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The new molecular classification for the *Amaranthus albus* plant in Iraq

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ABSTRACT

The molecular genetic index of *Amaranthus albus* species was investigated using the technology of random amplification regarding the polymorphic DNA (RAPD, where DNA fragments are included and multiplied by means of using the polymerase chain reaction (PCR). The National Centre for Biotechnology Information Gene Bank Graphics (NCBI) was utilized to determine the nitrogenous bases related to the two species in question via PCT using the primers ITs1 and ITs4. Results demonstrated that the nucleotide sequence of the worldwide isolate matches the *Amaranthus albus*, which was deposited in NCBI and registered under the numbers OP846011.1 and OP846010.1. The percentages of matching for the two isolates were 97 and 99%. Through adopting the molecular evidence represented through the genes, the phylogenetic tree data confirmed that there is a close relation between the *Amaranthus albus* in question (in Iraq) and the ones in South Korea, China, Canada and USA registered in NCBI.

التصنيف الجزيئي الجديد لمصنع *Amaranthus albus* في العراق

جيهان يحيى وانوار الطائي

قسم علوم الحياة – كلية التربية للبنات – جامعة الموصل - العراق

الخلاصة

تم التحقيق في المؤشر الجيني الجزيئي لأنواع *Amaranthus albus* باستخدام تقنية التضخيم العشوائي فيما يتعلق بالحمض النووي متعدد الأشكال (RAPD ، حيث يتم تضمين شظايا الحمض النووي وضربها عن طريق استخدام تفاعل البوليميراز المتسلسل (PCR). وقد استخدم المركز الوطني لمعلومات التكنولوجيا الأحيائية لبنك الجينات في تحديد القواعد النيتروجينية المتصلة بالنوعين المعنيين عن طريق معاهدة التعاون بشأن البراءات باستخدام البادئات (إيت1) و (إيت4). وأظهرت النتائج أن تسلسل النيوكليوتيدات للعزل في جميع أنحاء العالم يطابق *Amaranthus albus*، الذي أودع في NCBI ومسجلة تحت أرقام أوب 846011.1 و أوب 846010.1. كانت النسب المئوية للمطابقة للعزلتين 97 و 99٪. من خلال اعتماد الأدلة الجزيئية الممثلة من خلال الجينات ، أكدت بيانات شجرة النشوء والتطور أن هناك علاقة وثيقة بين *Amaranthus albus* المعني (في العراق) وتلك الموجودة في كوريا الجنوبية والصين وكندا والولايات المتحدة الأمريكية المسجلة في NCBI.

الكلمات المفتاحية: **، رابد ، العلاقة التطورية ، الجنرال الرسومات البنك**.

INTRODUCTION

Medicinal wild plants are considered as a rich source of secondary metabolites (Al-Hatem, 2018). *Amaranthus albus* is one of the plants that grows naturally in Mosul Governorate, Iraq. It belongs to the *Amaranthaceae* family, which comprises 165 genera and 2040 species distributed all over the world. Among these, approximately 70-80 species are recognized as medicinal or cultivated ornamental plants (Singh *et al*. 2023). Amaranth grains are a rich source of minerals such as calcium, magnesium and copper, as well as iron, sodium, zinc and phosphorus. It also contains vitamins such as riboflavin, thiamine, niacin and ascorbic acid (Joshi *et al*. 2018). The phytochemical examination of dried amaranth grains revealed that there is a presence of good-quality phenolics, alkaloids, saponins, and flavonoids (Barba de la Rosa *et al*. 2009). *Italic* is a promising agricultural crop and it is characterized with the ability to withstand negative growing conditions and it is resistant to pests, drought, salinity, heat and it is adaptable with various environments (Aderibigbe *et al*. 2020).

The genus *Italic* is characterized by its phenotypic diversity, which involves hybrid species and that makes the taxonomic identification challenging. Genetic markers and phenotypic markers were used to examine the phylogeny and taxonomy of the genus (Stetter and Schmid 2016).

