species based on IRAP markers are Percentage of polymorphic bands (P%): 61.19%, indicating a moderate level of polymorphism; Nei's Gene Diversity (H): 0.3597, suggesting a moderate level of genetic variation; and Shannon's Information Index (I) of 0.3672, reflecting a balanced genetic diversity distribution. These values highlight the efficiency of IRAP markers in detecting genetic diversity within *Tetraena* species, supporting their adaptability to varying environmental conditions.

2.3. Genetic Similarity Cluster Based on IRAP Analysis. The similarity index Euclidean is calculated among the eight samples of (five *T. hamiensis* and three *T. propinqua*) based on IRAP molecular marker polymorphism in Table 2. The genetic similarity among *Tetraena* species ranged from 0.77 to 0.92, indicating varying degrees of relatedness (Table 3). The highest similarity (0.92) was found between species 6 and 7 and between species 7 and 8, suggesting close evolutionary ties, while the lowest similarity (0.77) was observed between species 5 and both species 7 and 8, indicating genetic divergence. Species 3 and 4 showed moderate similarity to other species. These findings provide insights into species boundaries, evolutionary relationships, and potential adaptation contributing to conservation and genetic resource management.

Table 3. A Distance Unweighted Pair Group with Arithmetic Average Tree Illustrating the Relationships among the Eight Samples (Five *T. qatarensis* (1−5) and Three *T. propinqua* (6−8)) Based on the IRAP Analysis

	1	2	3	4	5	6	7
2	0.85						
3	0.88	0.83					
4	0.84	0.88	0.86				
5	0.85	0.88	0.86	0.85			
6	0.82	0.83	0.82	0.87	0.79		
7	0.81	0.78	0.84	0.80	0.77	0.92	
8	0.78	0.80	0.80	0.83	0.77	0.92	0.88

A UPMGA tree constructed using the PAST software (Figure 3) depicted the genetic diversity present in the sample based on IRAP polymorphism. The dendrogram analysis revealed that *T. propinqua* samples (6, 7, and 8) clustered together in a distinct group. The remaining five *T. hamiensis* samples formed a separate cluster, which was further divided into two subclusters: one consisting of populations 4, 2, and 5, and the other comprising populations 1 and 3. Pearson correlation analysis of *Tetraena* populations based on IRAP markers, conducted by using PAST software, is presented in Figure 4. A strong positive correlation was observed among populations 2, 4, and 5, as well as between populations 6 and 8. Conversely, a negative correlation was detected among populations 1, 3, and 7, indicating potential genetic divergence among these groups.

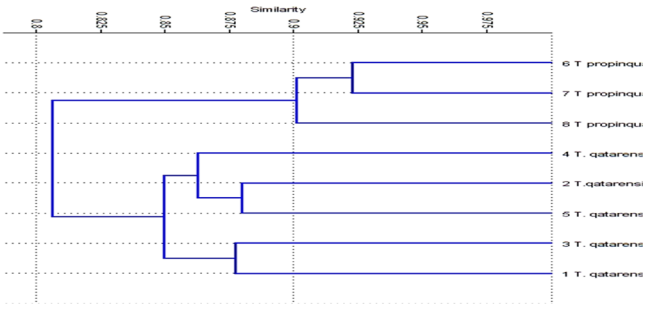

Figure 3. A distance unweighted pair group with arithmetic average tree illustrating the relationships among the eight populations (five *T. qatarensis* and three *T. propinqua*) based on the IRAP analysis.

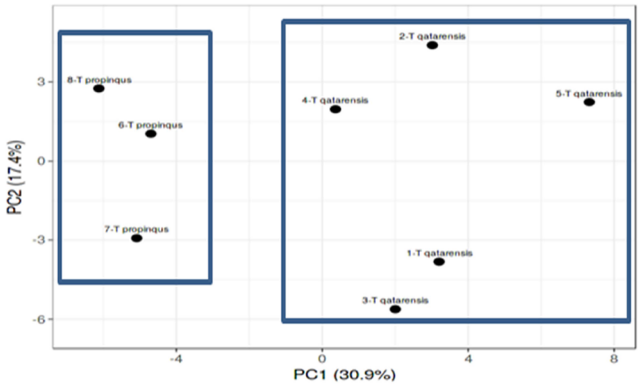

Figure 4. Pearson correlation analysis (PCA) between five *T. hamiensis*; 1−5 and three *T. propinqua*; 6−8 populations. Unit variance scaling is applied to rows; SVD with imputation is used to calculate the principal components. *X* and *Y* axis show principal component 1 and principal component 2 that explain 30.9 and 17.4% of the total variance, respectively. *N* = 8 data points.

