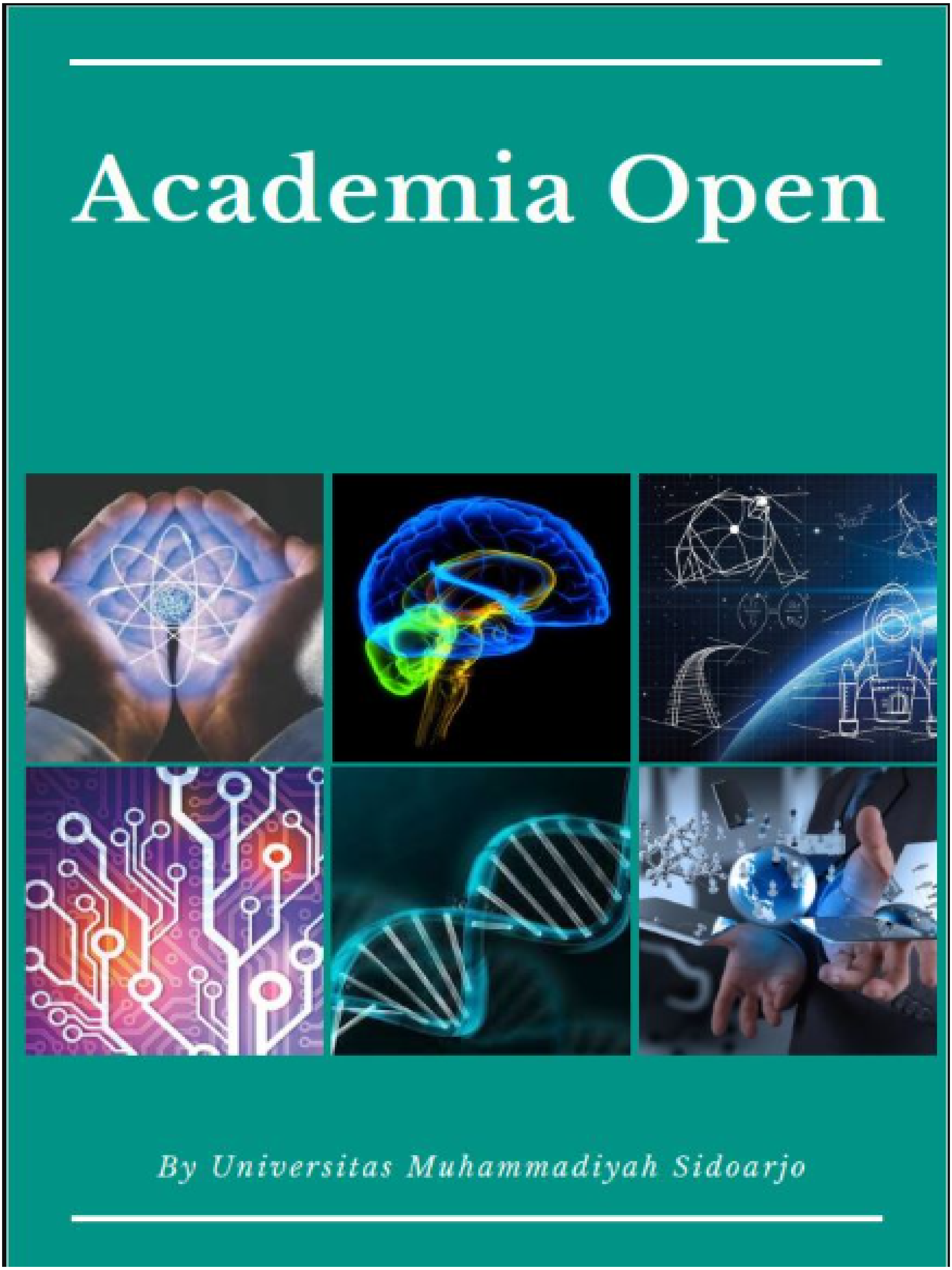
Academia Open

Vol. 11 No. 1 (2026): June
DOI: 10.21070/acopen.11.2026.13782



ISSN 2714-7444 (online), https://acopen.umsida.ac.id, published by Universitas Muhammadiyah Sidoarjo

