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THE USE OF RAPD MARKER FOR ASSESSING THE GENETIC DIVERSITY OF *BUNIUM PERSICU*M (BOISS.) B. FEDTSCH POPULATIONS

[1]Hoda Hashemi, [2]Abbas Safarnejad & [3]Abdolreza Bagheri
[1]Islamic Azad University- Ghochan Branch, Khorasan, Iran;
[*2]Khorasan Aagricultural and Natural Resources Research Center,P.O. Box: 91735/1148, Mashhad, Iran;
[3]Ferdowsi University of Mashad, Khorasan, Iran.

ABSTRACT
Persian Zira (*Bunium persicum*) is one of the most important medicinal plants. RAPD technology was used to study the genetic relationships amongst 20 wild Persian Zira populations. We examined 15 populations from Iran, 2 populations from India, 2 populations from Afghanistan, and 1 population from Europe. This is the first investigational report on DNA extraction and molecular variation of Persian Zira. DNA was extracted using new modified CTAB protocol. In RAPD experiments, 26 primers were used and all of them produced polymorphism. A total of 146 reproducible amplified bands were scored and 96% (141 bands) percentages of polymorphic bands (*PPB*) was found. Data were analyzed with NTSYS and POPGENE programs. The MXCOMP module was then used to compute the cophenetic correlation. The matrix of generated similarities was analyzed by the UPGMA, using the SAHN clustering module and the dandrogram was drawn. Cluster analysis of similarity data, grouping of the landraces were studied according to their geographic origin. The genetic similarity ranged from 0.37 to 0.95. As anticipated, European population was separated from the rest of populations. And two distinct subgroups were formed at 0.50 similarity for these 19 populations (Non-European populations). One subgroup consisted of Iranian populations, and the second, non-Iranian populations, which contained two populations from Afghanistan and two populations from India. At a higher level of similarity (0.70), Iranian group was divided in two clusters and the populations of the each province were classified in the same group. Furthermore, grouping by morphological characteristics was compared with the results from RAPD analysis, and a significant correlation was produced. Finally, the investigation of the genetic variation of this plant with RAPD indicated that this marker is suitable tool for assessing polymorphic loci and estimating the genetic distance.

KEY WORDS: *Bunium persicum*, Genetic diversity, RAPD, Medicinal plant, Persian Zira.

INTRODUCTION

Persian Zira (syn: Black Zira, Kerman Zira), *Bunium persicum* (Boiss.) B. Fedtsch, is a herbaceous perennial, self-pollinated, diploid (2n=2x=14) plant that belongs to the Apiaceae family. This plant grows extensively in Iran, Afghanistan, Pakistan, India and Mediterranean countries. In Iran, these self-growing plants are found in different altitudes. In North East of Iran (Khorasan, Khajee region) it grows in 920m altitude while they are also found in 2590m (Table1) in East of Iran, Kerman- Joopar region (Asgarzadeh *et al*, 2005).

Because of its pleasant mild aroma and taste, this plant use as a desire condiment cum medicine use. Several therapeutic effects including digestive disorders, urinary tract disorders, anti convulsion, antiasthma and also antimicrobial and antifungal effects have been confirmed in the *B. persicum* (Boskabady *et al*, 2004). In addition, there is no report regarding any side effects of this plant or its extract (Sardari, 1998).

For breeding and commercialization of promising Persian Zira populations a precise determination and evaluation of the populations is required. The first step for molecular researches is extraction the high quality DNA. The DNA isolation from tissue needs to be simple, rapid, inexpensive and effective when many samples are used in population studies. No paper has ever been published about its DNA isolation or/and using of molecular marker in this genus and that few Apiaceae have been evaluated for intrapopulation molecular variation. But morphological diversity (i.e seed yield, essence yield, seed length, number of established seedlings and 1000-seed weight) of some Persian Zira populations has been evaluated by Askarzadeh and his colleagues (2005). A wide range of variation has been reported by the authors and because all samples of seed populations were planted in the same environmental conditions, probably differences in morphological and yield investigations are due to their genetic profiles (Askarzadeh *et al*, 2005). Due to the effects of environmental factors on these investigations, their result can be ambiguous. Therefore, markers that are independent from the environment are necessary for reliable identification and discrimination of populations.