2.4. Phylogenetic Relationships. The phylogenetic analysis of trnH and ycf5 gene sequences provided valuable insights into the evolutionary relationships and genetic diversity within the *Tetraena* genus. The ycf5 and trnH-psbA gene regions were successfully amplified using specific primers. The ycf5 region was amplified by using the forward and reverse primers, producing a 475 bp fragment. Similarly, the trnH-psbA region was amplified with the forward and reverse primers, yielding a 462 bp fragment (Table 4). These results confirm the successful amplification of the targeted regions, which is essential for assessing genetic diversity and phylogenetic relationships among *Tetraena* species. The accession numbers for the *trnH* gene of *T. hamiensis* are as follows: 1−5 [PP505823], [PP505824], [PP505825], [PP505826], and [PP505827], while the three *T. propinqua* samples (6−8) are assigned [PP505828], [PP505829], and [PP505830]. Similarly, the accession numbers for the *ycf5*

Table 4. Primer Names and Sequences of Ycf5 and TrnH Barcoding Genes and Their Product Size in bps

no.	P. name	sequence	product size (bp)
1	*ycf5 F*	GGATTATTAGTCACTCGTTGG	
2	*ycf5 R*	CCCAATACCATCATACTTAC	475
3	*trnH-psbA F*	GTTATGCATGAACGTAATGCTC	
4	*trnH-psbA R*	CGCGCATGGTGGATTCACAATCC	462

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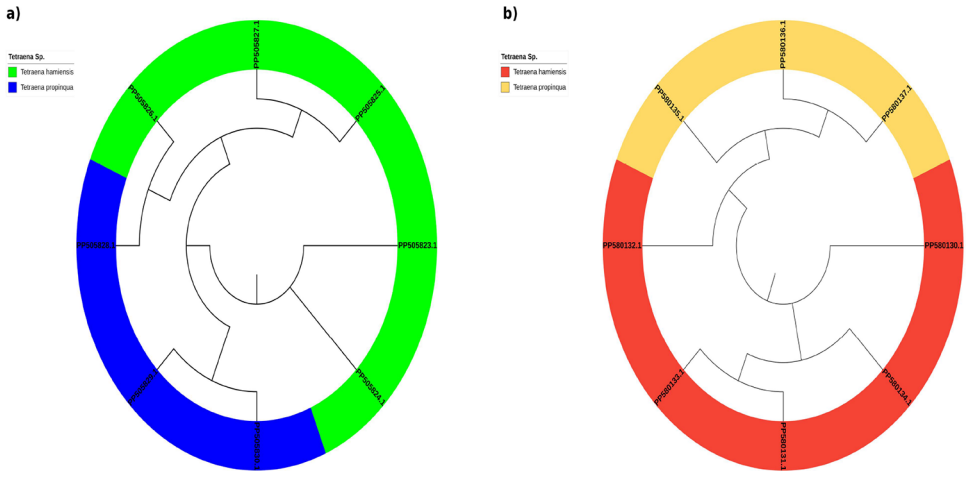

Figure 5. Phylogenetic tree generated using the *raxmlHPC* algorithm from (a) TrnH sequences and (b) Ycf5 sequences of *Tetraena* species.