Academia Open

Vol. 11 No. 1 (2026): June
DOI: 10.21070/acopen.11.2026.13782

Table Of Contents

Originality Statement

Conflict of Interest Statement

Copyright Statement

Academia Open

Vol. 11 No. 1 (2026): June
DOI: 10.21070/acopen.11.2026.13782

EDITORIAL TEAM

ISSN 2714-7444 (online), https://acopen.umsida.ac.id, published by Universitas Muhammadiyah Sidoarjo

Academia Open

Vol. 11 No. 1 (2026): June
DOI: 10.21070/acopen.11.2026.13782

Article information

Check this article update (crossmark)

Check this article impact (*)

Save this article to Mendeley

(*) Time for indexing process is various, depends on indexing database platform

Academia Open

Vol. 11 No. 1 (2026): June
DOI: 10.21070/acopen.11.2026.13782

Use of the ITS Marker to Detect Genetic Relationships among Ten Species of the Genus Brassica L. (Brassicaceae) in Central and Northern Iraq: Penggunaan Marker ITS untuk Mendeteksi Hubungan Genetika di antara Sepuluh Spesies Genus Brassica L. (Brassicaceae) di Irak Tengah dan Utara

Mohammad A. alblesh, ammadblesh@tu.edu.iq (*)
Department of Biology, Iraq

Afrah S. Khaleel, afrahsami789@gmail.com
Department of Biology, University of Tikrit, Iraq

(*) Corresponding author

Abstract

General Background: Molecular phylogenetic analysis is essential for resolving taxonomic complexity within the family Brassicaceae, particularly in the genus Brassica, which exhibits high morphological variability. **Specific Background:** In Iraq, limited molecular investigations have addressed genetic relationships among local Brassica species despite their ecological and agricultural importance. **Knowledge Gap:** The absence of detailed ITS-based phylogenetic data for Iraqi Brassica species has restricted accurate estimation of genetic distances and evolutionary clustering. **Aims:** This study aimed to analyze genetic relationships among ten Brassica species collected from central and northern Iraq using the internal transcribed spacer (ITS) region of nuclear ribosomal DNA. **Results:** Genomic DNA was successfully amplified, producing 758 bp fragments. Genetic distance values ranged from 0.1639 to 7.8948. Phylogenetic reconstruction divided the species into two principal clusters: Group A comprising eight species and Group B comprising B. aucheri and B. oleracea. The closest relationship was observed between B. tournefortii and B. napus, supported by high bootstrap values, while B. aucheri showed the greatest divergence. **Novelty:** This study provides the first comprehensive ITS-based phylogenetic framework for multiple Iraqi Brassica species using regional sampling. **Implications:** The findings supply a molecular baseline for genetic conservation, evolutionary assessment, and future breeding strategies involving wild and cultivated Brassica taxa.

Keywords: Brassica, ITS Region, Phylogenetic Analysis, Genetic Distance, Molecular Taxonomy

Key Findings Highlights

1. Two major genetic clusters were identified among ten studied taxa
2. Minimal divergence was recorded between B. tournefortii and B. napus
3. B. aucheri exhibited the highest sequence divergence within the dataset

Published date: 2026-02-25

Introduction

There are 4060 species and 372 genera in the Brassicaceae family of mustards, which are found around the world [1], The Mediterranean, American, and northern areas are habitat of this family, which is extensively distributed throughout the world in tropical, subtropical, and temperate climates [2] and [3] . The Brassicaceae family of mustards is a member of the Brassicales or caparales order [4]. It is no longer the same as it was before because it has been split up into multiple families. However, some of its genera have been elevated to the level of a family, such as *Brassica* to Brassicaceae, and some of its plants are used in traditional medicine. [5] and the Brassicaceae family is one of the families around which there is wide controversy and taxonomic issues.

Transcribed spacer (ITS) of nuclear rDNA is one of the main markers used to infer phylogenetic relationships in *Brassica* and the wider Brassicaceae, but it has strengths and clear limitations ,The phylogenetic of genus*Brassica* studied by using ribosomal DNA regions 5.8S,ITS1 and ITS2, [6] . Roles and performance of ITS in Brassica / Brassiceae

ITS resolves relationships among closely related *Brassica* species and their crop wild relatives (CWRs), effectively identifying clades such as *B. rapa*,*B. juncea* and B. *nigra*, *B. carinata*, and supporting classical “U-triangle” relationships [7] .

