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Genetic variation and cluster analysis of *Narcissus tazetta* L. genotypes using RAPD and ISSR markers

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Genetic variation and cluster analysis of *Narcissus tazetta* L. genotypes using RAPD and ISSR markers

Abstract: To evaluate the genetic diversity of Iranian *Narcissus* genotypes (e.g., Shomal, Shastpar, Shahla, Yasuj, Shiraz-1, Shiraz-2, Kuchak-e-Atri, Dutch, Khosf-1, Khosf-2, Birjand, and Tabas), different RAPD and ISSR primers were examined at the Plant Breeding Laboratory of the Faculty of Agriculture, University of Birjand, Iran. In sum, RAPD and ISSR primers produced 189 and 80 high-resolution bands. The average values of PIC, Ht, Hs, Nm, DST, FDT, NA, Ne, H, and I indices were 0.287, 0.369, 0.089, 0.486, 0.279, 0.760, 1.952, 1.459, 0.282, 0.437 for RAPD markers and equal to 0.297, 0.380, 0.099, 0.524, 0.278, 0.732, 1.978, 1.495, 0.303, 0.467 for ISSR markers, respectively. The analysis of molecular variance based on the RAPD and ISSR markers showed 23 and 13 % variations for intra-populations and 77 % and 87 % changes for inter-population variabilities, respectively. Cluster analysis based on the RAPD and ISSR markers grouped genotypes into four clusters. Based on RAPD and ISSR markers, the PCoA analysis also showed that the first three components justified equal to 96.9 % and 97.9 % of the total variance, respectively, indicating the dispersion of the primers used. In general, it was concluded that the genetic diversity of narcissus species could be employed for breeding programs.

Key words: cluster analysis; narcissus; genetic parameters; genetic diversity

Genetska raznolikost in klasterska analiza genotipov dvobarvnega narcisa (*Narcissus tazetta* L.) z uporabo RAPD in ISSR markerjev

Izvleček: Za ovrednotenje genetske raznolikosti iranskih genotipov narcisa (e.g., Shomal, Shastpar, Shahla, Yasuj, Shiraz-1, Shiraz-2, Kuchak-e-Atri, Dutch, Khosf-1, Khosf-2, Birjand, in Tabas), so bili preiskušeni različni RAPD in ISSR primerji v Plant Breeding Laboratory, Faculty of Agriculture, University of Birjand, Iran. Sumarno so RAPD in ISSR primerji dali 189 in 80 jasno razvidnih trakov. Poprečne vrednosti PIC, Ht, Hs, Nm, DST, FDT, NA, Ne, H in I indeksov so bile 0,287; 0,369; 0,089; 0,486; 0,279; 0,760; 1,952; 1,459; 0,282; 0;437 za RAPD markerje in 0,297; 0,380; 0,099; 0,524; 0,278; 0,732; 1,978; 1,495; 0,303; 0,467 za ISSR markerje. Analiza molekularne variance na osnovi RAPD in ISSR markerjev je pokazala 23 in 13 % variacij znotraj populacij in 77 % in 87 % sprememb med populacijami. Klasterska analiza na osnovi RAPD in ISSR markerjev je združila genotype v štiri skupine. Analiza glavnih komponent na osnovi RAPD in ISSR markerjev je pokazala, da so prve tri komponente razložile 96,9 % in 97,9 % celokupne spremenljivosti, kar kaže na razpršenost uporabljenih primerjev. V splošnem lahko zaključimo, da bi se genetska raznolikost vrst narcisa lahko uporabila v žlahtniteljskih programih.

Ključne besede: klasterska analiza; narcis; genetski parametri; genetska raznolikost

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1 INTRODUCTION

Narcissus (*Narcissus tazetta* L.), a member of the family of Amaryllidaceae, has many therapeutic and ornamental properties (Gotti et al., 2006). Narcissus is a beautiful plant of geophytic, monocotyledonous, and perennial plants whose genus has 65 species and 20,000 cultivars and hybrids (Saeedi, 2007). In general, the quality of this flower, especially in terms of color, odor, and characteristics such as the number of sepals and petals, are primarily controlled by genetic factors but environmental factors, such as stress and plant nutrition, have remarkable impacts on these characteristics (Zadebagheri et al., 2011). At present, numerous researches are going on the agronomic characteristics of this plant in Iran, but there are few reports on the plant genetic diversity. Since the study and determination of the genetic diversity of a plant is the first step in the next breeding program, the purpose of this study was to determine the kinship, diversity, and genetic structure of narcissus populations using RAPD and ISSR molecular markers.

Genetic resources can provide desirable genes and supply opportunities for breeders to produce new and desirable cultivars (Clegg, 1997; Bhandari et al., 2017). There are many methods to study the genetic diversity of plants. Among these ways, molecular markers (DNA-based techniques) are the most accurate and reliable method for determining and identifying genetic diversity and (along with multivariate statistical methods) has a significant potential for examining plants phylogenic, evolution, and genetic diversity (Pandey et al., 2008).

Among the molecular markers developed in recent years, RAPD (Random Amplified Polymorphic DNA) marker is a simple PCR-based technique that was developed by Williams et al. (1990) and Welsh & Mc-Clelland in 1990, and is based on the amplification of primer binding regions in DNA genome (Welsh et al., 1991, Ezzat et al., 2016, Shi et al., 2018). In general, this method is widely used due to the lack of basic information needed to design and construct primers, the possibility of simultaneously examining multiple loci in the sample genome, the lack of the need for a special probe in molecular analysis (especially genetic diversity assessment) (Williams et al., 1990). This marker has been used well to study the genetic diversity of narcissus (Jiménez et al., 2017), soybean (Sharma et al., 2018), oil palm (Basyuni et al., 2018), tomato (Maske et al., 2018) genotypes and etc.

ISSRs are semi-optional markers that are amplified by PCR in the presence of a primer that complements the target microsatellite. ISSR markers are useful in studies on genetic diversity, genome mapping, gene tagging, phylogeny and evolution (Pradeep Reddy Reddy et al., 2002). Such multiplication does not require sequential information and leads to the production of multi-position and highly polymorphic patterns. The ISSR fragments are 100- 3,000 bp length of DNA sequences that are located between two microsatellite regions with opposite directions. Microsatellite-based primers are designed and used to amplify the DNA sequence between two ISSRs (Kumar et al., 2009).

