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GENETIC DIVERSITY OF *ASTERACEAE* SPECIES IN DUHOK CITY, KURDISTAN REGION, IRAQ

D.Y.K. SINJARE[1*], J.J. MUHAMMED[2], and A.E. HUSSEIN[3]

[1]Department of Scientific Research Center, College of Science, University of Duhok, Kurdistan Region, Iraq
[2]Department of Forestry, College of Agricultural Engineering Sciences, University of Duhok, Kurdistan Region, Iraq
[3] Department of Science, College of Basic Education, University of Duhok, Kurdistan Region, Iraq
*Corresponding author's email: dalal.khudhur@uod.ac
Email addresses of co-authors: jotyar.muhammed@uod.ac, abdulqader.hussein@uod.ac

SUMMARY

The presented investigations aimed to elucidate the genetic diversity and phylogeny of nine species of the family Asteraceae. The research applied two types of DNA markers—the directed amplification of minisatellite DNA (DAMD) and the sequence-related amplified polymorphism (SRAP)—and the ribulose bisphosphate carboxylase (rbcL) barcode. The genetic distance and phylogenetic relationship estimations used the UPGMA (unweighted pair group method with arithmetic mean) algorithm and the Neighbor-Joining (NJ) tree clustering approaches. The total determined bands were 135 and 75, with an average of 13.5 and 9.37 for DAMD and SRAP markers, respectively. By analyzing the DAMD marker, the highest genetic distance (0.4666) was noticeable between species *Cousin* sp. and *Urospermum picroides,* while the lowest (0.785) was notable between the species *Tragopogon* sp. and *Carduus pycnocephalus*. In SRAP marker values, the lowest genetic distance (0.351) was evident in *Carthamus oxyacantha* and *Sonchus oleraceus*, while the highest (0.891) appeared between *Notobasis syriaca* and *Carduus pycnocephalus*. Based on combined data, the species *Carthamus oxyacantha* and *Onopordum carduchorum* emerged as highly diverse (0.483) compared with the species *Tragopogon* sp. and *Carduus pycnocephalus* (0.808). Four evolutionary clades were distinct, i.e., *Urospermum picroides, Lactuca serriola, Notobasis syriaca,* and *Carthamus oxyacantha*. Clade four underwent further subdivision into two subclades—*Sonchus oleraceus* and *Tragopogon* sp. with *Cousin* sp. and *Carduus pycnocephalus* and *Onopordum carduchorum*. The DAMD and SRAP markers with rbcL proved effective in analyzing genetic diversity in the family Asteraceae.

Keywords: *Asteraceae* species, DNA markers, DNA sequencing, genetic distance, genetic diversity, phylogenetic tree

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Key findings: The DAMD and SRAP markers with rbcL chloroplast sequencing occurred as considerable tools to estimate the genetic distance, as well as the evolutionary relationship among the *Asteraceae* species. The tribe of Cardueae members appeared most clustered together.

INTRODUCTION

Asteraceae has become one of the largest families of the order Asterales. This family is widespread globally from subpolar to tropical areas, including enormously economically important crops, vegetables, and ornamental plants (Simpson, 2009). The sunflower family is morphologically distinct due to the inflorescence, florets, individual flowers, and pappus, as well as the modified calyx. Accordingly, this family gained easy classification based on their distinct morphological features (Cassini, 1818; Bentham and Hooker, 1873; Xu *et al.,* 2017).

Although the family Asteraceae incurred further dividing into subfamilies and tribes, and their members and numbers were highly controversial because of the floral diversity and evolution (Jeffrey, 2009), Funk *et al.* (2009) proposed that this family has up to 1700 genera, including 24000–30000 species. In Iraq, the said family has around 123 genera, including approximately 433 species (Ghazanfar *et al.,* 2019; Rasheed *et al*., 2024).

By using DNA markers, various plant diversity and evolution attained speedy assessment, including the family Asteraceae (Yang *et al.,* 2006; Shao *et al.,* 2010). The sequence-related amplified polymorphism (SRAP) was considerably a crucial marker to amplify the open-reading frames (ORF) (Li and Quiros, 2001; Robarts and Wolfe, 2014). The *Carthamus* species identification and classification utilized the said novel marker (Peng *et al.,* 2008; Mokhtari *et al.,* 2013). Furthermore, the DAMD-PCR marker has the directed amplification of the minisatellite DNA. The DAMD DNA marker became often suggested to be a highly effective tool for evaluating the single locus in the plant genome, as well as assessing genetic diversity and evolution (Lamare and Rao, 2015; Hamdan, 2025).