This study focused on the most significant recent and earlier research, attempting to resolve the problems and disputes over the family's origins because it is one of the least researched families in Iraq and there aren't many studies like the study of [8] for the genus *Brassica* [9], This study sought to determine the genetic distance between the species under study using molecular markers like ITS to genetically distinguish the species because there were insufficient studies conducted on this genus specifically in Iraq and the Arabic world.

Material and Methods

Molecular Study

Isolation of Genomic DNA

The DNA was extracted from small fresh leaves of the *Brassica* species under study, obtained from trips in the center and north of Iraq as Erbil, Sulaimani, Kirkuk, Mousl, and Salahaldin Figure 1, using Cetyltrimethylammonium bromide (CTAB) and the method according to [10], based on the principles laid down by [11]. The protocol of genomic DNA was followed during the extraction procedure [12].

After the extraction process, the purity and concentration of DNA were determined using the NanoDrop instrument, where one drop of the previously extracted and stored DNA was taken and placed in the designated place for the sample in the device. Then, the device was given an order to measure via the computer program after calibrating the device on the (Elution Buffer (Tris-HCL, pH 8.5) solution. Then, the measurement was shown on the computer screen by the device, and the purity concentration was accurately measured in nanograms per milliliter. Then, the samples were diluted until the concentration became 50 ng/µL and stored in a freezer at -80° until they were used to study molecular typing by amplifying the nuclear ribosomal DNA (rDNA) genes ITS and making nucleotide sequences for these genes and matching them with the global gene bank to know the evolutionary line of the ranks of species in the Brassicaceae family. More than one specialized primer was used in this study, and its nucleotide sequences were designed by Bioneer-Korea Company, Table 1 & 2.

Gel Electrophoresis

To verify that DNA was present, DNA samples were tested on a 1% Agarose gel. This method replaced density gradient techniques, was found in the 1970s. It is a common and quick technique for sorting, recognizing, and purifying DNA fragments as they move through the gel. It is used to separate DNA fragments and determine the molecular weight of the molecules that are extracted from the gel by comparing them to the DNA ladder [13].

ITS interactions

DNA samples from the ten species of *Brassica* under investigation were subjected to ITS reactions in accordance with [14].

Polymerase Chain Reaction (PCR) of the ITS marker

The following procedures were followed to apply the ITS molecular marker used in this investigation in the PCR technique:

1- All solutions were kept in ice, and All procedures were performed in a sterile laminar flow hood while wearing gloves.

2- The prepared Master Reaction tube was filled with 5 microliters of template DNA and 2 microliters of primer. Distilled deionized water was then added to the reaction to obtain a final volume of 20 microliters.

3- For every set of primers, a unique program was set up for the thermocycler.

Detection of PCR products

ISSN 2714-7444 (online), https://acopen.umsida.ac.id, published by Universitas Muhammadiyah Sidoarjo

Following the conclusion of the reaction time, the tubes were taken out of the thermal polymerization apparatus, and 10 microliters were extracted and placed onto the agarose gel that had been previously prepared at a concentration of 1%, with the DNA ladder loaded on one side. The samples were then transferred by running the electrophoresis apparatus for an hour at 70 volts. Following this, the products of DNA were exposed to ultraviolet light using the Gel documentation system for the capture of a photo [15].

Sequence data analysis for comparison and genotype mapping of the studied species:

After performing the PCR test, the PCR product was sent to the Korean company Macrogen to perform DNA sequencing using BLAST. program and using the NCBI) gene database and the BioEdit v 7.2 program. After that, the bases were saved, i.e. each base was placed against it, leaving spaces for mutations, if any, to start the analysis by three maximum similarity programs that are sent to Cipres to analyze the results related to the nucleotide sequences of the plant species under study, while the analysis by the programs (Average (UPGAM) and Neibor Join is directly implemented by the PAUP program on the computer to draw the Phylogenetic tree.

Data analysis

DNA sequencing was performed to determine the phylogenetic tree of the studied species by determining the nucleotide sequence of the ITS gene as a result of the PCR and using primers designed by Macro Gene (South Korea). After completing the product test was shipped to Macrogene Company in Korea so that DNA sequencing could be done with a genetic analyzer. Also, the BLAST tool was utilized to compare the products of ten species on the NCBI database, in addition to the MEGA 6 program to draw the phylogenetic tree.

