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Phylogenetic Diversity of *Trifolium* L. Species in Iraq

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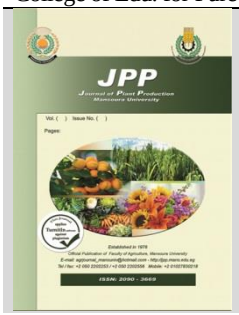

ABSTRACT

Thirteen species of *Trifolium* L. were tested by twenty different 10 mers RAPD primers. The results of the technique RAPD showed clear genetic variations among the species under study and the species were divided into two general groups at the level of similarity 1.42, while taking group I about 11 species, II was isolated with two species *T. dasynrum* and *T. pilulare*, the group I it was divided into branches A which separated from the rest of the species *T. leucanthum* and B which branched into B1 and B2, the B1 included 7 species, while B2 contained three species. The objective of this study was to determine *Trifolium* 's molecular phylogenesis based on the RAPD method as a genetic taxonomic tool to isolate and separate species.

Keywords: phylogenetic, diversity, *Trifolium*, Iraq

INTRODUCTION

The Leguminosae family (Fabaceae) consider the third greater family of flowering plants, Fabaceae consist of 727 genera and 19325 species (Lewis *et al.*, 2005), and the genus, *Trifolium* L. with 255 species is calculated one of the prime genera in the family (Gillett and Taylor, 2001). Based on the special leaves usually consist of three leaflets or the other name trifoliolate the *Trifolium* L. known as red clover about 10% of them being an important role in the agriculture field (Zohary, 1970).

Trifolium species spread in the world wild or cultivated crops as pure species or mixtures. Traditionally, the benefits of agriculture the *Trifolium* that are fixation the Nitrogen in the soil and refinement of the soil through legume rhizobium symbioses (Yates *et al.*, 2014). The *Trifolium* genus is spread across the temperate and subtropical regions of both hemisphere (Bisby *et al.*, 1994). *Trifolium* species are rich in the Mediterranean region (Zohary and Heler, 1984), in Turkey in particular where it is widespread, which recorded about103 species (Zohary, 1970).

In traditional medicine, some *Trifolium* species used to treat external skin and lung disease, nerve and sexual system disease (Figueiredo *et al.*, 2007), expectorants, residence, antiseptics, and against rheumatism aches (Baytop, 1984). It is also used for sheep and cattle feeding in the Mediterranean region (Acikgoz, 2001; Oleszek and Stochmal, 2002). The *Trifolium* extracts are marketed in the USA and in European supermarkets as dietary supplements (Polasek *et al.*, 2007).

The manifestations importance of this genus made it suffered from many genetic manipulations, including the transformation of the diploidy to polyploidy, this is true to the modification of some qualities such as resistance to the diseases, the hardiness of winter, and forage yield (Sattler *et al.*, 2016).As a result of heterogeneous and heterozygous genotypes, the genus is achieved peak levels of genetic diversity into populations of plants (Tucak *et al.*, 2009). Tanhuanpaa and Manninen (2012) mentioned that the genus has a higher genetic diversity but less differentiation among populations. The aim of this study that appears of the use of PCR method to isolate with genetic diversity of plant has been proposed as a clear tool than traditional taxonomic practices.

MATERIALS AND METHODS

1. Plants materials:

Thirteen species of *Trifolium* L. were collected in different parts of Iraq from their natural habitats and directly from the field for the period from March to September 2019. All species and place of collecting present in table 1. After samples were collected, dried and compressed, the samples were identified based on several books and flora, including Iraqi, Turkish, Iranian, Chinese, Kuwaiti and Saudi flora. The samples were deposited in the College of Pure Sciences Ibn ALHaitham, University of Baghdad after recording the scientific name, place and date of the collection.

2. DNA extraction:

Genomic DNA extraction was done according to modified Cetylrimethyl ammoniumbromide (CTAB) method of Doyle and Doyle (1990). The DNA sample is detected as 1 % agarose gel in electrophoresis and stained with ethidium bromide (0.5 mg / ml). Fifteen dicprimers RAPD in this study were tested (table 1 and 2) which provided by the company of Bioneer.

The master amplification Reaction existing in (table 3). The Polymerase chain reaction was started with a hot start-method by using the single strand cDNA template on Labnet Thermocycler (USA). The PCR reaction was done followed the program of 40 amplification cycles (95°C for 1 min, 43.7°C for 1 min, and 72°C for 1 min). The generated bands were compared.