The DNA barcode, the short DNA-targeted region for sequencing, is significantly used as a key to identify and discriminate various species (Hebert *et al.,* 2003; Hajibabaei *et al.,* 2007). The rbcL chloroplast gene region became regarded as a useful barcode due to sufficient variations within the genes (Newmaster *et al.,* 2006; Kress and Erickson, 2007; Dong *et al.,* 2014; Taher and Saeed, 2022).

Several studies have taken place on the family Asteraceae through the DNA barcode to the high-throughput sequencing (Olmstead *et al*., 1992; Gao *et al*., 2010; Mandel *et al.,* 2019; Pascual-Díaz *et al.,* 2021; Zhang *et al.,* 2024). However, the said family's subjections to few investigations focused mainly on interspecific variations (Abd, 2024; Ismail *et al.,* 2024), since non-considerable studies have transpired regarding the molecular markers as well as sequencing on the family Asteraceae in Iraq.

Thus, this study could be a guide to understanding the genetic diversity and species relationship within this family. Based on the above discussion, the presented study aimed to classify the genetic diversity and phylogeny of nine species of the family Asteraceae by using two types of DNA markers (DAMD and SRAP) and the rbcL (ribulose bisphosphate carboxylase) barcode.

MATERIALS AND METHODS

DNA extraction

The study used fresh leaves of nine species from *Asteraceae* (1S-*Lactuca serriola,* 2S-*Cousin* sp.*,* 3S-*Carthamus oxyacantha,* 4S-*Sonchus oleraceus*, 5S-*Tragopogon* sp., 6S-*Notobasis syriaca,* 7S-*Urospermum picroides,* 8S-*Carduus pycnocephalus,* and 9S-*Onopordum carduchorum*) to isolate the total

genomic DNA using the CTAB method, collected throughout May 2024 from two different locations (Semel and Barbeer villages, Atrosh) around Duhok City, Iraq (Weigand *et al.,* 1993).

Polymerase chain reaction (PCR)

The ABI Applied Biosystems PCR System 2720 thermal cycler was the treatment used to conduct the PCR amplification of 20 µl PCR reaction mixture. It contained 2 µl of genomic DNA (25-50 ng), 2 µl (10 pmol) for each forward and reverse oligonucleotide (primer), and 10 µl of Taq DNA Master Mix (2x conc.) (Addbio, Korea), along with 4 µl of DDs sterile water. Optimizing each set of the marker entailed separation with an appropriate program as follows: for rbcLa_F/ ATGTCACCACAAACAGAGACTAAAGC and rbcL724_R/GTAAAATCAAGTCCACCRCG gene region, 94 °C for 5 min, 40 cycles consisting of 94 °C for 30 sec (denaturation), 55 °C for half a min (annealing), 72 °C for half a min (primer extension), and final extension of 72 °C for 5 min. For amplification of the DAMD markers, 10 primers used incurred the optimized thermocycler conditions to a touch-down PCR reaction. This involved an initial 3 min hold at 94 °C, followed by 10 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 50 s, and an extension at 72 °C for 2 min. The annealing temperature sustained a reduction to 0.5 °C per cycle for the first 10 cycles, followed by up to 30 cycles at 45 °C for annealing, with a final extension at 72 °C for 10 min. Furthermore, the SRAP marker with the eight-primer combination set (Table 1) proceeded in an optimized PCR program of a 5-min hold at 94 °C, followed by 5 cycles of denaturation at 94 °C for 1 min, annealing at 35 °C for 60 s, and an extension at 72 °C for 1 min. The process continued to 35 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, extension at 72 °C for 1 min, and the final extension at 72 °C for 7 min (Joshi and Deshpande, 2010). The PCR results' visualization used a 2% agarose gel electrophoresis.

Data scoring and analysis

Each band has a marker representation. The DAMD and SRAP markers received manual scoring by the presence (1) or absence (0) of bands as a raw data matrix. The statistical analysis continued by using version 2.02 of NTSYS software (Rohlf, 1992). The discriminatory power of DAMD and SRAP markers could undergo evaluation based on the following three parameters. First, the polymorphic information content (PIC), which was calculated by using the formula PIC = $2f(1 - f)$, where f = the frequency of present bands in the developing gel, and $1-f$ = the frequency of absent bands (Roldan-Ruiz *et al.,* 2000). Second, the resolving power (Rp) was the ability of each primer to detect the level of variation among the species, calculated as $Rp = \Sigma Ib$, where Ib (band informativeness) takes the values of $1- [2|0.5-p|]$, where p is the proportion of individuals containing the band (Prevost and Wilkinson, 1999).

