Study of Different Wheat Cultivars Using the ISSR Marker

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Abstract

Genetic diversity and evaluation of wheat cultivars (Trictum) raised at the molecular level were conducted using the ISSR marker . For DNA extraction, modified Doyle and Doyle method was applied and in the next step, 9 genotypes with 14 primers were studied. In the ISSR primer, samples of certified Homa wheat and self-consumption Sayonz have the most similarity with the rest of samples. The minimum number of bands in the ISSR is 41 and the maximum number is 59. The highest percentage of ISSR is 61% and the lowest percentage is 27%. Samples of self-consumption Sayonz and certified Homa with 58% have the greatest similarity and self-consumption Sardari sample with 42% has the least similarity. Indeed, genetic diversity of self-consumption Sayonz and certified Homa is closer together.

Key words: genetic diversity, wheat cultivars, ISSR marker

Introduction

Although the human approach to medicinal products of plants has a profound background, the issue of increasing the production of these products at the level of farms and gardens assumed a new scientific form around the second half of the twentieth century. Effective use of genetic resources and hereditary reserves of plants requires knowledge of their heredity and genetic structure. To this end, researchers have used different morphological, cytogenetic and molecular markers.

Genetic diversity
One of the basic needs of plant breeding is the awareness of the diversity of hereditary reserves and genetic relationships among plants. Genetic diversity is considered as the most important factor in the survival of creatures, including plants, against changes in environmental conditions and pests (Bahra et al., 2008). Genetic diversity arises primarily from hereditary factors and is also transmitted to later generations or progenies. In contrast to genetic variation, we have environmental diversity which results from environmental factors. Genetic diversity may be simple and can be easily observed in grains and plants like the color of flowers or grains and the presence or absence of awn in cereals. Also, genetic diversity can be complex and manifest itself in complex inherited traits such as performance and drought resistance. The existence of hereditary diversity is essential for plant breeders and without it, permanent genetic modification is not possible (Slipper & Pullman, 2008). In general, diversity and selection are two basic principles of every breeding program and the selection is subject to the existence of a desirable variety in terms of the trait under investigation. Over thousands of years, natural selection and climatic conditions have caused genetic diversity in plant sources (Sa'eidi, 2003).

Thus, genetic diversity is a work tool for breeders and to take advantage of it, one can either use the diversity in populations or obtain the desired diversity for the desirable trait in the breeding program by the selection of the right parents and their confluence if there is no variation for the desired trait (Mohammadi, 2002).

Among the factors creating genetic diversity, mutation, hybridization and entering cultivars can be mentioned (Qaranjik, 2002). Plant breeding (or synthetic evolution by mankind according to Vavilov) like natural evolution is dependent on diversity and selection (Vavilov, 1951). Hence, genetic diversity is one of the fundamental requirements for progress in plant breeding (Ramanujam et al. 1974).

Study of genetic diversity and factors creating diversity

In the past, some believed that plants in the same species are not similar and thus assumed that this dissimilarity has a hereditary origin. But some others maintained that all kinds of plants and animals have been created uniformly from the beginning. This way of thinking caused some to believe that members of a species have been essentially the same without hereditary changes. Even Carl von Linné, the founder of systematic plant science, also initially thought that the plants within a stabilizing species are similar to each other. But with the emergence of evolutionary concepts and phenomena stated by Lamarck and Darwin, the possibility of variation among species was considered and it was confirmed that the evolution of species has not been possible simply and these changes have led to the creation of diversity within and between species.

Botany

Wheat with the scientific name of Triticum is among the most important cereals. This plant exists in wild and domestic species. Wheat is among the one-year-old flowering monocotyledons and is from the Gramineae family.

The importance of medicinal herbs and plants

Population growth and the urgent need to use medicinal plants as the raw material for drug production, failure to produce some essential drugs by the pharmaceutical industry and also the application of effective substances in medicinal plants in the food, cosmetic and health industries have meant that research on these types of plants have become important from the viewpoint of cultivation, production and consumption. Paying attention to the cultivation and development of medicinal herbs in the world began in 1986 since the 14th World Health Congress and has led to a growing increase in the global demand for these products. Following this, different countries have engaged in the planning for mass cultivation and production of medicinal herbs at industrial levels and productivity and use at cosmetic and health levels and at the same time, international trading of these products faced a great boom (Baqeri et al., 2005).

