# Classification of Iranian Garlic (*Allium sativum* L.) Ecotypes Using RAPD Marker

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## Abstract

**Background: Garlic is a valuable medicinal plant with variability in desirable morphological and physiological characteristics. The analysis of genetic diversity plays an important role in breeding programs. The RAPD technique could be very effective in detecting genetic variation in garlic.**

**Objective: The objective of the present work was to detect molecular polymorphism among Iranian garlic ecotypes by RAPD technique.**

**Methods: Random Amplified Polymorphic DNA (RAPD) marker utilized to assess the genetic diversity among twenty-two different ecotypes of the Iranian garlic. Genomic DNA was extracted from young leaves using Dellaporta method. RAPD amplification was performed using ten 10-mers arbitrary primers.**

**Results: Results indicated that five out of ten pair primers had no amplification. A total of 35 RAPD bands were produced, 31 of which (88.5%) were polymorphic. The similarity matrices and dendrogram were obtained using UPGMA algorithms.**

**Conclusion: In conclusion the entire population was grouped into four clusters with 3, 9, 1 and 9 ecotypes. No significant relationship between genetic diversity detected by RAPD technique and geographical origins.**

**Keywords:** ***Allium sativum*** **L., Ecotype, Genetic diversity, Polymorphism, RAPD marker, UPGMA**

# Introduction

Garlic (*Allium sativum* L.) is one of the most important bulb vegetables, which is used as spice and flavoring agent for foods [1]. Also, it has benefits in lowering total plasma cholesterol, reducing blood pressure and decreasing platelet aggregation [2]. The center of origin of garlic has been considered to be central Asia. From the center of origin, garlic has been spread to west, south and east [3]. In Iran cultivation and consumption of garlic has a long history and areas under its cultivation is estimated about 10000 ha [4]. Garlic is a sterile species and reproduces only by vegetative propagation. Although garlic is an asexually propagated crop, it displays great morphological diversity in bulb, leaf size, color, shape, scape presence, height, flower color, fertility and bulbil (topset) development [5]. Until now, characterization of garlic germplasm has been based primarily on morphological data. However, morphological characters may differ under varying environmental conditions. Many classification systems for garlic have been described [3]. Using molecular techniques could be used to evaluate diversity between ecotypes. Isozyme and RAPD markers have been utilized to classify and categorize genetic diversity in garlic germplasm [5, 6, 7, 8, 9, 10]. The random amplified polymorphic DNA (RAPD) technique provides high numbers of markers which can be used for genetic studies [11]. RAPD have the advantage that the material is processed by an efficient and inexpensive technique without requiring prior knowledge of the genome.

The purpose of this study was to detect molecular polymorphism among Iranian garlic by RAPD. We accomplished this by using total genomic extracts from diverse garlic ecotypes collected from the main cultivation areas of Iran, which will help to delimit varietals groups in Iranian garlic ecotypes.

# Materials and methods

### Plant material

Plant materials and the geographical origins of ecotypes are listed in Table 1 and included 22 garlic ecotypes existing in all over Iran. This study conducted in Institute of Medicinal Plants (ACECR), in cropping season 2007.

### DNA extraction

Total genomic DNA was extracted from fresh young leaves. After freezing by liquid nitrogen, leaves were grounded in a mortar with a pestle until no large pieces could be seen. 1g of the powder was used for DNA extraction by appropriate modifications of the method described by Dellaporta et al., (1983). DNA was air-dried, dissolved in TE buffer and stored at -20°C until polymerase chain reaction (PCR) amplification was performed. DNA quality was tested using 1% agarose gel electrophoresis. For quantifying the amount of DNA, 20 µl of nucleic acid was added to 1980 µl of TE buffer and mixed and absorption (OD) read in a spectrophotometer at 260 - 280nm.

### RAPD primers

Ten 10-mers arbitrary RAPD primers used in this study and the list of primers is shown in Table 2.