Phylogenetic analysis

The use of the Geneious software version 11 aided the sequence's alignment (Kearse *et al.,* 2012). Then, applying the Clustal W alignment helped extract sequences. The aligned data's transfer proceeded to the MEGA 11 software to find a robust phylogeny model (Tamura *et al.,* 2021). The phylogenetic tree reconstruction resulted in an appropriately selected evolutionary model.

Table 1. The SRAP primer combination set used in the research.

Reverse primer	Sequence	Forward primer	Sequence
EM 6	GACTGCGTACGAATT	ME 5	TGAGTCCAAACCGGTGC
EM 13	GACTGCGTACGAATTGGT	ME 6	TGAGTCCAAACCGGATG
EM 15	GACTGCGTACGAATTCTG	ME 8	TGAGTCCAAACCGGTGC
EM 16	GACTGCGTACGAATT	ME 15	TGAGTCCAAACCGGTCA
EM 17	GACTGCGTACGAATT	ME 17	TGAGTCCAAACCGGGTA

RESULTS AND DISCUSSION

The preliminary results showed 10 out of 14 tested DAMD primers were able to generate reproducible and clear polymorphic profiles for all the targeted species samples. In the case of the SRAP DNA marker evaluation, only eight out of 12 combinations exhibited successful amplification (Figure 1). Thus, the resulting data based on both markers have been used to estimate the genetic diversity in the *Asteraceae* genera (Tables 2 and 3). The analyzed DAMD and SRAP markers demonstrated the total number of scored polymorphic bands were 135 and 75, respectively. They revealed an average of 13.5 and 9.37 bands, ranging from eight bands in the URP13R to 19 bands in the URP6R, and six to 15 bands using SRAP, respectively.

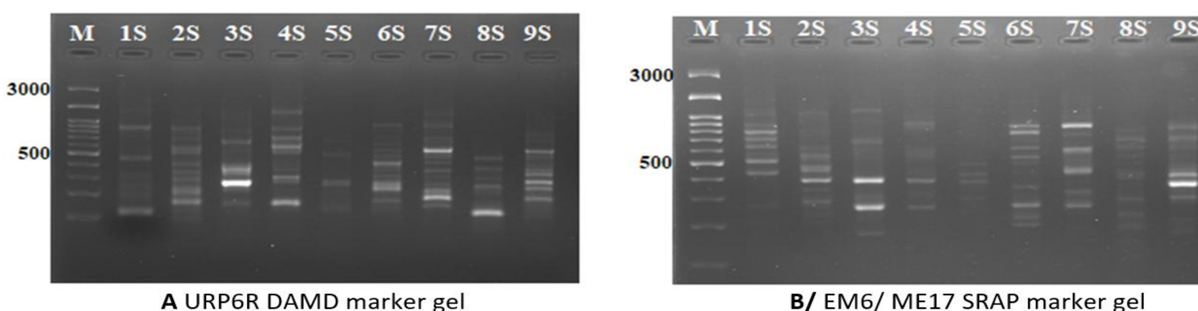

Figure 1. The result of both markers running in 2% agarose gel electrophoresis.

Table 2. Genetic diversity statistics for DAMD markers, including primer name and sequences, total number of bands, polymorphism information contents, and resolving powers in the family Asteraceae.

Primer name	Primer Sequences (5′- 3′)	Total number of bands	PIC	RP
URP2F	GTGTGCGATCAGTTGCTGGG	11	0.368	6.22
URP2R	CCCAGCAACTGATCGCACAC	15	0.365	8.22
URP4R	AGGACTCGATAACAGGCTCC	11	0.377	6.44
URP6R	GGCAAGCTGGTGGGAGGTAC	19	0.400	12.67
URP9F	ATGTGTGCGATCAGTTGCTG	15	0.286	5.77
URP13R	TACATCGCAAGTGACACAGG	8	0.358	4.44
URP17R	AATGTGGGCAAGCTGGTGGT	10	0.390	6
URP25F	GATGTGTTCTTGGAGCCTGT	13	0.429	10
URP30F	GGACAAGAAGAGGATGTGGA	17	0.403	11.11
URP32F	TACACGTCTCGATCTACAGG	16	0.364	8.88
Total		135	3.69	79.75
Means		13.5	0.369	7.97

Table 3. Genetic diversity statistics for SRAP markers loci, including primer name, total number of bands, polymorphism information contents, and resolving powers in the family Asteraceae.