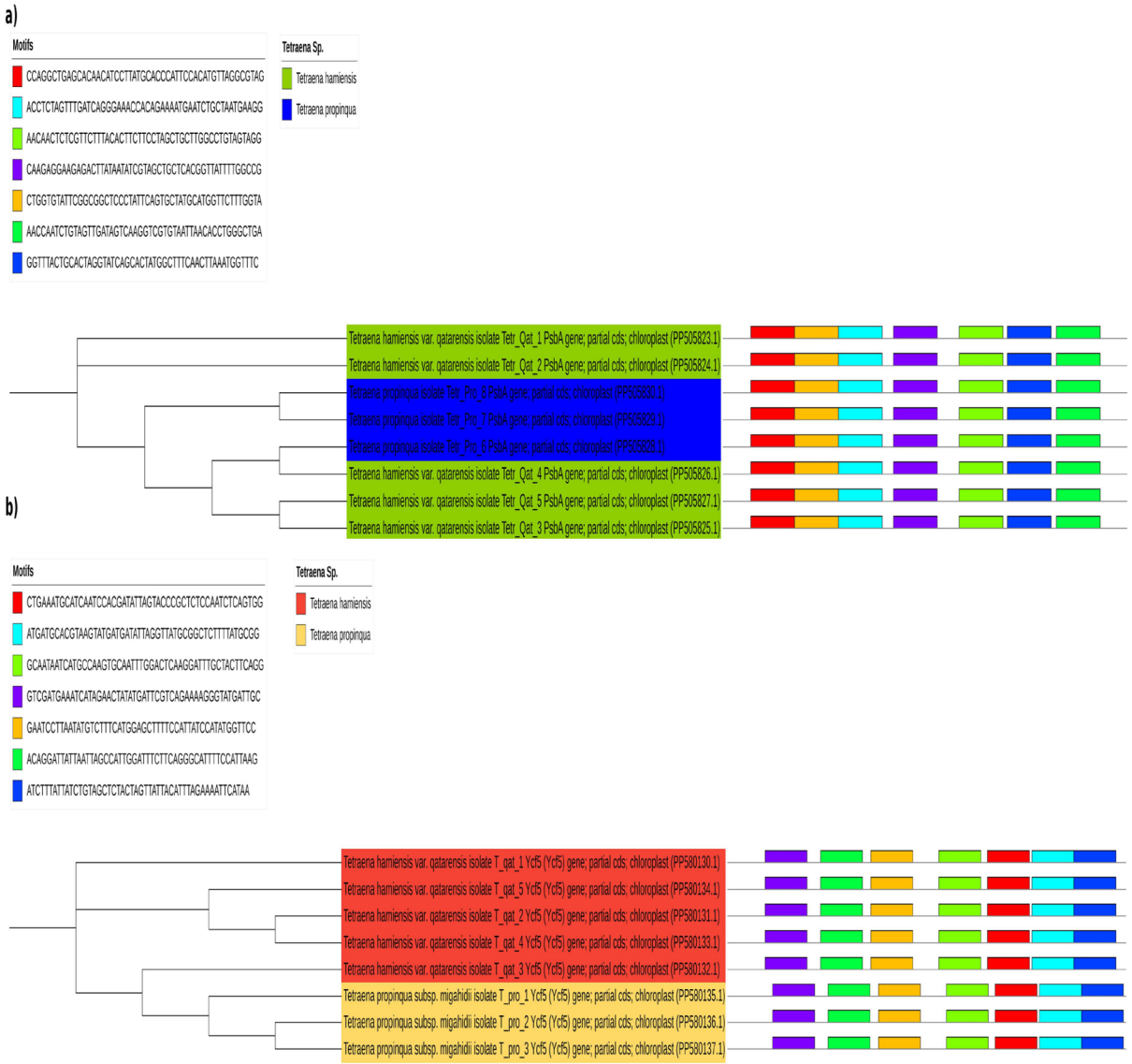

Figure 6. Phylogenetic tree generated using the *raxmlHPC* algorithm and Conserved Motifs analyses using the MEME suit algorithm from (a) TrnH sequences and (b) Ycf5 sequences *Tetraena* species.

gene of *T. hamiensis* (1−5) are [PP580130], [PP580131], [PP580132], [PP580133], and [PP580134], whereas those for *T. propinqua* (6−8) are [PP580135], [PP580136], and [PP580137]. The phylogenetic analysis based on the *trnH*

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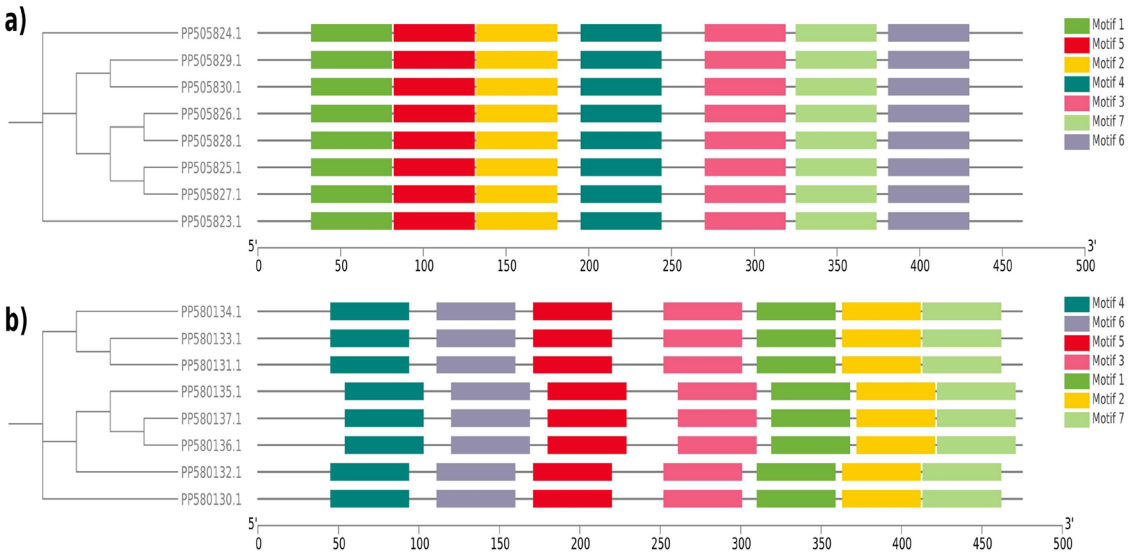