DNA markers are independent from environmental interactions, unlimited in number and show high level of polymorphism. Therefore, they are considered invaluable tools for determining genetic relationships/diversity. Various types of DNA markers are now used like RFLPs (Sambrook *et al*, 1989), RAPDs (Williams *et al*, 1990),

SSRs (Zietkiewcz *et al*, 1994), AFLPs (Vos *et al*, 1995) and ISSRs (Provostand and Wilkinson, 1999).

RAPD markers gained importance due to their simplicity, efficiency, the relative ease to perform the assay and non-requirement of DNA sequence information (Khanuja *et al*, 1999). They have been used in studies of genetic diversity among populations (Gustin, 2002; Chengxin, 2003), phylogeny and systematic (Sun *et al*, 1998), genetic linkage mapping (Cheung *et al*, 1997), and gene tagging (Tiwari *et al*, 1998). Efficiency of RAPD has been reported to be the same as AFLP markers (Ipek *et al*, 2003; Wen *et al*, 2004), SSR markers (Zahuang *et al*, 2004) and ISSR markers (Martins *et al*, 2003) in genetic diversity studies.

Here we describe a modified DNA isolation protocol that can be used for molecular biology applications in various medicinal plants. The isolated DNA proved amenable to PCR amplification. And also genetic diversity of 20 wild populations of Persian Zira was studied by using RAPD markers.

MATERIAL AND METHODS

Plant material

Seeds were collected from Agricultural and Natural Resource Research Center of Khorasan. By using common propagation method, the seeds of 20 wild populations (Table 1) were planted on B5 medium without hormones (Valizadeh *et al*, 2007) and they were sterificated at 4°C for 3 months to break the seed dormancy and germinate. After that, they were transferred to the culture room, at 25±2°C under 16 h photoperiod (30 µmols m^{-2} s^{-1}).

Table 1

DNA extraction

In order to evaluate the genetic variation and minimize the error rate, by using the CTAB protocol genomic DNA was extracted from a mixture of 40 plantlets of each wild population, to find possible range of bands on the gel. The extraction buffer contained; 100 mM Tris-Cl (pH 8), 25 mM EDTA(pH 8), 1.5 M NaCl and CTAB(2% [w/v]); however, 0.2% β- mercaptoethanol, 2% PVP and 0.06% sodium solfite were added just before use. In addition, some Washing buffers (76% ethanol + 10 mM ammonium acetate, and 76% ethanol +0.2 M sodium acetate) were used instead of 76% ethanol for washing and resuspending the DNA. Furthermore, precipitating the DNA pellet was performed by ice-cold isopropanol + 7.5M ammonium acetate, overnight at 25°C, instead of -20°C. After resuspending the DNA pellet in TE buffer, equal volume of phenol-chloroform-IAA (25:24:1) was added, then they were centrifuged and supernatant was transferred into new tubes. Cold washing buffer (76% ethanol and 0.2 M sodium acetate) was used to precipitate/wash DNA. Finally, after centrifuging, DNA was again dissolved in TE buffer. The purity and quantity of genomic DNA was determined spectrophotometrically and confirmed using 0.8% agarose gel electrophoresis against known concentrations of unrestricted lambda DNA.