Iran with a specific climate situation has more than 7,500 plant species, which are 2-3 times more than the vegetation of the entire continent of Europe and it is predicted that more than 750 medicinal species exist in Iranian vegetation (Omid Beigi, 1995). In spite of this potential power, Iran's important cultivated medicinal plants are less than 10,000 hectares and in terms of species diversity under cultivation, this figure is limited to below 40 species.

Definition of marker and its types

Use of genetic markers is as old as human history. The first humans (even those who have not still learned agriculture and had to collect seeds to continue their lives) unknowingly used morphological markers to recognize all kinds of seeds and fruits and wild beasts and preferred some to some others. But Mendel was perhaps the first one who used morphological or phenotypic markers in a codified and knowledge-based manner to study the inheritance of pea traits.

ISSR technique

ISSR technique is a PCR-based method which includes the amplification of DNA fragment present in the replicable space between two unique and repetitive microsatellite loci with opposite directions. This technique usually benefits from microsatellites with a length of 16-25 bp as the primer of a single-primer reaction which targets at multiple genomic loci for the amplification of inter-microsatellite sequences with different sizes. Microsatellite repeats used as the primer can have two, three, four or five nucleotides. The applied primers can be attached to any point in the DNA although they are attached to one to four bases at their 3'-end or 5'-end and expand accordingly. This technique has combined most of the advantages of AFLP and microsatellite with RAPD inclusiveness. High replicability of ISSRs is probably due to the use of longer primers (16 - 25 mers) compared to shorter primers (10 - mers) of RAPD, which provides the possibility for using high bonding temperature (45 to 60° C) leading to the increased probability of binding the primer to specific points in DNA (more replicability). Studies concerning replicability demonstrated that only the weakest bands are not replicable. About 92% to 95% of the rated fragments can be digitized over DNA samples and be replicated during separate periods of PCR when they were identified using polyacrylamide. 10 ng of DNA template will produce the same duplicate products that generate 25 or 50 ng of DNA template per 20 µl of PCR. Bonding temperature depends on the percentage of the primer used and usually ranges between 45 to 60° C.

Plant substances

In order to conduct molecular studies , 9 varieties of wheat species (available in Agriculture Jihad) were applied. A total of 9 leaf samples were collected (Table 3). These samples comprised fresh leaves of wheat species.

Primers

To investigate the genetic relationships , 14 ISSR primers were employed.

Genomic DNA extraction

To perform DNA extraction, the modified CTAB method of leaf samples before melting the ice of samples was used. To prepare 100 ml of 2% CTAB extraction buffer, the components of Table (3-4) were dissolved in 20 ml of distilled water and their PH reached 8 using 1M chloridric acid. Then, two grams of hexadecyl trimethyl ammonium bromide were dissolved in some heated distilled water and were added to the previous solution. Ultimately, the

Table 1: ISSR primers used in this research

No.	Primer name	Primer sequence	Bonding temperature (°C)
1	ISSR-1	5'-ACACACACACACACACG-3'	50
2	ISSR-2	5'-ACACACACACACACACT-3'	53
3	ISSR-3	5'-ACACACACACACACACC-3'	53
4	ISSR-4	5'-AGAGAGAGAGAGAGAGC-3'	47
5	ISSR-5	5'-AGAGAGAGAGAGAGAGT-3'	55
6	ISSR-6	5'-GAGAGAGAGAGAGAGAC-3'	47
7	ISSR-7	5'GAGAGAGAGAGAGAGAT-3'	51
8	ISSR-8	5'-CTCTCTCTCTCTCTCTG-3'	54
9	ISSR-11	5'-ACACACACACACACACGT-3'	52
10	ISSR-14	5'-ACACACACACACACACGG-3'	54
11	ISSR-15	5'-ACACACACACACACACGA-3'	47
12	RAMP-TAG	5'-TAGAGAGAGAGAGAGAGAG-3'	51
13	RAMP-GAC	5'-GACACACACACACACACAC-3'	51
14	LK7	5'-CCACTCTCTCTCTCTCTCT-3'	47

Table 2: Components of DNA extraction buffer

Buffer components	Amount	Final concentration
Tris-HCL	1.211 g	100 mM
NaCl	8.19 g	4 M
EDTA	0.744 g	0.2% (v/v)
CTAB	2 g	2% (w / v)
Merkapto etanol	200 µl	0.5 M
Pvp	2 g	2% (w / v)

the volume of the solution reached 100 ml. The DNA extraction steps are as in Table 2 above.

