African Journal of Biotechnology Vol. 11(15), pp. 3649-3655, 21 February, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.2169
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Genetic diversity in dill (*Anethum graveolens* L.) populations on the basis of morphological traits and molecular markers

M. Solouki[1], S. B. Hoseini[1], B. A. Siahsar[1] and A. Tavassoli[2]*

[1]Department of Agriculture, Agriculture Faculty, University of Zabol, Zabol, Iran.
[2]Department of Agriculture, Zahedan Branch, Islamic Azad University, Zahedan, Iran

Accepted 24 January, 2012

Dill (*Anethum graveolens* L.) is a green leafy medicinal and aromatic plant belonging to *Apiaceae* (*Umbelliferae*) family widely used as spice and medicine. In order to evaluate the genetic diversity in different landraces of dill based on morphological traits and molecular markers, the seeds of 37 accessions collected from different areas of Iran in addition to one European accession were cultured. Based on morphological traits assessment, the highest correlation coefficients were observed among the days to primary and secondary flowers which were 0.98. Cluster analysis classified the accessions of *A. graveolens* into four main groups. Cluster analysis framed by morphological traits was performed using Ward's cluster analysis and upon molecular markers the UPGMA and Jaccard's similarity coefficients was carried out in cluster analysis appeared by amplified fragement length polymorphism (AFLP) markers. Twenty 5-mer primers were used and 355 bands were detected with138 loci (39.8%) polymorphic. Principal coordinates analysis showed that 13 factors justified 69% of data variations. Mantel statistics revealed that the dendrogram was in conformity with similarity matrices (r=0.8). Results of cluster analysis showed that genetic diversity expressed by morphological traits and molecular markers did not completely correlate with geographical region.

Key words: Polymorphism, dill (*Anethum graveolens* L.), AFLP, morphological traits.

INTRODUCTION

Green leafy vegetables are good sources of minerals as well as vitamins. Dill (*Anethum graveolens* L.) a green leafy, widespread vegetable belongs to the family *Apiaceae* (*Umbelliferae*) that has an attractive flavor (Cankur et al., 2006). Dill originated from central Asia although wild and weedy types of dill are widespread in the Mediterranean basin and in West Asia (Zohary and Hopf, 2000). Dill classified as an aromatic vegetable has been widely used for flavoring numerous food products such as for soups and sauces and also flavoring salads and seafood (Lisiewska et al., 2006; Kmiecik et al., 2004). It has been reported that it is a possible source of antioxidant and also has anti-microbial properties against *Rhodotorula glutinis, Aspergillus ochraceus* and *Fusarium moniliforme* (Cankur et al., 2006; Nanasombata and Wimuttigosol, 2011).

The composition of dill essential oil changes markedly through the growing season. The characteristic of dill oil depends largely on the ratio of carvone and a-phellandrene (Callan et al., 2007).

Recently, molecular methods have been used for the identification and classification of different species of herbs and medicinal plants (Momeni et al., 2006; Solouki et al., 2008; Yu et al., 2011) and also evaluation of genetic diversity within and among species as well as plant populations (Raghu et al., 2007). These methods

*Corresponding author. E-mail: tavassoli_abolfazl@yahoo.com.

supported the classic methods such as morphological and physiological traits (Dolezalova et al., 2003) and reliable for genetic studies as environment does not affect them (Ali et al., 2007). Among DNA-based markers, the amplified fragment length polymorphisms (AFLPs) is a useful technique for breeders to hasten plant improvement for a variety of evaluation, by using molecular genetics maps and desirable cloning for special characters (Ali et al., 2007). Moreover, this method is a reliable molecular marker assay and the number of polymorphisms obtained per reaction is much higher than that revealed by restriction fragment linked polymorphisms (RFLP) or the PCR-based randomly amplified polymorphic DNA (Sharma et al., 2008). AFLP technique is established upon the selective amplification of restriction fragments obtained from the digestion of total genomic DNA. In this method, a large number of markers are easily and quickly available because, they are not depending on any prior knowledge about the genome. The resulting markers are dominant (Santos and Simon, 2002).