**Table 1: Plant materials and the geographical origins of ecotypes**

| Ecotype No. | Region originated | Climate[a] | Latitude, N | Longitude,E | Altitude (m) |
|---|---|---|---|---|---|
| 1 | Khaf | Temperate-arid | 34°34'N | 60°08'E | 970 |
| 2 | Tarom high | Temperate-semi arid | 36°57'N | 48°54'E | 620 |
| 3 | Tuyserkan | Cool temperate-arid | 34°32'N | 48°27'E | 1850 |
| 4 | Shevarin | Cool temperate-semi arid | 34°48'N | 48°33'E | 1850 |
| 5 | Gorgan | Temperate-semi arid | 36°50'N | 54°26'E | 160 |
| 6 | Alafi shiraz | Cool temperate-semi arid | 30°15'N | 51°59'E | 2240 |
| 7 | Dezful | Warm-arid | 32°22'N | 48°24'E | 140 |
| 8 | Talesh dane dorosht | Cool temperate-semi humid | 37°48'N | 48°54'E | 700 |
| 9 | Kalardasht | Cool temperate-semi humid | 36°39'N | 51°11'E | 1150 |
| 10 | Qasr-e- shirin | Warm-semi humid | 34°03'N | 45°34'E | 360 |
| 11 | Tafresh | Cool temperate-semi arid | 34°41'N | 50°01'E | 1900 |
| 12 | Tarome dwarf | Temperate-semi arid | 36°57'N | 48°54'E | 620 |
| 13 | Lobnani-e- shiraz | Cool temperate-semi arid | 30°15'N | 51°59'E | 2240 |
| 14 | Taleqan | Cool temperate-semi arid | 36°14'N | 50°49'E | 1900 |
| 15 | Barma | Temperate-semi humid | 36°44'N | 53°46'E | 200 |
| 16 | Behshahr | Warm-semi humid | 36°41'N | 53°32'E | 15 |
| 17 | Khoram Abad | Temperate-semi arid | 33°29'N | 48°21'E | 1200 |
| 18 | Talesh dane riz | Cool temperate-semi humid | 37°48'N | 48°54'E | 700 |
| 19 | Birjand | Temperate-arid | 32°53'N | 59°13'E | 1480 |
| 20 | Torbat-e- Jam | Temperate-arid | 35°14'N | 60°37'E | 910 |
| 21 | Gonbad-e- kavus | Warm temperate-semi arid | 37°15'N | 55°09'E | 45 |
| 22 | Langerud | Warm temperate-humid | 37°11'N | 50°09'E | 20 |

[a] Yearly mean temperature in warm, temperate and cool climates are respectively 15-25°C, 10-15°C and 0-5°C. Yearly mean rainfalls in semi humid, semi arid and arid climates are respectively 600-1400 mm, 300-600 mm and 100-300 mm.

**Table 2: Codes and sequences of the RAPD primers used for detection of genetic variation and number of fragments amplified by primers**

| Primer code | Sequence of nucleotides (5' to 3') | Total number of fragments | polymorphic number of fragments | Percent of polymorphic |
|---|---|---|---|---|
| OPJ-12 | 5´-GTCCCGTGGT-3´ | 6 | 6 | 100 |
| OPA-01 | 5´-CAGGCCCTTC-3´ | 9 | 8 | 88.8 |
| K-15 | 5´-CTCCTGCCAA-3´ | 9 | 8 | 88.8 |
| K-20 | 5´-GTGTCGCGAG-3´ | 2 | 2 | 100 |
| OPD-05 | 5´-GATGACCGCC-3´ | 9 | 7 | 77.7 |
| OPD-01 | 5´-ACCGCGAAGG-3´ | 0 | 0 | 0 |
| K-05 | 5´-TCTGTCGAGG-3´ | 0 | 0 | 0 |
| K-10 | 5´-GTGCAACGTG-3´ | 0 | 0 | 0 |
| OPB-16 | 5´-TTTGCCCGGA-3´ | 0 | 0 | 0 |
| OPB-17 | 5´-AGGGAACGAG-3´ | 0 | 0 | 0 |
| Total | | 35 | 31 | |

## RAPD analysis

Randomly amplified polymorphic DNA profiles were generated by using 10 different 10-mers at the final concentration of 0.4 µM. PCR reactions were performed in 25 µL final volumes containing 15 ng of DNA template, 50 µM of each dNTP, 1x reaction buffer, and 2 mM $MgCl_2$, 1.2 units/reaction DNA *Taq*

Polymerases. A range of annealing temperatures varying from 36-45°C was tested before establishing the optimum amplification conditions. For each primer reaction, tubes containing all components except DNA were included as negative control.

Amplifications were carried out using Eppendorf Mastercycler gradient Thermal Cycler programmed for 35 cycles as follows: 1 min at 94°C, 1 min at 38°C, 2 min at 72°C, with an initial melting of 6 min at 94°C, and a final extension of 8 min at 72°C. The amplification products were separated by electrophoresis in the gel of 1.5% agarose in TAE (40 mM Tris-Acetate and 1 mM EDTA, pH 8.0), visualized after ethidium bromide staining and under UV illumination, and photographed with a Polaroid apparatus. The 100 bp DNA molecular marker (GIBCO-BRL) was used as a standard molecular weight marker.