Primer name	Total number of bands	PIC	RP
EM17/ ME5	9	0.263	6.22
EM15/ ME8	11	0.260	5.11
EM6/ ME17	15	0.296	5.778
EM15/ ME15	6	0.246	1.778
EM16/ ME6	7	0.218	1.778
EM13/ ME8	11	0.323	4.667
EM16/ ME6	7	0.366	3.778
EM16/ ME17	8	0.351	4.667
Total	75	2.302	33.73
Means	9.37	0.287	4.216

The PIC value entailed calculation to estimate the discriminating power of a locus by considering not only the number of expressed alleles but also the relative frequencies of those alleles. The PIC values ranged between 0.286 and 0.429, with an average of 0.369 for DAMD markers. The PIC-recorded values for the SRAP marker were 0.218 to 0.366, with an average of 0.287. The PIC values appeared dependent on the genetic diversity of the selected genotypes (Manimekalai and Nagarajan, 2006).

The primer's ability (resolving power values, RP) to differentiate the species succeeded in their determination. The values ranged from six (primer URP17R) to 12.67 (primer URP6R), with an average of 7.97. In contrast, in SRAP markers, the RP showed 1.778 to 6.22, with an average of 4.216.
According to Dice's similarity matrix and the UPGMA clustering method, the dendrogram exhibited the clusters that were notable at the 0.79 similarity level. Thus, the results indicated both markers proved effective in analyzing the genetic diversity in the family *Asteraceae* genera.

The DAMD markers' application calculated the genetic distance among the various species of the family Asteraceae. The highest distance (0.4666) was evident between the species *Cousin* sp. (2S) and *Urospermum picroides* (7S), while the lowest distance (0.785) was noteworthy between the species *Tragopogon* sp. (5S) and *Carduus pycnocephalus* (8S). Despite the species *Tragopogon* and *Carduus* belonging to different tribes, however, they emerged as closely related in this examination. One expected that the species *Tragopogon* polyploidization causes this connection, which has also been a finding in past studies (Nazarova, 1991). Likewise, the highest and lowest distances reached estimation in the SRAP marker. Accordingly, 0.351 and 0.891 were the lowest and highest genetic distances between the species *Carthamus oxyacantha* (3S) and *Sonchus oleraceus* (4S), and *Notobasis syriaca* (6S) and *Carduus pycnocephalus* (8S), respectively.

By combining the scored data of both DNA markers, the generated results explained the existence of the highest genetic distance between the species *Carthamus oxyacantha* (3S) and *Onopordum carduchorum* (9S), with an estimated value of 0.483. Meanwhile, the species *Tragopogon* sp. (5S) and *Carduus pycnocephalus* (8S) showed the lowest genetic distance (0.808) (Table 4, Figure 2). The majority of the findings had previous determination in studying the tribes Cardueae and Cichorieae of the family Asteraceae (Petit, 1997; Kamel, 2004). Reducing the genetic distance between the genera *Carduus* and *Tragopogon* seemed to be the chromosome evolution of the genus *Carduus* (Ackerfield *et al.,*2020).

The chloroplast coding gene region, rbcL's application, reconstructed the phylogenetic tree of the nine different species of the family Asteraceae. The amplified amplicon result was 600 bp in the studied taxa. Since the study selected a single coding gene, only a portion of it sustained sequencing. Thus, the analyzed data showed a polytomous tree with four clades: *Urospermum picroides* (7S) and *Lactuca serriola* (1S) as the first clade. The *Notobasis syriaca* (6S) and *Carthamus oxyacantha* (3S) are the second and third clades, respectively. The last clade attained division into two subclades: *Sonchus oleraceus* (4S), *Tragopogon* sp. (5S), and *Cousin* sp. (2S), and the subclade of *Carduus pycnocephalus* (8S) and *Onopordum carduchorum* (9S) (Figure 3). The current results closely agreed with previous phylogenetic findings (Kim *et al.,* 1992; Zahra *et al*., 2024). Mentioning all details of such a big family like Asteraceae is difficult, but from selected species used for this study, it could reveal a high genetic diversity among all samples, although its collection came relatively from the same geographical area. The presented investigations will guide us to build up a robust phylogenetic tree as well as a genetic diversity determination at lower levels of taxonomical hierarchy in the Duhok Governorate and later, in Iraq.

Table 4. Genetic similarity matrix of studied species using the combined markers.