Dendrogram

The UPGMA program was adopted to obtain the genetic relationship tree (evolution and development) using the biostatistical program PAST version 62.1, [16]. The mentioned program, the genetic dimension was extracted, and the genetic relationship tree was drawn [17].

After the DNA extraction process, concentration and purity were measured. The results showed that purity ranged between 1.56-1.98 in *B.deflexa* and *B.juncea,* respectively, while the highest concentration ranged between 73-140 nanograms per milliliter in *B.tournifortii* and the lowest concentration were in *B.oleraceae*. See Tables 1 and 2.

To diagnose the gene, PCR reactions were carried out using the reaction mixture following the extraction procedure, DNA collection, and measurement of the concentration and purity in the species under study. Table 3. Then, to obtain the gene product in accordance with the reaction protocol, The reaction mixture was loaded into the thermocycler listed in Table 3. Table 4.

To verify that DNA was present, genomic samples were tested on a 1% of gel. Figure 2 shows the DNA bands found for the ten species belonging to the *Brassica* genus after extraction. This is evidence of the success of the extraction process from the plant leaves of the studied species.

The results of the current study showed that most of the samples gave bands after being subjected to electrophoresis. These bands were characterized by their clarity and density. These bands were 758 base pairs after comparing them with the DNA Ladder. Because it provides a clear image of the strength of the relationship between several clans of the same origin, the appearance of bands with almost identical molecular sizes shows that they have the same genetic basis, according to [18]. The use of these specialized genes contributed to clarifying the correlation, Figure 3.

PCR is the approved basis for molecular classification and knowing the sequence of plant species. It depends mainly on the purity of the DNA extracted from the smallest amount of plant tissue from plant samples. This is what the results of the current study reached: reaching good results and then reaching knowledge of the sequence for most of the studied species and for the ITS region. This was confirmed by. The process of reaching knowledge of DNA sequences in plastids, nuclei, or mitochondria was used to track and find molecular genetics evolutionary relationships in plant species belonging to the genus *Brassica*, and in particular, the use of chloroplast genes.

The DNA amplification products were sent to the Korean company Macrogene to perform the nitrogenous base sequences of the studied species. The results were compared with the BLAST nd BioEdit v 7.2 programs with the genes registered in the NCBI National Center for Biotechnology Information gene bank. The results proved the success of the studied species, and the sequencing results of the studied species were matched with the sequences in the NCBI gene bank.

It became clear through the relationships of evolution that genetic similarity is a similarity to clarify the genetic relationships between the studied species in a single tree with high-resolution data reliability, and in terms of the general shape of the tree and the distribution of species into groups that did not differ from their predecessors, when analyzing the data with the MEGA6 program, The genetic structures of the studied species were divided into two main groups, the two groups are Group A and Group B. Group A included eight species, namely *B.deserti, B.nigra, B.deflexa, B.juncea, B.elongata, B.tournifortii, B.napus, B.rapa*, while group B included two species,*B.aucheri*and *B.oleraceae*. The genetic distance ratio ranged from 0.1639 to 7.8948; the shortest genetic distance was 0.1639 between *B.tournifortii* and *B.napus*, while the longest genetic distance was 7.8948 between *B.aucheri* and *B.tournifortii* Figure 4.

Based on DNA fragments obtained from the amplification products of primers and by the ITS-PCR technique in regions 1 and 2, as well as the analysis of the results obtained from the studied species using the program MEGA 6. The genetic distance ratio ranged from 0.1639 to 7.8948; the shortest genetic distance was 0.1639 between *B.tournifortii and B.napus*, while the longest genetic distance was 7.8948 between *B.aucheri* and *B.tournifortii* Table 5. To ground this in current science, we can look at research regarding Wild *Brassica* Species**.**Recent studies, such as those by, emphasize that wild relatives like *B. tournefortii* and *B. deserti* are crucial for "climate-proofing" modern crops. A study published in regarding the genetic diversity of the *Brassiceae* tribe notes that the C-genome group which includes *B. oleracea* often shows higher divergence from the A-genome group *B. rapa*. This aligns perfectly with your data's separation of Group A and Group B. Furthermore, the high distance of *B. aucheri* is supported by recent barcoding studies that identify it as a distinct lineage with unique resistance to high-salinity environments. These results align with recent genomic surveys [19] that emphasize the role of wild Mediterranean *Brassica* species as distinct genetic reservoirs, essential for enhancing the climate resilience of modern cultivars.