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Table 1. The species under study and their regions

Species	Region
T. alexandrinum	Piramagrwn and Said Sadq and Halabja (dska)
T. campestre	Halabja (Qasrat) and Chnarok and Sarsang
T. cherleri	Qaradax
T. dasynrum	Nzik Grdabor and Sshaxi Bradost and Chnarok
T. echinatum	Tasluja and Darbandixan and Harir and Hawraman
T. leucanthum	Piramagrwn and Jafaran and Halabja (Mergaka)
T. pilulare	Qopi Qaradax and Benawa Swta Nzik Penjwen and Tawela
T. purpureur	Chnarok and Halabja (Qasrat)
T. respianthum	Penjwen and Grdabor and Sarsang
T. scabrum	Darbandixan- Shaxi hawraman and Penjwen
T. spumosunm	Kani Spi and Grdabor Said Sadq and Gali Warta.
T. subterranenum	Darbandixan.
T. syluticum	Benawa Swta Nzik Penjwen and Hawraman and Halabja (Nasr).

Screening of PCR:

In this study twenty different (10 mers) RAPD primers were selected and tested (Table 2). The ten primers which had already shown in the indicated results of band patterns, and the master amplification reaction existing appear in Table 2.

The Polymerase chain reaction began in the hot start-method by using the single strand (cDNA template) from Labnet Thermocycler (USA). The PCR reaction was done followed the program of 40 amplification cycles (95°C for 1 min, 43.7°C for 1 min, and 72°C for 1 min). Gel Electrophoresis (Agarose 1%) is used for the analysis of PCR products for 60 minutes. The generated bands were compared, the differential amplified bands were recorded as 0-1 due to the band's presence or lack, 150-1350 basic pairs (bp) shall be in the range of sizes.

Table 2. The sequences of RAPD primers used in this study including those produced amplified

Primers	Primer ID	5' – 3' Sequences	Primers	Primer ID	5' – 3' Sequences
1	OPA-02	TGCCGAGCTG	11	OPD-08	GTGTGCCCCA
2	OPA-04	AATCGGGCTG	12	OPE-02	GGTGCGGGAA
3	OPA-06	GGTCCCTGAC	13	OPG-19	GTCAGGGCAA
4	OPA-08	GTGACGTAGG	14	OPJ -17	ACGCCAGTTC
5	OPA-09	GGGTAAGGCC	15	OPL-19	GAGTGGTGAC
6	OPC-08	TGGACCGGTG	16	OPN-15	CAGCGACTGT
7	OPC-09	CTCACCGTCC	17	OPP-09	GTGGTCCGCA
8	OPC-12	TGTCATCCCC	18	OPP-10	TCCCGCCTAC
9	OPD-02	GGACCCAACC	19	OPS-19	GAGTCAGCAG
10	OPD-06	ACCTGAACGG	20	UBC 1	CCTGGGCTTC

3. Data analysis:

The analysis of the RAPD and ISSR matrix used the NTSYS-pc statistical package (version 2.1). In calculating the similarity of the genes between and within species, the data template was used based on Jaccard coefficients of similarity, and the dendrogram offers relationships among the 13 genotypes that have been built (Unweight Pair Group Method) with Arithmetic Mean (UPGMA).

RESULTS AND DISCUSSION

The results of the technique RAPD showed clear genetic variations among the species under study (figure 1.), the species were divided into two general groups and at the level of similarity 1.42, while taking group I about 11 species, II was isolated with two species were T. dasynrum and T. pilulare which were converging to 0.55. group, I it was divided into branches, A, which separated from the rest of the species T. leucanthum with a spacing of 0.31, and B which branched into B1 and B2 with a similarity rate of 0.21, B1 included 7 species, T. spumosunm isolated with a divergence 0.14 from other species, the two nearest species were T. alexandrinum and T. spumosunm with a similarity rate 0.03, while B2 contained three species, T. respianthum was isolated from its mates with a spacing of 0.62, while T. cherleri and T. purpureur have a rate of similarity were 0.48. all results showed in figure 2.