Phenotypic traits were utilized in the investigation of genus phylogeny and taxonomy. Three subgenera, comprising *A. Canada "Italic* , *A. albus "italic"* , and *Italic* , were all identified in the most recent taxonomic revision (Abdul Hassan *et al*. 2010). On the other hand, grain amaranths and their close relatives are presently well-known species and the phylogeny of such species, that depend on the genome genetic markers, has the potential to enhance the taxonomic evolution and the classification of the entire genus (Jimenez *et al*. 2013). Due to their high nutritional content and favorable amino acid compositions, Amaranthus leaves and seeds are both considered important commodities for the cultivations outside of their native ranges. Phylogenetic analysis may now make use of genome-wide polymorphisms from various species

as a result of the rapid advancement of the technologies of sequencing. For genetic studies, reduced representation sequencing techniques could yield thousands of single nucleotide polymorphisms (SNPs) (Poland *et al*., 2012 ; Elshire *et al*., 2011). SNPs in these species are discovered with the use of de novo sequencing read assembly (Catchen *et al*. 2013) or reference sequences of separate, yet closely related species (Maughan *et al*. 2009). Despite such drawbacks, phylogenetic studies of both distantly and closely related taxa were conducted using related RADseq techniques (Catchen *et al*. 2016 ; Nicotra *et al*. 2016). When a taxon's phylogenetic tree is available, theories about phenotypic traits or other interesting characteristics can be tested. Amaranthus species vary in several ways, including their reproductive systems (monoecious vs. dioecious) and the duplication of their genomes. As the genus Amaranthus is thought to be a paleoallotetraploid, due to undergoing a duplication of the genome between 18.40 and 34 Ma ago, the latter mechanism is frequently observed in plants (Clouse *et al*. 2016).

In the current work, genome size variation analysis was used to study the phylogeny of the genus Italic and to identify probable polyploidization events, which could have altered ecological traits or contributed to speciation. Using the GBS (i.e., genotyping by sequencing), the phylogeny related to the *A albus* genus was deduced. The UPGMA approach was used for inferring the evolutionary history. The optimum tree is shown with the branch length summation being equal to 3.95040582. The branch length values are expressed in identical units as those of the evolutionary distances that are utilized for estimating the phylogenetic trees, which are depicted to scale. Evolutionary distance values are expressed in base substitutions per site and have been estimated with the use of Maximum Composite Likelihood. There have been seven nucleotide sequences in the analysis, which are the 1st, 2nd, 3rd and noncoding codon positions that have been investigated. Every position with missing data and gaps was removed. So, the final dataset contained 229 positions in total. Moreover, MEGA6 was used for evolutionary studies. A genus-wide phylogeny could help determine the antecedents of this ancient crop and provide context for interpreting the data in relation to earlier domestication theories. Additionally, studying the evolution regarding herbicide resistance is made possible by understanding evolutionary relations that are between weedy amaranth species and their relatives.

MATERIALS AND METHODS

Plant material

The present research included the wild type, *A. albus*, which grows in Mosul and belongs to the Amaranthaceae family. Field visits took place in the late autumn Governorate of 2022 from various regions of Nineveh Governorate. The plant was initially identified based on diagnosis at the genetic level.

Genetic determinants amplification and sequencing alignment

10 µl of Genomic Master Mix (Gene All, S. Korea) has been used for extracting genomic DNA. More components have been 4 µl of gDNA (10 ng/ml), 1 µl (10 µM) of each forward and reverse primer set (Ella Biotech, Germany), and 4 µl of nuclease-free water to bring the total volume to 20µl. Table 1 contains a list of the primer sets for ITS1, ITS4, and other genes that were employed in this study.

Table1: Sequence of the primers that have been utilized in the present work.

Primer	Sequencew	Primer sequence	Tm (°C)	GC%	Size of Product (bp)
ITS	ITS1F	**5′- TCCGTAGGTGAACCTGCGG -3′**	60.3	50 %	**550**
	ITS14R	**5′ TCCTCCGCTTATTGATATGC-3′**	57.8	41 %	

The amplification process involved four minutes of initial denaturation at a a temperature of 95°C, and each denaturation took 30 seconds of at 95°C as well, thirty seconds of annealing at 60°C to hybridize the primers, 30 seconds for extension at 72°C and five minutes of final extension at 72°C (Rapley 2000). Amplification products have been sequenced by means of using a genetic lyzer (Applied Bio-systems 3500 USA) table (2 & 3) and purified utilizing a PCR purification kit (Gene All, S. Korea). In order to provide adequate sequencing output for each accession, two samples with distinct barcodes were generated for each accession and the fragment size was (490 bp). The UPGMA approach was used for inferring the evolutionary history (Freifelder 1983; Sneath and Sokal 1973).

