Genetic Diversity as Revealed by Intersimple Sequence Repeat Polymorphism in *Narcissus* Accessions to Identify the Tolerant Genotypes for Deficit Irrigation

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ABSTRACT. There are many species of *Narcissus* in diverse areas of the world in natural or cultured form and there is no complete information about their genetic status, especially the relatedness within a species. Thus, the current study applied intersimple sequence repeat (ISSR) markers to estimate the genetic diversity of 31 accessions, including 30 accessions of *Narcissus tazetta*, collected from 16 regions of Iran and one known exotic narcissus species that is being cultivated in Iran, and identification of tolerant genotypes for deficit irrigation by evaluation of their morpho-physiological characteristics. Seventeen anchored ISSR primers from a total of 19 tested ISSR primer pairs were used and produced 206 bands of different sizes. The average percentage of polymorphic bands was 96.02%. The maximum resolving power (8.32), polymorphic information content average (0.44), and marker index values (5.61) were observed for the primers of 811, 828, and 811, respectively. The unweighted pair group method with arithmetic mean based on Jaccard's coefficients was used to assign the genotypes to one of two major clusters. Both clusters were divided into two subclusters, with single and double flowers separating into subgroups. The results showed that ISSR markers can be used as a diagnostic tool to evaluate genetic variation in *Narcissus* genotypes and reveal their relationships. The results of screening study identified drought-tolerant accessions. They were clustered into two major groups: drought-tolerant accessions with single flowers and drought-sensitive accessions having double and semidouble flowers. The findings presented can be used in breeding programs for different *Narcissus* genotypes.

Flowering bulbs, or ornamental geophytes (Alam et al., 2013; De Hertogh and Le Nard, 1993), show variation in morphological, developmental, biological, and physiological reactions to environmental elements. They are significant contributors to the universal ornamental trade and profitably used for bulb and flower production of outdoor and potted plants and fresh-cut flowers, as well for landscaping in private gardens. Although ornamental geophytes constitute more than 800 genera, the industry is controlled by only seven: *Tulipa*, *Lilium*, *Narcissus*, *Gladiolus*, *Hyacinthus*, *Crocus*, and *Iris* (Kamenetsky and Okubo, 2013).

Narcissus is a member of the Amaryllidaceae family, which comprises 65 genera and more than 860 species. These species are broadly dispersed in tropical, subtropical, and temperate areas of the northern and southern hemispheres (Hanks, 2003). They possess highly decorative characteristics and have been used in floriculture and medicine since ancient times (Artyushenko, 1970; Khamidkhodzhaev, 1984). Amaryllids are important decorative ornamentals and are popular for indoor and outdoor use as both winter and spring coloring (Hanks, 2003). In addition, plants belonging to the Amaryllidaceae have been identified as possessing alkaloids that encompass a range of biological activity (Pigni et al., 2010).

Researchers have been able to isolate more than 200 alkaloids from the plants of this family (Hanks, 2003). Within this group, the genus *Narcissus* is used in traditional medicine to treat a variety of health problems. A range of pharmacological effects has been attributed to these alkaloids, and include antiviral, antifungal, and antitumor activity (Pigni et al.,

2010). Due to their delicate odor, the fragrance of narcissus flowers is popular for use in luxury perfumes. Among these species, *Narcissus poeticus*, *N. tazetta*, and *Narcissus jonquilla* are notable (Ruiz-Ramon et al., 2014). The complexity of *Narcissus* relates to the ease in which hybridization occurs naturally as well as by cultivation, breeding, selection, escape, and naturalization (Hanks, 2003).

Based on chromosomal studies of the genus, Fernandes (1968) separated *Narcissus* into two subgenera [*Hermione* ($x = 5$) and *Narcissus* ($x = 7$)] and 10 sections. Principally, *Narcissus* has a Mediterranean distribution with a center of diversity on the Iberian Peninsula. The genus also can occur in southwestern France, northern Africa, and eastward to Greece. *N. tazetta* is primarily found in Spain and northern Africa, as well as in a narrow band within China and Japan.

The development of effective conservation and management strategies requires a thorough understanding of the genetic diversity and relationships between the plant varieties to identify gaps in the germplasm collection (Nayanakantha et al., 2010). Identification of plant genotypes can be conducted through morphological, enzymatic, and genetic quantification strategies (Pivorienė and Pasakinskiene, 2008; Qian et al., 2001). Morpho-chemical characteristics are dependent on age and environment (Deore and Samantaray, 2015; Nayanakantha et al., 2010). Because they are largely free from environmental influence, DNA-based molecular markers have been applied extensively to measure genetic diversity (Deore and Samantaray, 2015).

Randomly amplified polymorphic DNA (RAPD) technology was used by Chehrizi et al. (2008) to discover the genetic relationship between the endemic and exotic narcissus. Nine main groups were identified. Using microsatellite

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Table 1. The accessions of *Narcissus tazetta* collected from different regions of Iran.

Accession no.	Accession	Collecting region	Latitude longitude altitude	Plant morphology
1	Behbahan1	Behbahan	30°37'11.56"N 50°16'07.98"E 329 m	Single flower—Cream, round, and wide tepal Medium, short, yellow, and cupped corona Light green leaves—4–6 flowers
2	Behbahan2	Behbahan	30°37'11.56"N 50°16'07.98"E 329 m	Double flower—Cream and lanceolate tepal Small, short, yellow, and wrinkled corona Light green leaves—4–6 flowers
3	Behbahan3	Behbahan	30°37'11.56"N 50°16'07.98"E 329 m	Semidouble flower—Cream and lanceolate tepal Small, short, yellow, and wrinkled corona Light green leaves—4–6 flowers
4	Behbahan4	Behbahan	30°37'11.56"N 50°16'07.98"E 329 m	Single flower—White and lanceolate tepal Small, short, yellow, and cupped corona Light green leaves—4–6 flowers
5	Behbahan5	Behbahan	30°37'11.56"N 50°16'07.98"E 329 m	Semidouble flower—Tepal and corona: green, wrinkled, impartible, and formless Light green leaves—4 flowers
6	Behbahan6	Behbahan	30°37'11.56"N 50°16'07.98"E 329 m	Single flower—Cream, round, and wide tepal Medium, short, orange, and cupped corona Light green leaves—4–5 flowers
7	Behbahan7	Behbahan	30°37'11.56"N 50°16'07.98"E 329 m	Double flower—Cream and lanceolate tepal Medium, short, yellow, and wrinkled corona Light green leaves—4 flowers
8	Behbahan8	Behbahan	30°37'11.56"N 50°16'07.98"E 329 m	Double flower—Cream and lanceolate tepal Big, short, yellow, and wrinkled corona Light green leaves—4 flowers
9	Ahvaz1	Ahvaz	31°20'03.94"N 48°44'12.17"E 21 m	Single flower—Cream, round, and wide tepal Medium, short, yellow, and cupped corona Light green leaves—4–6 flowers
10	Ahvaz2	Ahvaz	31°20'03.94"N 48°44'12.17"E 21 m	Double flower—Cream and lanceolate tepal Small, short, yellow, and wrinkled corona Light green leaves—4–6 flowers
11	Birjand	Birjand (Khusf)	32°46'35.81"N 58°54'00.82"E 1,329 m	Single flower—Cream, round, and wide tepal Medium, short, yellow, and cupped corona Light green leaves—4–6 flowers
12	Shiraz1	Shiraz	29°39'20.67"N 52°28'39.10"E 1,614 m	Single flower—Cream, round, and wide tepal Medium, short, yellow, and cupped corona Light green leaves—4–6 flowers
13	Shiraz2	Shiraz	29°39'20.67"N 52°28'39.10"E 1,614 m	Double flower—Cream and spoon-shaped tepal Small, short, yellow, and wrinkled corona Light green leaves—6–8 flowers
14	Mehran	Mehran	33°06'58.13"N 46°10'13.66"E 153 m	Single flower—Cream, round, and wide tepal Medium, short, yellow, and cupped corona Light green leaves—4–6 flowers
15	Dareh Shahr	Dareh Shahr	33°08'35.37"N 47°23'21.13"E 670 m	Single flower—Cream, round, and wide tepal Medium, short, yellow, and cupped corona Light green leaves—4–6 flowers
16	Jahrom1	Jahrom (Khafr)	28°58'02.23"N 53°11'53.14"E 1,284 m	Single flower—Cream, round, and wide tepal Medium, short, yellow, and cupped corona Light green leaves—5–6 flowers
17	Jahrom2	Jahrom (Khafr)	28°58'02.23"N 53°11'53.14"E 1,284 m	Double flower—Cream and lanceolate tepal Small, short, yellow, and wrinkled corona Light green leaves—6 flowers
18	Bavi1	Ahvaz (Bavi)	31°35'47.37"N 48°53'01.58"E 29 m	Single flower—Cream, round, and wide tepal Medium, short, yellow, and cupped corona Light green leaves—4–6 flowers
19	Bavi2	Ahvaz (Bavi)	31°35'47.37"N 48°53'01.58"E 29 m 29°19'40.75"N	Double flower—Cream and lanceolate tepal Small, short, yellow, and wrinkled corona Light green leaves—4–6 flowers Single flower—Cream, round, and wide tepal

