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ITS-Based technique characterization of Goosefeet weed (*Chenopodiastrum* spp) Across Different Regions in Al-Anbar Province, Iraq

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Abstract

The biodiversity of the Goosefoot weed plant (*Chenopodiastrum spp*) was investigated across thirteen ecologically diverse regions in western Iraq during the growing season of 2024. These regions cover diverse environments, such as agricultural lands, riverbanks, and arid zones. The Internal Transcribed Spacer (ITS) marker was employed in this study to investigate the characterization of this plant at the molecular level. The Internal Transcribed Spacer Ribosomal DNA (ITS rDNA) region sequencing and phylogenetic relationships revealed three major species, namely *Chenopodiastrum murale*, *Chenopodiastrum hybridum*, and *Chenopodium album*. Phylogenetic analysis divided the collected samples from the thirteen regions of Al Anbar governorate into two major clusters. The first cluster consolidated the arid regions, while the second cluster consolidated the agricultural and riverbank lands. Species such as *Chenopodiastrum murale* reflected halophytic adaptation as it grouped in the arid zones such as Hit and Qa'im, while *Chenopodium album* was dominant in the agricultural zones. Meanwhile, *Chenopodium hybridum* reflected high ecological plasticity in diverse environments. The genetic diversity was consistent with the effect of the environments on the structure of the goosefoot weed population, where minimum divergence was observed in the river-connected areas in comparison with isolated arid regions. The heterogeneity of goosefoot populations was shaped by the divergence of environments, which in turn suggests that the characterised species could act as reservoirs of stress-tolerant traits. In addition, findings also highlight the importance of ITS barcoding in the study of plant diversity. Further investigations regarding integrating geographical, climatic and phenological studies are required to better elucidate the biodiversity of this weed.

Keywords: Goosefoot, ITS barcoding, Biodiversity, phylogenetic analysis, *Chenopodiastrum* spp.

1. Introduction

Fundamentally, biodiversity is the most crucial pillar in protecting ecosystems. It contributes to maintaining stability and health, and a high level of biodiversity leads to high resilience in ecosystems against climate change. Biodiversity must also preserve natural resources, which are essential not only for the ecosystem but also to maintain a high level of sustainability. Although weed plants are considered a nuisance source for farmers and growers, they are important to nature and biodiversity. In addition, weed plants are beneficial in protecting the soil from erosion and can be used in grazing (Mousa et al., 2024). However, some weeds can be used in human nutrition. Weed plants contain various active compounds that might be utilized in pharmaceutical industries; this is further illustrated by studies on native Jordanian species such as *Chiliadenus montanus*, which exhibits notably high levels of Terpinen-4-ol and potent antibacterial activity, highlighting the pharmacological promise of wild flora in arid environments (Reham, 2024). Additionally, most weed plants are ancestors of domesticated plants. Therefore, maintaining them is essential as a gene bank for the domesticated plants. Hence, understanding their biodiversity leads to a better understanding of the growth and development of their counterparts, domesticated plants, which aligns with broader conservation strategies, such as cryopreservation, which have proven effective for preserving rare plant species and their genetic material (Saifan et al., 2024). Among these species is goosefoot weed (*Chenopodiastrum* spp.). Based on the Angiosperm Phylogeny Group-APG-III, it belongs to the Amaranthaceae family within the Chenopodioideae subfamily (Bremer et al., 2009). This family is one of the most widespread families around the globe (Raven & Wu, 2003) and contains numerous flowering plant species, some of which are perennial and others are annual. Chenopodioideae subfamily contains around 100 genera and 1700 species (Fuentes-Bazan et al., 2012) growing in temperate and tropical regions worldwide. Also, many species belonging to this subfamily are widespread in a wide range of environments, including arid and semi-arid areas, and most of them are halophytes and can grow in

