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Genetic Diversity and Population Structure of Iranian tulips revealed by EST-SSR and NBS-LRR Markers

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Abstract

The genus *Tulipa* L. (Liliaceae) comprises about 100 species and Iran is considered as one of the main origins of tulips. In this research, genetic diversity and population structure of 27 wild populations of tulips collected from Iran were studied by 15 highly polymorphic and reproducible expressed sequenced tag-simple sequence repeat (EST-SSR) markers and 8 nucleotide binding site (NBS)-enzyme combinations. According to EST-SSR genotyping, the average of gene diversity (GD) and polymorphism information content (PIC) were 0.66 and 0.62, respectively. However, the values of GD and PIC were equal for each NBS primer–enzyme combination which ranged between 0.85-0.95 with a mean value of 0.91. The mean value of resolving power (EST-SSR = 1.93; NBS-LRR = 17.39) indicated that the NBS markers had higher discriminatory power compared to the EST-SSR markers. UPGMA clustering confirmed the results of PCA which was further confirmed by Bayesian model-based STRUCTURE analysis. Population structure analysis detected 3 and 4 gene pools for 27 wild tulip germplasms with EST-SSR genotyping and NBS-LRR profiling, respectively. The AMOVA results indicated that molecular variation among populations (ΦPT = inter-population variation) was 82% and 93% of the total variation for EST-SSR and NBS-LRR markers, respectively. The results of this study will help the conservation and phylogenetic studies of tulips.

Key words: Bulbous crop, Genetic marker, Motif-directed profiling, Population genetics, *Tulipa* spp.

Introduction

Tulip is one of the most important ornamental bulbous crops in the world. There are more than 5000 registered tulip cultivars which most of them have been mainly developed in the Netherlands and are widely used in ornamental plants markets for cut flower, potted plant, gardening and landscaping (van Scheepen, 1996). The genus *Tulipa* L. (Liliaceae) comprises more than 100 species (Zonneveld, 2009). Tulips are native to

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temperate regions and wild populations occur naturally in the Greece, the southern Balkans to Siberia, the Caucasus, Iraq, Iran, Turkey and Central Asia to western China, Mongolia and the Himalayas (Veldkamp and Zonneveld, 2012; Christenhusz *et al*., 2013). The Pamir and Hindu Kush mountains and the steppes of Kazakhstan are reported as the center for tulips diversity (Veldkamp and Zonneveld, 2012). However, habitats of tulips extend from southern Europe to North Africa and some wild tulips are in the United States, Britain, The Netherlands, Italy, France, Sweden and Switzerland. Tulips from Western Europe are referred to as *Neotulipae* (Veldkamp and Zonneveld, 2012; Christenhusz *et al*., 2013). Due to the high levels of heterozygosity, there is a wide range of different characteristics in the progeny of the genus *Tulipa* which hampers taxonomy of tulips (Zonneveld, 2009).

Information upon genetic diversity and structure of tulips provide an estimation of dynamic behavior of the species in their habitat and subsequently help to assess individuals' capability to survive in extreme environmental conditions such as disease outbreak and climate changes. Thus, knowledge of genetic diversity allows successful management and conservation of endangered and rare wild species with proper population selection (Tabin *et al*., 2016). Moreover, genetic diversity is a source of traits which can be used in breeding programs to develop cultivars with high yield-potential and resistant to biotic and abiotic stresses (Wang *et al*., 2015). Knowledge of genetic diversity enable plant breeders to apply valuable wild germplasms preserving alleles of agricultural interest to develop new cultivars with desired traits. There is little information on genetic diversity of the genus *Tulipa*. However, molecular characterization of *Tulipa* species using RAPD, ISSR, and SNPs markers has been reported (Qi-fu *et al*., 2008; Kiani *et al*., 2012; Tang *et al*., 2013b).

Microsatellites, or simple sequence repeats (SSRs), represent co-dominant molecular genetic markers, which are abundantly distributed within genomes and are often highly polymorphic due to variation in the number of repeats (Amos and Pemberton, 1992; Chistiakov *et al*., 2006). These markers are widely used in population genetics analyses but their development from genomic DNA is expensive and time consuming for many researchers (Leigh *et al*., 2003). In contrast, expressed sequence tag-simple sequence repeats (EST-SSRs) can be developed directly from sequence resources and can often be transferred from one species to another. Due to their amplification in both species, when the same loci are evaluated in cross-species comparisons, one can include the inherent differences in the level of variation from one locus to another in statistical analyses. Thus, EST-SSRs provide more statistical power in paired comparisons. Moreover, these genetic markers produce pure results for scoring because of fewer null alleles (Leigh *et al*., 2003; Rungis *et al*., 2004) and fewer stutter bands (Leigh *et al*., 2003; Woodhead *et al*., 2003; Eujayl *et al*., 2004; Pashley *et al*., 2006). Therefore, EST databases are an attractive source of markers for the genetic analysis of understudied taxa (Ellis and Burke, 2007).

By motif-directed profiling, conserved domains in gene families are used to amplify the members of these families. Polymorphisms are detectable in form of length variation or absence/presence of bands caused by mutations in or near to genes of interest. In the first step, a pool of DNA fragments is created by cutting genomic DNA with a frequently cutting restriction enzyme. An adapter is ligated to the ends of fragments. PCR reaction is operated with a degenerate primer (selectively binds to domain-containing fragments) and an adapter primer which ultimately results in fragments that mostly originate from genes harboring the targeted

domain. The DNA fragments are separated on polyacrylamide gels and polymorphisms in the banding pattern are most likely to be associated with the function of the conserved motif. Nucleotide-binding sites (NBS) in many plant resistance genes that are numerous and well distributed all over the plant genomes (Wang *et al*., 2008) can be targeted by this approach. NBS profiling produces resistance gene targeted markers and recently has been used to assess genetic diversity in agricultural crops. Wang et al. (2008) used NBS markers to study systematic relationships of 49 tuber-bearing wild and cultivated *Solanum* species. They indicated that NBS profiling is a suitable technique for phylogeny reconstruction in the genus *Solanum* and could be better than AFLP system when more diverse materials are used. Analyzing functional genetic variation at NBS loci and *R* gene evolution in wild and cultivated accessions of *Zingiber* taxa revealed that low polymorphism at NBS loci is due to obligatory vegetative propagation in ginger. As a consequence, under high pressure of pathogen attacks the genetic vulnerability is observed (Nair and Thomas, 2012).