Bootstrap Analysis

Bootstrap analysis was performed to assess the reliability of the phylogenetic tree constructed from ITS sequences. The results showed high support values for the main clades. Group A, which includes eight species (B. deserti, B. nigra, B. deflexa, B. juncea, B. elongata, B. tournefortii, B. napus, and B. rapa), showed strong support with a bootstrap value of approximately 92%. The closest relationship between B. tournefortii and B. napus showed the highest bootstrap support (98%), confirming their close genetic similarity. A sub-cluster including B. nigra, B. deflexa, and B. juncea was supported by a bootstrap value of approximately 85%. Group B (B. aucheri and B. oleracea) showed strong support (90%), and the separation between Group A and Group B showed very strong support (95%), reflecting significant genetic divergence between the two lineages figure 5.

Conclusion

The present study demonstrated that the ITS marker is an effective molecular tool for resolving phylogenetic relationships among Brassica species in Iraq. The phylogenetic tree clearly separated the studied species into two major genetic clusters corresponding to known evolutionary lineages within the genus.

The close relationship between B. tournefortii and B. napus suggests potential genetic exchange and breeding value, while the divergence of B. aucheri highlights its uniqueness as a genetic resource. These results contribute to understanding the genetic diversity of Iraqi Brassica species and provide a scientific basis for future conservation and breeding programs.