Amplification reactions

26 RAPD's primers were used for PCR amplification. Polymerase chain reactions were performed in a total volume of 25 ml containing 50 ng template DNA, 1_ PCR buffer, 2 mM MgCl2, 200 mM dNTPs (CinnaGen Co, Iran), 0.2 mM of a single decamer primer and 1 U Taq DNA polymerase (CinnaGen Co, Iran). Error was minimized by making one large batch (master mix) of all reagents (except template DNA) for each primer. The reactions were performed in a thermocycler (iCycler, Bio Rad Co, USA) programmed as follows: 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 35° C for 30sec, 72°C for 2 min and a final extension at 72°C for 8 min. Amplified products were separated by 1.5% agarose (Roche Co, Germany) gel electrophoresis in TBE buffer. PCR products were visualized by ethidium bromide staining and photographed under UV light, by a Gel Doc system (UVP, Bio Doc Co, USA).

Data processing

Each gel was analyzed by scoring the present (1) or absent (0) polymorphic bands in individual lanes. The NTSYS-pc software ver. 2.02 (Roholf, 1998) was used to estimate genetic similarities with the Jaccard's coefficient. The matrix of generated similarities was analyzed by the unweighted pairgroup method with arithmetic average (UPGMA), using the SAHN clustering module. The cophenetic module was applied to compute a cophenetic value matrix using the UPGMA matrix. The MXCOMP module was then used to compute the cophenetic correlation, i.e, to test the goodness of fit of the cluster analysis to the similarity matrix.

RESULTS AND DISCUSSION

This is the first study conducted on DNA isolation and molecular variation of Persian Zira. In preliminary results showed that seed culture on B5 medium was a simple and quick method to germinate suitable seedling for propagation of this plant in laboratory conditions. Published methods of DNA isolation including those of Dellaporta *et al*. (1983), Doyle and Doyle (1987) and Porebski *et al*. (1997) proved unsuccessful and unreliable for *B. persicum* as the DNA obtained was dirty brown in appearance and with high viscosity. This may be due to high endogenous levels of secondary metabolites that interfere with DNA isolation and purification.

DNA contaminated with secondary compounds and carbohydrates is especially difficult to amplify by PCR. Hence we modified the original Doyle and Doyle protocol by precipitating the DNA in (isopropanol + acetate ammonium) and (absolute ethanol + accetate sodium), increasing washing steps, using some washing buffers, modifying extraction buffer and adding a phenol : chloroform : isoamyl alcohol step. During homogenization, polyphenols are released from vacuoles and they then react rapidly with cytoplasmic enzymes.

The addition of high concentration of PVP and β-mercaptoethanol were helpful to remove tannins and other polyphenolics due to preventing their interaction with DNA and sodium sulfite inhibits oxidation of polyphenols (Aljanabi *et al*, 1999). Beside, NaCl concentration of 1.5M was effective in removing polysaccharides.

Precipitation at room temperature indeed led to a clear reduction of coprecipitation of peptides, aromatic compounds, and polysaccharide and/or phenolic complexes during precipitation (Michiels *et al*, 2003). We found the combined use of ammonium acetate with isopropanol during the precipitation of DNA efficient in

removing most of the secondary metabolites and polysaccharides from the DNA.

For RAPD molecular analysis, the fresh leaves tissue was used as a plant material for all populations. In preliminary experiments of RAPD reproducibility, it was found that the concentration of template DNA was crucial to obtain the maximum number of reproducible bands. Varying the concentration of template DNA (20, 30, 40, 50 and 60 ng) revealed that 50 ng resulted to the maximum number of reproducible bands, and therefore 50 ng was used in all subsequent PCR reactions. These 26 primers produced 146 DNA fragments in all populations; among them 141 DNA fragments showed polymorphism. The average of total bands and percent of polymorphic bands (PPB), are showed in table2. Depends on used primers, band numbers ranged from 2 to 11. The highest polymorphism was observed by primer OPF16 with 5' TTC GAC ACG C 3' sequence (Figure1).The size of amplified fragments ranged between 100 and 4500bp for all primers. Nei's (1987) gene diversity and Shannon's Information index were calculated 0.34 and 0.52, respectively. The cophentic correlation coefficient indicated a correlation of r = 0.9 between the similarity matrix and the UPGMA dendrogram, indicating a good representation of the relationships among the populations.