ISSR markers are potential markers in identifying genetic diversity compared to other markers and allow the study of the diversity of specific regions of the genome at several locations simultaneously (Mengistu et al., 2004). ISSR markers has been used well for genetic diversity of cuscuta (Tajdoost et al., 2012), Persian violet (Asadi et al., 2016), narcissus (Jiménez et al., 2009), *Solanum nigrum* L. (Suganthi et al., 2018). Also, genetic diversity of different Turkish narcissus has been studied using SRAP markers (Zeybekoğlu et al., 2019). A study using markers (ISSR) investigated the genetic diversity of 31 narcissus species collected from 16 regions of Iran. The average percentage of polymorphic bands was 96.02 %. The highest resolution power (8.32), average polymorphic information content (0.44) and marker index values (5.61) were observed for primers 811, 828 and 811, respectively. They stated that ISSR markers can be used as a diagnostic tool to evaluate genetic diversity in narcissus genotypes and reveal them (Zangeneh and Salehi, 2019).

It should be noted that the Khosf (City of South Khorasan province) has a long historical tradition in narcissus cultivation and its area under cultivation has the highest area in the east of the country. The aim of this study was to investigate the genetic diversity of different narcissus ecotypes that were collected from different regions of Iran. It has also been the determination of the best genotype and its important traits for breeding purposes.

2 MATERIALS AND METHODS

This experiment was carried out in the research farm and laboratory of plant breeding and biotechnology of the Faculty of Agriculture, University of Birjand, Iran during 2017 growing season. In this study, 12 different genotypes of narcissus that been cultivated in Iran (consisted of Shomal, Shastpar, Shahla, Yasuj, Shiraz-1, Shiraz-2, Koochak-e-Atri, Dutch, Birjand, Khosf-1, Khosaf-2, and Tabas) were collected and cultivated both in pots and in the field for relevant experiments.

Leaf samples were taken after 60 days of planting in the four-leaf stage. The youngest leaves were selected for DNA extraction and stored in liquid nitrogen. The required solutions are prepared in stock with higher accu-

racy. DNA extraction was performed by CTAB method (Doyle and Doyle, 1987). For this purpose, 0.5 g of young leaves was used. The quantity and quality of the extracted DNA were evaluated using two methods of nano drop and 1 % agarose gel electrophoresis. In this experiment, 27 RAPD primers and 11 ISSR primers were used. The primers were purchased in freeze-dried form from Sinaclon Company and diluted 1:10 using double-distilled water. The final concentration of primer was considered to be one µl in each 20 µl PCR reaction (Tables 1 & 2).

The polymerase chain reaction was performed at a final volume of 20 µl (Table 3). 8 µl of the PCR product was stained with 2 µl of loading dye and 10 µl was poured into 1 % agarose gel (with 0.5X TBE buffer) wells. To better examine the bands, 5 µl of DNA size marker was injected into first the gel well. Samples were electrophoresed at voltages 90 for one hour. Amplified fragments were observed and photographed by GelDoc under ultraviolet light. Photos were scored using TL120 Lab Total software; was assigned to the presence of band, number 1and the absence of it, number zero. At first, according to geographical information, genotypes collected from different regions were divided into two groups (South Khorasan in one group and other genotypes in another group) and different statistical analyzes were performed.

In this study, the genetic similarity index was employed to determine genotype grouping, and cluster analysis, similarity coefficient and principal coordinate analysis were used to determine the distribution of primers. These calculations were also performed using NTSYS2.2 software. Molecular indices such as PIC (Sharma & Ghosh, 1955), population genetic index such as number of allels (Na), number of effective allels (Ne) (Kimura & Crow, 1973), gene diversity index of Nei (H) (Nei,

Table 1: RAPD and ISSR primers

Primer	Sequence	Primer	Sequence	Primer	Sequence
			RAPD		
R1	5'-TGCCGAGGTG-3'	R10	5'-AGGTGACCGT -3'	R19	5'-ACACCGATGG -3'
R2	5'-AGTCAGCCAC -3'	R11	5'- GTTGCGATCC -3'	R20	5'- CTTCTCGGAC -3'
R3	5'-AGGGGTCTTG -3'	R12	5'- GGAAACCCCT -3'	R21	5'-ACGGGACCTG -3'
R4	5'-GGTCCCTGAC -3'	R13	5'- TGGCGCACAC -3'	R22	5'-CCAGAACGGA -3'
R5	5'-GTGACGTAGG -3'	R14	5'-GGCACGCGTT -3'	R23	5'- GTGGCCGATG -3'
R6	5'-GGGTAACGCC -3'	R15	5'- GTTACGGACC -3'	R24	5'- ACGGAAGTGG -3'
R7	5'-GTGATCGCAG -3'	R16	5'- GGGCGACTAC -3'	R25	5'-GGACCCCTTAC -3'
R8	5'- CAATCGCCGT -3'	R17	5'-TCGCATCCAG -3'	R26	5'-TGAGTGGGTG -3'
R9	5'-TGGGCGATAG -3'	R18	5'-CTGGCGTTC -3'	R27	5'- GTTGCCAGCC -3'
			ISSR		
I1	5'-CTCTCTCTCTCTCTCTRC-3'	I5	5'-GAGAGAGAGAGAGA-I9 GAC-3'		5'-CTCTCTCTCTCTCTCTTG-3'
I2	5'-GAGAGAGAGAGAGAGAYG-3'	I6	5'-GAGAGAGAGAGAGAGA-I10 YA-3'		5'-CACACACACARY-3'
I3	5'-TCTCTCTCTCTCTCTCG-3'	I7	5'-GAGAGAGAGAGAGAGA-I11 YC-3'		5'-GACAGACAGACAGACA-3'
I4	5'-GAGAGAGAGAGAGAGYT-3'	I8	5'-ATCATCATCATCATC-3'		

Table 2: The PCR reaction temperature program for the ISSR and RAPD markers

	ISSR			RAPD		
Step	Tem.(°C)	Time	Cycle Num.	(°C) Te µLm.	Time	Cycle Num.
Initial denaturation	94	4min	1	95	4min	1
Denaturation	94	45s	40	94	1min	45
Annealing	45	5s	40	35	1min	45
Extension	72	55s	40	72	2min	45
Final extension	72	6min	1	72	6min	1

Table 3: Components of PCR reaction

Materials	Reaction components	The final concentration
Double distilled water	6.5 µl	.....
Master mix (Prepared from Yekta Tajhiz Azma Co.)	10 µl	2X
Primer	1.5 µl	0.67 pm
DNA	2.0 µl	20 ng μl^{-1}

PCR master mix includes the following details:
0.25 U/µl Taq DNA Polymerase; 2x PCR buffer; 0.4 mM dNTPs; 3.2 mM $MgCl_2$; 0.02 % bromophenol blue

1973), Shannon diversity index (I) (Lewontin, 1972), inter-population heterozygosity (Hs), intra-population heterozygosity (Dst), total heterozygosity (Ht), intra-population coefficient of variation (Gst) and fixation index (Fst) (Lynch & Milligan, 1994) was calculated using POPGEN-1.31. Molecular analysis of variance was also performed with Genalex 6.1 software.