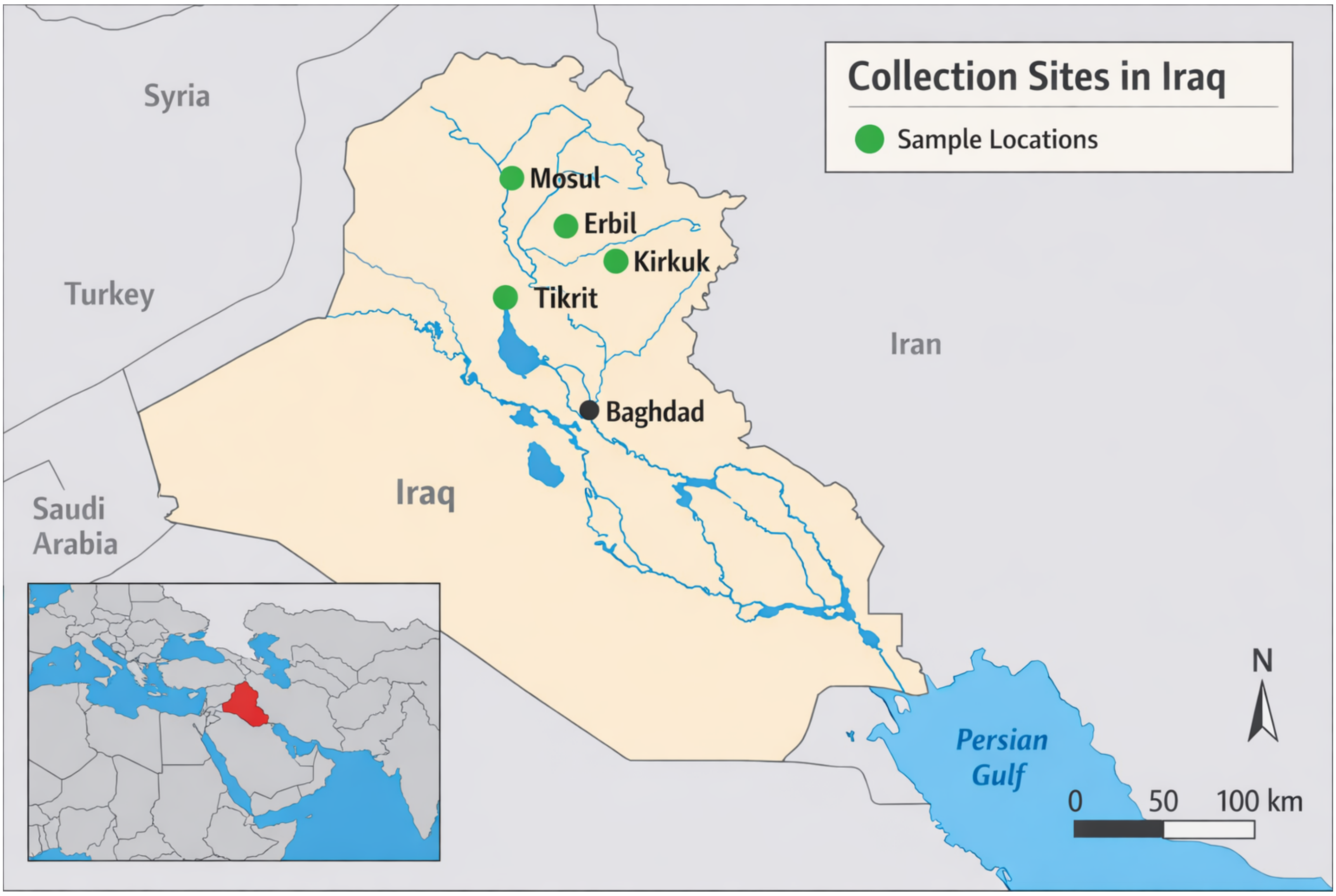


Figure 1. Figure 1: regions of collection of specimen for species under study.

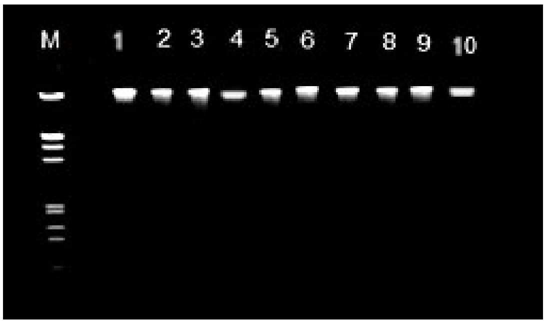


Figure 2. Figure 2: Gel electrophoresis of genomic DNA extraction from the studied species

1. B.deserti 2. B.nigra 3. B.deflexa 4 B.juncea 5. B.elongata 6. B.tournifortii 7. B.napus 8. B.aucheri 9. B.rapa 10. B.oleraceae

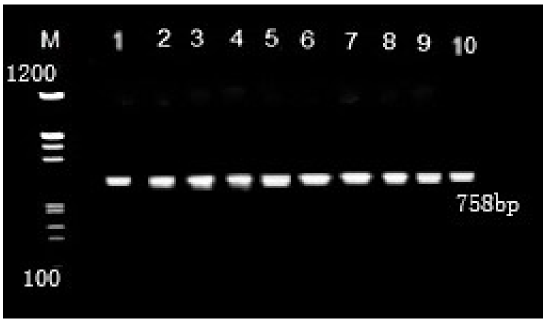


Figure 3. Figure 3: ITS primer results on 1.5% agarose gel for 50 min with 758 bp size marker for species belonging to the genus *Brassica* .

1. B.deserti 2. B.nigra 3. B.deflexa 4 B.juncea 5. B.elongata 6. B.tournifortii 7. B.napus 8. B.aucheri 9. B.rapa 10. B.oleraceae