Before everything, the CTAB buffer was heated at 65 °C in Bain-Marie.

100 ml of fresh, healthy and clean leaves were completely pounded in liquid nitrogen in Chinese mortar and were powdered. Mortar and its pestle (at least in the position of contact with the leaves) should be cool before doing this. This is a recommendation to disable the nucleases in the environment and can be done by adding a little liquid nitrogen or placing in a freezer.

The leaf powder is collected in the corner of mortar and immediately before melting its ice, 800 µl of CTAB buffer were added to the mortar and were fully mixed with the leaf powder. The contents of the mortar were transferred to a tube of 2 ml and were kept for half an hour at 60° C in Bain-Marie. During this time, the tubes were slowly inverted several times. Sample size (800 µL) of isoamyl alcohol / chloroform solution (24: 1 ratio) was added at room temperature. The tubes were gently and repeatedly inverted so that the tube contents were uniformly mixed. The contents were centrifuged at 13000 rpm for 10 minutes at room temperature. The upper clear part (about 500-600 µL) was removed and poured into another tube.

Two-thirds of the volume (about 350 µL) of cold isopropanol (-20° C) was added to each tube and the contents were centrifuged at 13000 rpm for 10 minutes at 4° C. The tube contents were gently removed and DNA cluster remained at the bottom of the tube. Empty tubes with DNA cluster were placed upside down on a completely clean surface for 10 minutes so that the clusters dried. 100 µl of TE buffer was added at room temperature.

800 µl of ammonium acetate plus cold ethanol were added (1.5 ml of 2.5 M ammonium acetate plus 3.5 ml of ethanol). The last stage was performed on ice and the tubes were gently inverted several times and were then transferred to the freezer. The contents were centrifuged at 13000 rpm for 15 minutes at 4° C.

The upper part became empty. The tubes were inverted to become dry. 150 µl of TE buffer was added to each tube. Samples were stored in the tubes at room temperature. Afterwards, subsequent works were done to evaluate quantity (spectrophotometry) and quality (electrophoresis on 1.5% agarose gel of the extracted DNA). DNA of the samples was diluted up to 100 ng / µl for subsequent use.

Determining the quantity of genomic DNA samples
By using the spectrophotometer device, absorption of diluted DNA solutions at a ratio of 1:61 (5 µl of DNA stock solution plus 295 µl of sterile double distilled water) was measured at a wavelength of 261 nm (absorption

wavelength of nucleic acids) and 280 nm (absorption wavelength of proteins) and finally, the ratio of light absorption at a wavelength of 261 nm to 280 nm (261/280) which is an index of DNA purity was obtained. Further, the DNA stack concentration was calculated using the following formula:

Formula 3-1: Dilution factor (61) × 50 × absorbance at 261 nm = DNA concentration (ng / µl)

In the spectrophotometric method of DNA, each absorption unit at a wavelength of 261 nm is equivalent to 50 mg / µl of double-stranded DNA and if the ratio of absorbance of DNA solution at a wavelength of 261 nm to the absorbance at 280 nm is within the range of 1.7-1.9, this indicates that absorption is mainly due to nucleic acids and the obtained DNA quality is desirable and it enjoys high purity.

Determining the quality of genomic DNA samples

By using the genomic DNA electrophoresis on 1.5% agarose gel, the DNA band quality of each sample was determined. For each sample, 10 µl of extracted DNA were loaded on 1.5% agarose gel wells. The agarose gel was electrophoresed for 45 minutes at the constant voltage of 90 and then, DNA was observed and photographed under UV light in the gel doc machine.