Figure 7. Conserved motif analyses using the MEME suite algorithm from (a) TrnH sequences and (b) Ycf5 sequences *Tetraena* species.

gene tree (Figures 5a and 6a) effectively differentiates between *T. hamiensis* and *T. propinqua*, a pattern that is similarly reflected in the *ycf5* gene tree (Figures 5b and 6b).

The *trnH* gene tree (Figures 5a and 6a) illustrates the differentiation of the eight *Tetraena* genotypes into one primary group and two distinct branches. In Group I, *T. propinqua* genotypes 7 and 8, with accessions [PP505829] and [PP505830], form a distinct cluster. Additionally, *T. hamiensis* genotypes 5 and 3, with accessions [PP505827] and [PP505825], are grouped together. Another cluster within the primary group consists of *T. hamiensis* genotype 4 and *T. propinqua* genotype 6, with accessions [PP505826] and [PP505828]. The two separate branches contain *T. hamiensis* genotypes 1 and 2, with accessions [PP505823] and [PP505824], indicating greater genetic divergence from the main group.

The *ycf5* gene tree (Figures 5b and 6b) is structured into two main groups with one isolated branch represented by *T. hamiensis* genotype 1 (accession [PP580130]). In Group I, *T. propinqua* genotypes 7 and 8, with accessions [PP580136] and [PP580137], form a distinct cluster, while *T. propinqua* genotype 6 (accession [PP580135]) appears as a separate branch. Additionally, *T. hamiensis* genotype 3 (accession no. [PP580132]) is positioned as an isolated branch. In Group II, *T. hamiensis* genotypes 2 and 4 (accessions [PP580131] and [PP580133]) form a cluster, whereas *T. hamiensis* genotype 5 (accession [PP580134]) is observed as an isolated branch.

This consistent clustering supports the taxonomic separation of *T. hamiensis* and *T. propinqua*, reinforcing their genetic distinction. The observed genetic variation within each species highlights the complex evolutionary processes driving the diversification of *Tetraena*. The integration of sequence data, phylogenetic analysis, and taxonomic inference provides valuable insights into the evolutionary dynamics and species boundaries within the genus. These findings establish a strong foundation for future research on *Tetraena*, particularly in understanding its genetic diversity, evolutionary history, and adaptation to diverse environmental conditions.

2.5. Conserved Motif Analysis. Conserved motif analysis of the *trnH* and *ycf5* genes identified seven distinct motifs, as illustrated in Figures 6a,b and 7a,b. These motifs shared a common region, spanning approximately 33−431 bp for *trnH* and 46−463 bp sfor *ycf5* (Figure 7a,b). The presence of these conserved motifs suggests functional significance in the genetic stability and evolutionary conservation of these regions across *Tetraena* species (refer to the supplementary MEME files).

These findings strongly support the potential use of the isolated *trnH* and *ycf5* genes as reliable genetic biomarkers. The presence of shared conserved motifs underscores the robustness and significance of these genetic markers, enhancing their applicability in molecular studies. Furthermore, these results contribute to a broader understanding of genetic relationships within *Tetraena* species, providing valuable insights into their evolutionary dynamics and taxonomic classification.

3. DISCUSSION

The genetic variation observed among *Tetraena* species in Saudi Arabia appears to be closely linked to differences in soil pH and electrical conductivity (EC), which are key environmental factors influencing plant adaptation in arid habitats. The sampled populations exhibited slightly alkaline soils, with pH values ranging from 7.8 to 8.49 across various locations, such as the Riyadh-Dammam and Riyadh-Al Kharj roads, where *T. hamiensis* (population 1) and *T. propinqua* (population 6) were found. Similar findings have been reported in other desert ecosystems, where slightly alkaline soils influence plant growth and distribution.[49]

Soil salinity, as indicated by EC values, varied significantly among populations, ranging from 822 μS/cm in *T. propinqua* (population 8) along Al Thumama road to 23,800 μS/cm in *T. hamiensis* (population 2) from the Al Jawhara-Dammam road. The strong correlation between EC levels and genetic clustering suggests that salinity plays a major role in shaping genetic differentiation. Studies have shown that salinity stress drives genetic variation in halophytic and xerophytic species, influencing their physiological and molecular responses to extreme environments.[50]