The purpose of the present study was to assess genetic diversity among landraces of dill (*Anethum graveolens* L.) using AFLP markers and morphological traits to take more information about genetic resources of this valuable species and accompany breeders for their selections

MATERIALS AND METHODS

Plant material

In order to evaluate the genetic diversity in different landraces of dill (*Anethum graveolens* L.) based on morphological traits and molecular markers, the seeds of 37 accessions collected from different areas of Iran in addition to one European accession, obtained from IARS (Iran-Isfahan Agriculture Research Station) were sown in the research field of Faculty of Agriculture and Natural Resources in Shirvan, Ferdowsi University, Iran (37°26˝ north and 57°45˝ east, 1067 m above sea level) during 2009. The field experiment was carried out in completely randomized blocks design with three replications. The seeds were sowed on three rows with 50 cm spacing and 15 cm distance between plants. The plots were irrigated every week. In order to estimate genetic diversity, 14 plants of central row of each plot were harvested.

Quantitative traits and essential oil

Twenty-two morphological traits were scored on the 38 dill landraces. To determine the essential oil, a sample of ground seeds (100 g) was mixed with 1000 ml of deionized water and it was distilled for 3 h using a Clevenger-type apparatus. The oil content was measured based on the volume of oil (ml) per 100 g dry seed.

All traits were subjected to analysis of variance (ANOVA) and correlation coefficient using SAS software (Version 9.1). The cluster analysis was performed using Ward's method based on squared Euclidean distances for standard variables using NTSYS pc software (Version 2.02) (Rohlf, 1998).

DNA extraction from leaves

Extraction of genomic DNA was done individually for 10 randomly selected plants from each accession. To optimize DNA extraction, 4 different methods, Doyle and Doyle (1990), Dellaporta et al. (1983) and modified method of Stein et al. (2001) were compared. Young leaves were ground with liquid nitrogen. Approximately 20 to 25 mg of leaf tissue powder from each plant was used for genomic DNA extraction. The extracted DNA was re-suspended in 100 µl of TE buffer and stored at -80°C. The concentration of the DNA was confirmed by 1% agarose electrophoresis for 30 min at 100 volts in 1x TBE and visualized under UV light after staining with ethidium bromide.

Amplified fragment length polymorphism (AFLP) profile

The genetic diversity of dills was evaluated using AFLP markers in Biotech Research Center, Faculty of Agriculture in Zabol University, Zabol, Iran. The genomic DNA (25 ng) was mixed with the restriction enzyme combinations *EcoR1* (50 mM) and *Mse1* (10 mM), tango buffer (10 X) and then, incubated for 3h at 37°C and 2 h at 65°C. The digested DNA fragments were then bound to *EcoR1-Mse1* adaptors with 1.5µl T4 DNA ligase (1unit/µl) for 2 h at 37°C and 2 h at 65°C. The ligated DNA template was diluted 3 times with TE buffer and stored at -20°C.

Pre-selective PCR reaction was performed in 25 µl reactions mixture with 3.75 (1:3) µl diluted ligation product using the protocol of Vos et al. (1995). The amplification was carried out in 25 µl reactions mixture containing 1x PCR buffer, 0.4 µmol/l of each primer, 2 mmol $MgCl_2$ and 0.2 mmol/l of dNTP mix and 1u/ml Taq DNA polymerase. The PCR was performed using an Eppendorf Cycler. It was set for 2 min at 94°C, 20 cycles of 30 s at 94°C, 1 min at 60°C, 1 min at 72°C, followed by a final extension of 2 min at 72°C and 15 min at 60°C. Amplification products were diluted in 100 µl deionized H_2O and 1 µl was used for selective amplification in a total volume of 20 µl containing 0.4 µM of each fluorescently labeled E- or M-specific primer extended by 3 selective nucleotides. Moreover, 2 mmol $MgCl_2$, 0.2 mmol/l of dNTP mix, and 1 u/ml of Taq DNA polymerase (Sinagen-Iran) were used. PCR was performed using a touchdown protocol with a initial denaturation of 2 min at 94°C, 10 cycles of 30 s at 94°C, 30 s at 63°C (decreasing the temperature by 1 after each cycle), and 2 min at 72°C, and 23 cycles of 30 s at 94°C, 30 s 54°C, 2 min at 72°C. The final PCR products were run on a 6% denaturing polyacrylamide gel in 1x TBE buffer. Silver-staining method was used for detecting AFLP markers.