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desert regions (Fuentes-Bazan et al., 2012). Based on the aforementioned variability of the Amaranthaceae family and subfamily (Chenopodioideae), the taxonomy of species in a particular area is fundamental, primarily if DNA-based techniques are used. The use of molecular methods to evaluate the genetic diversity of crop wild relatives and landraces has revolutionized plant taxonomy, resulting in many updates to angiosperm orders and families (Al Khateeb et al., 2024; Al-Kiyyam et al., 2024; Chase et al., 2016; Müller & Borsch, 2005). The diversity of nucleotides in the organism's genome can be exploited in the characterisation and taxonomy of organisms, including plants, and this is so-called DNA barcoding. DNA barcodes are beneficial as they can employ DNA fragments, besides their availability in all organisms' cells during all growth stages (Hebert et al., 2003). These barcodes are located in the conserved regions of the genome in the cells of the organisms. Therefore, they actively explore the diversity and distinguish between progenies (Wattoo et al., 2016). Many molecular methods exist to investigate the DNA barcodes, including the Internal Transcribed Spacer (ITS) and Ribulose Bisphosphate carboxylase large chain/maturase K (rbcl/matK), etc. The Internal Transcribed Spacer (ITS) marker was chosen for this study because it is a widely used and highly reliable molecular tool for plant species identification and phylogenetic analysis. ITS regions, located within the ribosomal RNA gene cluster, are highly conserved yet exhibit sufficient variability among species, making them ideal for distinguishing closely related plant species, such as those within the Chenopodiastrum genus. Additionally, ITS barcoding is effective in assessing genetic diversity, particularly in multi-part environments like western Iraq (Al Anbar province), where the ecological and environmental factors might affect the genetic diversity of plant populations (Chen et al., 2010; Zhu et al., 2022). Based on the information mentioned above, the current study highlighted the understanding of the biodiversity of goosefoot weed in different regions of the Al Anbar province, which is located in western Iraq, by employing the ITS method and studying the phylogenetic relationships between the various areas. The outcome of the study can help the policy makers in preserving the biodiversity and agricultural development in the specified region.

2. Materials and Methods

2.1. Study Area and Plant Samples:

The study included 13 regions in Al Anbar governorate, as shown in Table 1 and the weather data during the period of the study, as shown in Table 2. These regions belong to various ecological zones, including deserts, riverbanks, and agricultural lands. Several field trips were done during 2024 from January to April to collect plant samples at the flowering stage. Samples of goosefoot weed were collected from each region by taking a small part of its leaves and placing them directly into a tube full of TRIzol, then they were immediately placed in the fridge ($4 \pm 1°C$) before further molecular analysis.

Table 1. The Geographical Coordinates, Symbols and Names of the 13 Regions of Al Anbar Governorate

No	Location		Climatic Region	Symbol	English Name	Arabic Name
	Latitude	Longitude				
1	33˚23'55" N	43˚16'39" E	Semi-Arid	T1	Ramadi-Tash	الرمادي - الطاش
2	33˚26'56" N	43˚17'57" E	Semi-Arid	T2	Ramadi-Al-Jazeerah	الرمادي-الجزيرة
3	34˚22'26" N	41˚59'11" E	Semi-Arid	T3	Anah	عنه
4	34˚28'49" N	41˚54'52" E	Semi-Arid	T4	Rawa	راوة
5	33˚38'42" N	42˚49'31" E	Semi-Arid	T5	Hit	هيت
6	33˚29'14" N	43˚22'39" E	Semi-Arid	T6	Ramadi-Albu Aath	الرمادي-البوعيثة
7	33˚27'26" N	43˚23'43" E	Semi-Arid	T7	Ramadi-Al-Hamdhiyah	الرمادي -الحامضية
8	33˚27'03" N	43˚17'00" E	Semi-Arid	T8	Ramadi-Albu Faraj	الرمادي-البوفراج
9	34˚07'51" N	42˚22'26" E	Semi-Arid	T9	Haditha	حديثة
10	33˚02'16" N	40˚17'00" E	Desert	T10	Rutba	الرطبة
11	34˚19'23" N	41˚09'37" E	Desert	T11	Qa'im	القائم
12	33˚20'50" N	43˚46'52" E	Semi-Arid	T12	Al Fallujah	الفلوجة
13	33˚25'03" N	43˚27'16" E	Semi-Arid	T13	Khalidiya	الخالدية

Table 2. Data on Weather during the period of study (Nov 2023 to May 2024) showing the monthly mean for each parameter in Al-Anbar Governorate, Iraq