For instance, the distinct salinity conditions of *T. hamiensis* (population 2) and isolated *T. hamiensis* (population 3) were associated with their genetic divergence, indicating that high

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salinity stress may drive genetic variation. This is consistent with research on other halophytes, where extreme salinity levels contribute to significant genetic differentiation due to strong selective pressures.[51]

Conversely, populations experiencing similar salinity conditions, such as *T. hamiensis* (populations 4 and 5) and the genetically clustered group of *T. hamiensis* (population 1) with *T. propinqua* (populations 6, 7, and 8), exhibited greater genetic similarity. Previous studies suggest that stable salinity conditions promote genetic homogeneity within plant populations by maintaining consistent selection pressures.[52] These findings reinforce the role of soil salinity as a key driver of genetic diversity in desert plant populations, emphasizing its significance in shaping adaptive genetic variation in saline environments.[53,54]

In this study, IRAP markers were utilized to assess the genetic diversity of *Tetraena* species, demonstrating their effectiveness in detecting genetic variation. A total of 134 amplicons were generated with a polymorphism rate of 60.5% across 10 IRAP primers, highlighting significant genetic differentiation among the studied populations. The variation in the number of bands per primer, ranging from 7 (IRAP-2202) to 17 (IRAP-4351), underscores the differential capacity of each primer to reveal genetic differences. The average of 8.2 polymorphic amplicons per primer further supports the utility of IRAP markers in capturing intraspecies diversity, making them a valuable tool for genetic studies. These findings align with previous studies that emphasize the efficiency of IRAP markers in assessing plant genetic diversity and differentiation.[36]

The results suggest that IRAP markers provide a reliable approach for evaluating adaptive traits and species conservation in *Tetraena*. This is particularly relevant for assessing genetic variability in arid environments where plants must adapt to extreme conditions. The high polymorphic information content (PIC) values observed, particularly 0.374 for IRAP-2204 and IRAP-4351, indicate that these markers may be highly informative for future genetic diversity assessments. Studies have shown that higher PIC values correspond to greater discriminatory power in genetic studies, making IRAP markers valuable for phylogenetic and population genetic analyses.[55]

Furthermore, this study highlights the practical applications of fingerprinting techniques in biodiversity management, breeding programs, and conservation strategies, particularly for species thriving in extreme environments. Similar findings have been reported by Guan et al.,[56] who utilized start codon-targeted (SCoT) and IRAP markers to assess genetic diversity and relationships among *Diospyros* accessions from different regions in China. Their study further supports the use of IRAP markers as an effective tool for evaluating genetic diversity and phylogenetic relationships in plant species. The application of such molecular techniques contributes significantly to conservation efforts, genetic resource management, and understanding the evolutionary history of plant species, particularly those in harsh environments.[10,57]

The genetic diversity analysis of *Tetraena* species using IRAP markers revealed a moderate level of polymorphism (P % = 61.19%), indicating significant genetic variation. Nei's gene diversity (H = 0.3597) and Shannon's Information Index (I = 0.3672) further support this diversity, suggesting a well-distributed genetic structure. The mean PIC value (0.359) indicates that the markers used were moderately informative, with IRAP-2175, IRAP-2204, and IRAP-4351 being the most effective. Similar levels of genetic diversity have been reported in desert plant species, where adaptation to arid conditions drives genetic differentiation.[58] Studies indicate that genetic variation in desert plants is influenced by habitat fragmentation, ecological pressures, and reproductive strategies.[59] These findings highlight the genetic adaptability of Tetraena species to arid environments and emphasize the need for conservation efforts to maintain their genetic variability.

The study found high genetic similarity (92%) among *Tetraena propinqua* genotypes 6, 7, and 8, suggesting a close evolutionary relationship, while greater divergence (77%) was observed between *T. hamiensis* and *T. propinqua* genotypes 5, 7, and 8, likely due to adaptation to different ecological conditions. These findings highlight the role of genetic differentiation in species adaptation and conservation.[10] Similar patterns have been reported in *Diospyros*,[56] *Zygophyllum*,[23] and other desert plants, where molecular markers such as IRAP, ISSR, and SCoT have proven effective in assessing genetic diversity and adaptation mechanisms.[23,60] This research underscores the importance of genetic studies in conservation and evolutionary biology.[36,37,60]

The phylogenetic analysis of *trnH* and *ycf5* gene sequences provides crucial insights into the evolutionary relationships and genetic diversity of the *Tetraena* genus, aiding in the identification of unique lineages and genetic divergence among the species. This knowledge is essential for directing conservation efforts to preserve biodiversity. The *trnH* gene tree revealed that the eight *Tetraena* genotypes form two distinct branches and one main group, indicating varying degrees of genetic similarity. *T. hamiensis* (5, 3) and *T. propinqua* (7, 8) clustered together within the primary group, whereas *T. hamiensis* (4) and *T. propinqua* (6) formed a separate subgroup, suggesting a close genetic affinity. The two distinct branches, including *T. hamiensis* (1, 2), indicate significant genetic divergence, likely due to habitat-specific adaptations or unique evolutionary pressures.