Data analysis

The diversity analysis was carried out by scoring the bands as present (1) or absent (0) to form a raw data matrix. A square symmetric matrix of similarity was then obtained using Jaccard's similarity coefficient (Jaccard, 1908) to draw the dendrograms. The unweighed pair group method with arithmetic average (UPGMA) was used for cluster analysis using NTSYS pc software (Rohlf, 1998). Descriptive statistics and correlation coefficients analysis were done using Excel program. Factor analysis was performed to determine which trait contributed the highest variability. The correlation between the original similarity indices and cophenetic

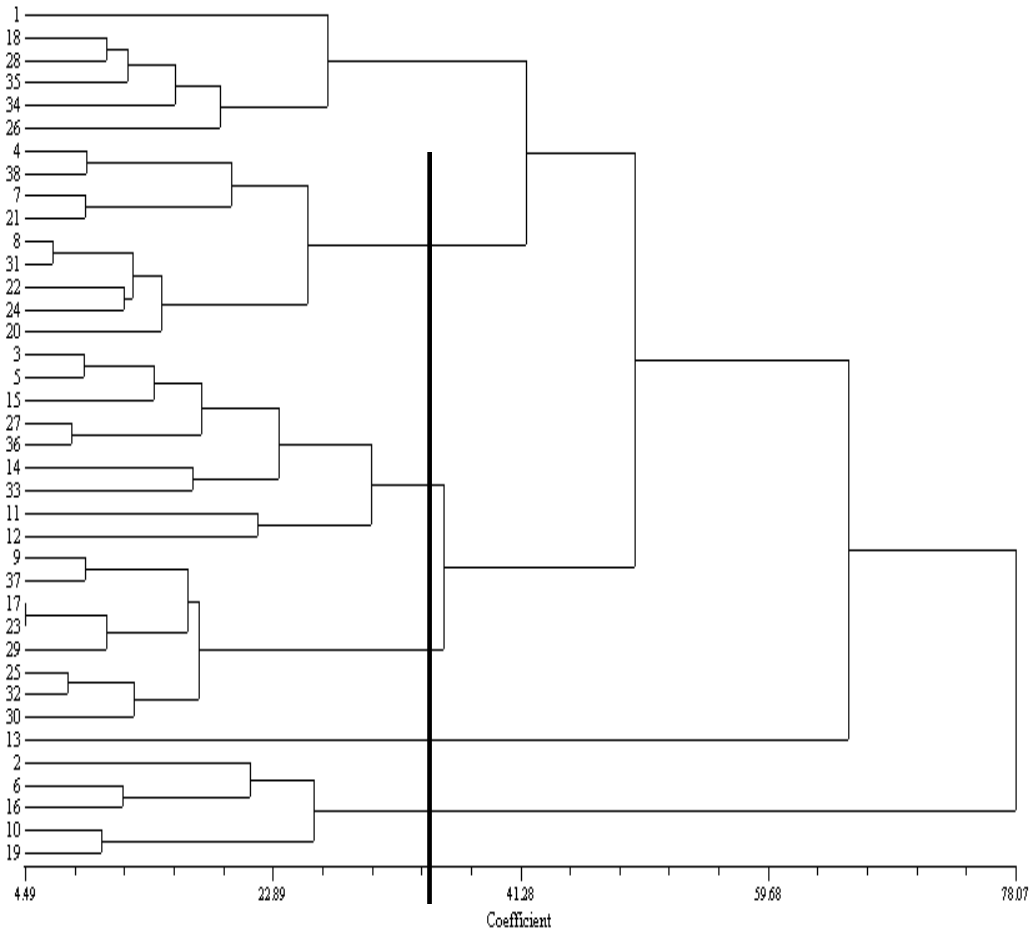

Figure 1. Dendrogram showing genetic dissimilarity among the dill accessions, using Ward's method based on morphological traits (1- Ghelyan-Shirvan, 2- Europein, 3- Shabestar, 4- Dorood-Lorestan, 5- Kermanshah, 6- Khoozestan-1, 7- Abade-1, 8- Baft, 9- Arak, 10- Birjand, 11- Esfahan-1, 12- Semnan-1, 13- Margh-Esfahan, 14- Bojnord-1, 15- Mohalat, 16- Kashmar, 17- Neyshaboor-1, 18- Shiraz-1, 19- Bojnord-2, 20- Gonabad, 21- Semnan-2, 22- Shiraz-2, 23- Shahrekord-2, 24- Najafabad, 25- Ajabshir, 26- Eslamabad-Esfahan, 27-Esfahan-2, 28- Ghoochan, 29- Yasooj, 30- Abade-2,31- Saman-Shahrekord, 32- Varamin-1, 33- Yazd, 34- Varamin-2, 35- Khoozestan-2, 36- Zoeram-Shirvan, 37- Neyshaboor-2, 38- Gheshm).

values, declared on each node of the dendrogram was computed by Mantel test statistics (Mantel, 1967) using the software package NTSYS pc software. Polymorphism information content (PIC) was performed using Excel 2003 software.