In this study 27 Iranian tulip populations were analyzed using EST-SSR genotyping and motif-directed profiling with NBS-LRR markers. The overall goal was to analyze the genetic diversity within and between some wild populations of Iranian tulips to help their conservation programs.

Materials and Methods

Field sampling and plant materials

Twenty seven populations of Iranian tulips were collected during their flowering time from March to May 2015 across their habitat in Iran. The geographical locations and altitude of the populations were recorded using a handheld Garmin Dakota 20 GPS. Characteristics of the populations (collecting locations, latitude, longitude and altitude) are listed in Table 1. A total of 216 genotypes were sampled (8 individuals per population). The map of collecting sites was conducted using Arc map 10.4.1 software based on latitude and longitude coordinates (Fig. 1). Nine leaf disks for each individual were collected into a microfuge tube, immediately snap-frozen in liquid nitrogen and stored at -80 °C until the DNA was extracted.

EST-SSR markers

The expressed sequence tags generated by Shahin *et al*. (2012) were used as the data source in this research. They used 454-pyro-sequencing technology to sequence and assemble transcriptomes of 5 tulip genotypes, including *T. fosteriana* 'Cantata', *T. fosteriana* 'Princeps', *T. gesneriana* 'Kees Nelis', *T. gesneriana* 'Ile de France', and *T. gesneriana* 'Bellona'.

For fluorescent dye labeling of PCR fragments in one reaction, three primers were used: 1) the forward primers were extended with an universal M13 sequence (AACAGGTATGACCATGA) at the 5' end (Schuelke, 2000); 2) the reverse primers were tailed with GTTT at their 5' end according to Brownstein *et al*. (1996) to reduce stutter bands (Brownstein *et al*., 1996); 3) and the universal fluorescent-labeled M13 primer with IRDye® 700 Phosphoramidite. The primers were ordered from Biolegio BV (Nijmegen, the Netherlands).

DNA extraction

Total genomic DNA was isolated from dried young leaves following the cetyl trimethylammonium bromide (CTAB) protocol introduced by Fulton *et al*. (1995) and followed by purification using DNeasy (Qiagen, Venlo, The Netherlands). DNA quantity and quality were determined by spectrophotometry and visual comparison of DNA electrophoresed on 1% agarose gel.

Table 1. Accessions of tulips collected from various regions of Iran.

Subgenus	Populations	Collection locations	Latitude - Longitude (N/E)	Altitude (m)
Eriostemons	*T. biebersteiniana*	Tale Zari, Sepidan - Fars	30º32'/51º57'	2094
	T. biebersteiniana	Margoon Waterfall, Sepidan - Fars	30º29'/51º53'	2128
	T. biflora	Dahaneh Zoqali, Baft - Kerman	28º48'/56º41'	2229
	T. biflora	Galoye Shahrbanoo, Neyriz - Fars	29º08´/54º15´	2386
	T. biflora	Derak Mountain, Shiraz - Fars	29º40'/52º25'	2068
	T. biflora	Safashahr, Khorrambid - Fars	30º41'/53º18'	2388
	T. biflora	Bishe (Dashte Laleh), Estahban - Fars	29º06'/53º59'	2413
	T. biflora	Bamu Prot. Reg., Shiraz - Fars	29º42'/52º36'	1877
	T. biflora	Pol-e Piran, Jiroft - Kerman	29º11'/57º23'	2550
	T. biflora	Margoon Track, Sepidan - Fars	30º18'/51º58'	2683
	T. biflora	Sarcheshmeh, Rafsanjan - Kerman	29º58´/55º46´	2650
Tulipa	*T. clusiana*	Khabr Prot. Reg. - Kerman	28º49'/56º17'	1918
	T. clusiana	Neyriz - Fars	29º09'/54º17'	1656
	T. clusiana	Bavan, Mamasani - Fars	30º02'/51º39'	1549
	T. clusiana var. *chrysantha*	Bezenjan, Baft - Kerman	29º15'/56º41'	2383
	T. clusiana var. chrysantha	Pol Piran, Jiroft - Kerman	29º11'/57º23'	2550
	T. systola	Zard Shehneh Mountain, Rostam - Fars	30º19'/51º32'	1528
	T. systola	Dashte Laleh, Estahban - Fars	29º06'/53º59'	2413
	T. systola	Bisheh, Estahban - Fars	29º06'/53º59'	2413
	T. systola	Derak Mountain, Shiraz - Fars	29º40'/52º25'	2278
	T. systola	Bamu Prot. Reg., Shiraz - Fars	29º40'/52º38'	2140
	T. systola	Margoon Track, Sepidan - Fars	30º18'/51º58'	2683
	T. systola	Margoon Waterfall, Sepidan - Fars	30º29' / 51º53'	2128
	T. systola	Siah Mountain, Eghlid (Sarhad) - Fars	30º40'/52º47'	2876
	T. systola	Chashmeh Chenar, Yasouj - Kohgiluyeh	30º42'/51º38'	2157
	T. systola	Marivan - Kurdestan	35º31´/46º06´	1436
	T. micheliana	Nasr Abad, Neyshabour - Khorasan Razavi	36º17´/58º23´	1512