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Table 1. Continued.

Accession no.	Accession	Collecting region	Latitude longitude altitude	Plant morphology
20	Kazerun	Kazerun (Bolbolak)	51°56'00.77"E 784 m	Medium, short, yellow, and cupped corona Light green leaves—4–6 flowers
21	Pol Dokhtar1	Pol Dokhtar	33°08'43.97"N 47°43'25.37"E 712 m	Single flower—Cream, round, and wide tepal Medium, short, yellow, and cupped corona Light green leaves—4–6 flowers
22	Pol Dokhtar3	Pol Dokhtar	33°08'43.97"N 47°43'25.37"E 712 m	Semidouble flower—Cream and lanceolate tepal Small, short, yellow, and wrinkled corona Light green leaves—4–6 flowers
23	Gachsaran1	Gachsaran	30°24'28.72"N 50°54'59.04"E 1,593 m	Single flower—Cream, round, and wide tepal Medium, short, yellow, and cupped corona Light green leaves—4–6 flowers
24	Gachsaran3	Gachsaran	30°24'28.72"N 50°54'59.04"E 1,593 m	Semidouble flower—Cream and lanceolate tepal Small, short, yellow, and wrinkled corona Light green leaves—4–6 flowers
25	Kerman1	Kerman (Jiroft-Darin)	28°38'32.04"N 32°02'03.60"E 1,845 m	Single flower—Cream, round, and wide tepal Medium, short, yellow, and cupped corona Light green leaves—4 flowers
26	Kerman2	Kerman (Jiroft)	28°26'02.90"N 52°08'19.40"E 1,350 m	Single flower—Cream, round, and wide tepal Medium, short, yellow, and cupped corona Light green leaves—4 flowers
27	Abdanan	Abdanan	32°59'39.60"N 47°25'31.74"E 890 m	Single flower—Cream, round, and wide tepal Medium, short, yellow and cupped corona Light green leaves—6 flowers
28	Ghaem Shahr	Ghaem Shahr	36°27'46.59"N 52°49'57.39"E 53 m	Single flower—Cream, round, and wide tepal Medium, short, yellow, and cupped corona Light green leaves—4–6 flowers
29	Rasht1	Rasht	37°17'27.22"N 49°32'57.76"E –11 m	Single flower—Cream, round, and wide tepal Medium, short, yellow, and cupped corona Light green leaves—4–6 flowers
30	Rasht3	Rasht	37°17'27.22"N 49°32'57.76"E –11 m	Semidouble flower—Cream and lanceolate tepal Small, short, yellow, and wrinkled corona Light green leaves—4–6 flowers
31	Exotic narcissus	—	—	Single flower—Yellow and lanceolate tepal Large, long, and yellow trumpet corona Dark green leaves—Only one flower

and chloroplast DNA, Hodgins and Barrett (2007) studied the population structure and genetic diversity of *Narcissus triandrus*. Lu et al. (2007) applied amplified fragment length polymorphism (AFLP) and RAPD markers to investigate the influence of irradiation on Chinese *Narcissus* for the purpose of analyzing its genetic variation.

Tucci et al. (2004) studied two species, namely *N. poeticus* and *Narcissus radiiflorus* populations originating from two geographically different locations. Each species was examined using AFLP markers. Interestingly, the results from cluster analysis and principal coordinate analysis (PCoA) of the computed similarity matrix showed that the genetic distance between individuals belonging to different populations of the same species was somewhat more than those between species.

Medrano et al. (2014) used AFLP markers for the purpose of studying genetic lines in an endemic population of daffodil (*Narcissus pseudonarcissus*) for which the taxonomic distinctions were problematic. The results identified well-defined ancestries of daffodil, which exist all over the southern highlands of the Iberian Peninsula. These findings strongly support the taxonomical definition of this lineage and its qualifying as a distinct part of conservation.

One method for the genetic mapping and genetic estimation of germplasm resources is using SSR markers (Estoup and Angers, 1998; McCough et al., 1997). A major limitation of this method is its requirement of distinguishing the flanking sequences to design species-specific primers for SSR polymorphism. Use of inter-SSR (ISSR) markers is an alternative method that overcomes most weaknesses of other markers (Gupta et al., 1994; Meyer et al., 1993; Pradeep Reddy et al., 2002; Wu et al., 1994; Zietkiewicz et al., 1994). This technique requires only a small amount of template DNA and does not need genome sequence information for the purpose of primer construction. ISSR markers also offer a random distribution all over the genome, resulting in the production of several fragments per reaction (Shi et al., 2010).

In ISSR, SSR motifs are primarily applied to amplify the ISSR regions (Tautz and Renz, 1984). The technique uses single primers of 16 to 25 nucleotides that are generally anchored at the 3' or 5' ends with one to four degenerate bases extended into the flanking sequences (Godwin et al., 1997; Zietkiewicz et al., 1994). ISSR–polymerase chain reaction (PCR) is commonly used in areas of genetic diversity, gene tagging, phylogenetic studies, genome mapping,

and evolutionary biology for a series of plant species (Pradeep Reddy et al., 2002). ISSRs have been effectively used to evaluate the degree of genetic diversity at the inter- and intraspecies levels for plant species such as aloe [*Aloe barbadensis* (Deore and Samantaray, 2015)]; onion, garlic, and leek [*Allium cepa*, *Allium sativum*, and *Allium porrum*, respectively (Mukherjee et al., 2013)]; an uncommon endemic orchid in China [*Calanthe tsoongiana* (Qian et al., 2013)], jasmine [*Jasminum* sp. (Ghasemi Ghehsareh et al., 2014)], three genera from the Iridaceae family (Raycheva et al., 2011), tall fescue [*Festuca arundinacea* (Salehi et al., 2014)], melon [*Cucumis melo* (Monforte et al., 2003)], tulip [*Tulipa* sp. (Kiani et al., 2012)], trifoliolate orange [*Poncirus trifoliata* (Fang et al., 1997)], and several other species of wild and cultivated plants. The objective of this investigation was to apply ISSR-PCR to determine the genetic diversity of available accessions of *Narcissus* species in Iran, and identification of tolerant genotypes for deficit irrigation by evaluation of their morpho-physiological characteristics.