Table-2, Figure 1

The lowest and the highest similarities are showed in the similarity matrix (Tables3,4). Assessment of this matrix through SAHN cluster analysis produced the dendrogram shown in Figure3. The surprising and striking comparisons can be found in the dendrogram. As we expected, comparison of populations by cluster analysis (Figure3) and PCA (Figure2) showed that Europe Zira populations (N16, group II) comprised a single cluster and that was very distinct from other populations (group I), in which Iranian group (group Ia) was separated (Figure-2,3).

Table 3,4 & Figure 2,3.

In the UPGMA analysis, significant correlation was observed between geographic distance and genetic diversity .Within group I, two distinct subgroups were formed at 0.50 similarity units. One subgroup consisted of Iranian populations (group Ia), and the second subgroup, non-Iranian populations (group Ib), contained two populations from Afghanistan (N17, N18) with 0.87 similarity unit, and two populations from India (N19, N20) with 0.95 similarity unit, which was the maximum similarity among all of the populations. A clear molecular similarity between samples belonging to India and Afghanistan has been found.

In the Iranian populations, more geographically closer populations had higher correlation values (Table-3). Iranian populations (group Ia), were separated in two main subgroups, at a similarity level of 0.70. In fact, grouping the Iranian populations by using RAPD markers resulted in separating Siahkooh population (N8) which is originally from Markazi province, in comparison with the rest of Iranian experimental populations. Although the main reason of this great divergence is not known, it is supposed to be a result of a considerable intrapopulation variation in the N8. Such a marker based genetic divergence could be potentially a good resource of agricultural significance in the breeding programs, subsequently.

The other 14 Iranian populations were grouped into two main clusters, Cluster I, contained N1, N7, N6, N9, N10, 11 and N12, Cluster II, consisted of N2, N3, N4, N5, N13, N14 and N15. Within Cluster I, two subclusters were separated, one consisting of N1, N7, N6, N9, and another containing N11 and N12 which were closely related to N10. These three populations were from Kerman province; also within Cluster II, two subclusters were separated, one consisting of N2, N3 (both of from Isfahan province) and another containing N4, N5, N13, N14 and N15.

Thus, there was a clear clustering pattern of geographically closer populations in the present study indicating that the association between genetic similarity and geographical distance was significant.

In other words, the RAPD markers revealed geographically concordant groupings. For instance the populations of the each province were classified in the same group, and also some populations like N1 and N7 which grow in adjacent provinces in South of Iran, were grouped together (Figure-3).

Clustering results in the present study was consistent with the relevant study (Asgarzadeh *et al*, 2005) on some morphological traits such as yield and yield components, seed yield, amount of seed essence, and…. which were studied by Asgarzadeh et al (2005). For instants, in our study the Lakhese Mehriz (N9) and Joopar (N11) populations showed high similarity (0.8) in dendrogram (Figure3), and also in pervious study which had been performed by Asgarzadeh and his colleague(2005) these two populations had the maximum similarity in seed yield. In addition, the Joopar (N11) and Khaje (N15) populations had low similarity (<0.7) in Figure3, and they had also revealed the highest differences in yield and yield components Asgarzadeh's research (2005). Moreover, according to Figure3, the Siahkooh (N8) and Khaje (N15) populations showed the lowest similarity (0.65), and also they had previously shown the highest differences ($p \leq 0.01$) in average of seed-essence percentage in Asgarzadeh's investigations (2005). Furthermore, the Zar (N4) and Khaje (N15) populations, which had the low similarity (<0.75) in dendrogram (Figure3), also had illustrated variation in some traits -like the seed yield, the number of established plants and the amount of seed essence- which were evaluated by Asgarzadeh and his colleagues (2005).