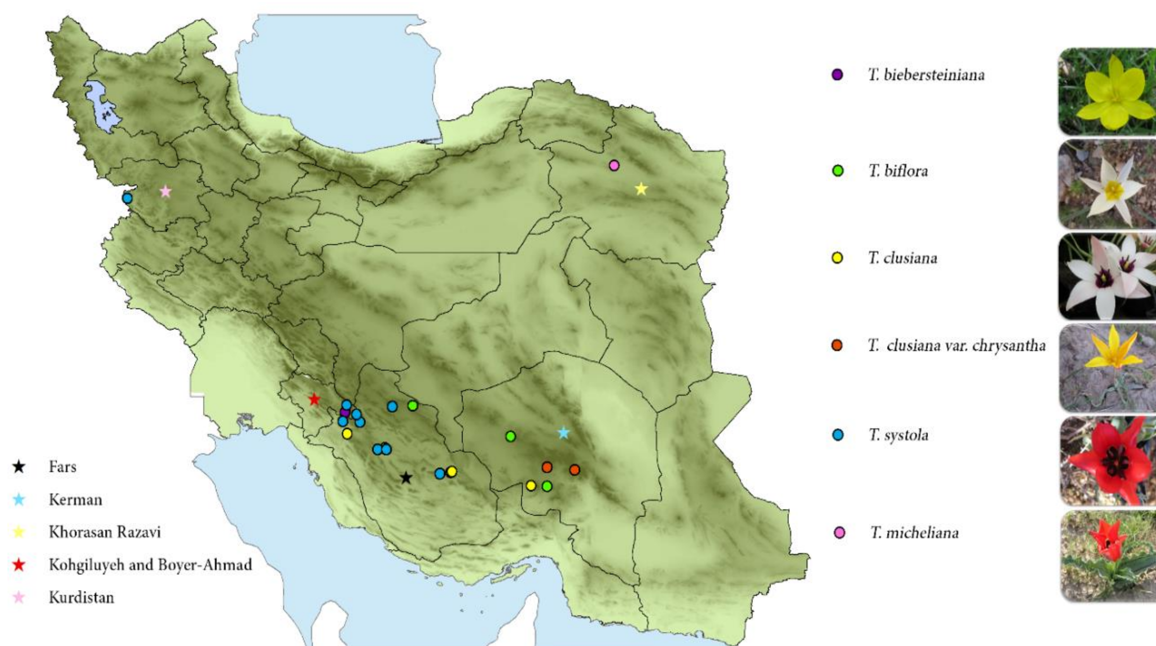

Fig. 1. The map of Iran indicating the geographical collecting sites of 27 wild tulip populations used in this study.

EST-SSR genotyping

Polymorphism of total 70 markers was first tested in tulip cultivars. Finally, 15 polymorphic SSRs that showed amplification in all wild and cultivated samples were selected, and polymerase chain reaction (PCR) conditions were optimized. The protocol of Schuelke (2000) was modified to perform PCRs. The PCR mix contained 30 ng template DNA, 8 pmol of each reverse and universal fluorescently labeled primer and 2 pmol of the forward primer in a final 20 µl reaction volume. Conditions of the PCR amplification were as follows: 94°C (5 min), then 30 cycles at 94°C (30 s) / 57°C (45 s) / 72°C (35 s), followed by 8 cycles 94°C (30 s) / 54°C (45 s) / 72°C (35 s), and a final extension at 72°C for 8 min. PCR products were labeled during the last 8 PCR cycles. After amplification, 25 µl water was added for dilution and increase the volume of the product. Subsequently, 5 µl of the diluted PCR product was added to 5 µl of loading dye and 0.8 µl of this mixture was run on a LI-COR 4300 DNA analyzer (LI-COR Corporate, Nebraska, USA). Markers were scored manually as co-dominant alleles compared to the size marker.

NBS profiling

The NBS profiling is a technique for DNA fingerprinting and expression profiling of *R*-genes based on conserved motifs in the nucleotide binding domain of resistance genes in plants. The technique involves three steps: 1) Restriction enzyme digest of (c) DNA and the ligation of adapters; 2) Selective amplification of fragments using a (degenerated) primer for the conserved domains; 3) Gel analysis of the amplified fragments.

In this research, nucleotide binding site profiling was performed based on the method described by van der Linden *et al.* (2004). Briefly, the extracted DNA was diluted to a final concentration of 50 ng/µl. The DNA was digested with two restriction enzymes (*RsaI* and *MseI*) with a four base recognition site and blocked adapters were ligated to the ends. In the first amplification round, the domain specific primer was annealed and elongated by the Taq polymerase resulted in an annealing site for the adapter primer not present previously. Conditions of the first PCR amplification were as follows: 95 °C (15:00), then 30 cycles at 95 °C (00:30) / 55-60 °C (01:40) / 72°C (02:00), and a final extension at 72 °C for 20 min. Conditions of the second PCR amplification round (with labelled primer) were as follows: 95 °C (03:00), then 15 cycles at 95 °C (00:30) / 55-60 °C (01:40) / 72°C (02:00), and a final extension at 72 °C for 20 min. To visualize the final PCR products, sample preparation and loading on LI-COR gel was performed as the protocols described for EST-SSR genotyping.

Data analysis

Genic microsatellites were co-dominantly or dominantly (based on the analysis) scored in separate data matrices. The matrices were used for calculation of population genetic variation indices and for assessment of the genetic distances among populations. On the other hand, the profiles developed by NBS-LRR markers were scored as 1 (present) or 0 (absent) for individual plant samples and binary matrix was generated for each genotype.

The informativeness of primer pairs in each molecular marker system as well as the efficiency of these two marker systems in genotyping and subsequent evaluation of genetic diversity and population structure was compared using the following parameters:

1. Gene diversity (GD) and polymorphism information content (PIC) of each primer pairs was calculated by PowerMarker v3.25 (Liu and Muse, 2005).