The findings indicate that previous phylogenetic *Trifolium* studies have been approved and rejected as well (Steele and Wojciechowski, 2003; Watson *et al.,* 2000). The sister group relationship between *Trifolium* and *Fabeae* was described by Steele and Wojciechowski (2003). The *Trifolium* species were also resolved and considered monophilous by Steele and Wojciechowski (2003) and obtained further support from other scientists. They sampled and resolved several of the same relationships between the 23 *Trifolium* groups, including the subgenus group of sister chromosomes, to the remaining American species and monophyletics but often received poor support. Opposes the results of the resulted of Watson et al. (2000) that explained the subgenus Chromosome and *Trifolium* genus are polyphyly. Liston *et al.,* (2006) set the *Trifolium* species as an unexpected position.

In fact, our study diversification the species in several rates of similarity. Based on our molecular phylogenetic results, the distance among most of these species was ranged from 0.1 in the same clade may be because these species native to that country (Thulin, 1983). This result agreed with Tanhuanpaa and Manninen (2012) that referred to the diversity among the *Trifolium* species.

All the North and South Americans and this genetic affinity as the deep taxonomic level is reflected in many phenotypes that clearing in the nomenclatural work of Hendrych (1988). We use data from the (DNA) genome for our phylogenetic analyzes. Usually, species and genera relationships are determined according to the rate and pattern (Hershkovitz *et al.,* 1999).