RESULTS

Morphological assessment

Assessment of 22 morphological traits using statistical analyses showed that the measured traits had significant differences at 5% probability level. The essential oil yield and height of first branch showed the maximum and phenological trait showed the minimum coefficient of variance. The European landrace with the average values of 2.88 ml per 100 g dry matters of seeds and Neyshaboor-2 with 1ml had the highest and lowest essential oil, respectively. The highest correlation coefficient was obtained between days to original and secondary flowers maturity with correlation more than 0.98. Step-wise regression showed that seed yield, essential oil percentage and height of first branch were entered the model, respectively by 98% of variation. The dendrogram obtained from morphological traits, classified 38 populations into 4 different groups (Figure 1). Based on principal component analysis (PCA) and factor analysis results, four factors justified 82 and 85% of data variations, respectively.

Table 1. Polymorphic bands obtained from amplified fragment length polymorphism (AFLP) primer combinations.

Primer combination	Total band	Polymorphic band	Polymorphism (%)	Polymorphism information content (%)
E+AGG/M+CAT	83	30	36	45.15
E+CTG/M+GAG	56	17	30	64.19
E+ACT/M+GAG	90	24	26	68.16
E+AAC/M+CAT	56	36	64	54.98
E+TAT/M+GAG	70	31	42	39.91
Total	355	138	39.8	54.47

The first cluster was included of short life and early ripening dill landraces and European one with the highest essential oil. Kashmar landrace (No. 1) showed the highest distance with other genotypes. In group 2, the long season growth and highest yield and plant height were recognized. Only Neyshaboor-2 was placed in the third cluster with the highest stem diameter and biological yield. The group 4 included genotypes by similar height and inflorescent diameter.

DNA extraction

Among different methods of DNA extraction, Stein et al. (2001) protocol gave the reliable quality and quantity which was confirmed by 1% agars gel electrophoresis that was used for the experiment.

AFLP analysis

Twenty primer combinations were selected for the analysis of genetic variability. Using amplified fragment length polymorphisms (AFLP), primers combinations (Table 1) generated a total of 355 bands. Among them, 138 loci (39.8%) were polymorphic among dill populations. The number of polymorphic bands generated per primer ranged from 56 to 90 bands with an average of 71 and 26.6, respectively. Polymorphic information content (PIC) of bands generated per primer varied from 39.91% (E+TAT/M+GAG) to 68.16% (E+ACT/M+GAG) and average of total PIC generated per primer was 54.47%. Results show that the combination of E+AAC/M+CAT primers produced maximum polymorphic bands as 64% of total detected bands (Figure 2). Generated fragments were at range of 150 to 800 bp. Cluster analysis performed based on marker study by using the AFLP categorized 38 populations to 4 different groups (Figure 3), based on Jacard's similarity coefficient. The correlation between the cophenetic values was high (0.8) indicating a good fit of the cluster analysis and calculated genetic distances.

DISCUSSION

Genetic diversity using morphological traits and molecular markers has been described in many species, especially in *Apium* (Castellini et al., 2006), chamomile (Solouki et al., 2008) and coriander (Lopez et al., 2008). Study of genetic diversity in the family *Apiaceae* using molecular markers has been reported in *Changium smyrnioides* (RAPD) by Fu et al. (2003), *Carum* L. (ITS) by Papini et al. (2007) and carrot (AFLP) by Santos and Simon (2002).

Study of the morphological traits is a useful tool for preliminary evaluation, because they offer a fast and useful approximate for estimating the extent of diversity. Analysis of variance among all traits showed significant differences at the levels of 0.01 and 0.05, but essential oil yield appeared significant difference at 0.05 probability level. The difference between traits could be influenced by genotype, environment and interaction among them (Lopez et al. 2008).

