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Araştırma Makalesi/*Research Article (Original Paper)*

The Determination of Moleculer Diversity Among Some Alfalfa (*Medicago sativa* L.) Ecotypes Using RAPD Markers

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Abstract: The relationships among total 76 ecotypes of alfalfa (*Medicago sativa* L.), 70 landraces and 6 cultivars, collected in Van and neighboring provinces were investigated. The experiment was established as an augmented design in 2009. In the molecular method, seventeen RAPD primers were used and 106 polymorphic bands were obtained. The genetic distances between the ecotypes were expressed by Euclidean coefficients. The genetic variation among alfalfa ecotypes was examined in 16 groups based on the localities, landraces and check cultivars. The highest genetic variations and polymorphisms were found in Ercis and Gurpinar localities H = 0.179, I = 0.277, H = 0.173, I = 0.267 and 62.26%, 59.43%, respectively. As a result, high genetic diversity was found out among the ecotypes and cultivars of allogamous *Medicago sativa* L.

Keywords: Alfalfa, Diversity*, Medicago sativa*, RAPD

Bazı Yonca (*Medicago sativa* L.) Ekotiplerindeki Moleküler Farklılıkların RAPD İşaretleyicileri Kullanılarak Belirlenmesi

Özet: Van ili ve çevre illerden toplanan 70 adet yonca (*Medicago sativa* L.) ekotipi ile 6 tescilli çeşit olmak üzere toplam 76 genotip arasındaki akrabalık ilişkilerinin belirlenmesi çalışması yürütülmüştür. Deneme augmented deseninde 2009 yılında kurulmuştur. Moleküler yöntem ile on yedi adet RAPD primerleri kullanılmış ve 106 polimorfik bant elde edilmiştir. Ekotipler arasındaki genetik uzaklıklar Öklid katsayısı yardımıyla belirlenmiştir. Yonca ekotiplerinin toplandığı bölgelere göre, ayrıca yerel çeşit olarak ve tescilli çeşitler olmak üzere toplam 16 grup altında genetik varyasyonu incelenmiştir. En yüksek genetik varyasyon, ekotip sayısı fazla olan Erciş ve Gürpınar bölgelerindeki ekotipler içerisinde sırasıyla H = 0.179, I = 0.277, H = 0.173, I = 0.267 ve polimorfizm %62.26, %59.43 olarak bulunmuştur. Sonuçta yabancı döllenen *Medicago sativa*'nın ekotip ve çeşitler arasında yüksek genetik çeşitliliğe sahip olduğu belirlenmiştir.

Anahtar kelimeler: Yonca, Çeşitlilik, *Medicago sativa*, RAPD

Introduction

Cultivated alfalfa (*Medicago sativa* L.) is one of the most important forage legumes, being an auto-tetraploid, outcrossing and perennial species (Crochemore et al. 1996). Alfalfa includes eight digestive enzymes facilitating of digestion of fat, protein and carbohydrate, and has very important functions such as removal of toxic substances from the body and struggle against infections. Additionally, in animals fed with alfalfa, there has a low level of LDL (Low Density Lipoprotein) and VLDL (Very Low Density Lipoprotein) (Demir et al. 2006). Alfalfa is originated in the Caucasus, northeastern Turkey, Northwestern Iran and Turkmenistan, and it is cultivated on over 32 million hectares worldwide (Michaud et al. 1988). Yields and other characteristics of alfalfa are greatly affected by ecological conditions. Therefore, for the cultivation of alfalfa, suitable varieties and genotypes should be selected according to various regions (Avcıoglu and Soya 1977). Complex members of *Medicago* species shows high morphological variations of reasons such as hybridization, breeding and polyploidy. As a result of the systematic with only morphological data systematic status of the unit complex has not been clearly demonstrated (Şakiroğlu et al. 2011). Several molecular methods have been used to determine the

relationship and differences among *Medicago sativa* L. (Touil et al. 2008). Considering the laboratory facilities, RAPD, SSR and ISSR are the methods which comfortably carry out researches with limited conditions due to the lack of use of radioactive substances (Özcan et al. 2004).

RAPD (Random Amplified Polymorphic DNAs) is a method used to estimate certain relationships in alfalfa. (Yu and Pauls, 1993; Bonnin et al. 1996; Crochemore et al. 1996; Denghan-Shoar et al. 1997; Gherardi et al. 1998; Jenczewski et al.1999; Paredes et al.2002; Tucak et al. 2008).