Table (2): Reaction components of PCR.

Component	25 µL (Final volume)
Taq PCR PreMix	5 µl
DNA	1.5µl
Reverse primer	10picomols/µl (1µl)
Forward primer	10picomols/µl (1µl)
Distilled water	16.5µl

Table3: optimal detection condition

No.	Phases	Tm (°C)	Time	Number of the cycle
1-	Initial Denaturation	95°C	5min	1 cycle
2-	Denaturation2	95°C	45 Sec	
3-	Annealing	52°C	1min	35 cycle
4-	Extension1	72°C	1min	
5-	Extension2	72°C	5min.	1 cycle

Data preparation and filtering

A custom (GBS) analysis pipeline was used for processing the raw sequence data. Initially, Python scripts were used to divide the scans into several files, based on their barcodes. Afterwards, fastQC {bioinformatics.babraham.ac.uk/projects/ fastqc/} was utilized to evaluate the reading quality. They were cut to (490 bp) because the reading quality decreased at the conclusion of the readings. In the case when a low-quality read has at least one N (which refers to an undefined base) or a score of quality no more than 20 in more than 10% of the bases following trimming, it was

eliminated. Each accession's replicated data merged and then it was examined as a single sample (Tamura *et al*. 2013).

De novo and reference-based SNP discovery:

For the purpose of calling SNPs from sequencing data, a de novo method with Stacks 1.35 was employed. The SNPs were called, directly from the processed data using the denovo map.pl pipeline that Stacks provided for this de novo technique (Catchen 2013). As for option-t, very repetitive (GBS) reads have been eliminated from the ustacks program. Furthermore, an analysis was conducted for the data that had two distinct minimum numbers of identical raw readings ({m = 7 and m = 3}) needed to form a stack. The two settings were selected for the comparison because (SNP) calling (Mastretta-Yanes *et al*. 2015) produced varied results in relation with these two settings. To process one individual, two mismatches between the loci were permitted; however, four mismatches between loci were allowed for constructing the catalog, which represents the collection of the non-redundant loci that are based upon all of the accessions and serves as a guide for the SNP calling. The SNPs were called using populations 1.35 and the Stacks tool, which filtered, to a varying degree, the missing values. Apart from the de novo method, sequence reads were aligned with bwa mem (Li, Durbin 2009). Using vcftools, the resultant SNPs were filtered for varying missing value degrees at a locus (Danecek *et al*. 2011).

Phylogenetic Analysis

The species trees were directly inferred from unlinked biallelic markers by utilizing the multi-species coalescent that is implemented in the SNAPP, which is a component of the BEAST package (Bouckaert 2014). Since the SNAPP technique is costly in terms of computation, the number of individuals per species was constrained to a maximum value of 4. Furthermore, Beagle (Browning & Browning 2016) was used for imputing the reference-map-based datasets. Subsequently, vcftools were used (Danecek *et al*. 2011) for thinning all four datasets to a 100 bp distance, hence excluding multiple Amaranthus phylogeny Page 8 SNPs per the GBS read. The BEAUti program was utilized for calculating the mutation rates, and SNAPP was run with default parameters. For the important parameters, the effective sample size (ESS) was higher than 200, nevertheless, it was smaller for some θ values. Since the low θ values shouldn't have an impact on the tree topology, the analysis continued (Nicotra *et al*. 2016). The "Maximum Clade Credibility" tree was created and annotated with posterior probability values using Tree Annotator (Tamura *et al*. 2004).

RESULTS AND DISCUSSION

DNA Sequencing

The molecular diagnosis results of the species *A. albus* (in question) with the use of PCR using a pair of specialized primers, showed the detection of genes in the ITs internal transcribed spacer region, which is the forward primer (ITS1F and ITS4R). In addition to that, the results of electrophoresis decomposition are shown in Figure (1) using agarose gel at a concentration of 1.5% (as it was exposed to ultraviolet radiation at a wavelength of 254 nm using a UV transilluminator to detect DNA bands, it was identical to what was illustrated in the design of the initiator responsible as a determinant of the amplification of the ITs1 and ITs4 genes and bands appeared during the migration with a size of 420 base pairs). The DNA product (420 bp–PCR product) produced by the PCR with primer pairs ITS1 and ITS4 is depicted in Fig. 1. One shouldn’t forget that M stands for the DNA ladder, or the molecular weight size marker, which displays the sizes

of each fixative as well as the quantity of nitrogenous base pairs (bp). Global isolate, which is identified by the accession numbers OP 846011.1 and OP 846010.1, matches the nucleotide sequence related to one of them, which is located on the left side of the figure. This confirms the diagnosis, as shown by the match percentage (97–99%) for the species *A. albus* and The global isolate has been deposited in GenBank.