1S	2S	3S	4S	5S	6S	7S	8S	9S
1.0000000								
0.5358852	1.0000000							
0.5119617	0.4880383	1.0000000						
0.6267943	0.5454545	0.4928230	1.0000000					
0.6315789	0.6459330	0.5933014	0.6411483	1.0000000				
0.5885167	0.6315789	0.5693780	0.6076555	0.7464115	1.0000000			
0.5980861	0.5645933	0.5502392	0.5980861	0.6602871	0.7033493	1.0000000		
0.6411483	0.6842105	0.5550239	0.6411483	0.8086124	0.7464115	0.6698565	1.0000000	
0.6076555	0.5837321	0.4832536	0.5885167	0.6411483	0.6076555	0.5789474	0.6315789	1.0000000

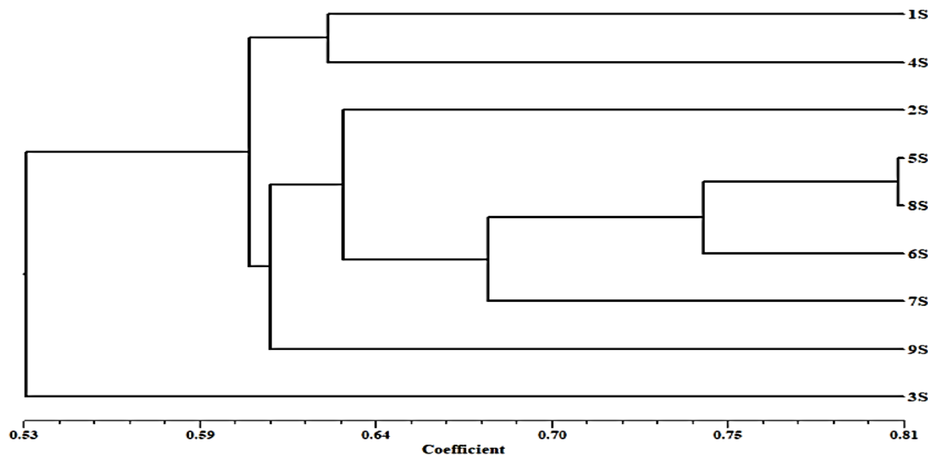

Figure 2. The illustrated dendrogram of the combined markers.

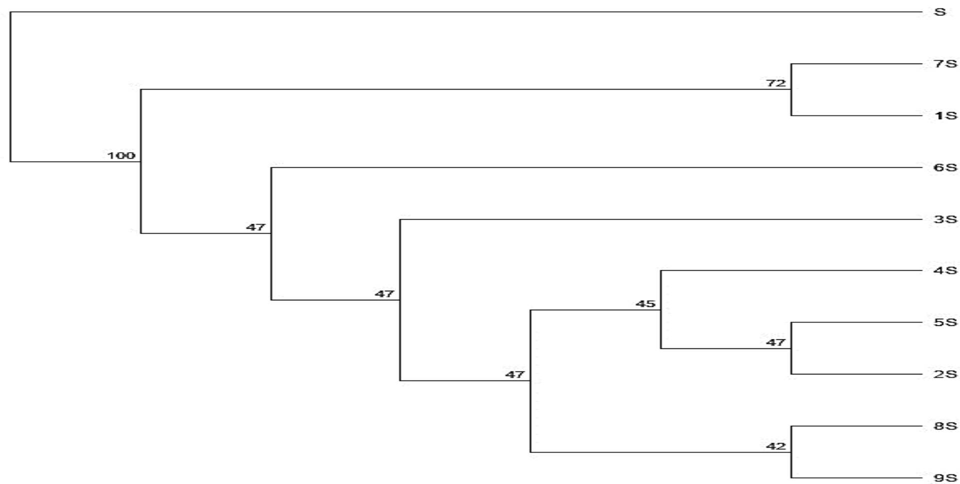

Figure 3. Neighbor-Joining tree of nine species of the family Asteraceae using the *rbcL* chloroplast gene. The value above the nodes is a bootstrap if it has > 40% support. S represents the outgroup *Famatinanthus decussatus*.

CONCLUSIONS

By studying the genetic diversity and phylogenetic relationship of the nine species of the family Asteraceae, the two DNA markers (DAMD and SRAP) achieved combining with a single chloroplast gene region (rbcL). Subsequently, both markers revealed the estimation of the genetic distances was appropriate. Moreover, the DNA barcode supports the findings by exhibiting the ability of a phylogenetic reconstruction within the family.

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