Drought is a major abiotic stress that affects the growth of plants and is caused by low water availability in arid and semiarid regions or due to changes in precipitation (Toker et al., 2007). During environmental stresses such as drought, reactive oxygen species (ROS) are increasingly produced. ROS negatively affect the growth and development of plants (Rahimizadeh et al., 2007) and is thought to be a contributing factor to stress damage and for quick cellular injury that occurs when plants are exposed to stress (Yong et al., 2006). ROS damages proteins, membrane lipids, and nucleic acids (Foyer et al., 1994). Activated oxygen also causes protein denaturation, lipid peroxidation, and DNA mutation at higher oxidative stress levels

(Baby and Jini, 2011). When plants are exposed to drought stress, antioxidants function as a protective mechanism against free radicals and increase the activity and amount of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), and an amino acid (proline) in plant cells (Rahimizadeh et al., 2007). Information about the significant morpho-physiological characteristics of *Narcissus* accessions can be applied to breeding programs to detect accessions resistant to drought stress; thus, we investigated the physiological and morphological traits of some *Narcissus* accessions under water-sufficient and water-deficient conditions.

Materials and Methods

EXPERIMENTAL SITE AND PLANT MATERIAL. The assay was performed in the Central Laboratory and Biotechnology Laboratory of the College of Agriculture at Ramin University, Ahvaz, Iran. Thirty accessions of *N. tazetta* from 16 regions of Iran and one commercial exotic species of *Narcissus* (*N. pseudonarcissus*) were used in this research (Table 1).

EXTRACTION OF GENOMIC DNA. Genomic DNA was extracted from young leaves using the standard CTAB method proposed by Doyle and Doyle (1990) with minor alterations. Leaf samples (0.1 g) were ground in liquid N₂ and the leaf powder was immediately transferred to two centrifuge tubes with 800 µL of extraction buffer [2% (w/v) CTAB, 1.4 M NaCl, 20 mM Na₂ EDTA (pH 8), 100 mM Tris-HCl (pH 8), 0.5% (w/v) polyvinylpyrrolidone, 1% (v/v) 2-mercaptoethanol] that had been prewarmed to 65 °C. The sample was kept at 65 °C for 30 min and the centrifuge tubes were inverted for 10 min. It then was mixed with 500 µL of chloroform–isoamyl alcohol (24:1)

Table 2. List of intersimple sequence repeat (ISSR) primers and results of experiments performed among 31 *Narcissus* accessions using ISSR markers.

Primer no.	Sequence (5'–3') ^z	Annealing temp (°C)	Fragment size range (bp)	Loci (no./primer)	Polymorphic loci (no.)	Pp (%) ^y	EMR ^y	PIC ^y	MI ^y	Rp ^y
807	(AG)8T	32.6	400–1,700	9	9	100	9	0.26	2.34	3.35
808	(AG)8C	36	390–1,000	6	6	100	6	0.32	1.92	3.09
809	(AG)8G	34.3	220–1,300	9	9	100	9	0.17	1.53	2
810	(GA)8T	34.3	290–1,100	15	14	93.33	13.06	0.21	2.74	4.19
811	(GA)8C	34.3	280–1,150	17	17	100	17	0.33	5.61	8.32
816	(CA)8T	—	—	—	—	—	—	—	—	—
825	(AC)8T	26.4	400–1,250	12	10	83.33	8.33	0.18	1.5	3.03
827	(AC)8G	42.7	320–1,400	10	7	70	4.9	0.15	0.73	2
828	(TG)8A	37	520–920	7	7	100	7	0.44	3.08	4.9
829	(TG)8C	43.4	570–1,400	6	6	100	6	0.31	1.86	2.77
830	(TG)8G	42.3	470–1,300	10	10	100	10	0.28	2.8	4.19
835	(AG)8YC	35	280–1,250	15	14	93.33	13.06	0.29	3.78	6.97
840	(GA)8YT	35	270–1,200	11	11	100	11	0.29	3.19	4.77
841	(GA)8YC	38.5	320–850	13	12	92.31	11.08	0.22	2.44	3.93
845	(CT)8RG	39.5	550–1,700	18	18	100	18	0.21	3.78	5.29
855	(AC)8YT	38.4	580–1,150	11	11	100	11	0.32	3.52	5.55
857	(ACA)5CYG	44.7	350–1,500	23	23	100	23	0.19	4.37	5.42
861	(ACC)6	—	—	—	—	—	—	—	—	—
ALO	(GTG)3GC	38.5	400–1,500	14	14	100	14	0.17	2.38	3.35
			Total	206	198	—	—	—	—	—
			Mean	12.11	11.65	96.02	—	—	—	—

^zY = cytosine (C) or thymine (T).

^yPp = polymorphic percentage; EMR = effective multiple ratio; EMR = np (np/n), where np = number of polymorphic loci and n = total number of loci; PIC = polymorphic information content [$= 1 - p^2 - q^2$ (Powell et al., 1996)], where p is frequency of visual alleles and q is the frequency of null alleles; MI = marker index [$= PIC \times EMR$ (Powell et al., 1996)]; Rp = resolving power [$= \sum I_b$, $I_b = 1 - (2 \times |0.5 - p|)$].

and slowly shaken for 30 min, then centrifuged at 13,000 g_n for 10 min at 4 °C. The supernatant was collected and the same amount of cold isopropanol was added. After incubation for 20 min at –20 °C, the mixture was centrifuged at 13,000 g_n for 10 min at 4 °C.

The aqueous phase was discarded and the pelleted DNA was washed twice with 500 μ L of 70% ethanol and again centrifuged

at 4 °C for 10 min at 13,000 g_n and then dried. Extracted DNA samples were resuspended with 50 μ L double-distilled water (ddH₂O) and were kept at –20 °C until used. The quantity and quality of DNA were determined using spectrophotometer (NanoDrop 2000; Thermo Fisher Scientific, Waltham, MA) and 1% agarose gel electrophoresis. The concentrations of the extracted DNA samples were measured by absorption at 260 nm, and the ratios A260/280 and A260/230 were used to estimate the amount of contamination, such as proteins, polyphenolic compounds, sugars, and lipids (Raycheva et al., 2011). Finally, the DNA samples were diluted to ≈ 50 ng· μ L^{–1} for use in the ISSR assay.

PRIMERS AND ISSR AMPLIFICATION. In this study, 19 ISSR primers (Takapouzist, Tehran, Iran) were selected that amplified *A. cepa*, *A. sativum*, and *A. porrum* accessions (Mukherjee et al., 2013); *Tulipa* accessions (Kiani et al., 2012); and *A. barbadensis* accessions (Deore and Samantaray, 2015). These di- and trinucleotide repeat primers were used to generate band profiles. The 19 anchored primers were screened for polymorphisms on DNA samples from 31 *Narcissus* accessions. The primers were selected based on the number of polymorphic fragments produced, band size, amplification intensity, and reproducibility. Two primers were excluded because their banding patterns were difficult to score accurately on 2% agarose

Fig. 1. *Narcissus tazetta* at (A) Flowering stage; 1 = water-sufficient condition [100% field capacity (FC)], 2 = drought-tolerant accession in water-stress condition (25% FC), and 3 = drought-sensitive accession in water-stress condition (25% FC). (B) Vegetative stage; 1 = water-sufficient condition (100% FC), 2 = drought-tolerant accession in water-stress condition (25% FC), and 3 = drought-sensitive accession in water-stress condition (25% FC).