2. The resolving power (Rp) of each primer (Prevost and Wilkinson, 1999) was calculated by Microsoft Excel software 2016 as follow:

$$\mathrm{Rp} = \sum Ib$$

where Ib is the informativeness of a band that is determined based on the proportion of genotypes containing it (p):

$$Ib = 1 - (2 \times |0.5 - p|)$$

Based on SSR alleles identified in the individuals, genetic diversity parameters were calculated. The number of observed alleles per locus (N_a), effective alleles per locus (N_e) (Kimura and Crow, 1964), observed heterozygosity (H_o), expected heterozygosity (H_e), Shannon's information index (I) (Lewontin, 1972), number of polymorphic loci (PL) and percentage of polymorphic loci (PPL) were calculated for each accession using software POPGENE 1.32 (Yeh *et al.*, 1999).

To assess the population structure, four different approaches were used:

1. Principal component analysis (PCA) was performed to investigate the genetic relationship of the accessions and to show the distribution of individuals in scatter diagram by drawing two-dimension PCA graph in Minitab 16 (Minitab Inc., State College, Pennsylvania, USA).
2. Genetic distances between all pairs of populations were calculated and an un-weighted pair group method with arithmetic mean (UPGMA) dendrogram was constructed based on Nei's genetic distance using PowerMarker v3.25.
3. Beyond that, a Bayesian clustering-based structure analysis was performed on the entire data set using STRUCTURE (version 2.3.4) to investigate the genetic structure among the tulip accessions. This software uses a Markov Chain Monte Carlo (MCMC) algorithm to detect population structure (Pritchard *et al.*, 2000) and estimate the admixture of individuals into subgroups (K) to assign individuals to one or more of these clusters (K). Two runs of analysis using the admixture model were performed. Initial runs were performed with a burn-in length of 10000 and 10000 MCMC (Markov Chain Monte Carlo) replicates for 10 times at each K from 1 to 10. The probable number of groups was estimated. The second run was 50000 for burn-in length and 75000 for MCMC replicates, 20 times for each K between 3 and 8. To estimate the best K value (number of groups) the Evanno test was performed on STRUCTURE results using 'Structure Harvester' (Evanno *et al.*, 2005). The accessions with membership coefficient ≥0.75 were assigned to the respective population and <0.75 were assigned to the admixture group. The results were summarized in a bar plot using DISTRUCT (Rosenberg, 2004).
4. GenAlEx 6.41 software was also used (Peakall and Smouse, 2012) for hierarchical analysis of molecular variance (AMOVA) to evaluate the variance among and within the accessions of studied tulip species (except *T. micheliana* that possessed only one accession) by FPT (analogous to FST) (Peakall and Smouse, 2012) using 9999 random repetitions.

Results

Molecular assessment of genetic diversity

- ***EST-SSR genotyping***

Out of the 70 EST-SSR primer pairs used for polymorphism validation, a total of 15 primer pairs were chosen for the study based on the results of preliminary assessment of quality, quantity, reproducibility, and polymorphism of amplicons generated with template DNA. These primer pairs resulted in 130 alleles with an average of 8.67 alleles per locus. Variation in numbers of alleles is shown in Table 2.

The informativeness of primer pairs was compared by calculating gene diversity (GD) and polymorphism information content (PIC). The GD ranged from 0.15 to 0.89 with an average value of 0.66. The PIC value of each primer pair was ranged from 0.14 to 0.88 with an average of 0.62. The resolving power (Rp) is a parameter that indicates the discriminatory potential of the primers. The highest (2.58) and the lowest (0.76) Rp values were observed with the primers Ca-5553 and Ca-6950, respectively. The average value of Rp per primer was 1.93 (Table 2).

The N_a ranged from 4 for Ca-3952 to 18 for Ca-15730 loci. Across the populations, N_a ranged from 1.27 for *T. clusiana* var. *chrysantha* Boiss. (Pole-Piran) to 2.60 for *T. systola* Stapf (Derak). The N_e ranged from 1.17 for Ca-6950 locus to 9.10 for Ca-15730 locus with an average of 3.69 alleles per locus. Across the populations, N_e ranged from 1.22 for *T. clusiana* var. *chrysantha* (Pole-Piran) to 2.41 for *T. systola* (Derak) (Tables 2 and 3).

NBS profiling

Allelic data obtained from whole individuals were used to calculate the indexes of markers effectiveness. The values of GD and PIC were equal for each NBS primer–enzyme combination which ranged between 0.85-0.95 with a mean value of 0.91. The Rp had values from 8.83 to 27.46 with a mean of 17.39 (Table 4).

A summary of the genetic diversity parameters calculated for 8 NBS primer-enzyme combinations is presented in Table 4. Totally 508 bands were amplified, 500 of which were polymorphic (98.34%). The size of most amplified bands ranged between 100 to 500 bp. The highest (91) and the lowest (38) number of bands were achieved for NBS6-*MseI* and NBS1-*RsaI*, respectively.

Table 2. List of the fifteen genic microsatellite markers (EST-SSRs) used in this study and their efficiency parameters.