Similarly, the *ycf5* gene tree divided *Tetraena* genotypes into two major groups and one distinct branch, further supporting genetic divergence patterns. *T. propinqua* (7, 8) clustered within Group I, while *T. propinqua* (6) and *T. hamiensis* (3) formed separate branches. Group II contained *T. hamiensis* (4, 2), while *T. hamiensis* (1) appeared as an isolated branch, reinforcing its distinct genetic lineage. These findings underscore the genetic diversity within *Tetraena* and provide valuable insights into the evolutionary history and conservation strategies of these species.

This study represents the first attempt to investigate the roles of the *trnH* and *ycf5* genes within the *Tetraena* genus. Both genes have previously been identified as effective markers for assessing genetic diversity in plants. For instance, Loera-Sánchez et al.[61] evaluated three plant DNA barcodes (*rbcLa*, *matK*, and *trnH-psbA*) to discriminate 16 key grass and legume species in temperate subalpine grasslands. Their results demonstrated that *trnH-psbA* achieved a 100% correct assignment rate (CAR) for the five analyzed legumes, confirming its reliability in plant identification. Similarly, a study on three *Allium* species using five barcode regions (*ndhJ*, *rpoC1*, *rpoB*, *ycf5*, and *rbcL*) found that the *ycf5* region had the highest species resolution success rate (SRSP) at 93.33%.[62] These studies support the selection of *trnH* and

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ycf5 as suitable barcoding markers for assessing the genetic diversity in *Tetraena* species.

The phylogenetic relationships observed in this study confirm the potential of *trnH* and *ycf5* genes as reliable barcoding markers for the *Tetraena* species. These findings are consistent with previous studies that have employed multiple DNA barcode regions to assess genetic relationships among plant species. For instance, Hani et al.[63] utilized *rbcL*, *matK*, *ycf5*, *psbA-trnH*, and *ITS* markers for DNA barcoding of six date palm (*Phoenix dactylifera*) cultivars—Hayani, Sakkoty, Gondila, Bartamoda, Malkaby, and Amhaat—demonstrating the effectiveness of these markers in distinguishing genotypes through phylogenetic analysis.

Hani et al.[63] performed sequence data analysis from *rbcL*, *matK*, *ycf5*, *psbA-trnH*, and *ITS* genes, uploading them to the NCBI GenBank and using BLASTn for sequence comparison. Multiple sequence alignment was conducted with CLUSTAL Omega, and phylogenetic analyses—including pairwise distance, transitional/transversional substitutions, and the maximum likelihood (ML) substitution matrix—were executed using MEGA 6.2 software. The study employed the ML, neighbor-joining (NJ), and unweighted pair group method with arithmetic mean (UPGMA) clustering methods to construct phylogenetic trees. Their results identified four distinct clusters: (1) Sakkoty, (2) Hayani and Malkaby, (3) Gondila, and (4) Bartamoda and Amhaat, highlighting genetic differentiation among the cultivars.

Similarly, Bhatt and Thaker[64] conducted phylogenetic analysis in *Poaceae* species and *Calligounum* species,[60] selecting *matK* and *rpoC1* as DNA barcode regions to assess species relationships. Their emphasis on conserved DNA motifs aligns with the present study's approach in using *trnH* and *ycf5* for genetic differentiation in *Tetraena* species. These studies collectively support the use of chloroplast gene markers for evaluating genetic diversity and evolutionary relationships, reinforcing the significance of *trnH* and *ycf5* as effective molecular barcodes in *Tetraena*.

4. MATERIALS AND METHODS

4.1. Collection of Plant Samples. *Tetraena hamiensis* var. *qatarensis* and *Tetraena propinqua* subsp. *migahidii* samples were collected from various habitats across Saudi Arabia (Table 1). *Tetraena hamiensis* var. *qatarensis* specimens were gathered from multiple locations in Dammam, including Al Jawhara (site 2) and Ash Sharqiyah (sites 1 and 3), as well as Al Jubayl Industrial City (sites 4 and 5). Meanwhile, *Tetraena propinqua* subsp. *migahidii* samples were obtained from sites 6, 7, and 8 in Riyadh. The collected plant specimens were identified and authenticated at the Department of Botany and Microbiology, King Saud University Herbarium (KSU) in Saudi Arabia, where voucher specimens were deposited under accession numbers for *Tetraena hamiensis* var. *qatarensis* and *Tetraena propinqua* subsp. *migahidii*.