3 RESULTS AND DISCUSSION

3.1 DETERMINATION OF DNA QUANTITY AND QUALITY AND ANALYSIS OF MOLECULAR VARIANCE

In this study, the amount of extracted DNA ranged from 83.5 to 565 ng μl^{-1}, which was reduced to less than 12 ng after dilution. Also, in all samples the wavelength ratio of 260:280 was 1.90 to 2.04, which indicated that it was suitable. Results also showed that from 27 RAPD primers produced 189 bands (Figure 1), of which nine bands were single-shaped and 180 had polymorphic bands. An average of seven bands were obtained per primer, so the used primers showed more than 95 % polymorphism. Out of a total of 11 ISSR primers, 80 bands with high-resolution were counted. Of the amplified bands, one band was single-shaped, and 79 bands were polymorphic. An average of 7.27 bands were obtained per primer. Overall, the ISSR primers that were used showed 98 % polymorphism. In general, RAPD and ISSR primers showed more than 95 % and 98 % polymorphism, respectively, and indicated the efficiency of primers in showing polymorphism. Chehrazi (2007) reported that out of 14 RAPD markers used to study the genetic diversity of narcissus genotypes, 105 bands were amplified, of which 92 were polymorphic bands. Consistent with our findings, the average rate was 7.5 bands per primer, and the polymorphism rate was equal to 96 %. Sargazi et al. (2016) studied the genetic diversity of fifteen ajowan populations using 25 RAPD primers and observed that all primers showed good polymorphism. In the study of genetic diversity of fennel using ISSR markers (Taheri et al., 2016), a total of 813 primers were reproduced from six primers, and the number of bands of each primer varied between 7-15, which is the average number of bands per primers consistent with this study.

3.2 GENETIC PARAMETERS

The polymorphism data exhibit notable variations between individuals and populations and are important indicators to compare different primers in terms of their power to differentiate genotypes. According to the presented results, it was found that the primers used had a relatively good PIC index, which indicates their acceptable efficiency in detecting and distinguishing genotypes (Table 4). In RAPD primers, the polymorphic content index differs from 0.15 to 0.47. The highest polymorphic content index (PIC) belonged to R13 (0.47), R1 (0.40), and R27 (0.40) primers, which showed their high efficiency in differentiating these genotypes. The lowest PIC also belonged to R2 (0.16) and R3 (0.15) primers. The polymorphism ratio index differs from 2.25 to 11.00. The lowest value belonged to R19 (2.25) primer, and the highest amount was for the R6 (11.00) primer. Also, the lowest and the highest values of the marker index were obtained for R14 (0.27) and R13 (3.78) primers, respectively. The lowest and the highest values of resolving power belonged to R26 (0.44) and R10 (1.67) primers, respectively. The mean values of total heterozygosity, intra-population heterozygosity, gene flow (Nm), intra-population heterozygosity (Dst), and Fst index were achieved equal to 0.37, 0.09, 0.49, 0.28, and 0.76, respectively.

Overall, the highest and the lowest values of the total heterozygosity were gained equal to R13 (0.46) and R2 (0.20) primers, respectively. The lowest heterozygosity inter-populations were for primers R20 (0) and R10 (0.01), while the highest amount of heterozygosity inter-populations was for R27 (0.22). The highest amount of gene flow belonged to R25 (1.14), and the lowest amount of gene flow was to R20 (0) and R10 (0.02). The highest heterozygosity intra-populations belonged to R10 and R7 (0.38), and the lowest was related to R2 (0.14). Primer R20 (1)

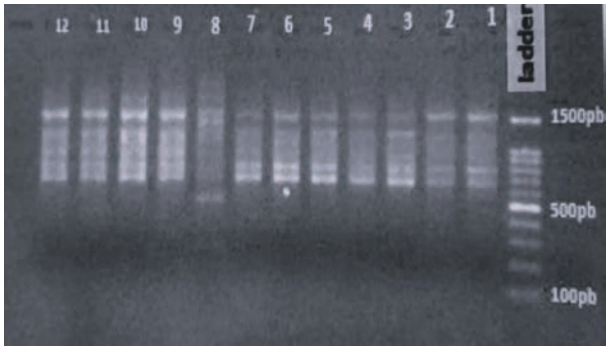

Figure 1: Separation pattern of amplified bands of 12 narcissus genotypes using OPA09 primers

had the highest Fst, and the lowest value with an average value of 0.50 was observed for R27. The maximum number of effective alleles (Ne) calculated for primers R2, R3, R16, R17, R19, and R20 were 1.67, 1.67, 1.89, 1.91, 1.75, and 1.83, respectively, and for other markers, it was equal to 2.0. The lowest number of effective alleles (Ne) related to R19 and R3 (1.21), and the highest these were obtained for primers R27 (1.71), and the average number of effective alleles (Ne) was 1.46. The highest gene diversity index of Nei (H) and Shannon diversity index (I) belonged to R13 primer (0.46 and 0.65), and the lowest was related to R3 primer (0.15 and 0.25), and their average was 0.28 and 0.47, respectively (Table 4).