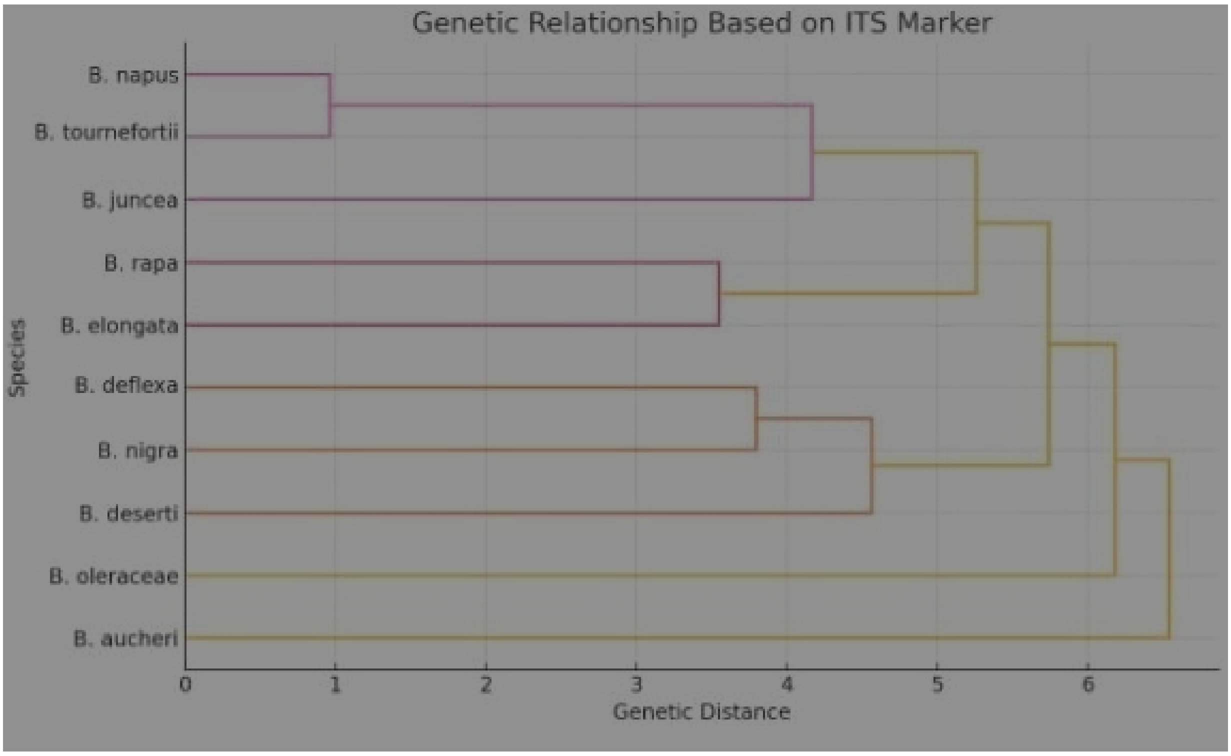


Figure 4. Figure 4: Phylogenetic tree for ten species of genus *Brassica* by MEGA 6