Considering morphological data in our study revealed that the landraces were grouped into four clusters. Group 1 includes an early-season landraces which showed the lowest height and biological yield. These results are similar with the results of Petraityte and Dastikaite (2007) on caraway. Group 2 includes long-season landraces that had the highest height and lowest essential oil yield. Group 3 includes Margh-Esfahan landrace, with lowest seed weight and highest stem diameter, essential oil yield and biological yield, and group 4 includes landraces with similar inflorescent. Lopez et al. (2008) were the three main groups distinguished in coriander. In the study of Petraityte and Dastikaite (2007) on caraway, the populations were grouped into four clusters which varied in the morphological and biochemical parameters. Results of cluster analysis showed that genetic diversity based on morphological traits was not according to the geographical region. These results are similar with the study conducted by Solouki et al. (2008) on chamomile. The four first principal components (PCA) had Eigen values greater than 1.0 and together, they explained 82.4% of the total variation for this assemblage of

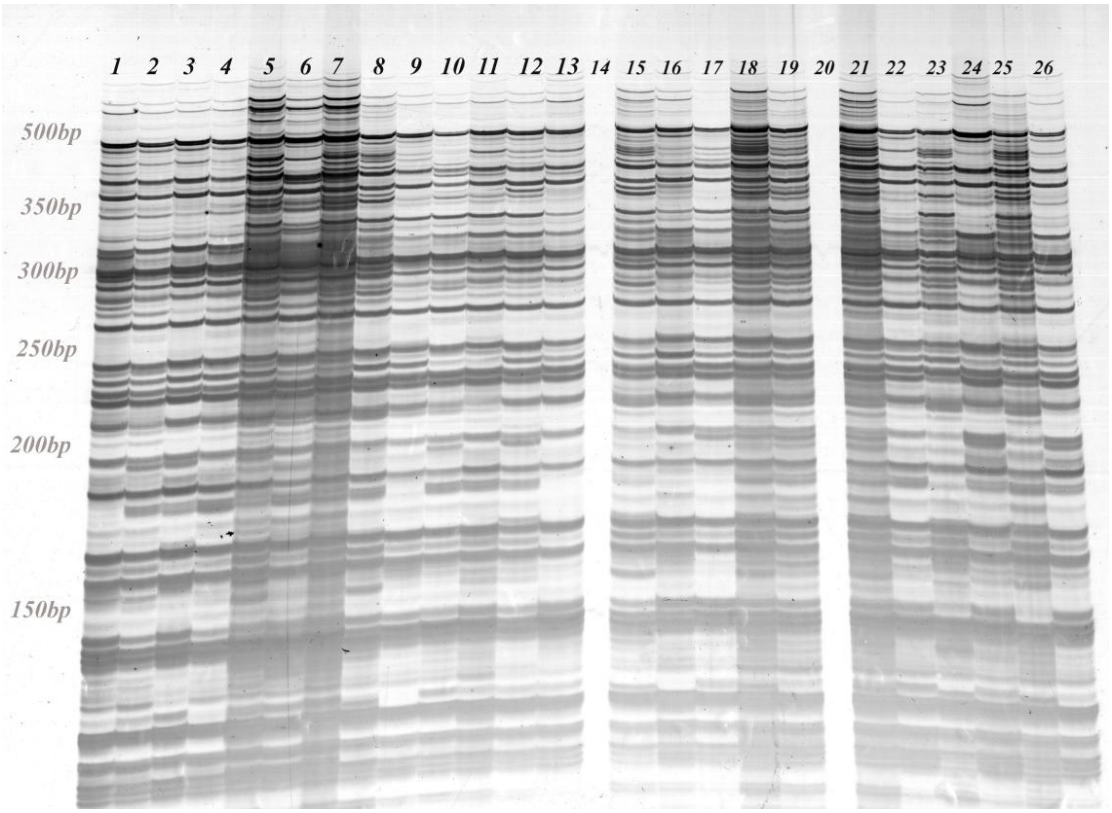

Figure 2. Photograph of silver-stained gel showing amplified fragment length polymorphism amplified with primer combination of E+AGG/M+CAT.

morphological traits. In the PCA, the group 1 principal component of biological yield is 48.6%, the group 2 principal component of plant height is 18.5%, the group 3 principal component of phenological parameter is 10.3% and the group 4 principal component of essential oil yield is 5%. Similar results have been reported on coriander whit 73.5% of the total variation greater than 1.0 (Lopez et al., 2008). In the study carried out by Campos et al. (2005), the 5 principal components accounted 56.1% of the total variability and the first two showed 37.3% of the variance. Raghu et al. (2007) also observed three PCA that were explained 68% of the total variation in which PCA1, PCA2 and PCA3 variations were 48.1, 12.7 and 7.4, respectively. The differences among total variations may be due to variation of species, selection, adaptation, migration, genetic drift and the method of pollination. It is also most related to the environment and human activity over the time (Solouki et al., 2008).