Table 4: Genetic diversity parameters in narcissus genotypes with RAPD markers

Number	Ht	Hs	Dst	Fst	Na	Ne	H	I	PIC	EMR	MI	RP
R1	0.38	0.16	0.22	0.58	2.00	1.79	0.42	0.60	0.40	8.00	3.19	1.08
R2	0.20	0.07	0.14	0.67	1.67	1.28	0.17	0.28	0.16	2.67	0.42	1.22
R3	0.27	0.03	0.24	0.90	1.67	1.21	0.15	0.25	0.15	4.00	0.62	1.37
R4	0.39	0.10	0.29	0.74	2.00	1.62	0.36	0.53	0.36	8.00	2.89	0.83
R5	0.43	0.08	0.35	0.81	2.00	1.30	0.23	0.37	0.19	6.00	1.67	0.67
R6	0.35	0.06	0.30	0.84	2.00	1.33	0.23	0.38	0.24	11.00	2.68	0.86
R7	0.43	0.04	0.38	0.89	2.00	1.59	0.35	0.52	0.31	5.00	1.55	1.13
R8	0.42	0.16	0.26	0.62	2.00	1.60	0.36	0.54	0.33	6.00	2.01	1.25
R9	0.40	0.11	0.29	0.72	2.00	1.57	0.34	0.51	0.36	6.00	2.24	0.89
R10	0.39	0.01	0.38	0.97	2.00	1.25	0.19	0.33	0.18	8.00	1.44	1.67
R11	0.36	0.14	0.22	0.60	2.00	1.45	0.29	0.45	0.29	5.00	1.43	0.83
R12	0.36	0.12	0.24	0.66	2.00	1.29	0.21	0.37	0.25	5.00	1.28	1.00
R13	0.46	0.14	0.33	0.71	2.00	1.86	0.46	0.65	0.47	8.00	3.78	1.00
R14	0.42	0.14	0.27	0.65	2.00	1.67	0.37	0.55	0.34	8.00	0.27	1.22
R15	0.33	0.06	0.27	0.80	2.00	1.46	0.28	0.44	0.30	6.00	1.80	0.89
R16	0.3	0.08	0.22	0.73	1.89	1.30	0.21	0.35	0.24	8.11	1.70	0.94
R17	0.33	0.04	0.29	0.88	1.91	1.34	0.23	0.37	0.25	9.09	2.31	0.86
R18	0.26	0.07	0.35	0.83	2.00	1.55	0.32	0.49	0.30	7.00	2.11	0.76
R19	0.29	0.06	0.23	0.81	1.75	1.21	0.16	0.27	0.28	2.25	0.41	1.58
R20	0.31	0.00	0.31	1.00	1.83	1.28	0.18	0.30	0.18	4.17	0.76	1.47
R21	0.40	0.06	0.33	0.83	2.00	1.47	0.28	0.44	0.28	7.00	1.97	1.08
R22	0.43	0.06	0.37	0.86	2.00	1.52	0.30	0.47	0.31	7.00	2.17	1.13
R23	0.41	0.10	0.30	0.74	2.00	1.41	0.27	0.43	0.26	8.00	2.11	0.92
R24	0.35	0.14	0.21	0.60	2.00	1.44	0.30	0.42	0.30	7.00	2.72	0.57
R25	0.37	0.10	0.26	0.71	2.00	1.41	0.27	0.42	0.29	9.00	2.62	0.71
R26	0.30	0.05	0.25	0.82	2.00	1.47	0.29	0.45	0.30	3.00	0.92	0.44
R27	0.45	0.22	0.22	0.50	2.00	1.71	0.39	0.57	0.40	6.00	2.40	0.92
Mean	0.37	0.09	0.28	0.76	1.95	1.46	0.28	0.47	0.29	1.90	0.64	1.00

In the ISSR, the polymorphic indices content (PIC) changed between 0.15 and 0.37. The highest PIC belonged to primers I10 and I11 (0.37), which indicates its high efficiency in differentiating used genotypes. The lowest value belonged to the I2 primer (0.15). The polymorphism ratio index was between 3 and 14, with the lowest value belonging to primer I2 and the highest to primer I1. The lowest marker index was related to primer I5 (0.08), and the marker index was related to primer I8 (3.28). The lowest resolution index was related to primer I8 (0.27), and the highest value was related to primer I7 (1.55). The highest total heterozygosity was for primer I6 (0.43), and the lowest was related to primer I9 (0.31). The lowest heterozygosity inter-populations were obtained for primer I7 (0), and the highest value was for primer I10 (0.16). The highest level of gene flow was obtained for primers I1 (0.96) and I11 (0.94), while the lowest level was related to I2 (0). The highest heterozygosity intra-populations belonged to primer I2 (0.37), and the lowest was related to primer I9 (0.17). The highest and the lowest value of Fst were in primer I2 (1) and primer I9 (0.55), respectively. The average number of alleles for 11 primers of ISSR was 1.98. The lowest number of effective alleles (Ne) related to I2 (1.18) and the highest these were obtained for primers I10 (1.81). The means values for Nei's genetic diversity index (H) and Shannon diversity index (I) were 0.30 and 0.47, respectively. The highest value of Nei's genetic diversity index (H) and Shannon diversity index (I) belonged to primer I10 (0.43 and 0.62), and the lowest of these were related to primer I2 (0.15 and 0.29) (Table 5).

Table 5: Genetic diversity parameters in narcissus genotypes with ISSR markers

Number	Ht	Hs	Dst	Fst	Na	Ne	H	I	PIC	EMR	MI	RP
I1	0.37	0.14	0.23	0.62	2.00	1.42	0.28	0.45	0.35	14.00	0.85	0.80
I2	0.37	0.00	0.37	1.00	2.00	1.18	0.15	0.29	0.15	3.00	0.35	1.28
I3	0.35	0.11	0.24	0.70	2.00	1.40	0.27	0.43	0.30	5.00	1.49	0.70
I4	0.42	0.07	0.36	0.84	2.00	1.47	0.29	0.47	0.27	8.00	2.17	0.83
I5	0.41	0.10	0.31	0.75	2.00	1.47	0.30	0.46	0.31	10.00	0.08	1.00
I6	0.43	0.07	0.36	0.83	2.00	1.54	0.33	0.50	0.30	4.00	1.19	1.08
I7	0.33	0.06	0.27	0.81	1.83	1.46	0.28	0.43	0.24	4.16	1.02	1.55
I8	0.37	0.12	0.25	0.68	1.93	1.51	0.30	0.46	0.27	12.07	3.28	0.27
I9	0.31	0.14	0.17	0.55	2.00	1.56	0.33	0.50	0.34	4.00	1.35	0.96
I10	0.41	0.16	0.25	0.61	2.00	1.81	0.43	0.62	0.37	8.00	2.99	1.43
I11	0.37	0.13	0.25	0.66	2.00	1.62	0.36	0.53	0.37	5.00	0.83	1.07
Mean	0.38	0.10	0.28	0.73	1.98	1.49	0.30	0.47	0.30	7.02	2.15	1.09