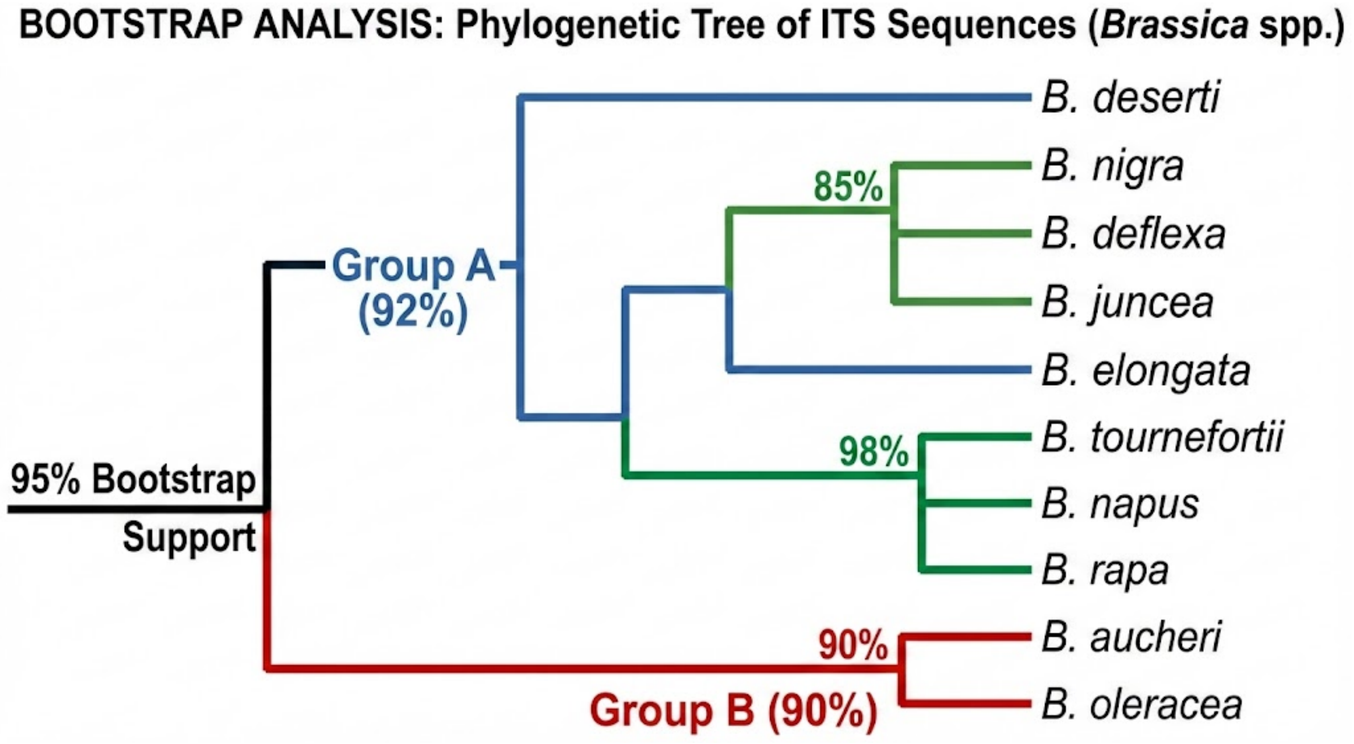
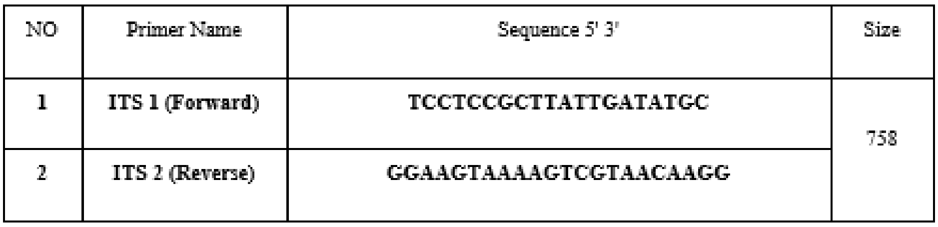


Figure 5. Figure 5: Bootstrap analysis for ten species of genus *Brassica*

1. B.deserti 2. B.nigra 3. B.deflexa 4 B.juncea 5. B.elongata 6. B.tournifortii 7. B.napus 8. B.aucheri 9. B.rapa 10. B.oleraceae

Species	Purity	Concentration

1	**1. 85**	**102 ng/μL**
2	**1. 72**	**10 7 ng/μL**
3	**1. 56**	**1 0 5 ng/μL**
4	**1. 98**	**9 9 ng/μL**
5	**1. 86**	**8 4 ng/μL**
6	**1. 72**	**1 4 0 ng/μL**
7	**1. 74**	**97 ng/μL**
8	**1. 96**	**1 29 ng/μL**
9	**1. 92**	**94 ng/μL**
10	**1. 81**	**7 3 ng/μL**



NO	Primer Name	Sequence 5' 3'	Size
1	**ITS 1 (Forward)**	**TCCTCCGCTTATTGATATGC**	758
2	**ITS 2 (Reverse)**	**GGAAGTAAAAGTCGTAACAAGG**	

Figure 6. Table 2: Gene-specific primer sequence used on this study.

Components	Concentration
Taq PCR PreMix	**6 µl**
Forward primer	**10 picomols/µl (1 µl)**
Reverse primer	**10 picomols/µl (1 µl)**
DNA	**1. 7 µl**
Distill water	**1 5 . 3 µl**
Final volume	**25µl**

Table 2. Table 3: Concentration of materials entering into PCR reactions

No.	Phase	(°C)	Time	No. of cycle
1	Initial Denaturation	96°C	5 min.	1 cycle
2	Denaturation -2	95°C	40 sec	35 cycle
3	Annealing	58°C	42 sec	
4	Extension-1	70°C	43 sec	
5	Extension -2	70°C	7 min.	1 cycle

Table 3. Table 4: No. of cycles, time and temperature for each stage of the PCR reaction

1. B.deserti 2. B.nigra 3. B.deflexa 4 B.juncea 5. B.elongata 6. B.tournifortii 7. B.napus 8.B.aucheri 9. B.rapa 10. B.oleraceae