Locus	Repeat motif	Product size (bp)	N_a	Ne	I	GD	PIC	Rp
Ca-2572	(GAGAAG)4	213	10	2.51	1.29	0.60	0.57	1.76
Ca-3952	(CAG)4	189	4	2.62	1.08	0.62	0.55	2.10
Ca-5526	(GAG)6	242	11	5.37	1.94	0.81	0.79	2.54
Ca-5553	(TTG)9	168	12	4.30	1.75	0.77	0.74	2.58
Ca-6950	(GAT)4	198	4	1.17	0.33	0.15	0.14	0.76
Ca-7862	(CGC)4	131	5	2.88	1.19	0.65	0.59	2.01
Ca-8508	(GTT)10	325	8	2.84	1.25	0.65	0.59	1.63
Ca-13333	(GAT)4	242	8	3.29	1.46	0.70	0.65	2.04
Ca-15730	(CGC)8	213	18	9.10	2.46	0.89	0.88	2.28
Kn-834	(AT)8	221	4	2.29	0.96	0.56	0.50	1.51
Kn-1412	(GGA)10	242	13	5.57	1.96	0.82	0.80	2.03
Kn-2291	(GAGAAG)4	275	10	2.52	1.19	0.60	0.55	1.83
Kn-7108	(TTTC)4	231	9	4.18	1.63	0.76	0.72	1.96
Kn-7480	(GAC)9	268	5	2.19	0.89	0.54	0.44	1.60
Kn-30956	(CTC)6	237	9	4.56	1.74	0.78	0.75	2.31
		Mean	8.67	3.69	1.41	0.66	0.62	1.93

Kn: 'Kees Nelis' contig; **Ca:** 'Cantata' contig; **Na:** number of amplified bands; **N_e:** effective alleles per locus; **I:** Shannon's information index; **GD:** gene diversity; **PIC:** polymorphism information content; **EMR:** effective multiplex ratio; **MI:** marker index; **Rp:** resolving power.

Table 3. Genetic diversity parameters in the studied tulip accessions.

Accession	N_a	N_e	I	H_o	H_e	PL	PPL
T. biebersteiniana (Zari-Sepidan)	1.33	1.31	0.22	0.30	0.17	5	33.33
T. biebersteiniana (Margoon)	1.47	1.36	0.26	0.34	0.19	6	40.00
T. biflora (Zoqali)	2.33	1.48	0.44	0.31	0.26	9	60.00
T. biflora (Neyriz)	1.60	1.51	0.37	0.48	0.27	8	53.33
T. biflora (Derak)	1.73	1.53	0.40	0.46	0.29	9	60.00
T. biflora (Dehbid)	1.87	1.61	0.45	0.50	0.32	10	66.67
T. biflora (Estahban)	2.13	1.51	0.45	0.27	0.29	10	66.67
T. biflora (Bamu)	1.73	1.28	0.29	0.15	0.18	8	53.33
T. biflora (Pole Piran)	1.40	1.35	0.24	0.30	0.18	5	33.33
T. biflora (Margoon Track)	1.53	1.25	0.24	0.20	0.16	6	40.00
T. biflora (Rafsanjan)	1.80	1.50	0.37	0.18	0.23	8	53.33
T. clusiana (Khabr)	1.47	1.29	0.23	0.24	0.16	6	40.00
T. clusiana (Neyriz)	1.60	1.26	0.24	0.25	0.16	8	53.33
T. clusiana (Bavan)	2.27	1.73	0.55	0.39	0.36	12	80.00
T. clusiana var. *chrysantha* (Bezenjan)	2.47	1.85	0.61	0.40	0.38	11	73.33
T. clusiana var. *chrysantha* (Pole Piran)	1.27	1.22	0.16	0.22	0.12	4	26.67
T. systola (Zard Shehneh)	2.33	1.71	0.56	0.47	0.36	11	73.33
T. systola (Dashte Laleh- Estahban)	1.67	1.38	0.33	0.20	0.23	8	53.33
T. systola (Bisheh - Estahban)	1.53	1.39	0.31	0.22	0.23	7	46.67
T. systola (Derak)	2.60	2.41	0.76	0.38	0.47	11	73.33
T. systola (Bamu)	1.93	1.55	0.42	0.30	0.28	10	66.67
T. systola (Margoon track)	2.20	1.64	0.50	0.44	0.32	12	80.00
T. systola (Margoon Waterfall)	1.80	1.60	0.47	0.52	0.34	11	73.33
T. systola (Eghlid)	1.93	1.47	0.40	0.37	0.27	10	66.67
T. systola (Yasouj)	2.10	1.54	0.44	0.38	0.28	10	66.67
T. systola (Kurdistan)	1.87	1.68	0.45	0.36	0.31	8	53.33
T. micheliana (Nasr Abad)	2.27	1.74	0.56	0.44	0.36	11	73.33
Mean	1.86	1.52	0.40	0.34	0.27	8.67	57.78

N_a: number of observed allele per locus; **N_e:** effective alleles per locus; **I:** Shannon's information index; **H_o:** observed heterozygosity; **H_e:** expected heterozygosity **PL:** number of polymorphic loci; **PPL:** percentage of polymorphic loci.

Table 4. Polymorphism results of 8 NBS primer-enzyme combinations for functional motif-directed profiling of wild and cultivated tulip accessions.

NBS primer-enzyme combination	Bands			GD	PIC	Rp
	Amplified	Polymorphic	Polymorphic (%)			
NBS1-RsaI	38	38	100	0.89	0.88	8.83
NBS2-RsaI	53	53	100	0.86	0.85	14.21
NBS3-RsaI	68	67	98.53	0.93	0.93	18.01
NBS6-RsaI	64	62	96.88	0.94	0.93	16.08
NBS1-MseI	45	43	95.56	0.92	0.91	10.64
NBS2-MseI	64	62	96.88	0.91	0.91	20.90
NBS3-MseI	85	85	100	0.89	0.88	27.46
NBS6-MseI	91	90	98.90	0.95	0.95	22.97
Total	508	500				
Mean	63.5	62.5	98.34	0.91	0.91	17.39

GD: gene diversity **PIC:** polymorphism information content; **EMR:** effective multiplex ratio; **MI:** marker index; **Rp:** resolving power.

Table 5. Analysis of molecular variance (AMOVA) of the studied tulip species.

Source of variation	df	SS	EV	Total variation	Φ_{PT}
EST-SSR genotyping					
Among accessions within species	26	2681.005	12.554	82%	0.824
Among individuals within accessions	189	507.250	2.684	18%	
Total	215	3188.255	15.238	100%	
NBS profiling					
Among accessions within species	26	404.685	1.927	93%	0.927
Among individuals within accessions	189	28.625	0.151	7%	
Total	215	433.310	2.078	100%	

df: degree of freedom; **SS:** sum of squared observations; **MS:** mean of squared observations; **EV:** estimated variance; **Φ_{PT}:** proportion of the total genetic variance among individuals within an accession, ($P < 0.0001$).