With this study, relationships among total 76 ecotypes of alfalfa (*Medicago sativa* L.), 70 landraces and 6 cultivars, collected in Van and neighboring provinces were investigated in order to assist the future breeding programs.

Material and Methods

Alfalfa (*Medicago sativa* L.) genotypes (landraces) were collected in Central, Caldıran, Saray, Ozalp Muradiye, Ercis, Catak, Başkale, Gurpinar, Gevas counties of Van from 62 localities in a range of altitudes of 1680-2353 m. Four genotypes were from Bitlis province and two were from Siirt province, and two genotypes growing naturally were collected from University campus. Six cultivars (Planet, MA324, Kalender, Alsancak, Elci, and Bilensoy-80) were included in the experiment as check cultivars. These genotypes were cultivated by cultivator. A total of 76 genotypes were sown in an augmented design on the 3rd of April in 2009.

Table1. Information about alfalfa landraces

No	Province	Town	Village	Latitude	Longitude	Altitude (m)
1	Van	Ozalp	Aksurguc	38 49 12	43 59 09	2047
2	Van	Ozalp	Merkez	38 39 17	43 59 09	2008
3	Van	Merkez	Kıratlı	38 31 56	43 27 34	1959
4	Van	Merkez	Ercek	38 29 21	43 38 55	1823
5	Van	Merkez	Degirmenarkı	38 38 37	43 44 07	1922
6	Van	Merkez	Seed dealer	-	-	-
7	Van	Merkez	Seed dealer	-	-	-
8	Van	Caldıran	Yukarıyanıktaş	39 13 54	43 52 36	2203
9	Van	Caldıran	Doyumalan	39 03 36	43 53 19	2066
10	Van	Caldıran	Kurtoglan	39 04 09	43 53 20	2038
11	Van	Caldıran	Bogulukaynak	39 00 10	43 58 41	2046
12	Van	Caldıran	Salhane	39 00 15	43 57 24	2057
13	Van	Caldıran	Kılavuz	39 11 39	43 54 40	2091
14	Van	Caldıran	İncealan	39 02 49	43 54 15	2078
15	Van	Başkale	Albayrak	38 08 34	44 12 31	2069
16	Van	Başkale	Barış	38 01 13	43 59 05	2290
17	Van	Başkale	Merkez	38 02 33	44 01 19	2353
18	Van	Başkale	Yolmacayır	38 09 45	44 03 11	2252
19	Van	Muradiye	Merkez	38 59 43	43 45 59	1708
20	Van	Muradiye	Yenişehir mah.	38 59 50	43 45 35	1708
21	Van	Muradiye	Yumaklı	38 55 58	43 46 07	1724
22	Van	Muradiye	Yumaklı	38 55 52	43 46 14	1724
23	Van	Erciş	Taşlıcay	39 02 03	43 07 16	2042
24	Van	Erciş	Merkez	39 01 41	43 21 32	1693
25	Van	Erciş	Merkez	39 01 41	43 21 32	1693
26	Van	Erciş	Pay	39 06 17	43 30 43	1957
27	Van	Erciş	Keklikova	39 04 12	43 26 47	1918
28	Van	Erciş	Kozluca	38 59 46	43 32 13	1732
29	Van	Erciş	Karlıyayla	39 00 01	43 05 10	2051
30	Van	Erciş	Merkez	39 01 41	43 21 32	1693
31	Van	Erciş	Karlıyayla	39 00 01	43 05 10	2052
32	Van	Erciş	Taşevler	39 02 12	43 09 25	1980
33	Van	Erciş	Kocapınar	39 05 58	43 12 12	1777
34	Van	Erciş	Merkez	39 01 41	43 21 32	1693

Table1. Information about alfalfa landraces(continued)