3.3 DETERMINING SIMILARITIES BETWEEN GENOTYPES

The results showed that in both markers, UN1 similarity coefficient is the best coefficient and UPGMA algorithm is the best clustering pattern due to having the highest correlation coefficient (Table 6). Chehrazi et al. (2007) reported a cophenetic correlation coefficient between native and non-native narcissus genotypes was 0.97. Examination of similarity matrix in RAPD marker showed that the highest similarity was between Khosf-2 and Tabas genotypes (0.95). Genotypes such as Shomal with Shastpar, Shiraz-1 with Shiraz-2 and Birjand with Khosf-1 had high similarity (0.93). Shahla with Yasuj and Shahla with Shiraz-1 also had high similarity (0.92). Shastpar with Shahla and with Shiraz-1 and Yasuj with Shiraz-1 had a similarity in 0.91. Shahla with Shiraz-2, Yasuj with Shiraz-2, Shiraz-2 with Kuchak-e-Atri, Birjand with Khosf-2, and Khosf-1 with Khosf-2 had a relatively high similarity (0.90). The lowest similarity between genotypes was belonged to Dutch genotype with other genotypes, so that the similarity coefficient of this genotype with Birjand (0.43), with Shahla (0.45) and with other genotypes was less than 0.50 (Table 7). In general, the similarity coefficients showed that the similarity between most genotypes except Dutch was more than 0.80 and there was a lot of similarity between genotypes

Examination of similarity matrix of ISSR showed that the highest similarity coefficients were obtained between Shomal and Yasuj genotypes (0.92), Shastopar with Shahla and Yasuj with Shiraz-1 (0.91) and Shomal with Shastopar, Shomal with Shiraz-1 and Yasuj with Shiraz-2 (0.90). The similarity between all genotypes except Dutch was relatively high and averaged above 0.80. The lowest

Table 6: Cophenetic correlation coefficient based on SIM, J, DICE, UNI in RAPD and ISSR

	RAPD			ISSR		
Cophenetic coefficients	UPGMA	Single	Complete	UPGMA	Single	Complete
SM	0.99**	0.98**	0.96**	0.96**	0.96**	0.65**
J	0.99**	0.97**	0.94**	0.95**	0.94**	0.91**
DICE	0.98**	0.98**	0.96**	0.96**	0.95**	0.93**
UN1	0.99**	0.98**	0.97**	0.97**	0.60**	0.96**

Table 7: Similarity matrix based on UN1 similarity coefficient for RAPD marker in different genotypes of narcissus

Genotypes	1	2	3	4	5	6	7	8	9	10	11	12
1- Shomal	1											
2-Shastpar	0.93	1										
3-Shahla	0.88	0.91	1									
4-Yasuj	0.87	0.87	0.92	1								
5-Shiraz-1	0.88	0.91	0.92	0.91	1							
6-Shiraz-2	0.87	0.88	0.90	0.90	0.93	1						
7- Kuchak-e-atri	0.83	0.83	0.86	0.84	0.89	0.90	1					
8-Dutch	0.49	0.49	0.45	0.49	0.48	0.47	0.50	1				
9-Birjand	0.81	0.83	0.83	0.82	0.83	0.82	0.84	0.43	1			
10-Khosf-1	0.81	0.80	0.86	0.83	0.84	0.84	0.82	0.49	0.93	1		
11-Khosf-2	0.80	0.82	0.87	0.88	0.84	0.85	0.85	0.47	0. 90	0.90	1	
12-Tabas	0.78	0.80	0.85	0.87	0.84	0.84	0.86	0.49	0.88	0.89	0.95	1

Table 8: Similarity matrix based on UN1 similarity coefficient for ISSR marker in different genotypes of narcissus

Genotypes	1	2	3	4	5	6	7	8	9	10	11	12
1- Shomal	1.00											
2-Shastpar	0.90	1.00										
3-Shahla	0.86	0.91	1.00									
4-Yasuj	0.92	0.88	0.87	1.00								
5-Shiraz-1	0.90	0.86	0.85	0.91	1.00							
6-Shiraz-2	0.83	0.79	0.82	0.90	0.87	1.00						
7- Kuchak-e-atri	0.84	0.80	0.77	0.86	0.87	0.85	1.00					
8-Dutch	0.51	0.60	0.59	0.51	0.50	0.47	0.48	1.00				
9-Birjand	0.85	0.79	0.78	0.87	0.86	0.79	0.80	0.50	1.00			
10-Khosf-1	0.85	0.79	0.82	0.87	0.84	0.84	0.82	0.44	0.87	1.00		
11-Khosf-2	0.87	0.79	0.67	0.82	0.86	0.86	0.80	0.53	0.83	0.84	1.00	
12-Tabas	0.84	0.83	0.83	0.84	0.87	0.83	0.84	0.46	0.83	0.88	0.88	1.00

similarity coefficient was related to the Dutch with others, so that the similarity coefficient between the Dutch with Yasuj, Shastpar, Shahla, Kouchak-e-Atri, Shiraz-1, Shiraz-2, Birjand, Khosf-1, Khosf-2 and Tabas were estimated as 0.51, 0.60, 0.59, 0.48, 0.50, 0.47, 0.50, 0.44, 0.53 and 0.46; respectively (Table 8). In general, it can be deduced from the similarity coefficients, there is a huge difference similarity between internal genotypes and internal and external genotypes and led to its diversity.

3.4 CLUSTER ANALYSIS AND PRINCIPAL COORDINATE ANALYSIS (PCOA)

Based on the RAPD marker, the genotypes were grouped into four clusters (Figure 2). The first cluster was divided into two sub-clusters. The first sub-cluster included Shomal and Shastpar. The second sub-cluster, which also had two subgroups, included Shahla, Yasuj, Shiraz-1 and Shiraz-2. The Kuchak-e-atri was the only genotype of the second cluster. In the third cluster, there were two sub-clusters that Birjand and Khosf-1 were under the first cluster and Khosf-2 and Tabas were under the second cluster. The Dutch genotype was placed independently in the fourth cluster, which shows a clear difference from others. Based on the ISSR marker (Figure 3), the dendrogram divided the genotypes into four clusters. The first cluster was divided into three sub-clusters. The first sub-cluster included Shomal and Yasuj genotypes, the second sub-cluster included Shiraz-1, and the third sub-cluster included Shastpar and Shahla. The second cluster included two genotypes of Shiraz-2 and Kuchak-e-Atri. The third cluster had three sub-clusters. The first sub-cluster included Birjand genotype, the second sub-cluster included Khosf-2 and the third sub-cluster included Khosf-1 and Tabas genotypes. The Dutch genotype independently formed the fourth cluster. This result is consistent with the results of Zanganeh and Salehi (2019) that a foreign genotype formed a separate group independent of the rest.