Molecular assessment of population genetic structure

• *EST-SSR genotyping*

The genetic structure of populations was analyzed using principal component analysis (PCA). The PCA analysis was performed to assess inter-species genetic relationship among 6 tulip species based on allele frequencies of the EST-SSR markers and revealed a clear differentiation between the tulip species (Fig. 2A). The first 2 principal components explained 36.60% and 23.30% of the total variance, respectively.

Structure of genetic differentiation among accessions was further studied using EST-SSR data based on Bayesian clustering algorithm as implemented in software STRUCTURE. According to the method described by Evanno et al. (2005) three optimal groups or clusters (K) were identified and majority of the individuals were clearly assigned into these specific groups. Assignment of genotypes to specific accessions was based on the threshold value of membership coefficients (≥0.75). Setting the threshold value of membership coefficients as 0.75, 24 out of 216 (11.11%) individuals were admixture and the remaining 216 individuals were non-admixed and assigned into either P1, P2, or P3 genetic clusters. Individuals of *T. biebersteiniana*, *T. clusiana* and *T. clusiana* var. *chrysantha* formed the first cluster. The second cluster comprised of individuals which all belong to *T. biflora*. Ten populations of *T. systola* along with *T. micheliana* population were separated into cluster four (Fig. 3B).

The genetic differentiation was determined by hierarchical AMOVA that produced significant genetic difference among the populations. The results revealed that molecular variation among accessions (ΦPT = inter-population variation) was 82% of the total variation.

UPGMA clustering analysis clearly discriminated the genotypes into different clusters. The UPGMA tree shows 2 main clades. The first clade consists of *T. biebersteiniana* and *T. biflora* Pall, populations which belong to the subgenus *Eriostemones*. The second clade contains species of the subgenus *Tulipa*. This clade included two subclades of 15 populations as follows: 1) Ten populations of *T. systola* in section *Tulipanum*; 2) Three populations of *T. clusiana* plus two populations of *T. clusiana* var. *chrysantha* belonging to section *Clusianae*. However, *T. micheliana* was placed in between two main clades.

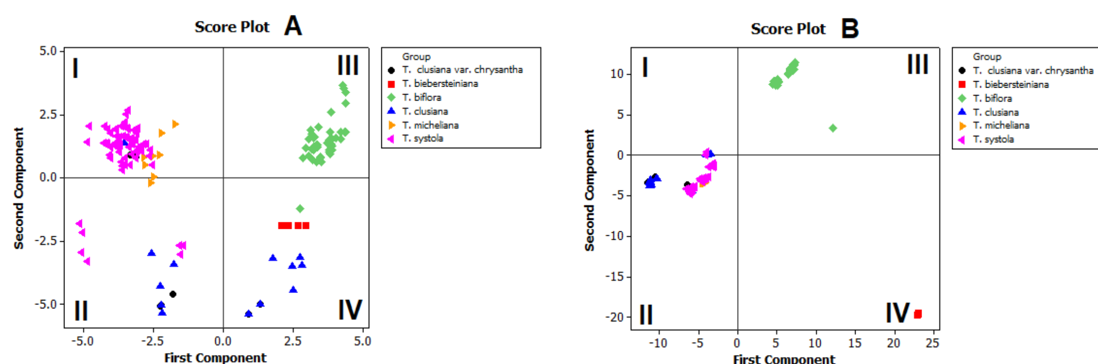

Fig. 2. Distribution of *Tulipa* L. populations between factors PC1 (x-axis) and PC2 (y-axis) calculated by PCA of 15 EST-SSR loci (A) and 8 NBS-LRR markers (B).

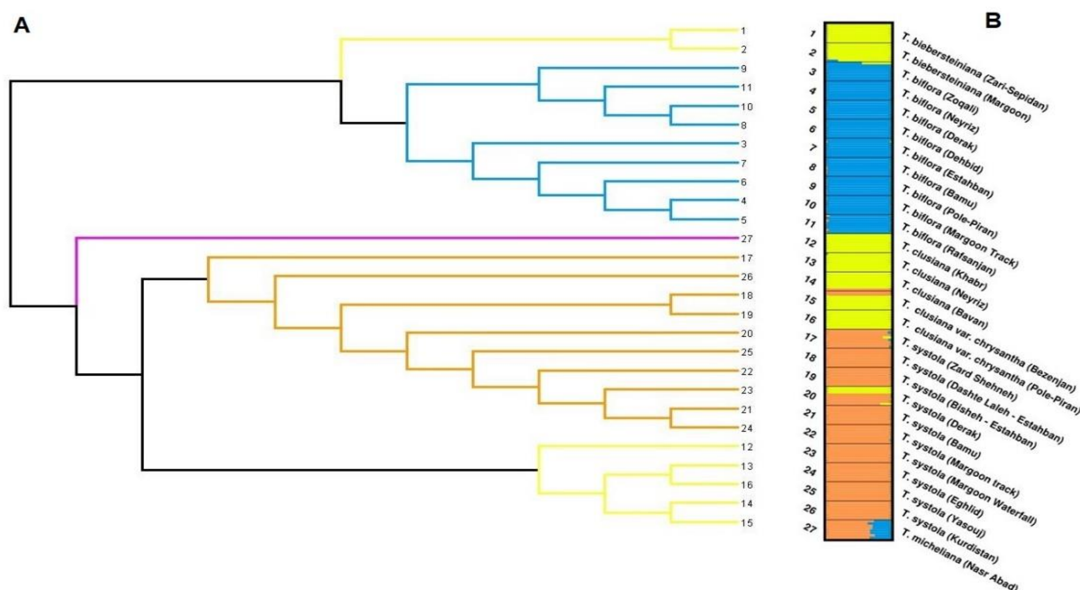

Fig. 3. A) Dendrogram of 27 tulip populations revealed by UPGMA cluster analysis based on Nei's genetic distance derived from EST-SSR data of fifteen markers analysis. B) Population structure inferred by Bayesian cluster analyses (Structure) for Iranian populations of tulip. Three optimal clusters (K) were identified based on Evanno *et al*. (2005) prediction.