Parameter	2023		2024				
	November	December	January	February	March	April	May
Temperature	17.4	11.97	10.34	10.36	14.04	22.35	25.87
Relative Humidity(%)	49.73	62.02	63.55	62.22	53.01	41.61	34.57
Precipitation (mm day^{-1})	0.52	1.22	0.22	1.09	1.05	0.46	0.28
Wind speed (m sec^{-1})	2.42	2.36	2.42	2.58	2.88	2.53	3.12
Soil Moisture in the root zone (%)	0.42	0.45	0.46	0.47	0.48	0.46	0.43

Ref: https://power.larc.nasa.gov/data-access-viewer/

2.2. Molecular study

2.2.1. DNA Extraction

The preserved samples from goosefoot were homogenized in liquid nitrogen using a mortar and pestle. Total genomic DNA was extracted with CTAB reagents following the manufacturer's protocol. Finally, the quality was assessed through 1.5% agarose gel electrophoresis in 1x TEB buffer and stained with ethidium bromide dye.

2.2.2. Identification of ITS using PCR

Primers used for amplification of the internal transcribed spacer (ITS) were provided by Macro Gene (South Korea), including: ITS1F: 5'-TCCGTAGGTGAACCTGCGG-3' and IT4R: 5'-TCCTCCGCTTATTGATATGC-3' (Wang et al., 2015).

The reaction mixture consisted of 12.5 µl ddH_2O, 2.5 µl Thermopol (10X), 2.5 µl $MgCl_2$ (25 mM), 5 µl dNTPs (10 mM), 0.5 µl each primer (10 µM), 0.5 µl Taq polymerase (5 U/µL), and 1 µL of DNA sample. The mixture was then well vortexed and subjected to the following thermal cycles: Initial denaturation at 95 °C for 5 min, followed by 30 cycles involving denaturation at 95 °C for 30 s, annealing at 56 °C for 45 s, and extension at 72 °C for 1 min. A final extension of 1 cycle was carried out at 72 °C for 7 min, after which the reaction was held at 4 °C.

The PCR product was visualized by 1.5% agarose gel (Figure 1). The product was then cleaned to remove the proteins, primers-dimers and dNTPs. DNA (ITS1 and ITS4) extracted from the gel was sequenced to determine the phylogenetic relationship between the plant samples according to Macrogene Company, Korea, using a Genetic Analyzer. DNA sequences were then subjected to the Multivariate Statistical Package (MVSP v.3.22) to construct the phylogenetic tree.

3. Results

Goosefoot (*Chenopodiastrum spp*) from thirteen locations in Al Anbar governorate was subjected to molecular analysis. Figure 1 shows that all amplified bands located between 600 and 700 bp validated the quality and integrity of DNA extracted from goosefoot weed plants collected from the above-mentioned regions. Therefore, the gel electrophoresis analysis confirms the successful ITS region amplification, and this will provide the fundamental data for the subsequent analysis, e.g. sequencing, and phylogenetic relationship.

Table 3 showed that 10 out of 13 samples of goosefoot weed plants were registered in the NCBI database. The analysis also revealed that there was considerable biodiversity among them. ITS regions of rDNA Sequences identified three species, including Chenopodium album, *Chenopodiastrum murale* and *C. hybridum* (Table 3). Table 3 results indicated a high identity percentage (97-100%) associated with a low expected value (E value), and this confirms the close genetic relationship to the reference sequence. It also appeared that *Chenopodiastrum murale* dominated regions such as Ramadi-Tash, Hit, and Qa'im, while *Chenopodium hybridum* spread in regions like Anah and Haditha. However, Ramadi-Al-Hamdhiyah and Rutba were dominated by *Chenopodium album*. This suggests the adaptability to various ecological zones.