Table 3. Sample size (N), number of alleles (Na), number of effective alleles (Ne), Nei's genetic diversity (I), Shannon's information index (He), and percentage of polymorphic loci (Pp), of *Narcissus* populations collected from different regions of Iran.

Population	N	Na	Ne	I	He	Pp	Bands (no.)
			(SE)				
Behbahan	8	1.218 (0.061)	1.325 (0.027)	0.275 (0.020)	0.185 (0.014)	51.94	144
Ahvaz	2	0.733 (0.054)	1.144 (0.020)	0.123 (0.017)	0.084 (0.012)	20.39	109
Shiraz	2	0.660 (0.053)	1.124 (0.019)	0.106 (0.016)	0.072 (0.011)	17.48	100
Birjand (Khusf)	1	0.466 (0.035)	1.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.00	96
Kerman (Giroft)	2	0.718 (0.055)	1.144 (0.020)	0.123 (0.017)	0.084 (0.012)	20.39	106
Mehran	1	0.374 (0.034)	1.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.00	77
Dareh Shahr	1	0.505 (0.035)	1.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.00	104
Abdanan	1	0.461 (0.035)	1.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.00	95
Ghachsaran	2	0.937 (0.055)	1.199 (0.022)	0.170 (0.019)	0.117 (0.013)	28.16	135
Pol Dokhtar	2	0.650 (0.049)	1.096 (0.017)	0.082 (0.014)	0.056 (0.010)	13.59	106
Ghaem Shahr	1	0.485 (0.035)	1.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.00	100
Rasht	2	0.845 (0.056)	1.182 (0.022)	0.156 (0.018)	0.107 (0.013)	25.73	121
Bavi	2	0.733 (0.050)	1.110 (0.018)	0.094 (0.015)	0.064 (0.010)	15.53	119
Gahrom (Khafr)	2	0.772 (0.053)	1.141 (0.020)	0.120 (0.017)	0.082 (0.012)	19.90	118
Kazerun (Bolbolak)	1	0.466 (0.035)	1.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.00	96
Exotic Narcissus	1	0.466 (0.035)	1.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.00	96
Mean	1.938	0.656 (0.012)	1.092 (0.004)	0.078 (0.004)	0.053 (0.002)	—	—

gels; thus, 17 primers were selected for further study (Table 2).

The primers were applied to amplify the interrepeat motifs in the genomic DNA of the *Narcissus* accessions using a standard PCR protocol with some alterations. PCR was performed with 25- μ L reactions, including 17.05 μ L sterile ddH₂O, 2.5 μ L of 10X assay buffer, 0.75 μ L MgCl₂, 0.5 μ L dNTP (10 mM total, 2.5 mM each), 2 μ L primer, 0.2 μ L (5 U- μ L⁻¹) Taq-DNA polymerase (Sinnagen, Tehran, Iran), and 2 μ L genomic DNA (50 ng- μ L⁻¹). A sample without genomic DNA was used as the negative control in each assay. The amplification reactions were carried out in a thermal cycler (iCycler; Bio-Rad Laboratories, San Diego, CA) under the following conditions: initial denaturation at 94 °C for 4 min followed by 40 amplification cycles of denaturation at 94 °C for 45 s; primer annealing for 45 s at optimized temperature (26.4 to 44.7 °C depending on the kind of primer), and elongation at 72 °C for 1 min with a final extension at 72 °C for 4 min.

The PCR products were electrophoresed on 2% agarose gels in 0.5x tris borate EDTA buffer stained with DNA safe stain dye (0.025 μ L- μ L⁻¹) by horizontal gel electrophoresis (22 $\times$ 18 cm with 25 wells) at 100 V for 4 h, then the band profiles were visualized under ultraviolet light and photographed with a gel documentation and analysis system (JS-6800; UVITEC, Shanghai, China). The size of the amplified products was evaluated by comparing them with a 100-base pair (bp) ladder as the molecular size standard (Fermentas, St. Leon-Rot, Germany).

DATA ANALYSIS. For genetic diversity analysis, each ISSR band was treated as a dominant allele for a particular fragment. Amplified bands of each fragment were scored

based on the presence (1) and absence (0) of the bands. Only clear and reproducible fragments were scored and the data matrices of the ISSR fragments were accumulated for further investigation. These data were applied to generate a similarity matrix based on Jaccard's coefficients by using the SIMQUAL program in the NTSYS-PC software package version 2.02e (Rohlf, 1998). To construct the dendrogram, clustering was performed using the unweighted pair group method with arithmetic mean (UPGMA) based on Jaccard's coefficients. A Mantel test was used to calculate the coefficient of cophenetic correlation, which is a measure of the congruence of the similarity matrix and the dendrogram (Mantel, 1967).

The ability of each primer to differentiate between accessions was assessed by calculating their resolving power [Rp (Deore and Samantaray, 2015; Gilbert et al., 1999)], marker index [MI (Ghasemi Ghehsareh et al., 2014; Powell et al., 1996)], and polymorphic information content [PIC (Deore and Samantaray, 2015; Ghasemi Ghehsareh et al., 2014; Weising et al., 2005)]. PIC was calculated as $PIC = 1 - p_i^2 - q_i^2$, where p is the band rate and q is no band rate (Chadha and Gopalakrishna, 2007; Deore and Samantaray, 2015). MI was calculated as $MI = PIC \times \text{effective multiplex ratio (EMR)}$ and $Rp = \sum I_b$, I_b (band informativeness) = $1 - (2 \times [0.5 - p])$, where p is the proportion of each accession comprising the band (Ghasemi Ghehsareh et al., 2014). GenAEx 6.4 (Peakall and Smouse, 2006) was used to calculate the percentage of polymorphic fragments, and the parameters of PIC, EMR, MI, and Rp were calculated by Excel (2010 for Windows; Microsoft Corp., Redmond, WA).

The diversity measures were calculated with Nei's (1978) genetic diversity index (He) and Shannon information index

Fig. 2. Dendrogram based on unweighted pair group method with arithmetic mean of Jaccard's similarity matrix represents the genetic relatedness among 31 *Narcissus* accessions from different regions of Iran based on intersimple sequence repeat data. Regions of Iran: 1 = Behbahan1, 2 = Behbahan2, 3 = Behbahan3, 4 = Behbahan4, 5 = Behbahan5, 6 = Behbahan6, 7 = Behbahan7, 8 = Behbahan8, 9 = Ahvaz1, 10 = Ahvaz2, 11 = Birjand, 12 = Shiraz1, 13 = Shiraz2, 14 = Mehran, 15 = Dareh Shahr, 16 = Jahrom1, 17 = Jahrom2, 18 = Bavi1, 19 = Bavi2, 20 = Kazerun, 21 = Pol Dokhtar1, 22 = Pol Dokhtar3, 23 = Gachsaran1, 24 = Gachsaran3, 25 = Kerman1, 26 = Kerman2, 27 = Abdanan, 28 = Ghaem Shahr, 29 = Rasht1, 30 = Rasht3, 31 = exotic narcissus.