NBS profiling

The PCA plot graph of 8 NBS marker-enzyme combinations was constructed to assess inter-species genetic relationship among 6 tulip species based on allele frequencies of the NBS markers which revealed differentiated populations and greatly confirmed the patterns of genetic diversity obtained among the species (Fig. 2B). The first two principal components explained 21% and 18.10% of the total variance, respectively.

The NBS profiling data were used in software STRUCTURE and based on Bayesian clustering algorithm the structure of genetic differentiation among accessions was evaluated. According to the method described by Evanno et al. (2005), four optimal groups or clusters (K) were identified (Fig. 4B) and majority of the individuals were clearly assigned into these specific groups. Setting the threshold value of membership coefficients as 0.75, 16 out of 216 (5.71%) individuals were admixture and the remaining 216 individuals were non-admixed and assigned into either P1, P2, P3, or P4 genetic clusters. Individuals of *T. biebersteiniana* formed the first cluster. The second cluster comprised of individuals which all belong to *T. biflora*. Individuals of *T. clusiana* and *T. clusiana* var. *chrysantha* formed the third cluster. Ten populations of *T. systola* plus *T. micheliana* individuals were separated into fourth cluster (Fig. 4B).

The hierarchical AMOVA analysis was used to produce significant genetic differences among the accessions. The AMOVA results indicated that molecular variation among populations (ΦPT = inter-population variation) was 93% of the total variation.

Genetic relationship among 27 wild tulip populations is depicted in Fig. 4A. Cluster analysis clearly discriminated the 216 genotypes into four clusters at the DNA level. The group I consisted of two populations of *T. biebersteiniana*. All eleven accessions of *T. biflora* were placed into group IV. However, three populations of *T. systola* formed a separate group (clade III) and the other seven populations of *T. systola* along with populations of *T. clusiana* and *T. clusiana* var. *chrysantha* formed the three subclades of clade II.

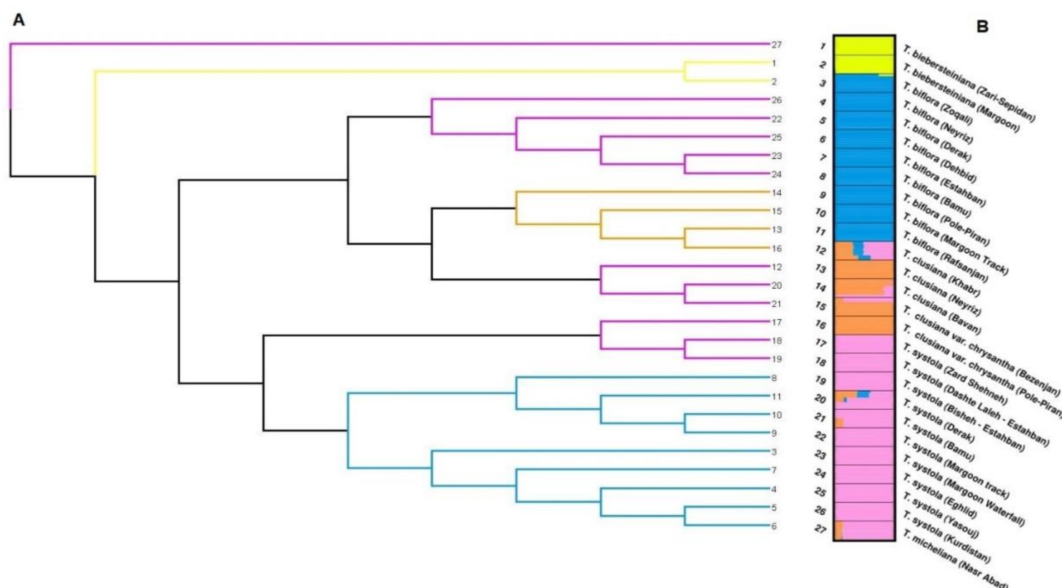

Fig. 4. A) Dendrogram of 27 tulip populations revealed by UPGMA cluster analysis based on Nei's genetic distance derived from NBS profiling data. B) Population structure inferred by Bayesian cluster analyses (Structure) for Iranian populations of tulip. Four optimal clusters (K) were identified based on Evanno *et al*. (2005) prediction.

Discussion

Discriminatory power of molecular markers

In this study, fifteen SSR loci were developed from a collection of ESTs as a useful tool to identify SSRs in non-model plants (Shahin *et al*., 2012). Moreover, a total of 8 NBS marker-enzyme combinations were used to perform genetic analyses. Informativeness of a marker can be quantitatively measured by GD, PIC, and Rp values. The PIC values range from 0 for markers with only one allele to 1 for markers with infinite number of alleles (Hildebrand et al., 1992). When the PIC value is ≥ 0.5, the SSR marker is classified as informative (Sharma *et al*., 2009). Highly informative markers indicate a PIC value greater than 0.7. Based on our findings, high mean values of GD, PIC, and Rp demonstrated that both molecular marker systems used in this research were highly informative by representing of high discriminatory power to evaluate the genetic diversity and discriminate between tulip populations. Therefore, the obtained results in this investigation are in agreement with the findings of Mantovani *et al*. (2006) and Caser *et al*. (2010) reported a high discriminatory power of NBS markers.