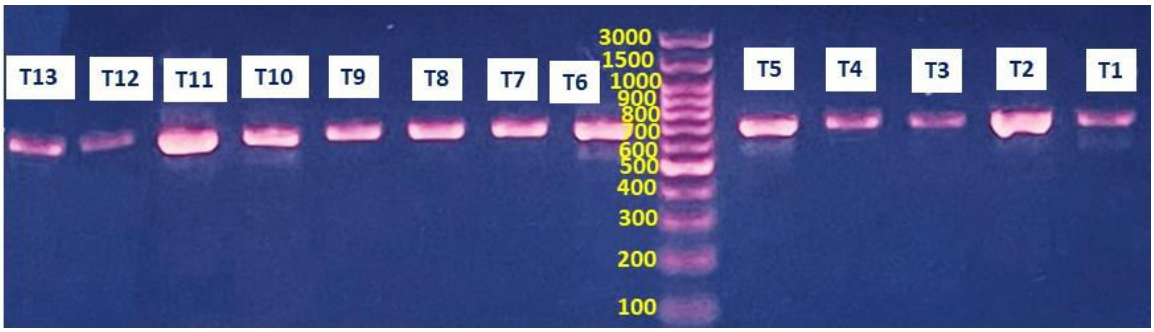

Figure 1. Amplification of ITS region of rDNA (Agarose gel 1.5%) Extracted from Goosefoot weed Collected from 13 Regions of Al Anbar Governorate. (T1-Ramadi-Tash, T2-Ramadi-Al-Jazeerah, T3-Anah, T4- Rawa, T5-Hit, T6-Ramadi-Albu Aath, T7- Ramadi-Al-Hamidiyah, T8-Ramadi-Albu Faraj, T9-Haditha, T10- Rutba, T11- Qa'im, T12- Al Fallujah, T13- Khalidiya).

Table 3. Accession number of 10 out of 13 plant samples collected from 10 regions of Al Anbar Governorate.

Locations	Accession No.	Identified Species	Identity(%)	Query Coverage (%)	E-value
Ramadi-Tash	PV418216.1	*Chenopodiastrum murale*	99.4	100	0
Anah	PV418217.1	*Chenopodiastrum hybridum*	98.9	98	3×10^{-120}
Rawa	PV418218.1	*Chenopodium album*	97.8	99	1×10^{-95}
Hit	PV418219.1	*Chenopodiastrum murale*	99.1	100	0
Ramadi-Al-Hamdhiyah	PV418220.1	*Chenopodium album*	98.5	97	2×10^{-110}
Haditha	PV418221.1	*Chenopodiastrum hybridum*	99	99	0
Rutba	PV418222.1	*Chenopodium album*	98.2	96	5×10^{-100}
Qa'im	PV418223.1	*Chenopodiastrum murale*	99.3	100	0
Al Fallujah	PV418224.1	*Chenopodium album*	98.7	98	1×10^{-105}
Khalidiya	PV418225.1	*Chenopodiastrum hybridum*	99.2	100	0

The phylogenetic analysis indicated that plant samples from different regions were ordered into two clusters. The top cluster consisted of T7, T12, T4, T3, T1, T11, T2, and T9, indicating that the goosefoot population in those regions is genetically similar because of the gene flow since they are located close to the Euphrates riverbank. The second cluster included T10, T8, T6, T5, and T13. Goosefoot weed plants from Ramadi-Al-Hamidhiyah and Fallujah scored the minimal distance (close to 0.00), indicating the same ITS sequences. Goosefoot collected from Hit and Rutba were grouped in one cluster, which is located in the desert region, and this reflects the unique genetic background.

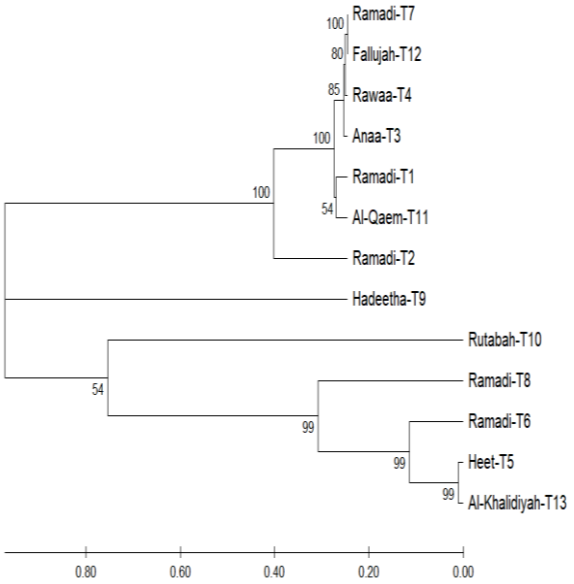

Figure 2. Phylogenetic relationship of ITS sequences among goosefoot weed from 13 regions of Al Anbar Governorate using UPGMA Method (MEGA v. 3.22)