Clustering of all Iranian experimental populations indicates that they may share similar germplasms. Whether or not they bring substantially different genetics to a breeding program it needs to be further investigated. Certainly, the environmental conditions under which they were grown differ considerably, and the mild selection pressure used in their development may have selected them for different traits.

We showed a relatively good polymorphism among Persian Zira populations in Iran, however, we suggested that use of other biochemical and molecular markers could supply complementary useful information. In general, RAPDs were useful for identification of Persian Zira and, compared with the simple analysis of morphological characteristics, gave much more information about the genetic background of Persian Zira populations. RAPD analysis also has certain limitations. The low

reproducibility of RAPDs introduces a problem for their use in cultivar identification. Moreover, it is worth noting that putatively similar bands originating from RAPDs in different individuals are not necessarily homologous although they may share the same size in base pairs. This situation may lead to error when calculating genetic relationships. However, the ease of RAPD analysis, which only requires PCR technology, offers advantages for high throughput breeding programs.

In conclusion, it is difficult it presume extraction of DNA from *B. persicum* due to the presence of large amount of secondary metabolites and polyphenols. But we have established a reliable method for isolation high-quality DNA free of polysaccharides and secondary metabolites, and it could be adopted as a standard method for the medical plant for isolation DNA for molecular biological studies.

In addition, the results demonstrate the utility of using RAPD markers to detect relationships among populations from Iran, India, Afghanistan and Europe. Also, this technique evaluated germplasm diversity in *B. persicum*, to identify potential sources of unique germplasm material. Mass selection and Pure-line selection are two important methods for breeding of self pollination plants without hybridization and its efficiency depends on range of genetic variation. With respect to obtained variation among seed populations under study, and also because Persian Zira is a self-pollination plant, in future researches on the basis of results obtained from these data, in order to breed native plants, these two selection methods will be utilized.

Furthermore, the information result can be used as guidelines for improved germplasm collection and breeding. The results reported here also support relationships predicted by using traditional morphologic evaluation. Also, RAPD data obtained for these populations are strongly concordant with the regional geographic relationships. Taking this into considerations, as well as the small number of existing populations in *B. persicum*, efforts should be made to protect the populations and to collect seeds in all of them.

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Table1: Populations used as source of DNA

Population No	(origin) Country-Province-location	Altitude (m)	Population No	(origin) Country-Province-location	Altitude (m)
N 1	South of Iran- Fars-Shiraz	1860	**N 11**	East of Iran- Kerman-Joopar	2590
N 2	Center of Iran-Isfahan- Khonch	1000	**N 12**	East of Iran- Kerman-Bardsiir	2500
N 3	Center of Iran-Isfahan- Jandagh	950	**N 13**	North East of Iran-Khorasan- Chenarn	1680
N 4	North of Iran-Semnan- Damghan	1400	**N 14**	North East of Iran-Khorasan- Dargaz	1300
N 5	North East of Iran -Khorasan- Bojnoord	1250	**N 15**	North East of Iran-Khorasan- Khaje	920
N 6	Center of Iran -Ghazvin- Alamoot	1500	**N 16**	Europe*	
N 7	South of Iran -Hormozgan- Ganoo	2050	**N 17**	Afghanistan 1*	
N 8	West of Iran-Makkazi- Siahkooh	1100	**N 18**	Afghanistan 2*	
N 9	Center of Iran- Yazd-Mehriz	1040	**N 19**	India 1*	
N 10	East of Iran- Kerman-Sirch	2300	**N 20**	India 2*	

*Means samples have been sent from these countries

Table-2 Analysis of the Polymorphic amplified bands with 26 random primers

Bands Populations (Numbers of populations)	Number of Total Bands (percent per primers)	Number of Polymorphic Bands (percent per primers)	Percent of Polymorphic Bands (*PPB*)
All Populations (20)	146 (5.61%)	141 (5.42%)	96.5%
Group I* (19)	139 (5.34%)	122 (4.69%)	87.7%
Iranian Group, Ia* (15)	128 (4.92%)	89 (3.42%)	69.1%

*Groups referred to those are in Fig 3.