In the present study using AFLP, the number of bands generated per primer were 56 to 90, that E+AAC/M+CAT primers produced maximum polymorphic bands (64%), and E+ACT/M+GAG made minimum polymorphic bands (26%) with average polymorphic bands of 39.8%. The achievement of polymorphic bands was relatively low (39.8) compared with other plants such as (77.3%) in taro (Sharma et al., 2008), (75.8) in *Pinellia teranata* (Zhang et al., 2007), and (53.7%) in celery (Castellini et al., 2006). But this results was relatively high in comparison with ginger accessions (11.45%) (Wahyani et al., 2003). Hence, the percentage of polymorphism is significantly lower, that reflecting the poor genetic diversity between populations.

It has been reported that the numbers of amplification products in *Withania somenifera* varied from 53 to 101 in E+ACG/M+CAT and E+AAC/M+CAA primer combinations, respectively. The percentage of polymorphism ranged from 79 in the primer combinations of E+ACC/M+CTC and E+AAC/M+CAA to 89 which were illustrated by E+ACG/M+CAT with an average of 82% bands being polymorphic (Negi et al., 2006).

Principal coordinates analysis based on AFLP showed that thirteen factors justifies 69 percentages of data variations. Mantel statistics revealed that the dendrogram was in conformity with similarity matrices (r=0.8). Results of cluster analysis showed that genetic diversity based on morphological traits and molecular markers was not