4.2. Chemical Analysis of Soil. Chemical analysis was conducted on soil samples collected from the habitats of eight *Tetraena* species across different regions of Saudi Arabia. Soil pH was measured using a calibrated pH meter following standard protocols for soil material testing. EC was determined using an EC meter to assess salinity levels.[65] To ensure accuracy and reliability, all measurements were performed at a controlled room temperature, and triplicate readings were recorded for each sample.

4.3. DNA Extraction. Genomic DNA was extracted from fresh, young leaves of *Tetraena* species using the DNeasy Plant Mini Kit (QIAGEN, Santa Clarita, CA, USA), following the manufacturer's protocol. The concentration and purity of the extracted DNA were assessed by using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Germany).

4.4. Amplification of IRAP Markers. The IRAP assay was performed using ten primers for screening, following the protocol described in.[66] IRAP PCR amplifications were conducted in a total reaction volume of 20 μL, consisting of 6 μL of dH_2O, 2 μL of DNA template (10 ng/μL), 2 μL of primer (10 pmol/μL) (Table 2), and 10 μL of 2× Master Mix (One PCR; GeneDireX, Inc., Taipei, Taiwan).

The amplification was carried out using a PerkinElmer/GeneAmp PCR System 9700 (PE Applied Biosystems, Waltham, MA, USA) with the following program: an initial denaturation at 94 °C for 3 min, followed by 35 cycles for IRAP and 40 cycles for IRAP. Each cycle consisted of denaturation at 94 °C for 40 s, annealing at 50 °C for 50 s, and elongation at 72 °C for 60 s. The final extension was performed at 72 °C for 7 min. The amplification products were separated by electrophoresis on a 1.5% agarose gel containing ethidium bromide (0.5 μg/mL) in 1X TBE buffer at 95 V. The PCR products were visualized under UV light and documented by using a Gel Documentation System (BIO-RAD 2000).

4.5. Data Analysis. The DNA banding patterns obtained from the IRAP-PCR reactions were scored as present (1) or absent (0) using the Gel Analyzer 3 application. The percentage of polymorphism for each primer was calculated by dividing the number of polymorphic bands by the total number of bands scored. Each population consisted of three samples, and a binary statistical matrix was constructed based on the banding patterns. Dice's similarity matrix coefficients were computed between genotypes using the unweighted pair group method with arithmetic averages (UPGMA). A phylogenetic tree (dendrogram) was generated based on the Euclidean similarity index using PAST3 software (Version 1.91).[67] The polymorphic information content (PIC) value for each primer was calculated following the standard formula https://irscope.shinyapps.io/iMEC/.[68]

Genetic diversity can be assessed using several parameters, including:

1. Percentage of Polymorphic Bands (*P*%): $P\% = (\sum PB/\sum TNB) \times 100$. Where PB = Number of polymorphic bands per primer, TNB = Total number of bands per primer.
2. Polymorphic Information Content (PIC): $PIC = 1 - \sum pi^2$. Where *pi* = Frequency of each allele at a given locus.
3. Shannon's Information Index (*I*): $I = -\sum(pi \ln pi)$. Where *pi* is the frequency of each allele.
4. Nei's Gene Diversity (*H*): $H = 1 - \sum pi^2$. Where *pi* is the allele frequency.

4.6. DNA Barcoding Analysis (trnH and ycf5 Genes). The trnH and ycf5 regions were subjected to DNA barcoding analysis.[60] PCR amplification was performed using a reaction mixture containing 1× buffer (Promega), 15 mM $MgCl_2$, 0.2 mM dNTPs, 20 pmol of each primer (Table 4), 1 unit of Taq DNA polymerase (GoTaq, Promega), 40 ng of genomic DNA, and ultrapure water, in a final reaction volume of 50 μL. Amplification was carried out using a PerkinElmer/

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GeneAmp PCR System 9700 (PE Applied Biosystems) under the following thermal cycling conditions: initial denaturation at 94 °C for 5 min, followed by 40 amplification cycles consisting of denaturation at 94 °C for 10 s, annealing at 47 °C for trnH and 50 °C for ycf5 for 30 s, and extension at 72 °C for 1 min, with a final extension at 72 °C for 7 min. The amplified PCR products were separated through electrophoresis on a 1.5% agarose gel containing 0.5 μg/mL ethidium bromide in 1X TBE buffer at 95 V. A 100 bp DNA ladder was used as a size standard. The bands were visualized under UV light and documented using a Gel Documentation System (BIO-RAD 2000). The amplified products were subsequently purified using the EZ-10 Spin Column PCR Purification Kit, following the manufacturer's instructions.