Table 4 indicates the distances among goosefoot weed sequences from the thirteen regions of Al Anbar governorate, which were calculated using the model of Maximum Composite likelihood using MEGA12 (V. 3.22) (Kumar et al., 2024). However, 790 nucleotides were included in this analysis after deletion of the ambiguous sites. Data presented in Table 3 indicated the divergence and the relationship among goosefoot weeds from the regions mentioned above. Notably, samples from arid areas such as Rutba and Hit showed higher genetic distances (>1.50) in comparison with other areas, such as Ramadi regions, reflecting the adaptability of those plants to the prevailing environment, while more considerable distances, e.g. 1.70, suggest high divergence and less gene flow or even environmental isolation. Contrarily, between Rawa and Ramadi- Al-Hamdhiyah, minimal divergence was observed (0.01 and 0.02, respectively), indicating gene flow or ancestral lineage. Also, Hit and Khalidiya showed a notably low distance (0.02) despite the variation of the geography of those two regions. Distances among sub-regions of Ramadi (Ramadi-Tash, Ramadi-Al-Jazeerah, Ramadi-Albu Aath, and Ramadi-Albu Faraj) exhibited intermediate to high divergence (0.31-1.70), explaining that the diversity might also occur even within the same geographical zone. An identical ITS region sequence (0.00) was observed between the goosefoot plants from Fallujah and Ramadi-Al-Hamdhiya.

Table 4. Matrix of Distances among ITS region sequences collected from the 13 regions of Al Anbar Governorate using MEGA 12 (v. 3.22)

	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12
Ramadi-Tash-T1												
Ramadi-Al-Jazeerah-T2	0.31											
Anah-T3	0.06	0.31										
Rawa-T4	0.06	0.31	0.02									
Hit-T5	1.70	1.70	1.70	1.70								
Ramadi-Albu Aath-T6	1.70	1.70	1.70	1.70	0.23							
Ramadi-Al-Hamdhiyah-T7.	0.06	0.31	0.02	0.01	1.70	1.70						
Ramadi-Albu Faraj-T8	1.70	1.70	1.70	1.70	0.61	0.61	1.70					
Haditha-T9	1.45	1.45	1.45	1.45	1.70	1.70	1.45	1.70				
Rutba-T10	1.70	1.70	1.70	1.70	1.51	1.51	1.70	1.51	1.70			
Qa'im-T11	0.05	0.31	0.06	0.06	1.70	1.70	0.06	1.70	1.45	1.70		
Al Fallujah-T12	0.06	0.31	0.02	0.01	1.70	1.70	0.00	1.70	1.45	1.7	0.06	
Khalidiya-T13	1.70	1.70	1.70	1.70	0.02	0.23	1.70	0.61	1.70	1.51	1.7	1.7

4. Discussion

Goosefoot weed (*Chenopodiastrum* spp) is a widely spread weed plant, especially in Al Anbar governorate, which is characterized by various ecosystems ranging from arid and semi-arid areas to riverbanks (Alfalahi & Saleh, 2018; Dawood et al., 2020). Plant biodiversity plays a crucial role in sustaining ecosystem functions, particularly in arid and semi-arid environments. Goosefoot weed species (*Chenopodiastrum* spp.) contribute significantly to biodiversity maintenance in Al Anbar governorate by stabilizing soil and preventing erosion in desert regions. These species, belonging to the Chenopodiaceae family, are well-adapted to arid conditions and are among the most abundant plant families in desert ecosystems, highlighting