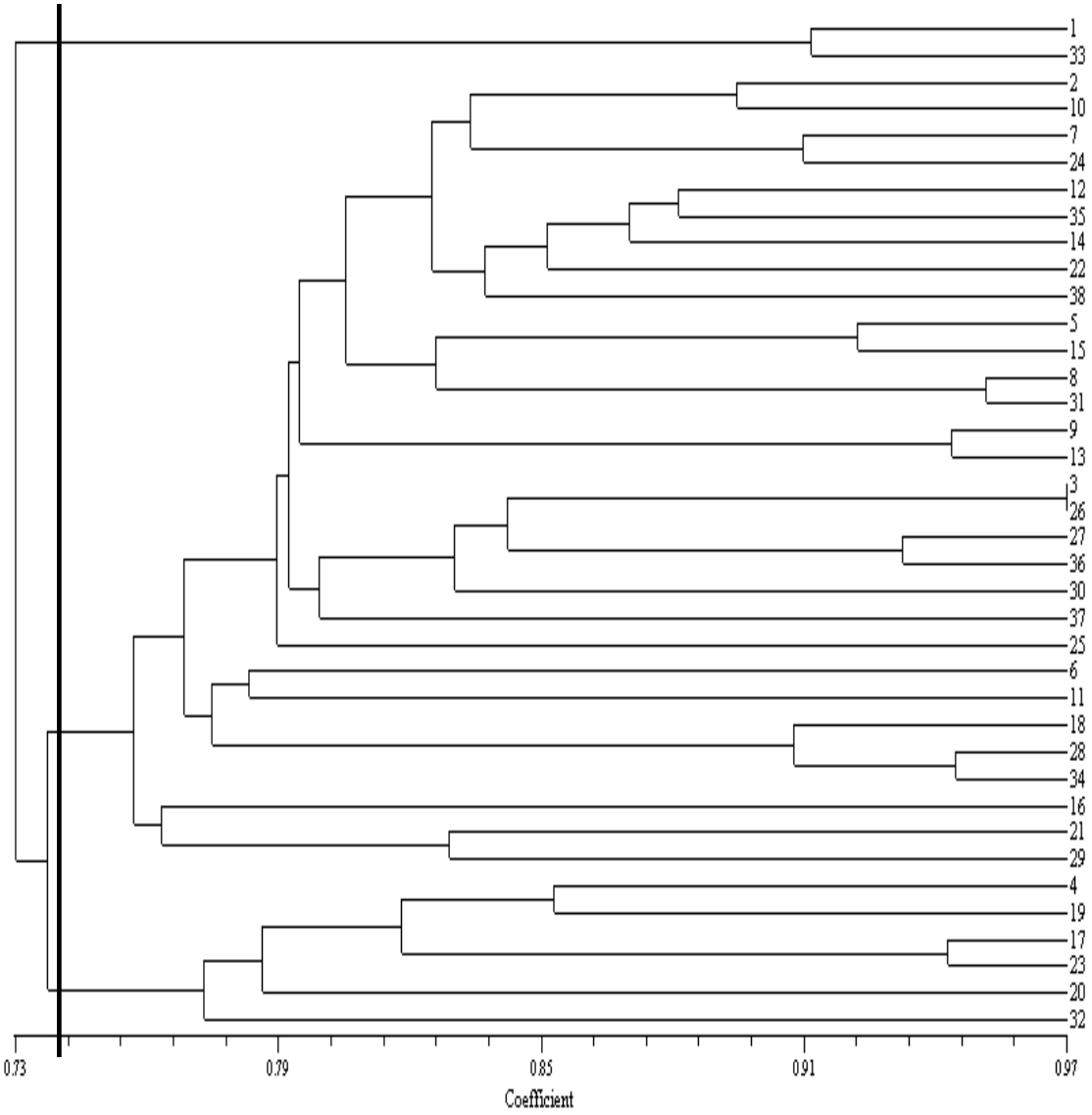

Figure 3. Dendrogram showing genetic dissimilarity among 20 populations of dill derived from AFLP analysis (populations are recognized by number as shown in Figure 1).

according to the geographical region. But in the study of Torre et al. (2008) genetic (AFLP) and geographical distances were significantly correlated. Also the experiment of Ali et al. (2007) on the sesame showed that different geographical regions could be characterized by the presence of AFLP fragments, and a possible correlation between some morphological characters and geographic origin was also evident. Similar results also have been reported by Lopez et al. (2008) on coriander. Results of cluster analysis showed that the genetic diversity based on morphological traits and molecular markers presented a little relationship between the two dendrograms (0.06). Raghu et al. (2007) reported a correlation between simple sequence repeats (SSRs) matrices and morphological traits (0.077) obtained on cassava germplasm.

Our study is the first report on the use of DNA-based markers and morphological markers for the cluster analysis on dill. Morphological markers show a high degree of variation among the dill accessions, but molecular markers show a low variation. These results may be related to the phenotypic plasticity of dill, phenological traits which may be controlled by few genes, plant biological characteristics, and its low numbers of

primers used for AFLP markers. This may be derived that the primers have not made the expected fragments related to the morphological similarity. Similar results for other species have been reported (Ali et al., 2007; Lopez et al., 2008).

REFERENCES

Ali GM, Yasumoto S, Katsuta MS (2007). Assessment of genetic diversity in sesame (*Sesamum indicum* L.) detected by Amplified Fragment Length Polymorphism markers. Electronic. J. Biotech. 10: 12-23.

Callan W, Janson l, Wesstcott P, Welty E (2007). Herb and oil composition of Dill (*Anethum graveolens* L.). Effects of crop maturity and plant density. Ind. Crop. Prod. 25: 282-287.

Campos ET, Espinosa MAG, Warburton ML, Varela AM, Monter AV (2005). Characterization of mandarin (*Citrus* spp.) using morphological and AFLP markers. Formato Documento Electrónico. 30: 687-693.

Cankur O, Yathavakilla E, Santha K, Caruso V, Goseph A (2006). Selenium speciation in dill (*Anethum graveolens* L.) By ion pairing reversed phase and cation exchange HPLC with ICP-MS detection. Talanta. 70: 784-790.

Castellini G, Torricelli R, Albertini E, Falcinelli M (2006). Morphological and molecular characterization of celery landrace from central Italy, *Apium graveolens* L.Var. Dulce (Miller) Pers. ISBN., 88-900622-7-4.

Dellaporta SL, Wood J, Hicks JB (1983). A plant DNA minipreparation: Varsion II. Plant Mol. Biol. Rep. 1: 19-21.

Dolezalova L, Lebeda A, Dziechciarkova M, Kristkova E, Astley D, Wiel C (2003). Relationships among morphological characters, isozymes polymorphism and DNA variability-the impact on *lactuca* germplasm taxonomy. Czech J. Genet. Plant Breed. 39(2): 59-67.

Doyle JJ, Doyle JL (1990). Isolation of plant DNA from fresh tissue. Focus. 12: 13-15.

Fu C, Qiu Y, Kong H (2003). RAPD analysis for genetic diversity in *changium smyrnioides* (*Apiaceae*), an endangered plant. Bot. Bull. Acad. Sin. 44: 13-18.

Jaccard P (1908). Nourvelles recherches Sur la distribution florale. Bull. Soc. Vaudoise Scie. Nat. 44: 223-270.