**Data Analysis**

Polymorphic bands were identified using electrophoresis imaging. Only bands clearly polymorphic and reproducible were selected as markers. The bands were scored as present (1) or absent (0) by Gene Tools software. A similarity matrix was generated according to the coefficient of Jaccard [13]:

$sij = a/(a+b+c)$

Where sij=similarity coefficient; a=number of 1-1 matches; b=number of 1-0 matches and c=number of 0-1 matches. The data in the similarity matrix was used to perform a cluster analysis using the un-weighted pair-group method average with an arithmetic average (UPGMA) [14] obtained using the software NTSYS-pc (Numerical Taxonomy and Multivariate Analysis System version 1.80). After which a dendrogram indicating the calculated relatedness among garlic was constructed with the tree program of NTSYS-pc.

## Results

Electrophoresis of PCR products on 1.5% agarose gels revealed different degrees of polymorphism for different primers (Table 2). For example, primer OPJ-12 produced 6 bands that all of them were polymorphic (Fig. 1) and 8 out of 9 bands produced by primer K-15 were polymorphic. In OPA-01, 8 bands out of 9 were noted as polymorphic bands, where as in OPD-05, 7 out of 9 bands were polymorphic. The highest percentage of polymorphism was observed using OPJ-12 and K-20 (100%).

Grouping the ecotypes cluster analysis was carried out by the UPGMA method using Jaccard's genetic similarity coefficient. The position of the genotypes in different clusters is presented in Figure 2. The dendrogram constructed with UPGMA based on Jaccard's coefficient revealed that 22 ecotypes clustered into four distinct groups. The main cluster A included 3 ecotypes: Khaf, Tarom high, Langerud. Cluster B included 9 ecotypes: Tuyserkan, Shevarin, Gorgan, Dezful, Kalardasht, Tafresh, Lobnani-e-shiraz, Talesh dane dorosht and Alafi shiraz. Cluster C included 1 ecotype: Torbat-e-jam and finally Cluster D included 9 ecotypes: Qasre-e-shirin, Tarome dwarf, Taleqan, Barma, Talesh dane riz, Gonbad-e-kavus, Birjand, Behshahr and Khoram Abad.

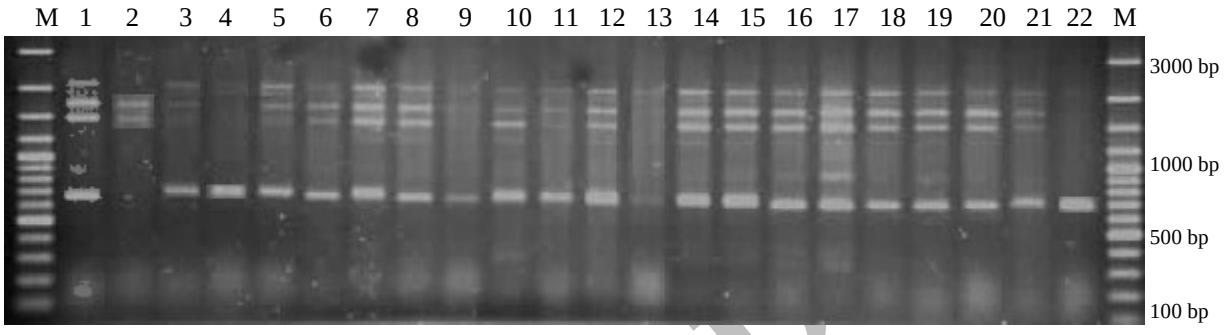


**Fig. 1: Electrophoretic pattern of PCR-amplified DNA products from genomic DNAs of 22 Iranian garlic ecotypes with primer OPJ-12. M. Lanes 1kb DNA ladder; 1-22 Garlic ecotypes**

## Discussion

The analysis of genetic diversity plays an important role in breeding programs. In this order, molecular techniques could be used to evaluate diversity between ecotypes. The RAPD technique could be effective in detecting the genetic variation in garlic. Garlic is a sterile species with variability in desirable morphological and physiological characteristics. However, the vegetative mode of multiplication of the species restricts breeding methods. A solution is sought in the use of domestic ecotypes, which are fully adapted to local conditions and are important genetic resources and initial breeding material (Gvozdanovic-Vagar et al., 2002). The preliminary analysis of the variability indicators in garlic ecotypes, originating form the main cultivation areas, showed similar and dissimilar properties among them. Isozyme and RAPD markers have been utilized to classify and categorize genetic diversity in garlic germplasms [5, 6, 7, 8, 9, 10].