Table3: Similarity matrix for 20 Persian Zira populations

	N1	N2	N3	N4	N5	N6	N7	N8	N9	N10	N11	N12	N13	N14	N15	N16	N17	N18	N19	N20
N1	1																			
N2	0.72	1																		
N3	0.73	0.85	1																	
N4	0.77	0.74	0.78	1																
N5	0.73	0.72	0.74	0.82	1															
N6	0.82	0.72	0.75	0.77	0.8	1														
N7	0.82	0.72	0.74	0.73	0.71	0.78	1													
N8	0.70	0.70	0.70	0.67	0.68	0.72	0.70	1												
N9	0.78	0.72	0.79	0.76	0.79	0.80	0.77	0.79	1											
N10	0.72	0.70	0.77	0.73	0.75	0.78	0.74	0.70	0.80	1										
N11	0.73	0.70	0.75	0.75	0.77	0.74	0.74	0.71	0.80	0.91	1									
N12	0.71	0.67	0.73	0.72	0.73	0.73	0.72	0.67	0.78	0.89	0.93	1								
N13	0.72	0.77	0.80	0.77	0.82	0.78	0.72	0.70	0.80	0.78	0.77	0.76	1							
N14	0.70	0.75	0.78	0.77	0.84	0.75	0.68	0.71	0.76	0.75	0.77	0.72	0.88	1						
N15	0.71	0.74	0.72	0.74	0.80	0.74	0.67	0.65	0.75	0.72	0.69	0.68	0.77	0.77	1					
N16	0.42	0.48	0.43	0.41	0.41	0.39	0.43	0.43	0.42	0.44	0.43	0.41	0.40	0.40	0.39	1				
N17	0.52	0.56	0.60	0.55	0.59	0.60	0.54	0.53	0.61	0.56	0.53	0.53	0.54	0.56	0.56	0.45	1			
N18	0.51	0.56	0.58	0.53	0.57	0.62	0.54	0.46	0.59	0.53	0.50	0.51	0.54	0.53	0.56	0.43	0.87	1		
N19	0.44	0.44	0.43	0.46	0.46	0.45	0.43	0.38	0.43	0.43	0.42	0.44	0.42	0.43	0.49	0.37	0.51	0.58	1	
N20	0.45	0.46	0.47	0.45	0.49	0.46	0.44	0.41	0.43	0.44	0.43	0.43	0.44	0.47	0.50	0.38	0.55	0.59	0.95	1

T**able4**: Analysis of the polymorphic amplified bands with 26 random primers

Similarity Group (Number of populations)	Maximum	Minimum
All samples (20)	Between N19 and N20 (0.95)	Between N16 and N19 (0.37)
Iranian Group, Ia (15)	Between N11 and N12 (0.93)	Between N8 and N15 (0.65)
Non-Iranian Group, Ib (4)	Between N19 and N20 (0.95)	Between N16 and N19 (0.37)
Compare between Ia and Ib groups (19)	Between N6 and N18 (0.62)	Between N8 and N19 (0.38)