Kmiecik W, Lisiewska Z, Slupski J (2004). Effects of freezing and storage of frozen products on the content of nitrate, nitrites and oxalates in dill (*Anethum graveolens* L). Food. Chem. 86: 105-111.

Lisiewska Z, Kmiecik W, Korus A (2006). Content of vitamin C, carotenoids, chlorophylls and polyphenols in green parts of dill (*Anethum graveolens* L.) depending on plant height. J. Food. Compos. Anal. 19: 134-140.

Lopez PA, Widrlechner MP, Simon PW, Boylston SRTD, Isbell TA, Bailey TB, Gardner CA, Wilson LA (2008). Assessing phenotypic, biochemical, and molecular diversityin coriander (*Coriandrum sativum* L.) germplasm. Genet. Resour Crop. 55: 247-275.

Mantel NA (1967). The detection of disease clustering and a generalized regression approach. Cancer Res. 27: 209-220.

Momeni S, Shiran B, Razmjoo K (2006). Genetic variation in Iranian mints on the bases of RAPD analysis. Pak. J. Biol. Sci. 9(10): 1898 - 1904.

Nanasombata S, Wimuttigosol P (2011). Antimicrobial and antioxidant activity of spice essential oil. Food. Sci. Biotech. 20(1): 45-53.

Negi MS, Sabharwal V, Wilson N, Lakshmikumaran1 MS (2006). Comparative analysis of the efficiency of SAMPL and AFLP in assessing genetic relationships among *Withania somnifera* genotypes. Curr. Sci. 91(4): 464-471.

Papini A, Banci F, Nardi E (2007). Molecular evidence of polyphyletism in the plant genus *Carum* L. (*Apiaceae*). Genet. Mol. Biol. 30(2): 475-482.

Petraityte N, Dastikaite A (2007) Agrobiological assessment of wild *Carum carvi* L. cenopopulation biodiversity ex situ. Biologija. 53: 74-79.

Raghu D, Senthil N, Saraswathi T, Raveendran M, Gnanam R, Wnkatachalam R, Shanmugassundaram P, Mohan C (2007). Morphological and Simple Sequence Repeats (SSR) based Finger printing of South Indian Cassava Germplasm. Int. J. Integrative. Biol. 1(2): 141-149.

Rohlf FJ (1998). NTSYS- pc Numerical taxonomy and multivariate analysis system ver 2.02. Newyork: Exeter software, setauket.

Santos CAF, Simon PW (2002). Some AFLP amplicons are highly conserved DNA sequences mapping to the some linkage groups in tow F2 populations of carrot. Genet. Mol. Biol. 25(2): 195-201.

Sharma K, Mishar AK, Misra RS (2008). Analysis of AFLP variation of Taro population and markers associated with leaf blight resistance gene. Academic. J. plant sci. 1(3): 42- 48.

Solouki M, Mehdikhani H, Zeinali H, Emamjomeh AA (2008). Study of genetic diversity in Chamomile (*Matricaria chamomilla*) based on morphological and molecular markers. Scientica Horticulturae, 117: 281-287.

Stein N, Herren G, Keller B (2001). A new DNA extraction method for high-throughput marker analysis. Plant Breeding, 120: 354-356.

Torre A, Lopez S, Yglesias E, Cornelius JP (2008). Genetic (AFLP) diversity of nine *Cedrela odorata* populations in Madre de Dios, southern Peruvian Amazon. Forest. Ecol. Manag. 255: 334-339.

Vos P, Hogers R, Bleeker M, Reigans M, Theovan DL, Hornes M, Frijers A, Kuiper M, Zabeau M (1995). AFLP: A new technique for DNA fingerprinting. Nucleic Acid Res. 23: 4407-4414.

Wahyani S, Xu DH, Bermawie N, Tsunematsu H, Ban T (2003). Genetic relationships among ginger accessions based on AFLP marker. Biotechnol Pertanian. 8(2): 60-68.

Yu HH, Yang ZL, Sun B, Liu RN (2011). Genetic diversity and relationship of endangered plant magnolia officials assessed with ISSR polymorphisms. Biochem. syst. ecol. 39 (2). 71-78.

Zhang J, Xu T, Zhang DM, Yang YJ, Chen JS (2007). AFLP analysis of genetic diversity of *Pinelia ternate* (Thunb.) Breit. in Gansu province. Medic. Sci. 33(2): 38-44.

Zohary D, Hopf M (2000). Domestication of plants in the Old World, third edition (Oxford: University Press), p.206.