4.7. TrnH and Ycf5 Sequencing. PCR products were sequenced using an ABI PRISM 3730XL Analyzer and Big Dye Terminator Cycle Sequencing Kits, following the manufacturer's instructions.[60] Single-pass sequencing was performed for each template using forward primers trnH and ycf5. Fluorescently labeled fragments were separated from unincorporated terminators by ethanol precipitation. After purification, the samples were resuspended in distilled water and subjected to capillary electrophoresis on an ABI 3730XL sequencer at The American University in Cairo, Egypt, School of Sciences and Engineering.

4.8. Phylogenetic Relationships. The MAFFT algorithm[69] was used to align *Tetraena* species' trnH and ycf5 gene sequences to infer evolutionary relationships and construct a phylogenetic tree. To infer evolutionary relationships and construct a phylogenetic tree from the aligned sequences, we employed the raxmlHPC algorithm using the maximum likelihood method.[70] Renowned for its accuracy and reliability in phylogenetic analysis, raxmlHPC was chosen to elucidate the evolutionary context of our sequences. The resulting phylogenetic tree file was subsequently uploaded and visualized using the Interactive Tree of Life (iTOL) software platform (Bailey et al., 2015), allowing for a clear and scientifically robust representation of the evolutionary relationships among the sequences.[71]

4.9. Conserved Motif Analysis. To identify conserved motifs shared between our isolated genes and those documented in NCBI-supported genes, we utilized the MEME Suite algorithm.[71] This analysis encompassed the trnH and ycf5 genes from all *Tetraena* species as well as our isolated genes. The parameters were set to detect a maximum of five motifs. The conserved motifs were then visualized using TBtools software.[72] These results were further integrated with phylogenetic tree analysis to provide a comprehensive understanding of motif conservation across diverse evolutionary lineages.

5. CONCLUSIONS

Both ambient salinity and species-specific genetic adaptations play crucial roles in shaping the genetic diversity and clustering patterns among populations of *Tetraena hamiensis* and *Tetraena propinqua*. The strong genetic cohesion observed among populations with similar salinity levels suggests that soil salinity is a key factor influencing genetic structuring. This pattern is particularly evident in *T. propinqua*, where populations 6, 7, and 8 exhibit strong genetic similarities and cluster closely across multiple analyses. Conversely, the IRAP, *trnH*, and *ycf5* gene trees reveal distinct subclusters and isolated branches within *T. hamiensis*, indicating a higher degree of genetic differentiation. Notably, the *ycf5* gene tree distinguishes two primary groups with several isolated branches, suggesting unique evolutionary trajectories within *T. hamiensis*. This divergence underscores the significance of both genetic variation and environmental pressures potentially arising from localized adaptations or historical population separations. Overall, these findings highlight the interplay between genetic lineage and environmental constraints in shaping the evolutionary dynamics of *T. hamiensis* and *T. propinqua*. The results provide valuable insights into the adaptive responses of these populations to ecological stresses, reinforcing the importance of both intrinsic genetic factors and external environmental conditions in determining genetic diversity and species differentiation.

■ ASSOCIATED CONTENT

Data Availability Statement

All data generated or analyzed during this study are included in this published article. The sequence of five isolates were submitted to NCBI. The accession numbers for trnH gene of *T. hamiensis* var. *qatarensis* are as follows 1- 5: [PP505823], [PP505824], [PP505825], [PP505826], [PP505827], and three *T. propinqua* subsp. *Migahidii* from 6 to 8: [PP505828], [PP505829], [PP505830], and ycf5 gene of *T. hamiensis* var. *qatarensis* are as follows 1- 5: [PP580130], [PP580131], [PP580132], [PP580133], [PP580134], and three *T. propinqua* subsp. *Migahidii* from 6 to 8: [PP580135], [PP580136], [PP580137].

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Author Contributions

Conceptualization, methodology, software, validation, M.A.-E. and S.A.; formal analysis, investigation, resources, data curation, S.A.; writing—original draft preparation, writing—review and editing, visualization, M.A.-E.; supervision, S.A.; project administration, M.A.-E.; funding acquisition, S.A. All authors have read and agreed to the published version of the manuscript.

Notes

The authors declare no competing financial interest.

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