No	Province	Town	Village	Latitude	Longitude	Altitude (m)
36	Van	Catak	Kayabogazı	38 08 17	43 10 59	2243
37	Van	Catak	Uzuntekne	38 09 28	43 04 51	2249
38	Van	Catak	Alacayar	38 01 33	43 09 33	1813
39	Van	Catak	Alacayar	38 01 33	43 09 33	1813
40	Van	Catak	Alacayar	38 01 33	43 09 33	1813
41	Van	Catak	Teknecik	38 08 30	43 12 37	2312
42	Van	Catak	Agaclık	38 09 29	43 11 27	2348
43	Van	Gevas	Yuva	38 19 35	42 54 25	1820
44	Van	Gevas	Merkez	38 17 45	43 06 27	1689
45	Van	Gevas	Kocak	38 15 59	42 54 40	1883
46	Van	Gevas	Göründü	38 20 37	42 54 55	1680
47	Van	Gevas	Yemişlik	38 17 48	42 55 42	1789
48	Van	Gevas	Abalı	38 16 55	43 12 35	1837
49	Van	Gevas	Balaban	38 21 06	42 50 16	1687
50	Van	Saray	Caybagı	38 31 07	44 08 36	2299
51	Van	Saray	Degirmigol	38 35 09	44 09 25	2159
52	Van	Saray	Sırımlı	38 40 27	44 12 44	2159
53	Van	Saray	Degirmigol	38 35 09	44 09 25	2159
54	Van	Gurpinar	Merkez	38 19 36	43 24 44	1748
55	Van	Gurpinar	Yukarıkaymaz	38 19 09	43 24 06	1750
56	Van	Gurpinar	Koyunyatagı	38 19 25	43 23 29	1745
57	Van	Gurpinar	Degirmendüzü	38 19 33	43 24 22	1745
58	Van	Gurpinar	Bozyigit	38 23 14	43 34 19	1745
59	Van	Gurpinar	Degirmendüzü	38 19 33	43 24 22	1745
60	Van	Gurpinar	Sakalar	38 17 03	43 20 5	1758
61	Van	Gurpinar	Merkez	38 19 36	43 24 44	1748
62	Van	Gurpinar	Bozyigit	38 23 14	43 34 19	1919
63	Bitlis	Ahlat	Güzelsu	38 45 29	42 11 26	-
64	Bitlis	Ahlat	Güzelsu	38 45 29	42 11 26	-
65	Siirt	Merkez	Country Agr. Dep	37 55 29	41 56 33	-
66	Siirt	Merkez	Tarım İl Md.	37 55 29	41 56 33	
67	Van	Merkez	Campus	38 33 53	43 16 49	1665
68	Van	Merkez	Campüs	38 33 38	43 16 48	1670
69	Bitlis	Hizan	Hizan	38 13 27	42 25 35	-
70	Bitlis	Hizan	Hizan	38 13 27	42 25 35	-

Table 2. Information about alfalfa cultivars

No	Cultivar	No	Cultivar
71	Elci	**74**	Bilensoy-80
72	Alsancak	**75**	MA-324
73	Kalender	**76**	Planet

Genomic DNA was extracted from young leaves of each population (15 plants in each population) following the method by Doyle and Doyle (1987) with minor modification. DNA concentration was determined by spectrophotometer at 260 nm. The measurement of the OD 280 nm is used to define the content. The ratio OD260/OD280 were between 1.8 and 2.0 (Touil et al. 2008).

PCR reactions were performed in total volumes of 20 µl final amount. The total volume used each assay was 10X buffer (2.5 µl), 200 mM of each dNTP (2.0 µl), 50 mM $MgCl_2$ (0.75 µl), 5 mM of primer (1.0 µl), 1 U Taq polymerase (0.2 µl), 12.50 µl sterile water and 1 µl (50 ng) of DNA (Paredes et al.2002). Seventeen RAPD primers were selected from the previous studies on Medicago sativa L. (Denghan-Shoar et al. 1997; Gherardi et al. 1998; Mengoni et al. 2000; Paredes et al. 2002; Touil et al. 2008; Tucak et al. 2008; Petolescu and Nedelea, 2009). Amplification was performed in thermo-cycler starting with 4 min of denaturation at 92 C^o followed 35 cycle of 1 min at 92 C^o, 1 min at 36 C^o and 2 min at 72 C^o and final extension of 6 min at 72 C^o. The RAPD fragments were separated by 1.5% agarose-gel electrophoresis

with TAE 1X buffer at 90V for 3 hours and visualized with ethidium bromide. Gels were photographed under UV light to score band. Bands were analyzed in binary form for absence (0) or presence (1) (Sensoy et al. 2007; Ünverdi, 2007; Alınca, 2008; Tucak et al. 2008). Monomorphic bands were excluded from data analysis (Tucak et al. 2008).