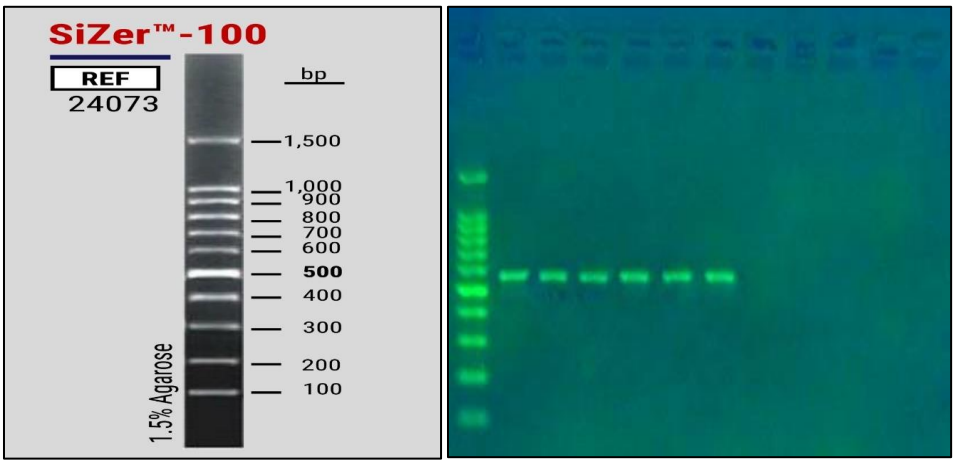

Figure (1) PCR product band size, the product has been under the impact of the electrophoresis on 1.5 % agarose at 5volt.cm^{-2}. 1 x TBE buffer for 1:30 hours. M: DNA ladder (100).

Phylogenetic Trees

The genotype tree diagram reveals that the standard cultivars registered with NCBI originated from nations that are distant from Iraq and the surrounding area. This suggests that the present investigation was among the first investigations that focus on the genetic characteristics of *A. albus atalic* at the local level. However, in Figure (2) it is observed that the genetic tree of the species *A. albus atalic* has a high degree of similarity between the Iraqi isolate of the species in question and was registered with the number ID: OP846010.1, OP846011.1 and the species registered in the NCBI International Gene Bank. The match percentage was 99.98% for China isolate. The number is JF975853, AF210918.1, while the match percentage was 97% for the American isolates MG685200.1, Canada isolates MG237223.1 and the South Korea isolate KP318852.1, and the match percentage was 97% for the Chinese isolate 4KY968956.1.

	1	2	3	4	5	6
1. OP846010.1 Amaranthus albus:IRAQ						
2. OP846011.1 Amaranthus albus:IRAQ	0.00					
3. 1 JF975853.1:Amaranthus albus:China	0.01	0.01				
4. 2 AF210918.1:Amaranthus albus:China	0.01	0.01	0.00			
5. 3 KP318852.1:Amaranthus albus:South Korea	0.37	0.37	0.35	0.35		
6. 4 MG685200.1:Amaranthus albus:USA	1.93	1.97	1.87	1.87	2.15	
7. 5 MG237223.1:Amaranthus albus:Canada	2.54	2.48	2.62	2.62	2.85	3.58

Table (4): Molecular diagnosis of the species *A. albus* depends on the percentage of identifying the nucleotide sequences with the global isolate in the World Gene Bank

	Accession	Country	Source	Isolation source	Compatibility
1.	ID: JF975853.1	China	*Amaranthus albus*	----------	99%
2.	ID: AF210918.1	China	*Amaranthus albus*	----------	98%
3.	ID: KP318852.1	South Korea	*Amaranthus albus*	----------	97%
4.	ID: MG685200.1	USA	*Amaranthus albus*	----------	97%
5.	ID: MG237223.1	Canada	*Amaranthus albus*	----------	97%