In general, several factors such as altering life cycles, environmental changes, climate change, and topography can affect the structure, diversity, and genetic similarity of different populations of a plant species in different ways (Sintayehu, 2018, Zhao et al., 2021). In addition, according to the principle of natural selection, plant populations in similar ecological conditions that grow in different geographical locations have more genetic similarities than plant populations grown in various ecological conditions. Our findings were in sequence with the principle, and the results showed a good relationship between genetic diversity and geographical diversity in most cases, and genetic diversity was followed by geographical diversity. The findings indicate a logical relationship between genetic diversity and geographical location. Similar results related to the relationship between geographical and climatic location with genetic diversity of different plant populations have been reported by other researchers using RAPD and ISSR markers (Gharibi et al., 2011, Abou El-Nasr et al., 2013).

In addition, the obtained results of RAPD marker indicated that the first three components explained 96.9 % of the total variance. In the meantime, the first, second, and third components were justified 87.6, 6.3, and 3.1 % of the total variance. The ISSR data also showed that the first three components explained 97.9 % of the total variance, in which the first component justified 89.3 %, the second component equal to 5.7 % and the third component equal to 2.9 % of the total variance.

In general, molecular markers allow detecting variations and polymorphisms for specific regions of DNA among individuals of a population, and the lower percentage of variance assigned to main factors indicates a more appropriate distribution of markers. It means that molecular markers have examined wider regions of the genome. Thus, they have targeted different parts of the genome that contain gene controlling various traits (Mohammadi & Prasanna, 2003, Marwal & Gaur, 2020). Therefore, it can be concluded that in the present study,

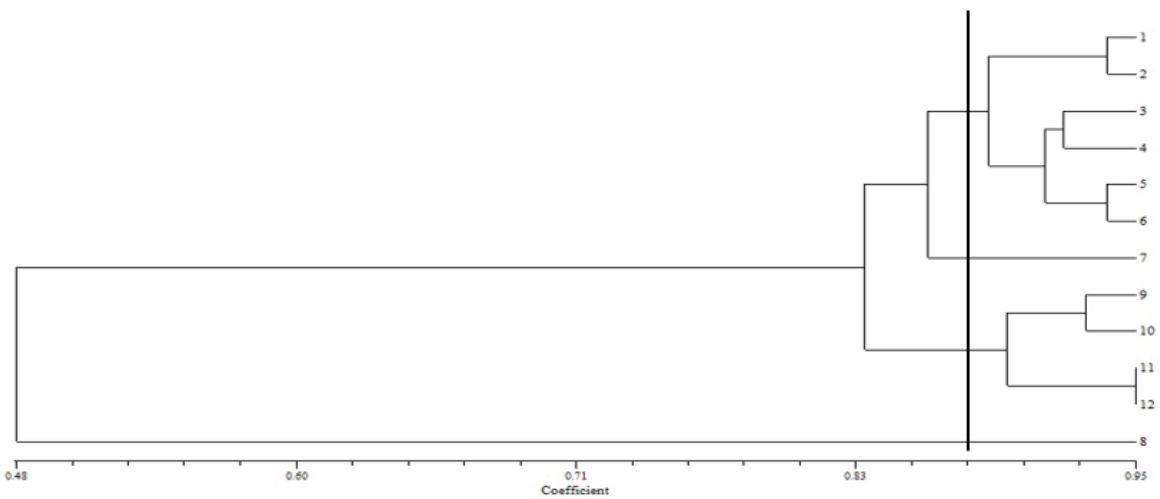

Figure 2: Clustering of Narcissus genotypes based on RAPD markers. 1: Shomal, 2: Shastpar, 3: Shahla, 4: Yasuj, 5: Shiraz-1, 6: Shiraz-2, 7: Kuchak-e-atri, 8: Dutch, 9: Birjand, 10: Khosf-1, 11: Khosf-2, 12: Tabas

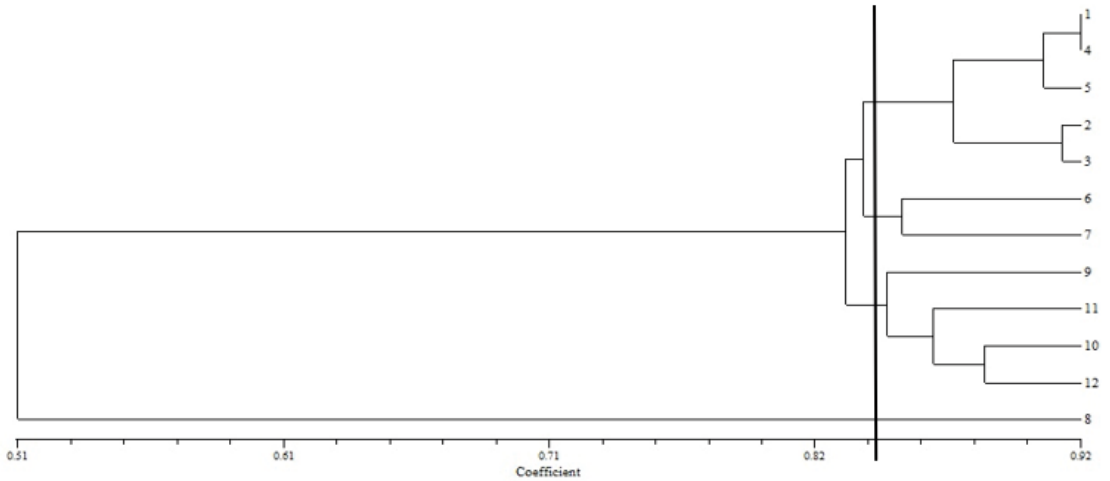

Figure 3: Clustering of Narcissus genotypes based on ISSR markers. 1: Shomal, 2: Shastpar, 3: Shahla, 4: Yasuj, 5: Shiraz-1, 6: Shiraz-2, 7: Kuchak-e-atri, 8: Dutch, 9: Birjand, 10: Khosf-1, 11: Khosf-2, 12: Tabas

RAPD and ISSR markers had good performance and were able to cover a large area of the genome. Hence, it can be concluded that the RAPD and ISSR markers examined in the present study had good performance and were able to cover a large area of the genome. These results are consistent with the results of Zanganeh and Salehi (2019).