Genetic distances between genotypes were determined with the help different coefficients of similarity index (Euclidean coefficient) and a dendrogram was created by NTSYSpc-2.02k package program by non-peer group mainly arithmetic mean method (UPGMA: Unweighted Pair Group Method with Arithmatic Mean) (Rohlf 1997).

Genetic variation between varieties and genotype of alfalfa were determined with used POPGENE package program according to the kinds of genotypes in populations separated by region (Yeh *et al.*1997; Labate, 2000).

Genetic diversity index and polymorphism rates of Nei (Nei, 1973) and Shannon (Shannon and Weaver, 1949) were determined with POPGENE program (Yeh et al. 1997). The genetic variation among alfalfa ecotypes was examined in 16 groups based on the localities, landraces and registered cultivars.

Results and Discussion

Genetic variation

Alfalfa genotypes were examined under the 16 groups according to collected regions, landraces and check cultivars. Taking into account the data of RAPD obtained from the statistical variation criteria, the differences were involved according to the regions. The high genetic diversity (H = 0.179 and I = 0.277) and polymorphism 62.26% were observed in the genotypes of Ercis. Genetic variation between Gurpinar genotypes had rather high values. Among the landrace genotypes and check cultivars were found as H = 0.243, I = 0.382 and polymorphism 99.06% and H = 0.171, I = 0.253 and polymorphism 47.17%, respectively. Genetic diversity of landraces was considerably higher than the check cultivars. Considering all genotypes and varieties H = 0.217, I = 0.352 and 100% polymorphism were determined (Table 4).

Table 3. Genetic variation between the groups as measured on the basis of Alfalfa landrace genotypes and check cultivars

Genotypes	N*	H	I	% Polymorphism
Van/Ozalp	2	0.090	0.131	21.70
Van/Merkez	5	0.138	0.206	38.68
Van/Caldıran	7	0.176	0.264	50.94
Van/Başkale	4	0.100	0.152	29.25
Van/Muradiye	4	0.125	0.185	33.96
Van/Ercis	13	0.179	0.277	62.26
Van/Catak	7	0.150	0.229	46.23
Van/Gevaş	7	0.184	0.276	54.72
Van/Saray	4	0.154	0.231	43.40
Van/Gurpınar	9	0.173	0.267	59.43
Bitlis/Ahlat	2	0.109	0.160	26.42
Siirt	2	0.082	0.120	19.81
Van/Campus	2	0.090	0.131	21.70
Bitlis/Hizan	2	0.066	0.097	16.04
Check cultivars	6	0.171	0.253	47.17
Landrace	70	0.243	0.382	99.06
Entire Genotypes	76	0.217	0.352	100

*N= Number of Genotype in Populations; H= Nei's genetic diversity index; I= Shannon's genetic diversity index

Degree of Relationship

A total of 156 bands were obtained with 106 polymorphic bands used as markers. The most polymorphic bands were obtained from the primer SD1 and the least obtained from the primer OPB6. The maximum

total number of bands was obtained from the primer RAPD35 and the least one was obtained from the primer RAPD1. The equal of number of bands, either polymorphic or monomorphic, were obtained from the primers OPB8, OPB10 with OPB11 and OPA8 with OPB7 and OPA11 with OPA19. Depending on the number of the plant and different species, the number of bands acquire as obtained less than those of Tucak et al. (2008) but more than those of Mengoni et al. (2000) who worked with the same primers on *Medicago sativa*.

Table 4. Nucleotide sequence of the primers used in RAPD analysis

Primer	Sequence (5'→3')	Number polymorphic bands	Number total band	Percentage of polymorphic bands (%)
RAPD1	CGTCTGCCCG	3	5	60.00
RAPD4	CTGGCGGCTG	7	9	77.77
RAPD8	GTGCGTCCTC	5	7	71.43
RAPD11	CAAACGGCAC	6	8	75.00
RAPD35	GGGCATCGGC	11	17	64.70
OPB11	GTAGACCCGT	5	8	62.50
RF1	GTAGCTGACG	6	7	85.71
OPB18	CCACAGCAGT	5	9	55.56
OPA8	GTGACGTAGG	4	8	50.00
OPB6	TGCTCTGCCC	3	9	33.33
OPB7	GGTGACGCAG	4	8	50.00
OPB8	GTCCACACGG	5	8	62.50
OPB10	CTGCTGGGAC	5	8	62.50
OPB13	TTCCCCCGCT	9	11	81.82
SD1	GGTACTCCAG	12	12	100.00
OPA11	CAATCGCCGT	8	11	72.72
OPA19	CAAACGTCGG	8	11	72.72
Total	17	106	156	67.95