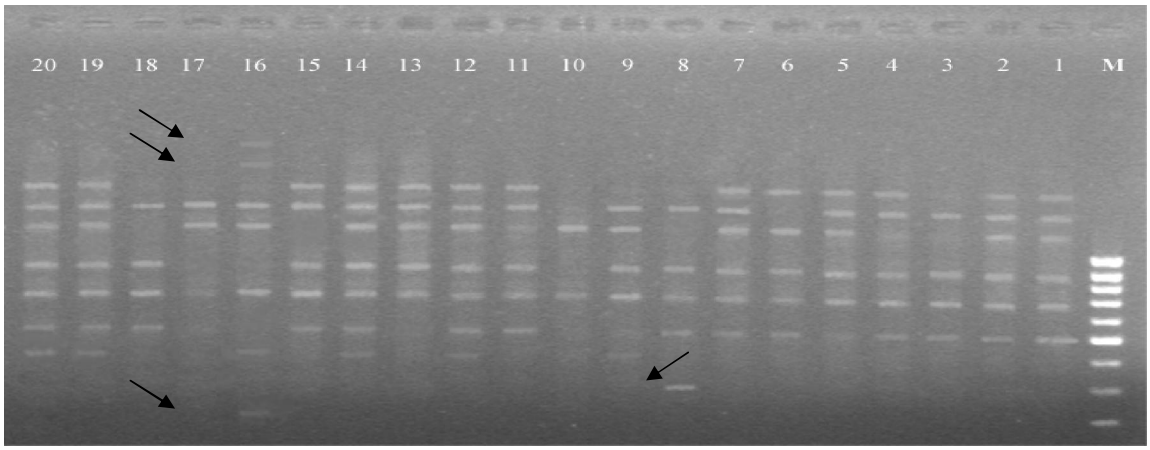

Figure1: PCR profile of the *Bunium persicum* amplified with OPF16 primer (5' TTC GAC ACG C 3') on 2% agarose gel. M; DNA ladder. Unique bands are shown by pointers.

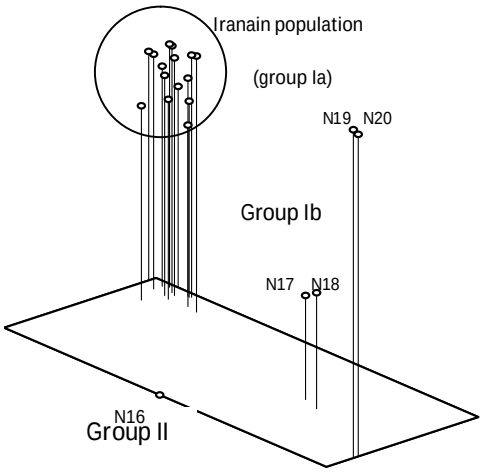

Figure2: Three-dimensional representation of NTSYS based PCA, among 20 populations.

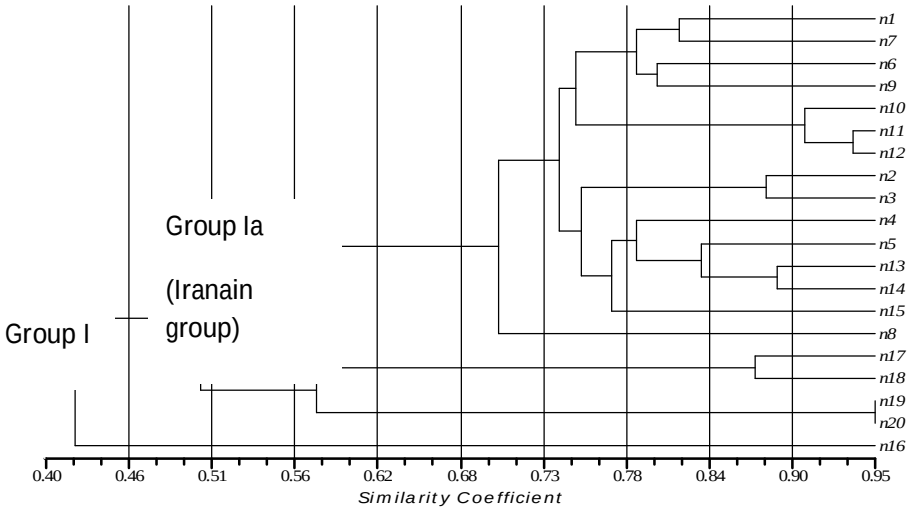

Figure3: UPGMA dendrogram (nested clusters) of 20 Persian Zira (*Bunium persicum*) populations, showing their possible relationships.