(I). GenAlEx 6.4 software was used to calculate the following genetic diversity parameters: pairwise genetic distance using Nei's genetic identity between populations, He, I, effective number of alleles (Ne), and the percentage of polymorphic fragments. NTSYS-pc software (version 2.02e) was also used for PCoA to show the genetic relationships between accessions.

Fig. 3. Biplot derived from principal coordinate analysis (PCoA) of genetic distance matrix based on intersimple sequence repeat data in 31 *Narcissus* accessions (PC 1 vs. 2) from different regions of Iran: 1 = Behbahan1, 2 = Behbahan2, 3 = Behbahan3, 4 = Behbahan4, 5 = Behbahan5, 6 = Behbahan6, 7 = Behbahan7, 8 = Behbahan8, 9 = Ahvaz1, 10 = Ahvaz2, 11 = Birjand, 12 = Shiraz1, 13 = Shiraz2, 14 = Mehran, 15 = Dareh Shahr, 16 = Jahrom1, 17 = Jahrom2, 18 = Bavi1, 19 = Bavi2, 20 = Kazerun, 21 = Pol Dokhtar1, 22 = Pol Dokhtar3, 23 = Gachsaran1, 24 = Gachsaran3, 25 = Kerman1, 26 = Kerman2, 27 = Abadan, 28 = Ghaem Shahr, 29 = Rasht1, 30 = Rasht3, 31 = exotic narcissus.

EXPERIMENTAL DESIGN TO DETERMINE DROUGHT STRESS RESPONSES. Endemic *N. tazetta* bulbs were harvested in July. Uniform-sized bulbs, after treatment with benomyl fungicide (0.2%), were planted in the plastic containers (filled with clay loam soil) in last week of August and moved to a greenhouse under $25 \pm 5^\circ\text{C}$ at planting time and gradually reached to $10 \pm 5^\circ\text{C}$ at flowering time under 3000 lx, 12 h per day and 70% to 80% relative humidity. The assay was organized as a completely randomized design with three replications of two samples each. The plants were exposed to one of the following regimens: water-sufficient condition [100% field capacity (FC)] and water stress (25% FC) (Fig. 1). At the end of the vegetative stage, fresh leaves were excised to calculate their physiological traits of CAT (Dhindsa et al., 1981), SOD (Beauchamp and Fridovich, 1971), and POX (Chance and Maehly, 1955) activity, proline and total protein contents, and relative water content (RWC). Morphological characteristics including fresh and dry weight ratio, number of bulblets, and dry weight of bulb were assessed as

Table 4. Nei's genetic identity between *Narcissus* populations collected from different regions of Iran by intersimple sequence repeat markers.

Population no. ^z	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	1.000															
2	0.903	1.000														
3	0.885	0.833	1.000													
4	0.880	0.849	0.834	1.000												
5	0.914	0.878	0.892	0.862	1.000											
6	0.891	0.810	0.874	0.830	0.882	1.000										
7	0.742	0.733	0.726	0.680	0.735	0.723	1.000									
8	0.789	0.793	0.685	0.782	0.776	0.699	0.714	1.000								
9	0.873	0.816	0.822	0.833	0.859	0.847	0.784	0.850	1.000							
10	0.831	0.770	0.816	0.774	0.809	0.797	0.788	0.806	0.900	1.000						
11	0.791	0.770	0.735	0.748	0.768	0.743	0.757	0.820	0.836	0.852	1.000					
12	0.853	0.790	0.816	0.781	0.816	0.809	0.776	0.817	0.903	0.930	0.835	1.000				
13	0.845	0.828	0.833	0.812	0.842	0.808	0.794	0.823	0.911	0.877	0.810	0.853	1.000			
14	0.842	0.793	0.816	0.803	0.849	0.816	0.767	0.769	0.879	0.869	0.796	0.863	0.860	1.000		
15	0.829	0.768	0.771	0.854	0.810	0.772	0.660	0.811	0.838	0.772	0.767	0.815	0.806	0.833	1.000	
16	0.517	0.486	0.459	0.398	0.450	0.432	0.427	0.461	0.459	0.455	0.417	0.460	0.426	0.407	0.388	1.000

^z1 = Behbahan (8 accessions), 2 = Ahvaz (2 accessions), 3 = Shiraz (2 accessions), 4 = Birjand (1 accession), 5 = Kerman (2 accessions), 6 = Mehran (1 accession), 7 = Dareh shahr (1 accession), 8 = Abadan (1 accession), 9 = Ghachsaran (2 accessions), 10 = Pol Dokhtar (2 accessions), 11 = Ghaem shahr (1 accession), 12 = Rasht (2 accessions), 13 = Bavi (2 accessions), 14 = Jahrom (2 accessions), 15 = Kazerun (1 accession), 16 = exotic narcissus.

well. The morpho-physiological traits were measured for accession clustering as either drought tolerant or drought sensitive using between-groups linkage of hierarchical cluster analysis by using the SPSS statistical package (version 16.0; IBM Corp., Armonk, NY).

Results

The selected primers generated 206 fragments (12.11 fragments per primer) ranging from 220 to 1700 bp (Table 2). The highest number of fragments (23) was observed with primer 857 and the minimum number (6) was generated by primers 808 and 829. In total, 198 fragments (96.02%) were polymorphic. The maximum Rp value was 8.32 for primer 811 and the minimum value was for primers 809 and 827 (2). The major PIC value (0.44) was observed for primer 828 and the minor (0.15) for primer 827. The MI value varied from 0.73 (for primer 827) to 5.61 (for primer 811) (Table 2).

The maximum number of bands was generated for accession 19 (108) and the minimum were for 15 and 25 (77 and 78,

respectively). Behbahan's population showed the highest percentage of polymorphic fragments (51.94%) and the lowest percentage (0.00%) was observed in Birjand, Mehran, Abadan, Dareh Shahr, Kazerun, Rasht, and the exotic populations (Table 3).

The cophenetic coefficient of ISSR clustering from Jaccard's similarity matrix was 0.8882 ($P = 0.001$). To evaluate the genetic similarities, the 0/1 matrix was used to construct a similarity matrix using Jaccard's coefficients. The similarity matrices then were applied to create a dendrogram using UPGMA. At the 0.23 level of similarity, the accessions formed two groups. All of the endemic narcissi from the different distribution areas of Iran were clustered in one group, and the exotic narcissus formed a separate cluster. Moreover, at the similarity coefficient of 0.55, accession 4 (Meskin variant) separated from other accessions and formed a separate cluster. The dendrogram indicated that other endemic accessions were grouped into two main clusters. The initial main cluster was separated into two subclusters (A and B). Subcluster A contained 13 accessions of single-flower genotypes (Shahla; 6 accessions), semidouble flowers (2 accessions) and double

Table 5. Evaluation of physiological characteristics of 30 accessions of *Narcissus tazetta*, collected from different regions of Iran, under water-sufficient condition. The data represent the average of six plants per treatment.