4 CONCLUSIONS

In general, based on different coefficients of similarity between genotypes, placement of genotypes in different and distinct clusters and other information, it was been indicated the existence of genetic diversity between the studied genotypes. In most cases, there was a good relationship between genetic diversity and geographical diversity, therefore genetic diversity followed geographical diversity. The study of genetic diversity of narcissus genotypes using 27 RAPD primers and 11 ISSR primers showed that these markers can be useful in identifying polymorphic regions and managing germplasm and can be used to study and determine the genetic distance and differences between narcissus genotypes. The results obtained from RAPD marker were highly consistent with ISSR and conformed the genotypes in clusters according to a distinct pattern. In cluster analysis, placement of genotypes with short geographical distance in close clusters and even one cluster showed that genetic diversity is largely consistent with geographical diversity, and genotypes collected from one geographical area were placed in the same clusters and sub-clusters. Based on principal coordinate analysis, the first three components explained many variances. These results showed that these primers could not evaluate different parts of the sample genome and therefore it is necessary to use more and different primers in future studies.

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6 REFERENCES

Abou El-Nasr, T. H. S., Sherin, A., & Mahfouze, A. K. M. (2013). Assessing phenotypic and molecular variability in fennel (*Foeniculum vulgare*) varieties. *Australian Journal of Basic and Applied Sciences*, *7*(2), 715-722.

Asadi, N., Babaei, A. R., & Naghavi, M. R. (2016). Genetic diversity among some Iranian viola (*viola* Spp.) Genotype using molecular markers ISSR. *Iranian Journal of Horticultural Science and Technology*, *17*(1), 103-116.

Basyuni, M., Prayogi, H., Putri, L. A. P., Syahputra, I., Siregar, E. S., Risnasari, I., ... & Arifiyanto, D. (2018, March). RAPD markers on genetic diversity in three populations of pisifera type of oil palm (*Elaeis guineensis*). In *IOP Conference Series: Earth and Environmental* Science, *130*, 1-5. https://doi.org/10.1088/1755-1315/130/1/012050

Bhandari, H. R., Bhanu, A. N., Srivastava, K., Singh, M. N., & Shreya, H. A. (2017). Assessment of genetic diversity in crop plants-an overview. *Advances in Plants & Agriculture Research*, *7*(3), 279-286. https://doi.org/10.15406/apar.2017.07.00255

Chehrazi, M. (2007). *Study of genetic diversity of endemic and exotic daffodils (Narcissus sp.) by using morphological,*

RAPD and cytogenetically markers. PhD Thesis, Faculty of Agriculture, University of Tehran, 130pp.

Clegg, M. T. (1997). Plant genetic diversity and the struggle to measure selection. *Journal of Heredity*, *88*(1), 1-7. https://doi.org/10.1093/oxfordjournals.jhered.a023048

Doyle, J. J. and Doyle, J. L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin*, *19*, 11-15.

Ezzat, S. M., El Sayed, A. M., & Salama, M. M. (2016). Use of random amplified polymorphic DNA (RAPD) technique to study the genetic diversity of eight *Aloe* species. *Planta Medica*, *82*(15), 1381-1386. https://doi.org/10.1055/s-0042-108208

Gharibi, S., Rahimmalek, M., Mirlohi, A., & Majidi, M. M. (2011). Assessment of genetic diversity in *Achillea millefolium* subsp. *millefolium* and *Achillea millefolium* subsp. *elbursensis* using morphological and ISSR markers. *Journal of Medicinal Plants Research*, *5*(13), 2413-2423. https://doi.org/10.5897/JMPR2021.7203

Gotti, R., Fiori, J., Bartolini, M., & Cavrini, V. (2006). Analysis of Amaryllidaceae alkaloids from *Narcissus* by GC–MS and capillary electrophoresis. *Journal of Pharmaceutical and Biomedical Analysis*, *42*(1), 17-24. https://doi.org/10.1016/j.jpba.2006.01.003 Jiménez, J. F., López-Romero, C., Rosselló, J. A., & Sánchez-Gómez, P. (2017). Genetic diversity of *Narcissus tortifolius*, an endangered endemic species from Southeastern Spain. *Plant Biosystems-An International Journal Dealing with all Aspects of Plant Biology*, *151*(1), 117-125.https://doi.org/10.1080/11263504.2015.1108937

Jiménez, J. F., Sánchez-Gómez, P., Guerra, J., Molins, A., & Rosselló, J. A. (2009). Regional speciation or taxonomic inflation? The status of several narrowly distributed and endangered species of *Narcissus* using ISSR and nuclear ribosomal ITS markers. *Folia Geobotanica*, *44*(2), 145-158. https://doi.org/10.1007/s12224-009-9040-2

Kimura, M., & Crow, J. F. (1963). The measurement of effective population number. *Evolution, 17*(3), 279-288. https://doi.org/10.2307/2406157

Kumar, M., Mishra, G. P., Singh, R., Kumar, J., Naik, P. K., & Singh, S. B. (2009). Correspondence of ISSR and RAPD markers for comparative analysis of genetic diversity among different apricot genotypes from cold arid deserts of trans-Himalayas. *Physiology and Molecular Biology of Plants*, *15*(3), 225-236. https://doi.org/ 10.1007/s12298-009-0026-6

Lewontin, R. C. (1972). The apportionment of human diversity. In *Evolutionary biology* (pp. 381-398). Springer, New York, NY. https://doi.org/10.1007/978-1-4684-9063-3_14

Lynch, M. M. B. G., & Milligan, B. G. (1994). Analysis of population genetic structure with RAPD markers. *Molecular Ecology*, *3*(2), 91-99. https://doi.org/10.1111/j.1365-294x.1994.tb00109.x