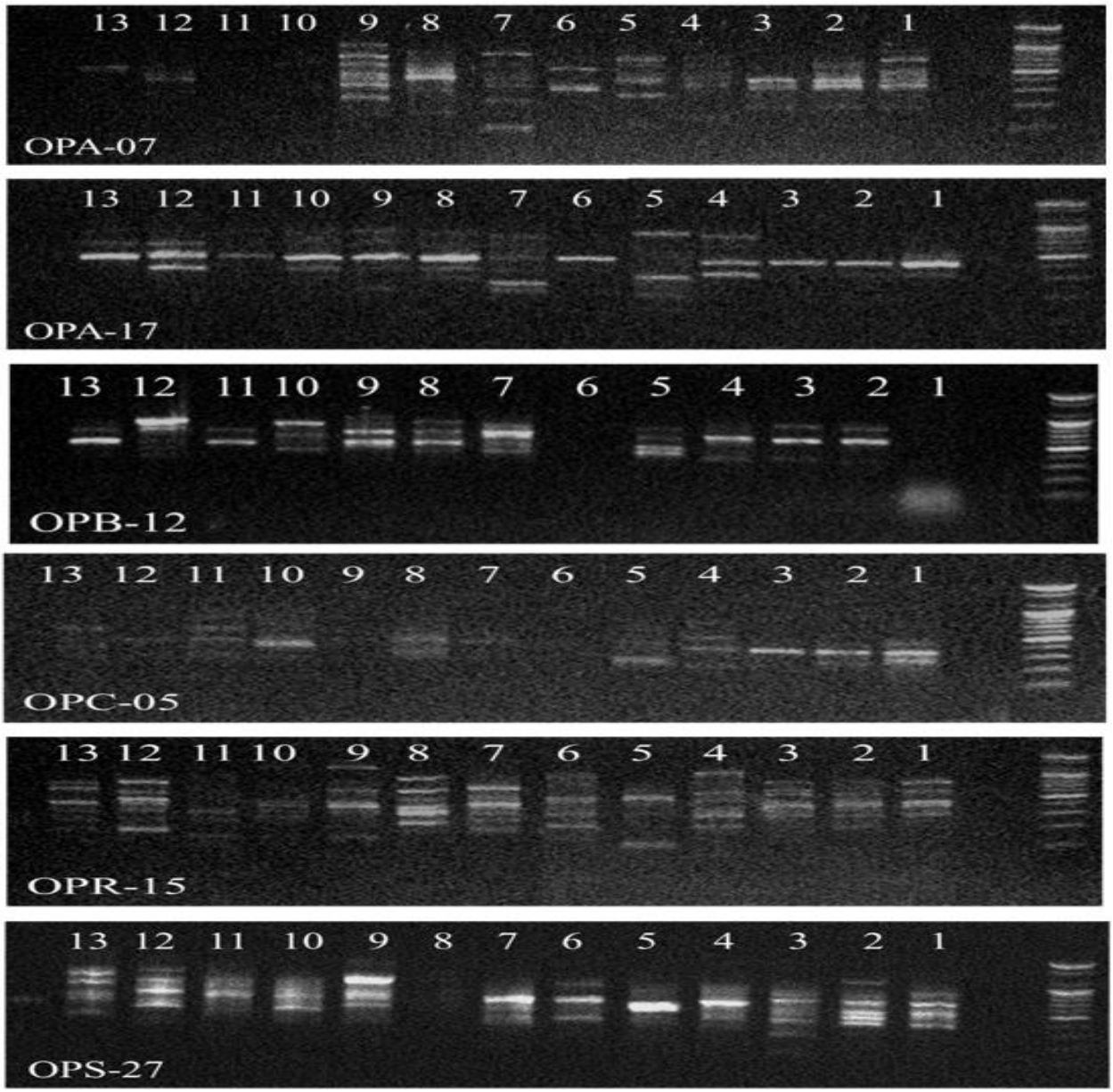

Figure 1. Spectrum of DNA amplification products of 13 species of *Trifolium* genus.molecular weight marker 1500-bp DNA Ladder

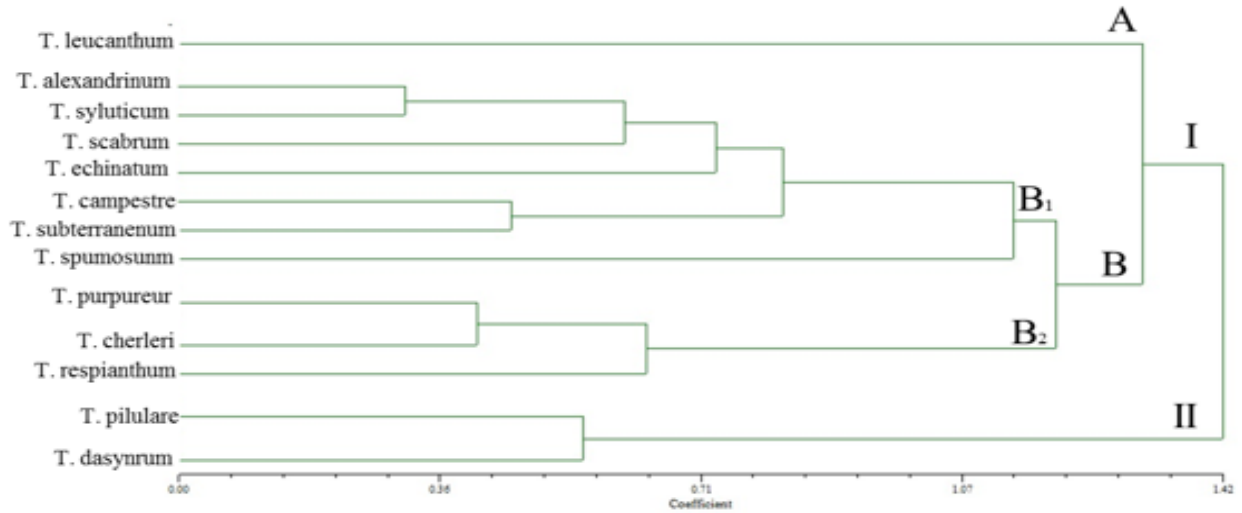

Figure 2. Dendrogram showing genetic relationships among of 13 species of *Trifolium* genus.

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التنوع الوراثي لأنواع *Trifolium* L. في العراق

لة نجه هيوا مصطفى خال[1] ، اسيل كاظم الانباري[2] ، معزز عزيز الحديثي[3] و روباك توفيق عبد الرزاق[1]

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تم جمع ثلاثة عشر نوعًا من *Trifolium* L خلال شهر نيسان 2019 من مناطق مختلفة من السليمانية ، وتم استخراج الحمض النووي الجيني واختبار خمسة عشر نوعًا من RAPD في هذه الدراسة بحيث تم اختبار عشرين نوعًا مختلفًا من RAPD. أظهرت نتائج تقنية RAPD اختلافات وراثية واضحة بين الأنواع قيد الدراسة وتم تقسيم الأنواع إلى مجموعتين رئيسيتين وتم عزل النوع الثاني بنوعين *T. dasynrum* و *T. Pilulare* ، المجموعة الأولى تم تقسيمها إلى مجموعة A التي انفصلت عن بقية الأنواع بالنوع *T. leucanthum* ومجموعة B والتي تفرعت إلى B1 و B2 ، وتضمنت مجموعة B1 سبعة أنواع ، بينما احتوت مجموعة B2 على ثلاثة أنواع. كان الهدف من هذه الدراسة هو تقييم النشوء والتطور الجزيئي لـ *Trifolium* بناءً على طريقة RAPD كأداة تصنيف وراثي لعزل وفصل الأنواع.