Accession no. ^z	CAT ($\Delta OD/min/mg$) ^y	SOD ($\Delta OD/min/mg$) ^y	POX ($\Delta OD/min/mg$) ^y	Proline ($mg \cdot g^{-1}$)	Protein contents ($mg \cdot g^{-1}$)	RWC ^y
1	0.038	28.59	0.027	1.05	9.70	85
2	0.018	19.98	0.016	4.42	3.54	82.33
3	0.014	20.44	0.016	4.10	3.18	79.14
4	0.034	28.32	0.026	2.21	7.63	85.98
5	0.010	17.69	0.013	2.74	0.82	78.81
6	0.017	28.84	0.030	1.93	9.15	81.72
7	0.018	19.54	0.017	3.17	3.37	73.63
8	0.017	18.74	0.016	3.18	3.15	71.33
9	0.036	33.53	0.026	1.73	9.41	83.54
10	0.017	19.45	0.015	7.02	3.55	77.44
11	0.026	40.95	0.026	10.17	6.07	84.66
12	0.036	30.91	0.021	10.77	4.41	84.20
13	0.014	16.43	0.015	9.34	2.80	77.90
14	0.036	34.85	0.024	1.51	6.89	77.98
15	0.023	52.32	0.020	10.10	8.82	79.51
16	0.045	32.53	0.022	3.63	4.62	84.05
17	0.028	26.17	0.012	9.73	4.20	80.97
18	0.035	27.09	0.026	1.27	8.15	83.54
19	0.018	22.60	0.018	8.63	3.76	81.66
20	0.037	33.47	0.026	4.78	8.89	85.35
21	0.035	44.47	0.022	2.35	8.20	77.11
22	0.020	30.08	0.015	6.18	4.39	75.16
23	0.036	40.47	0.026	2.46	9.05	80.64
24	0.013	26.75	0.013	6.70	4.63	77.66
25	0.013	31.76	0.026	4.30	8.74	79.92
26	0.019	32.30	0.031	7.59	8.62	79.45
27	0.023	30.61	0.029	2.50	3.34	85.41
28	0.038	35.13	0.033	1.54	5.25	83.64
29	0.041	28.18	0.030	1.95	9.49	80.63
30	0.016	20.60	0.017	4.47	3.06	74.66
LSD ^x (5%)	0.006	5.74	0.005	3.02	1.96	5.46

^z1 = Behbahan1, 2 = Behbahan2, 3 = Behbahan3, 4 = Behbahan4, 5 = Behbahan5, 6 = Behbahan6, 7 = Behbahan7, 8 = Behbahan8, 9 = Ahvaz1, 10 = Ahvaz2, 11 = Birjand, 12 = Shiraz1, 13 = Shiraz2, 14 = Mehran, 15 = Dareh Shahr, 16 = Jahrom1, 17 = Jahrom2, 18 = Bavi1, 19 = Bavi2, 20 = Kazerun, 21 = Pol Dokhtar1, 22 = Pol Dokhtar3, 23 = Gachsaran1, 24 = Gachsaran3, 25 = Kerman1, 26 = Kerman2, 27 = Abadan, 28 = Ghaem Shahr, 29 = Rasht1, 30 = Rasht3.

^yCAT = catalase, OD = optical density, SOD = superoxide dismutase, POX = peroxidase, RWC = relative water content.

^xFisher's least significant difference (LSD) test at $P = 0.05$.

flowers (5 accessions). Subcluster B contained two accessions of Shahla with 60% similarity (Fig. 2). In each subcluster, single and double flowers formed separate clusters. Accession 5 fell between 3 (semidouble flower) and 7 (double flower) accessions. Based on Jaccard's similarity matrices, the similarity of accession 5 to 3 and 7 was 69% and 74%, respectively. The accessions of Behbahan showed high diversity with the similarity of accessions of this region being 55% (accession 4) to 85% (between accessions 7 and 8). The similarity of accession 6 to 1 (both single flowers collected from Behbahan) was 60% and formed an individual group that confirmed the morphological trait differences between accessions 6 and 1.

The second main cluster was separated into two subclusters (C and D). Subcluster C comprised only accession 16 (single flower) and subcluster D comprised accessions with single flowers (18, 20, 21, 23, 26, 27, 28, and 29), semidouble flowers (22, 24, and 30) and double flowers (17 and 19), which formed separate groups.

The PCoA was applied based on the genetic distance matrix to better visualize the genetic relationships among the studied

Narcissus accessions. The overall grouping pattern in PCoA was similar to the clustering pattern in the dendrogram and confirmed the patterns of genetic variation observed among the accessions (Fig. 3). The exotic population did not group with the accessions in PCoA. The two principal axes showed 25.23% and 23.43% of the genetic variation, respectively.

Behbahan's population displayed the maximum percentage of polymorphism (51.94), the highest Ne (1.325), I (0.275), and He (0.185). The minimum percentage of polymorphism (0.00), the lowest Ne (1.000), I (0.000), and He (0.000) were found in the populations of Mehran, Dareh Shahr, Abdanan, Ghaem Shahr, Kazerun, and the exotic narcissus (Table 3). On the basis of pairwise population matrix of Nei's (1978) unbiased genetic identity at the collecting region level, the greatest similarity (0.914) was perceived among the Behbahan and Kerman populations and the lowest similarity (0.388) was observed between the Kazerun and exotic populations (Table 4).

The results indicate that the CAT, SOD, and POX contents, and also the proline and protein contents in response to water deficit increased (Tables 5 and 6). The UPGMA dendrogram of

Table 6. Evaluation of physiological characteristics of 30 accessions of *Narcissus tazetta*, collected from different regions of Iran, under water-deficient condition. The data represent the average of six plants per treatment.

Accession no. ^z	CAT ^y (ΔOD/min/mg) ^y	SOD (ΔOD/min/mg) ^y	POX (ΔOD/min/mg) ^y	Proline (mg·g ⁻¹)	Protein contents (mg·g ⁻¹)	RWC ^y
1	0.154	92.13	0.093	50.72	21.85	67.16
2	0.131	65.67	0.058	21.21	13.95	64.88
3	0.130	92.25	0.059	20.35	13.44	63.82
4	0.140	98.68	0.077	48.02	19.36	66.50
5	0.121	67	0.058	25.77	12.06	61.96
6	0.136	92.61	0.080	52.34	21.61	68.15
7	0.133	73.54	0.069	16.43	14.30	40.41
8	0.133	73.02	0.065	16.69	14.87	40.14
9	0.149	82.48	0.100	31.46	21.89	67.25
10	0.128	63.21	0.059	19.85	13.88	57.06
11	0.135	70.19	0.086	34.25	18.02	65.50
12	0.153	206.75	0.084	36.05	17.21	61.19
13	0.128	65.89	0.046	20.96	13.38	55.68
14	0.135	113.63	0.074	35.14	18.98	57.53
15	0.128	122.51	0.063	29.24	20.08	59.97
16	0.125	101.09	0.163	29.47	17.58	51.64
17	0.114	57.84	0.046	19.23	14.31	42.98
18	0.156	96.04	0.082	41.28	20.50	62.61
19	0.128	66.99	0.046	20.43	12.68	59.05
20	0.250	215.02	0.092	47.68	20.79	63.56
21	0.139	94.03	0.068	39.33	25.49	49.62
22	0.127	70.74	0.046	20.22	16.70	42.98
23	0.142	81.46	0.072	32.56	24.99	57.81
24	0.122	64.89	0.051	19.46	13.94	46.37
25	0.131	154.65	0.055	29.41	16.81	42.54
26	0.134	156.89	0.104	24.13	15.61	46.69
27	0.132	86.84	0.088	44	15.93	65.36
28	0.166	191.03	0.109	53.54	20.45	63.29
29	0.147	95.12	0.114	47.81	21.45	61.16
30	0.133	68.39	0.062	18.38	15.54	52.19
LSD ^x	0.008	29.34	0.022	11.49	3.04	4.99

^z1 = Behbahan1, 2 = Behbahan2, 3 = Behbahan3, 4 = Behbahan4, 5 = Behbahan5, 6 = Behbahan6, 7 = Behbahan7, 8 = Behbahan8, 9 = Ahvaz1, 10 = Ahvaz2, 11 = Birjand, 12 = Shiraz1, 13 = Shiraz2, 14 = Mehran, 15 = Dareh Shahr, 16 = Jahrom1, 17 = Jahrom2, 18 = Bavi1, 19 = Bavi2, 20 = Kazerun, 21 = Pol Dokhtar1, 22 = Pol Dokhtar3, 23 = Gachsaran1, 24 = Gachsaran3, 25 = Kerman1, 26 = Kerman2, 27 = Abdanan, 28 = Ghaem Shahr, 29 = Rasht1, 30 = Rasht3.