Marwal, A., & Gaur, R. K. (2020). Molecular markers: tool for genetic analysis. In *Animal Biotechnology*. Academic Press. pp. 353-372. https://doi.org/10.1016/B978-0-12-416002-6.00016-X

Maske, J. M., Keshavrao, Z. R., & Amarsing, R. C. (2018). Analysis of Genetic Diversity of Commercial Tomato Varieties using Molecular Marker viz. RAPD. *International Journal of Current Microbiology and Applied Sciences 7*(6), 3559-3565. https://doi.org/10.20546/ijcmas.2018.706.418

Mengistu, L. W., Messersmith, C. G., & Christoffers, M. J. (2005). Genetic diversity of herbicide-resistant and-susceptible *Avena fatua* populations in North Dakota and Minnesota. *Weed Research*, *45*(6), 413-423. https://doi.org/10.1614/WS-06-108.1

Mohammadi, S. A., & Prasanna, B. M. (2003). Analysis of genetic diversity in crop plants—salient statistical tools and considerations. *Crop science*, *43*(4), 1235-1248. https://doi.org/10.2135/cropsci2003.1235

Nei, M. (1973). Analysis of gene diversity in subdivided populations. *Proceedings of the national academy of sciences*, *70*(12), 3321-3323. https://doi.org/10.1073/pnas.70.12.3321 Pandey, S., Kumar, S., Mishra, U., Rai, A., Singh, M., & Rai, M. (2008). Genetic diversity in Indian ash gourd (*Benincasa hispida*) accessions as revealed by quantitative traits and RAPD markers. *Scientia Horticulturae*, *118*(1), 80-86. https://doi.org/10.1016/j.scienta.2008.05.031

Pradeep Reddy, M., Sarla, N., & Siddiq, E. A. (2002). Inter simple sequence repeat (ISSR) polymorphism and its application in plant breeding *Euphytica*, *128*(1), 9-17. https://doi.org/10.1023/A:1020691618797

Saeedi H. (2007). *Systemic herbal*. Jahad daneshgahi of Isfahan press.

Sargazi, A., Fakheri, B., Soloki, M., & Fazelinasab, B. (2016). Genetic diversity of some population of medicinal Ajowan (*Carum copticum*) using RAPD marker. *Journal of Medicinal Plants Biotechnology*, *2*(2), 22-36.

Sharma, A. K., & Ghosh, C. (1955). Cytogenetics of some of the Indian umbellifers. *Genetica*, *27*(1), 17-44. https://doi.org/10.1007/BF01664152

Sharma, R., Sharma, S., & Kumar, S. (2018). Pair-wise combinations of RAPD primers for diversity analysis with reference to protein and single primer RAPD in soybean. *Annals of Agrarian Science*, *16*(3), 243-249. https://doi.org/10.1016/j.aasci.2018.03.002

Shi, X., Waiho, K., Li, X., Ikhwanuddin, M., Miao, G., Lin, F., & Ma, H. (2018). Female-specific SNP markers provide insights into a WZ/ZZ sex determination system for mud crabs *Scylla paramamosain*, *S. tranquebarica* and *S. serrata* with a rapid method for genetic sex identification. *BMC Genomics*, *19*(1), 1-12. https://doi.org/10.1186/s12864-018-5380-8

Sintayehu, D. W. (2018). Impact of climate change on biodiversity and associated key ecosystem services in Africa: a systematic review. *Ecosystem Health and Sustainability*, *4*(9), 225-239. https://doi.org/10.1080/20964129.2018.1530054

Suganthi, S., Rajamani, K., Renuka, R., Suresh, J., Joel, A. J., & Suganthan, R. N. (2018). Genetic diversity analysis using ISSR markers in black nightshade (*Solanum nigrum* L. complex). *Medicinal Plants-International Journal of Phytomedicines and Related Industries*, *10*(2), 111-119. https://doi.org/10.1080/19315260.2020.1829768

Taheri, S., Zabet, M., Izanlou A., & Izadi Darbandi, A. (2016). Assessment of genetic diversity of fennel ecotypes using RAPD and ISSR markers. *Agricultural Biotechnology Journal*, *7*(4), 113-128. https://doi.org/10.22103/JAB.2016.1394

Tajdoost, S., Ali Khavari-Nejad, R., Meighani, F., Zand, E., &

Noormohammadi, Z. (2012). Assessment of genetic diversity in *Cuscuta campestris* Yunker ecotypes based on their molecular and protein markers. *Environmental Sciences*, *9*(4), 93-108.

Welsh, J., Honeycutt, R. J., McClelland, M., & Sobral, B. W. S. (1991). Parentage determination in maize hybrids using the arbitrarily primed polymerase chain reaction (AP-PCR). *Theoretical and Applied Genetics*, *2*(4), 473-476. https://doi.org/10.1007/BF00588601

Williams, J. G., Kubelik, A. R., Livak, K. J., Rafalski, J. A., & Tingey, S. V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research*, *18*(22), 6531-6535. https://doi.org/10.1093/nar/18.22.6531

Zangeneh, M., Salehi, H. (2019). Genetic diversity as revealed by intersimple sequence repeat polymorphism in narcissus accessions to identify the tolerant genotypes for deficit irrigation. *Journal of the American Society for Horticultural Science, 144*(2), 92-106. https://doi.org/10.21273/JASHS04583-18

Zadebagheri, M., Sohrabnejad, A., Abutalebi Jahromi, A., & Sharafzadeh, S. (2011). The effects of nitrogen and phosphor on some physico-chemical characteristics and after harvest life of *Narcissus* flower. *New Finding in Agriculture*, *6*(1 (autumn 2011)), 39-51. https://doi.org/10.22059/ijhst.2019.271662.267

Zeybekoğlu, E., Asciogul, T. K., Hulya, I. L. B. I., & Ozzambak, M. E. (2019). Genetic diversity of some daffodil (*Narcissus* L. spp.) genotypes in Turkey by using SRAP markers. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca*, *47*(4), 1293-1298. https://doi.org/10.15835/nbha47411664

Zhao, W., Wang, X., Li, L., Li, J., Yin, H., Zhao, Y., & Chen, X. (2021). Evaluation of environmental factors affecting the genetic diversity, genetic structure, and the potential distribution of *Rhododendron aureum* Georgi under changing climate. *Ecology and Evolution*, *11*(18), 12294-12306. https://doi.org/10.1002/ece3.780