0.8% and 1.5% agarose preparation method to determine the quantity and quality and separation of amplified fragments

Agarose powder was weighed and poured into a besher . EDTA 10 X was diluted with distilled water with a ratio of 9: 1 and EDTA 1 X was prepared in this way.

EDTA 1 X was poured into a besher by a graduated cylinder based on the desired volume of electrophoresis tank and was slowly shaken so that the contents were mixed completely. The solution inside the besher was placed in the microwave such that the agarose particles inside it were completely dissolved and the solution became uniform. 0.2 µl of DNA Safe Stain was added for gel staining and after that, the intended gel was poured into the gel mold whose shoulder was also ready. The solution inside the besher was gently poured into the electrophoresis tray. 30 minutes must pass for the agarose gel to become firm. The gel together with the tray was placed inside the electrophoresis tank and became fixed. Then, inside the tank became full of EDTA 1 X up to slightly above the gel. Components of the polymerase chain reaction

To perform PCR in a volume of 12 µl, the components provided in Table (3.5) were used.

Table 3: Components used for PCR

Component	Amount (µl)
PCR Kit (Mastermix)	5
Deionized water	5
Primer	1.1
Genomic DNA	1
Total	12.1

Time cycle and steps of polymerase chain reaction

Polymerase chain reaction was performed in the thermocycler machine (Bio Rad) with a 4-minute program of initial denaturation at 94° C, 10 primary cycles in the form of touch down (so that the temperature of the primer bonding to the template strand was considered to be 5° C higher than the actual bonding temperature) and for each cycle, 0.5 degree was reduced from the bonding temperature in order to reach the actual bonding temperature. This partly decreases the creation of pseudo-microsatellite bands which cause difficulties in scoring in normal cycles of PCR) and 30 cycles including 30 seconds of denaturation at 94° C, the recommended temperature for each primer for 45 seconds (for connecting the primers) and 2 minutes at 72° C for extension and the final extension was done at 72° C for 7 minutes.
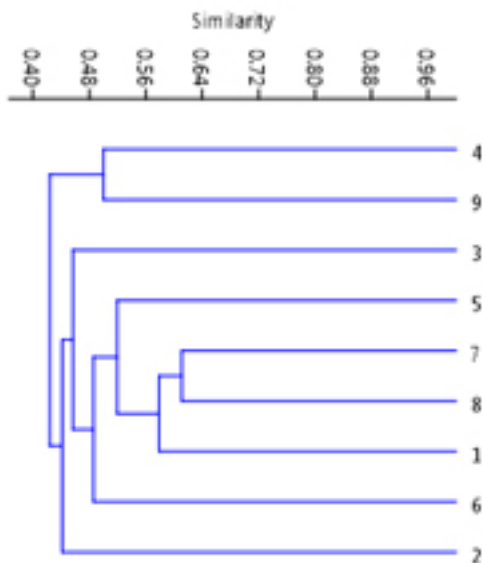
Power and time required for PCR product electrophoresis The time needed for electrophoresis is dependent on the required temperature and the distance between the two positive and negative poles. With regard to performing the optimal separation of fragments from each other, a voltage of 90 volts was used. The electrophoresis time also depends on the weight of the bands produced by the PCR in terms of the base pairs. In this study, considering the results of initial testing and optimization of electrophoresis conditions, 2.5 hours were found to be an appropriate time for separating PCR products. The buffer required for electrophoresis is TAE with a concentration of 1 X, which is obtained through properly diluting 10 X TAE buffer with distilled water.

Software used in this research consists of:
1)   Darwin 6 software
2)   Past 3 Software

Phylogenetic tree diagram using the ISSR molecular marker

Drawing the evolutionary tree shows that if we put the commentary line (or in other words the cutoff line) in one place, the self-consumption Sardari sample at a genetic distance of 0.43 is separated from studied cultivars and genotypes. At a genetic distance of 0.45, the cultivars under study are divided into two main groups. The first group encompasses the samples of certified Pishtaz and certified Mihan and the second group includes Azar 2, self-consumption Sayonz, certified Homa, Parsi Madari, Sirvan Madari and certified Parsi. The Sardari sample is the farthest sample and the two samples having the greatest similarity with each other are self-consumption Sayonz and certified Homa.