Close resemblance was determined between the genotypes #19 and #20 of Muradiye district with the Euclidean coefficient of 3.16E+00. The most distant resemblance was determined with 6.56E+00 Euclidean coefficient between genotype #40 from Catak and Gurpinar genotype #58, and Alsancak cultivar. On average, the genotype having the highest and the lowest mean similarities were determined as the genotype #5 of Van (4.36E+00 Euclidean coefficient) and as the genotype #68 of Campus (#68 with 5.46E+00 Euclidean coefficient). After the genotypes #19 and #20 of Muradiye, the closest resemblance to each other within the regions was followed by the genotypes #16 and #18 of Baskale (3.32 E+00 Euclidean coefficient) and the genotypes #5 and #6 of Van (3,46 E+00 Euclidean coefficient). The remotest genotypes to each other in within the regions were followed by the genotypes #56 and #58 of Gurpinar (5.92 E+00 Euclidean coefficient), genotypes #27 and #33 of Ercis (5.57 E+00 Euclidean coefficient), the genotypes #8 and #13 of Caldıran and the genotypes #50 and #52 of Saray (5.48 E +00 Euclidean coefficient). The closest resemblance among the varieties was determined among the Alsancak, Kalender and Planet (4.24 E+00 Euclidean coefficient), but the remotest resemblance was between the Elci and the varieties of MA-324 (5,39 E+00 Euclidean coefficient). By the examination of the dendrogram obtained from Euclidean coefficient matrix, the genotype #52, #58, #64, # 68 and #33 showed a very different branching from the other genotypes possible were located in certain groups. In general, registered cultivars were close to each other but not completely, and did not gather around a group with any other landraces.

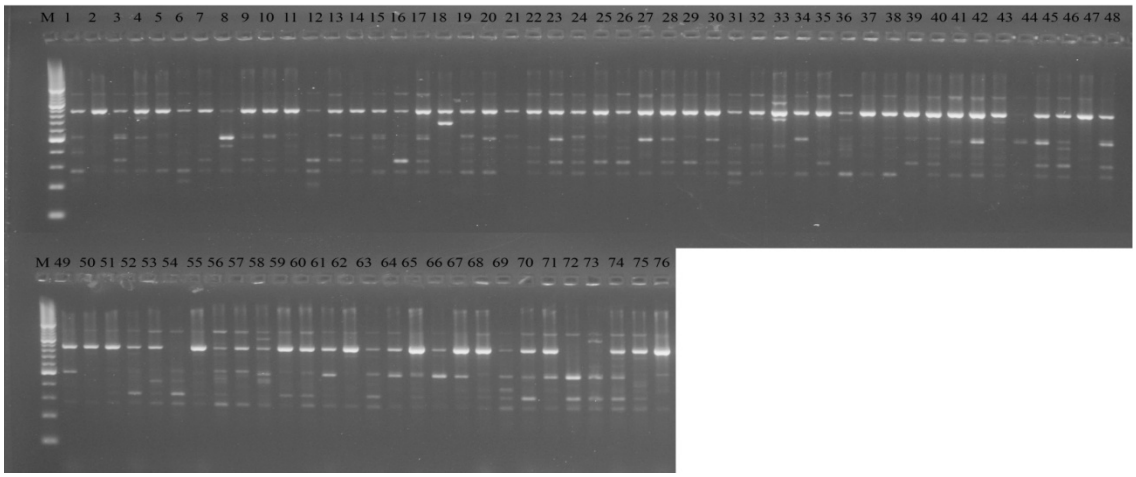

Figure 1. The band profile obtained from OPB13 RAPD primers.