their importance in maintaining species diversity and ecosystem stability (Sun et al., 2023). In addition, they provide suitable habitats for the microorganisms and insects. Therefore, the availability of those plants improves the ecosystem's resistance to climate change. *Chenopodium murale* can easily grow in soil affected by salinity. Hence, this makes it vital to exploit degrading soils. Besides, some species of goosefoot are considered wild ancestors of the domesticated plants such as spinach and quinoa (Areces-Berazain, 2022; Ihuoma, 2021). This could make the Al Anbar regions as a gene bank for breeding important crops, particularly for traits such as drought and salt tolerance (Sharef et al., 2024; Alfalahi et al., 2022). Such genetic reservoirs are increasingly critical, especially when combined with conservation biotechnologies like cryopreservation to prevent biodiversity loss (Saifan et al., 2024). The current investigations on goosefoot weed (*Chenopodiastrum spp*) from 13 regions of Al Anbar governorate revealed important biodiversity, which was shaped by the ecology's heterogeneity (Salih et al., 2023). ITS-based molecular analysis uncovered mainly three goosefoot species, including *Chenopodiastrum murale*, *Chenopodiastrum hybridum*, and *Chenopodium album*, which originated in distinct regions of Al Anbar. The use of ITS-based molecular markers in this study aligns with previous investigations that utilized DNA markers to reveal eco-geographical genetic diversity in wild plant species *Gundelia tournifortii* in Jordan (Saifan et al., 2020). Furthermore, phylogenetic relationships and genetic distance highlight the effect of environmental elements on the genetic backgrounds. This, in turn, gives an insight into the evolution and adaptation in the diverse regions of Al Anbar. The species *Chenopodiastrum murale* was dominant in the arid regions such as Hit and Qa'im. The survival of this species in such areas provides it with halophytic traits (Flowers & Colmer, 2008). On the contrary, Chenopodium album prevailed in agricultural lands such as Ramadi-Al-Hamidiyah, indicating that this species adapted to grow in such soils, while *Chenopodiastrum hybridum* occupied regions such as Anah and Haditha, which reflects a wide range of ecological plasticity. The habit specialization within the subfamily Chenopodiodeae was consistent with the global pattern observed in the family of Amaranthaceae (Bremer et al., 2009). On the other hand, phylogenetic analysis indicated two main clusters, including the riverbank and agricultural lands group and the arid and semiarid group. Also, the minimal genetic distance within the first cluster between samples collected from Fallujah and Ramadi-Al-Hamidhiyah suggests that gene flow was facilitated by the Euphrates River, which connects the two regions, and this maintains the genetic homogeneity in the agricultural lands. In contrast, the second group showed higher divergence between Rutba and Ramadi-Tash (1.70), indicating the isolation of environments between those two regions. It also showed high adaptability to the local habitat.

It is worth mentioning that the lower genetic distance (0.02) between Hit and Khalidiya, despite the geographical variation, might reflect convergent evolution due to the similar desert environment or anthropogenic activities. The genetic diversity of the goosefoot population in arid regions such as Rutba highlights the role of these plants as a gene bank of adaptive traits, which is necessary for tolerance against climate change (Bhargava et al., 2006). While the homogeneity in agricultural lands increases the risk of genetic erosion, this urges policymakers to preserve ecosystems by promoting sustainable agriculture, which will ultimately protect biodiversity. ITS-based DNA barcoding has proven to be an effective method to highlight the genetic relationships among species belonging to the same genus in various geographical regions (Chen et al., 2010). Alternatively, the 97.8% identity found in Rawa may be a result of intraspecific variation or frequent interbreeding, which is common in species such as *Chenopodium* (Mandák et al., 2018). The biodiversity observed in the current investigations highlights the importance of maintaining those species as a gene bank for domesticated plants, particularly in regions affected by climate change.

5. Conclusions

The results of the present study underscore the influence of environmental heterogeneity in Al Anbar on the genetic diversification of goosefoot weed (*Chenopodiastrum spp*.), suggesting that such ecological variability may drive adaptive evolutionary processes across arid and agricultural habitats. In addition, findings provide the foundation for preserving genetic resources, and this is vital for sustaining biodiversity in the study area, which is characterized by insubstantial ecosystems. It is recommended to incorporate more advanced DNA barcoding approaches, such as *matK* or genome-wide SNP analysis, to further resolve biodiversity patterns (Ancona et al. 2023). Further investigations integrating geographical, climatic, and phenological data are essential to comprehensively elucidate the biodiversity and ecological adaptation of this weed.

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