^yCAT = catalase, OD = optical density, SOD = superoxide dismutase, POX = peroxidase, RWC = relative water content.

^xFisher's least significant difference (LSD) test at $P = 0.05$.

the physiological characteristics under the water-sufficient condition grouped the accessions into two main clusters. The first group had low antioxidant enzyme activity and low proline and protein contents. These comprised 12 accessions that included all of the double and semidouble flowers. Other accessions (single flowers) fell into the second group and showed more antioxidant enzyme activity and higher proline and protein contents (Fig. 4).

The UPGMA dendrogram of the morpho-physiological traits under drought stress indicate that accessions 16 and 20 (single flowers) were separate from the other accessions and formed a separate cluster. The dendrogram showed that other accessions were grouped into two major clusters. The first group was composed of 12 accessions with double and semidouble flowers, whereas the second group was composed of 16 accessions with single flowers. The first group was drought-sensitive and showed lower antioxidant activity,

proline and protein contents, RWC, dry and fresh weight ratios, number of bulblets, and dry weight of bulb. The second group and accessions 16 and 20 were drought tolerant and had more distinct traits than the first group (Fig. 5).

A two-dimensional projection of the morphological and physiological characteristics of the Iranian *Narcissus* accessions grown under water-sufficient and water-stress conditions is shown in Figs. 6 and 7. The single and double flowers displayed considerable separation between the two water-accessibility conditions.

Discussion

An understanding of the degree of genetic variation is important for prebreeding and breeding of germplasms and germplasm collection (Deore and Samantaray, 2015; Langridge

and Chalmers, 2004). The current study is the first application of the ISSR method used to estimate genetic diversity and evaluate genetic relationships among 31 *Narcissus* accessions in Iran. The present investigation demonstrated a high degree of polymorphism among populations (70% to 100%, with an average of 96.02%; Table 2). These primers also revealed similar results for *Allium* (Mukherjee et al., 2013) and *Tulipa* (Kiani et al., 2012), as well as some *Iris* (Raycheva et al., 2011) and *Aloe* (Deore and Samantaray, 2015) populations. The primers included (AG)8T, (AG)8C, (AG)8G, (GA)8T, (GA)8C, (CA)8T, (AC)8T, (AG)8YC, (GA)8YC, (ACA)5CYG, and (ACC)6, and were applied to estimate genetic diversity among important species of *Allium*, which generated an average 99.17% polymorphism. In species of *Tulipa*, the primers of (GA)8C, (AC)8T, (AC)8G, (TG)8A, (TG)8C, (TG)8G, (AG)8YC, (GA)8YT, (CT)8RG, and (AC)8YT produced a total of 97 polymorphic bands. Hence, we decided these primers could be effective in determining the genetic diversity of other geophytes (e.g., *Narcissus*).

Polymorphism is produced by ISSR markers whenever one genome loses the sequence repeat or has a deletion, insertion, or translocation that changes the distance between two primer sites (Vijayan, 2005). The amount of polymorphism differs with the nature of the primer used (unanchored, 3'-anchored or 5'-anchored) and the sequence of the repeats (motif) in the primer. Whenever only SSRs (unanchored)

Fig. 4. Dendrogram of the physiological relationships under water-sufficient condition between 30 accessions of *Narcissus tazetta* collected from different regions of Iran: 1 = Behbahan1, 2 = Behbahan2, 3 = Behbahan3, 4 = Behbahan4, 5 = Behbahan5, 6 = Behbahan6, 7 = Behbahan7, 8 = Behbahan8, 9 = Ahvaz1, 10 = Ahvaz2, 11 = Birjand, 12 = Shiraz1, 13 = Shiraz2, 14 = Mehran, 15 = Dareh Shahr, 16 = Jahrom1, 17 = Jahrom2, 18 = Bavi1, 19 = Bavi2, 20 = Kazerun, 21 = Pol Dokhtar1, 22 = Pol Dokhtar3, 23 = Gachsaran1, 24 = Gachsaran3, 25 = Kerman1, 26 = Kerman2, 27 = Abadan, 28 = Ghaem Shahr, 29 = Rasht1, 30 = Rasht3, 31 = exotic narcissus.

are applied as primers, the primer tends to slide within the repeats during amplification and produce smears instead of clear bands [such as primer (ACC)₆ (Table 2)] (Pradeep Reddy et al., 2002).

Generally, a high degree of polymorphism was observed in dinucleotide repeat units anchored either at the 3' or 5' ends (Blair et al., 1999; Joshi et al., 2000). The primers anchored at the 3' end generate clearer bands than those anchored at the 5' end (Blair et al., 1999; Nagaoka and Ogiwara, 1997). Overall, primers based on the (AC), (CA), (CT), (TC), (AG), and (GA) repeat revealed higher polymorphism than primers with other di-, tri-, or tetranucleotide repeats. AT repeats were the most frequent dinucleotides in the plants; however, the primers with (AT) motifs tended to self-anneal and not amplify. ISSR primers with AT repeats are self-annealing and, because of sequence complementarity, they create dimers during PCR amplification (Blair et al., 1999; Mukherjee et al., 2013). Because of the decrease in

abundance of the tri- and tetranucleotide repeats in plants, their application in primers is less than the dinucleotides (Pradeep Reddy et al., 2002; Vijayan, 2005). The findings of the present study illustrate that primers with (AG), (GA), and (CT) repeats produced clear band profiles. It suggests that these dinucleotides are the most abundant motifs in *Narcissus*. Similar results for poly(AG) and poly(GA) have been found in *Allium*, but (CT) repeats have not generated good profiles in *Allium* (Mukherjee et al., 2013). Primers synthesized from (CA) and (ACC) motifs did not amplify, even at a low annealing temperature. A feasible explanation for this is that the dispersion of these motifs in the *Narcissus* genome was outside the range of amplification of Taq-DNA polymerase (Vijayan, 2005). The trinucleotide repeat primers based on (ACA) and (GTG) motifs generated excellent amplification.

In this investigation, PIC means ranged from 0.15 to 0.44. PIC depends on the number of visible alleles and the distribution of their frequency corresponds to the gene diversity. The maximum limit of PIC in dominant markers is 0.5 (Botstein et al., 1980; De Riek et al., 2001). EMR ranged from 4.9 to 23. Higher EMR values indicate that the primer/marker method is operative.