RAPD markers could obtain relatively acceptable results to determine the relationship among the genotypes. It may be possible to reach to more satisfactorily results by using more samples and markers (Denghan-Shoar et al. 1997). Alfalfa is an allogamous and tetraploid species. It is concluded that genetic variation was very high among landraces and also among the varieties used. As reported by Gherardi et al. (1998) and Mengoni et al. (2000), it is considered that co-dominant marker systems and more than one marker systems will give more effective results of the calculation of genetic distance.

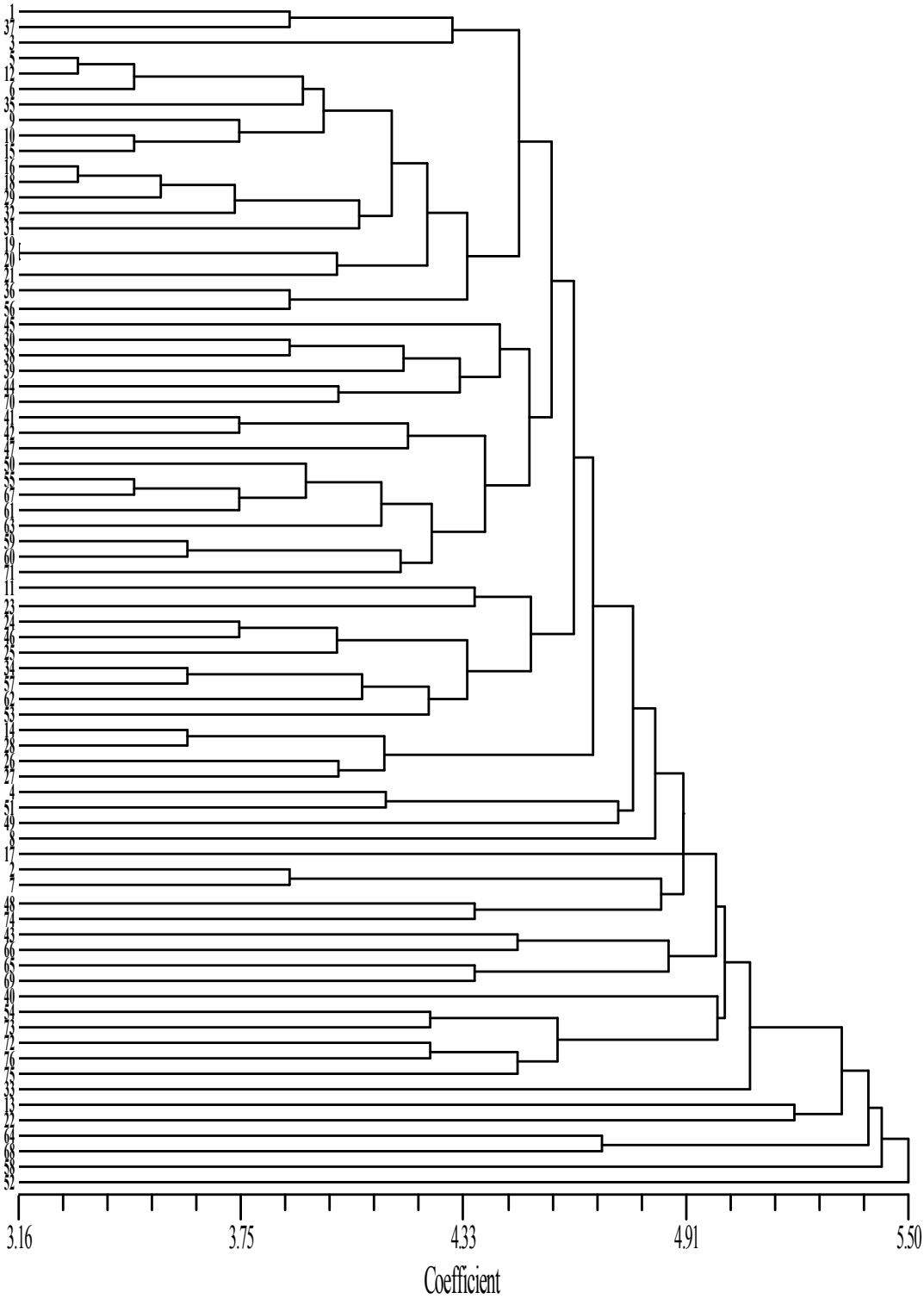

Figure 2. Dendrogram showing the relationship between 76 population, based on 17 RAPD primers.

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