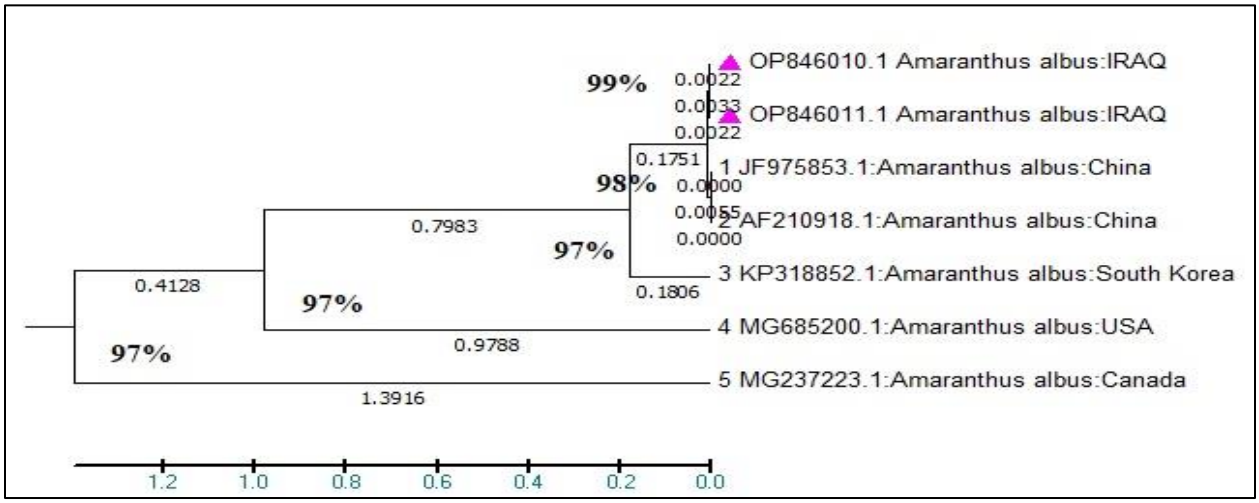

Figure (2): Dendrogram clustering of *A. albus* generated by analyzing RAPD data using the UPGMA method.

The DNA of the species in question was obtained by isolating it according to the method listed on the kit, which was highly efficient and easy to use in isolating DNA. The process of isolating DNA in plants is relatively difficult compared to other living organisms, due to the presence of the thick cell wall surrounding the plant. On the one hand, the plasma membrane contains different amounts of polysaccharides, phenolic compounds, essential oils, and other compounds that affect the purity of the extracted DNA, which consequently affects PCR reactions (Xu and Sun 2001). SNPs can be called without a reference genome thanks to de novo techniques. We compared the two to ascertain which method was more effective at identifying SNPs. Thus, there were few SNPs that could be used in phylogenetic research. All of the data were utilized in de novo assembly, and more SNPs were found than when the mapping against the *A. albus* genome was done (Clouse *et al*. 2016). Genetic similarity and dissimilarity were determined in this study using the RAPD indicators due to their simplicity and low cost. The genetic distance has been determined based upon the appearance or absence of duplication bands, as well as the difference in the molecular sizes of these bands. These bands differ according to the number of sites complementary to the primer sequences. On the DNA strand, it also varies depending on the distance between one site and another (Park *et al*. 2014). This pattern showed the production of binding sites with bands varying in molecular size and number within the products of the double strand of genetic DNA of the studied variety using the primers ITS1 and ITS4 with a variation rate

of (99) %. These primers are of great importance for finding genetic variation because their capabilities and efforts are reduced with the fewest interactions (Kolano *et al*. 2016; Al-Annaz and Al-Hatim 2023).

The phylogenetic analyses regarding the relation between the species in genus *A. albus* shed a new spot light on the relation of certain species about which morphological data were ambiguous, and they validate the ties that presumably exist between certain clades and species. Relations between species in South Korea, China, Iraq, the United States and Canada. The worldwide isolate that was deposited in GenBank had a match percentage of 97–99%. Generally, the clade is poorly resolved in our phylogenies, yet the few connections that are well-resolved have some precedent in the taxonomy and phylogenetic literature on *A. albus* These results are consistent with his findings (Stetter and Schmid 2017).

Data Availability

The corresponding author can provide data-sets that are utilized and/or analyzed in the present work upon reasonable request.

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