MI value ranged from 0.73 to 5.61. MI is a product of expected heterozygosity and EMR (Mukherjee et al., 2013). It is applied to estimate the overall effectiveness of a marker system and validate the effectiveness of the technique (Perez de la Torre et al., 2012; Powell et al., 1996). In this study, Rp varied from 2 to 8.32. Rp is used to represent the ability of a primer/method to differentiate among large numbers of genotypes (Gilbert et al., 1999). Prevost and Wilkinson (1999) found that Rp can be used to evaluate the ability of each primer for differentiating among several accessions. In general, primers with a higher Rp value can better distinguish among accessions (Ghasemi Ghehsareh et al., 2014).

The results obtained with PCoA confirms that the ISSR primers were very effective for discriminating between the accessions. The results of PCoA and the dendrograms showed primarily similar clustering patterns. For instance, the PCoA plot placed accession 7 very close to 8. On the basis of Jaccard's similarity matrices, the similarity of accessions 7 and 8 was 85%. The close relationship between these could be the result of naturally occurring hybridization or mutation.

Behbahan's population showed a high percentage of polymorphism, Ne, I, and He. These results

Fig. 5. Dendrogram of the physiological and morphological relationships under water-deficient condition between 30 accessions of *Narcissus tazetta*, collected from different regions of Iran: 1 = Behbahan1, 2 = Behbahan2, 3 = Behbahan3, 4 = Behbahan4, 5 = Behbahan5, 6 = Behbahan6, 7 = Behbahan7, 8 = Behbahan8, 9 = Ahvaz1, 10 = Ahvaz2, 11 = Birjand, 12 = Shiraz1, 13 = Shiraz2, 14 = Mehran, 15 = Dareh Shahr, 16 = Jahrom1, 17 = Jahrom2, 18 = Bavi1, 19 = Bavi2, 20 = Kazerun, 21 = Pol Dokhtar1, 22 = Pol Dokhtar3, 23 = Gachsaran1, 24 = Gachsaran3, 25 = Kerman1, 26 = Kerman2, 27 = Abdanan, 28 = Ghaem Shahr, 29 = Rasht1, 30 = Rasht3, 31 = exotic narcissus.

demonstrated that Behbahan is the eldest and premier *Narcissus* diversity center in Iran.

Principal component analysis is a common explorative method applied to decrease multivariate data complicity.

This method helps to identify similarities and differences between samples (Goodacre et al., 2000). The results showed that activity of antioxidant enzymes, such as CAT, SOD, and POX, in the accessions significantly increased during water stress (Tables 5 and 6). Similar results have been reported for maize [*Zea mays* (Bai et al., 2006)], chickpea [*Cicer arietinum* (Mafakheri et al., 2011)], and barley [*Hordeum vulgare* (Salekjalali et al., 2012)].

Fig. 6. Principal component analysis of physiological characteristics in 30 accessions of *Narcissus tazetta* under water-deficient condition collected from different regions of Iran: 1 = Behbahan1, 2 = Behbahan2, 3 = Behbahan3, 4 = Behbahan4, 5 = Behbahan5, 6 = Behbahan6, 7 = Behbahan7, 8 = Behbahan8, 9 = Ahvaz1, 10 = Ahvaz2, 11 = Birjand, 12 = Shiraz1, 13 = Shiraz2, 14 = Mehran, 15 = Dareh Shahr, 16 = Jahrom1, 17 = Jahrom2, 18 = Bavi1, 19 = Bavi2, 20 = Kazerun, 21 = Pol Dokhtar1, 22 = Pol Dokhtar3, 23 = Gachsaran1, 24 = Gachsaran3, 25 = Kerman1, 26 = Kerman2, 27 = Abadan, 28 = Ghaem Shahr, 29 = Rasht1, 30 = Rasht3.

Fig. 7. Principal component analysis of morphological and physiological characteristics in 30 accessions of *Narcissus tazetta* under water-deficient condition collected from different regions of Iran: 1 = Behbahan1, 2 = Behbahan2, 3 = Behbahan3, 4 = Behbahan4, 5 = Behbahan5, 6 = Behbahan6, 7 = Behbahan7, 8 = Behbahan8, 9 = Ahvaz1, 10 = Ahvaz2, 11 = Birjand, 12 = Shiraz1, 13 = Shiraz2, 14 = Mehran, 15 = Dareh Shahr, 16 = Jahrom1, 17 = Jahrom2, 18 = Bavi1, 19 = Bavi2, 20 = Kazerun, 21 = Pol Dokhtar1, 22 = Pol Dokhtar3, 23 = Gachsaran1, 24 = Gachsaran3, 25 = Kerman1, 26 = Kerman2, 27 = Abadan, 28 = Ghaem Shahr, 29 = Rasht1, 30 = Rasht3.

In response to environmental stresses such as drought, ROS such as hydroxyl radicals, superoxide, hydrogen peroxide, and singlet oxygen are produced and accumulate in plant cells (Jiang and Zhang, 2004). The protective mechanisms of tissues against ROS accumulation is related to their ability to break ROS down by increasing the activity of the antioxidant enzymes during water deficit. CAT, SOD, and POX are common and significant antioxidant enzymes that can destroy ROS (Capell et al., 2004; Saba et al., 2001). Reports have demonstrated that these antioxidant enzymes are higher in tolerant plants than in sensitive ones during water deficit (Bergmann et al., 1999; Gogorcena et al., 1995; Mafakheri et al., 2011; Shao et al., 2005).

The results of this study indicate that the CAT, SOD, and POX contents increased under drought stress, especially for tolerant accessions (Tables 5 and 6). Also, in response to water deficit, the proline and protein contents increased (Tables 5 and 6). When plants are exposed to drought stress, proline increases to high concentrations to protect cytoplasmic structures. Proline acts as an osmotic adjustment material and plays an important role as an osmoprotectant (Handa et al., 1983) for resistance and adaptation under water-deficient conditions (Hsiao, 1973).

The clustering pattern of the molecular markers showed that genotypes of different geographical regions could be grouped in one cluster and genotypes from similar ecological areas could be collected into one group or different clusters. This signifies that there was no proper connection between geographical variety and genetic variety. Other researchers (De and Rao, 1987; Garg and Kumar, 2017; Singh et al., 1987) have shown that

geographical diversity is not necessarily correlated to genetic diversity. On the other hand, most single and double flowers in the same geographical area were grouped together into major groups and then formed separate subclusters. The current phylogenetic study showed that the morphological and physiological traits under water stress and the origin of accessions could be used to cluster them into three groups: very drought-tolerant accessions collected from Fars province, Jahrom, and Kazerun regions (single flower); drought-tolerant accessions including other single flowers; and drought-sensitive accessions with double and semidouble flowers.

We found that accessions with a very low degree of similarity were not closely related genotypes and had different agronomic characteristics under water deficit. Similar results have been reported by Barakat et al. (2010) for Saudi wheat (*Triticum aestivum*) genotypes under drought stress.

Conclusion

The current study demonstrated that ISSR is an effective and informative molecular marker method for identifying genetic diversity of *Narcissus* accessions. The present study found that genetic variation did not necessarily coincide with morphological trait differences among *Narcissus* accessions. These results can be used in breeding programs on *Narcissus* genotypes. The genetic relationships among *Narcissus* accessions can be displayed more accurately by application of multiple DNA marker methods.

The morpho-physiological traits of the Iranian accessions can be clustered into two major groups: drought-tolerant accessions including single flowers and drought-sensitive accessions containing double and semidouble flowers. The findings of the present investigation can be used in future breeding programs for different *Narcissus* genotypes.

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