Figure: Phylogenetic tree diagram using the ISSR molecular marker

Table of similarity coefficient using the ISSR molecular marker

Jaccard's Similarity Table has been provided below. The numbers represent the distance between the samples. The greater the numbers, the smaller the distances will be. Investigation of Jaccard calculation demonstrates that the samples of certified Homa and self-consumption Sayonz have the greatest similarity and the sample of self-consumption Sardari has the least similarity relative to other cultivars. In fact, the genetic diversity of certified Homa and self-consumption Sayonz are closer to each other.

Similarity coefficient using the ISSR molecular marker (See Table below)

Principal Component Analysis (PCA)

Alongside cluster analysis, PCA is among the most common multivariate statistical methods in the studies about the genetic relationships of genotypes. The purpose of this analysis is to find combinations of variable P of x_1, x_2,..., x_p to create independent (non-correlated) indices of z_1, z_2,... z_p called principal components (PC). PCs express different aspects of the properties of the initial data due to lack of correlation and are arranged in the way that z_1 has the greatest amount of changes and z_2 is placed in the next position and so on. PCA is carried out using variance-covariance matrix or correlation matrix. But regarding the molecular data, similarity or distance matrix between individuals is applied as the PCA input. In using the molecular data in principal component analysis, there is a possibility of negative stagnant roots and to eliminate this problem, Franco et al. (1997) proposed that the similarity or distance matrix should be converted using the formula $s'_{ij} = s_i - s_j + s$, in which s'_{ij} is the similarity matrix between the individual $S_{i,j}$, i and s_j is respectively the average similarity coefficients for the individual ith and jth and S.. is the average of total similarity coefficients.

As to the quantitative properties, in most cases, the first two or three PCs justify the highest amount of changes related to the initial data (around 75%-80%) and these PCs are used for graphic display for grouping the genotypes. But in connection with the molecular data, the first two or three PCs justify a maximum of about 10% -20% of the changes related to the initial changes in the markers. Although these results may not be statistically appropriate for PCA and graphic display, they genetically suggest the proper sampling of the markers from the whole genome. Accordingly, each of the markers used is from different genome sections and thus has less correlation. In principal component analysis using the molecular data, it should be considered that the graphic display and grouping based on two or three PCs cannot represent the total changes of the initial variables. As a result, it is recommended that grouping is done based on the great number of PCs which justify more changes. Selecting the number of PCs in this state can be based on the stagnant roots of PCs. PCs with the stagnant root larger than one should be used as PCs effective in grouping (Izony & Prits, 1991). Another problem in PCA is related to the missing data, which is common with molecular data. In PCA, missing data is simply replaced by the average of the relevant attribute (variable) when calculating the distance or similarity matrix. Consequently, genotypes with more missing data are located near the

	1	2	3	4	5	6	7	8	9
1	1	0.512195	0.526316	0.545455	0.5	0.485714	0.578947	0.578947	0.428571
2	0.512195	1	0.414634	0.378378	0.425	0.333333	0.5	0.463415	0.388889
3	0.526316	0.414634	1	0.342857	0.432432	0.411765	0.513514	0.4	0.277778
4	0.545455	0.378378	0.342857	1	0.4375	0.464286	0.441176	0.53125	0.5
5	0.5	0.425	0.432432	0.4375	1	0.424242	0.571429	0.486486	0.451613
6	0.485714	0.333333	0.411765	0.464286	0.424242	1	0.5625	0.470588	0.37931
7	0.578947	0.5	0.513514	0.441176	0.571429	0.5625	1	0.611111	0.411765
8	0.578947	0.463415	0.4	0.53125	0.486486	0.470588	0.611111	1	0.454545
9	0.428571	0.388889	0.277778	0.5	0.451613	0.37931	0.411765	0.454545	1

center of the relevant group. To overcome this problem in PCA, it is recommended to calculate the similarity or distance coefficient of two separate individuals only by using the attributes or variables that have full data for both individuals and remove the variables or attributes with missing data for one or both individuals from the calculation of the coefficient for those two individuals.