In this study the numbers of amplified DNA fragments were different in primers and ecotypes. With 10 arbitrary 10-mer primers, 35 discrete fragments of DNA were amplified, with average of 3.5 DNA fragments per primer. Fragment sizes ranged from 0.5 to 3.0 kb, with a majority between 1.0 to 3.0 kb. The cluster analysis was shown as a dendrogram indicating the estimated relations between the garlic ecotypes. A dendrogram created by UPGMA, using 22 ecotypes, showed four main groups (Fig. 2). Twenty-one of the 22 ecotypes were included in cluster groups A, B, and D, while Torbat-e-Jam was presented in cluster group C. A large variation in allicin yield and botanical traits of Iranian garlic was recorded previously by Baghalian *et al.* (2005). Earlier Bradley *et al.* (1996) investigated 20 Australian garlic accessions using RAPD analysis resulting in 65 marker bands. The approach was well suited to group the major Australian cultivars according to bolting behavior, early and late types, and places of origin. They identified uniquely named ecotypes with identical genotypes. Using RAPD analyses, Bradley *et al.* (1996) also separated flowering and non flowering or incomplete flowering garlic ecotypes [7]. Maass and Klaas (1995) used RAPD analyses to further distinguish softneck types from the Caucasus and Mediterranean regions (incomplete bolting artichoke types) from softneck types in east and middle Asia [10].

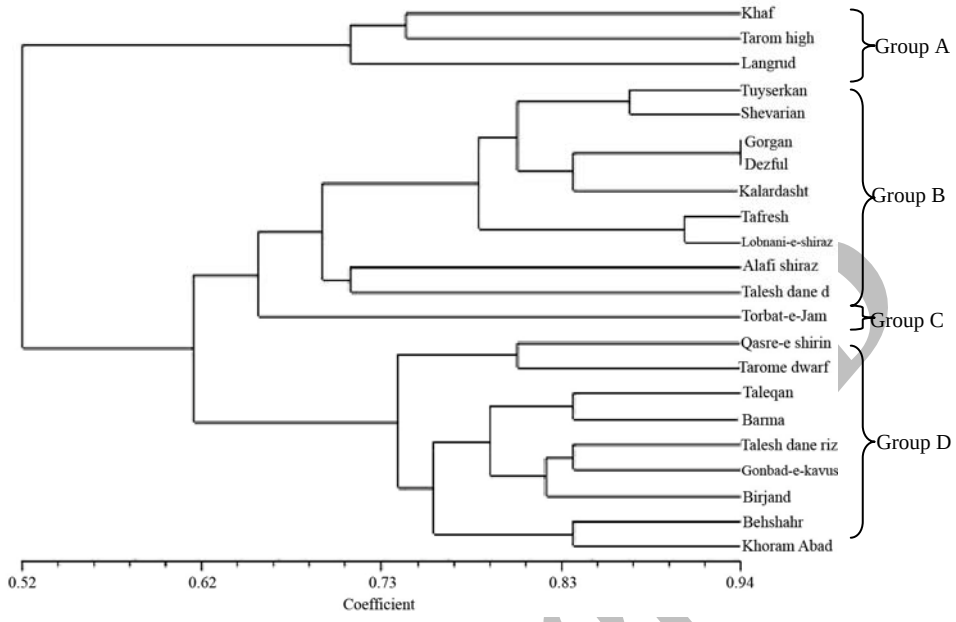


**Fig. 2: Dendrogram among 22 garlic ecotypes resulting from UPGMA cluster analysis based on Jaccard coefficient**

Al-Zahim *et al.* (1997) correlated morphological variation with RAPD patterns and could differentiate rocamboles and most softnecks from other hardnecks [6].

In general, we found that there was no relationship between genetic diversity and geographical origins, as ecotypes from one origin were clustered in more than one group. Ecotypes of groups were originated from different areas in the center, northeast and northwest of Iran. Ipek *et al.* (2003) and Pooler and Simon (1993) were unable to correlate the geographical origin of their samples to phylogenetic relationships [15, 16]. The diversity observed by collectors has occasionally led to the renaming of ecotypes as they are exchanged among growers and gardeners. Baghalian *et al.* (2005) believed that the classification of the ecotypes according to morphological and phytomedical characters does not correspond with the geographical grouping of Iranian garlic ecotypes [4]. It seems that this material has been exchanged among farmers of different regions. For example, Ecotypes No. 1 and 22 are clustered together (Fig. 2) even though the environmental conditions are sharply different. Using isozyme patterning, previous investigators [9] linked ecotypes to their points of origin, however this is disputed by other findings [5].

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