Principal component analysis can be employed as a method to determine the desirable number of clusters. The desirable number of clusters is the number by which the first PC in each cluster can justify the maximum changes, meaning that all individuals are initially regarded as a cluster and are then integrated so that in all the clusters formed, the changes justified by the second PC are less than the specified amount.

Results and Discussion

In the research conducted by Rai et al. (2007) on the genetic relationships of Amaranthus from Caryophyllales family, ISSR and RAPD molecular markers were used. For ISSR, 18 primers were applied and for the RAPD, 15 primers were used. The similarity coefficient of ISSR was obtained to be 0.45 and its cophenetic coefficient was 0.83 and for the RAPD, the similarity coefficient was 0.47 and its cophenetic coefficient was estimated to be 0.83, which indicate the good fit between the similarity matrix and the dendrogram. Both dendrograms showed good similarity between species, suggesting that ISSR and RAPD act efficiently for determining the genetic relationships and are good tools for classification between species (Rai et al., 2007).

Yousefi and colleagues examined the genetic diversity of the herb "thyme" using the ISSR molecular marker. In this study, genetic diversity of 14 thyme ecotypes received from the Research Institute of Forests and Rangelands and belonging to different geographical regions of Iran was investigated using the ISSR marker. The seven primers selected for analysis produced almost 79 polymorphic bands (95/018%) out of the 83 bands that appeared. The highest number of formed polymorphic bands belonged to the UBC-786 primer. The used primers revealed a total of 83 bands within the range of 200-2600 base pairs and there were approximately 79 polymorphic bands. According to the cluster analysis using Jaccard's genetic similarity coefficient, ecotypes were divided into three distinct groups. Principal component analysis approved the results of the cluster analysis. The least genetic similarity was between ecotypes 13206 and 27814 and the greatest genetic similarity was between ecotypes 27800 with 27814.

Phylogenetic relationships of some word species were studied using the ISSR marker. The word is one of the important and economical ornamental plants in the world. ISSR markers were used to determine the phylogenetic relationships among 47 word genotypes. Of the 15 primers used in this research, 11 primers showed acceptable polymorphism. Totally, 193 fragments were added and

173 fragments among the species were polymorphic. The additive products were electrophoresed on 1.5% agarose gel. The similarity matrix was formed and the dendrogram of grouping the cultivars was drawn using Jaccard algorithm in NTSYS 2.02 software. In the obtained dendrogram, in the similarity coefficient, more than 52% of the species studied were divided into four groups (Jabbarzadeh et al., 2011).

Conclusion

Minimum and maximum distances are different in various analyses. It seems that the analysis used as the basis for conclusion is the one that has similarity and overlap. Besides, based on our studies, it was found that the ISSR molecular marker has more efficiency than other markers.

References

Chamani Mohasses, F., Sami'zadeh, H., Rabi'ei, B. & Sohani, M. (2012). Evaluating the genetic diversity of 9 rice lines using the ISSR molecular markers. 12th Iranian Congress, 6 (1-12). Journal of seed and sapling breeding, 1-26: 331-350.

Naqavi, M. R., Qare Yazi, B. & Hoseini Salkadeh, V. Q. (2007). Molecular markers. Second Edition, Tehran University Press, Tehran, 340 pages.

Nabovvati, S., Aqaei Sarbarzeh, M., Choukan, R., Qanavati, F. & Najafian, G. (2010). Genetic variation of crop characteristics and traits related to grain quality of Durum wheat genotypes.

Khajepour, M. H. (2004). Fundamentals of Agriculture. Jahad Daneshgahi Press, Isfahan industrial unit, 386 pages.

Rahimian, H. & Banaeiyan Avval, V. M. (1996). Physiological foundations of plant breeding. Mashhad Jahad Daneshgahi Press. Rastegar, M. 'A. (1991). Industrial Plants Cultivation, University of Tehran Publishing Center.

Amin, A. (1981). Islam, a doctor without medicine. Young Generation Publishing.

Barimani, L. (1987). Traditional medicine, traditional drugs. Gutenberg Publishing.

Khoshkhouy, M., Sheibani, B., Rohani, A. & Tafazzoli, 'A. Principles of gardening. Shiraz University Press.