Molecular assessment of genetic diversity

Generally, there is rare information with regard to genetic diversity of the genus *Tulipa*. However, molecular characterization of *Tulipa* species using molecular markers has been reported in recent years. Using RAPD marker, genetic diversity of four tulip varieties in Xinjiang province and 10 cultivars of tulip were analyzed. All tulips were placed into four clusters and cluster 1 included four tulip varieties which were far from the cultivars in genetic distance (Luan *et al*., 2008). Kiani *et al*. (2012) studied genetic relationships among Iranian tulips from Khorasan and Yazd provinces using inter simple sequence repeat (ISSR) primers. A total of 39 wild individuals of seven *Tulipa* species were collected during their flowering time. The results showed that 24 accessions of *Tulipa* from different distribution areas that were recognized as *T. micheliana* based on morphological

observations clustered in one group. Three accessions of *T. biflora* showed high diversity, as each accession made a separate cluster. *T. biebersteiniana* and *T. clusiana* also formed distinct clades. They concluded that molecular clustering analysis was in agreement with traditional taxonomy of the genus *Tulipa* at the section level.

Knowledge of genetic diversity allows successful management and conservation of threatened and rare wild species with proper population selection (Tabin *et al*. 2016). Moreover, information on genetic diversity will assist germplasm utilization in breeding programs. Thus, evaluating the genetic diversity and structure of wild tulips in Iran is of great importance in conservation and breeding programs of this ornamental crop. Moreover, knowledge of genetic diversity enables tulip breeders to use valuable wild germplasm preserving alleles of agricultural interest to develop new tulip cultivars with desired traits.

Geographical distribution, mating system, dispersal of propagating materials (seeds and other vegetative propagules), and life style affect distribution and amount of genetic diversity in natural populations (Tang *et al*. 2013a; Ballesteros-Mejia *et al*. 2016; Nam *et al*. 2016). Based on EST-SSR genotyping data, individuals of *T. clusiana* var. *chrysantha* (Pole-Piran) showed the lowest genetic diversity among Iranian wild tulip populations. This might be a consequence of habitat impacts and propagation system. This population was collected from wheat fields in foothills of Pole-Piran. It is assumed that most of the tulip individuals in this location are propagated vegetatively by plowing wheat fields and this type of propagation maintains their homozygosity and prevents the chance of propagation by seed.

NBS profiling results revealed *T. biebersteiniana* (Zari-Sepidan) the lowest number of observed alleles per locus, Shannon's information index, and percentage of polymorphic loci among six wild tulip species. This finding is in line with Kutlunina *et al*. (2013) research. They used morphological and AFLP markers to assess genetic diversity among four closely related species of tulips and reported that populations of *T. biebersteiniana* show low levels of genetic diversity. As they concluded, this species has natural vegetative propagation by forming plagiotropic stolons with a bulb at the end. This type of reproduction leads to increase in genetic diversity. On the other hand, self-incompatibility prevents effective pollination and fertilization inside the clonally propagated populations of *T. biebersteiniana*. Beside reproductive system, it could be a consequence of ecological factors. As individuals of this tulip species grow under the forest canopy or in a bushy area, pollination may be complicated due to less pollinators visiting. All these factors result in high levels of homogeneity by the prevention of seed propagation.

Natural populations is threated by the low level of genetic diversity . It has been shown that Laar population of *Rheum emodi* which possess low level of genetic diversity are under extinction situation in adverse environmental conditions and plant pathogens attack (Tabin *et al*., 2016). Similarly, low level of diversity is a threat for *T. clusiana* var. *chrysantha* and *T. biebersteiniana* which can increase their susceptibility to adverse environmental conditions and diseases outbreak. It should be noted that as preliminary evidence, these two tulip species were severely infected by rust possibly as a consequence of increase in their homozygosity along with high pressure of pathogen. These conditions can put these populations in extinction situation. Therefore, efficient cross breeding programs are required to increase the level of heterozygosity in this species.

According to both EST-SSR genotyping and NBS profiling techniques, *T. systola* individuals exhibited the highest levels of genetic diversity. Most populations of *T. systola* grow on high mountains and impassable areas where the conditions are

suitable for seed production and dispersal. Thus, it could be concluded that habitat differences that affect reproductive system and propagating material dispersal may be the reason of increase in genetic diversity of *T. systola* populations.

Molecular assessment of genetic structure

The PCA plot graph of EST-SSR and NBS loci revealed differentiated populations and greatly confirmed the patterns of genetic diversity observed among the species. Similar to findings by Kiani et al. (2012), the present results indicated that PCA obviously indicated the position of species in the subgenera and sections of *Tulipa*. In the PCA plot obtained based on NBS profiling data, PCA-1 clearly distinguished populations of the subgenus *Eriostemones* (*T. biebersteiniana* and *T. biflora*) clustered on the right side of the diagram (quadrants III and IV) from the accessions belong to the subgenus *Tulipa* (*T. clusiana*, *T. clusiana* var. *chrysantha*, *T. systola*, and *T. micheliana*), which were placed on both quadrants I and II. In total, it could be concluded that discriminations between the two tulip subgenera and their species was clearer with NBS profiling data in comparison with data obtained by EST-SSR genotyping.

Population structure and differentiation of the 216 tulip individuals from 27 populations was investigated using a Bayesian Markov chain Monte Carlo approach as implemented in Structure version 2.3.4. This analysis is used to identify distinct genetic groups and admixed individuals and also to display the ancestry of individuals. With EST-SSR genotyping, three distinct groups were found. Individuals of *T. biebersteiniana*, *T. clusiana*, and *T. clusiana* var. *chrysantha* were grouped in one cluster which is in accordance with their morphological traits such as flower shape, flower color, plant height, etc. Grouping all accessions of *T. systola* in a distinct cluster was completely in line with their genetic background. With NBS profiling, 4 distinct groups were identified. Two accessions of *T. biebersteiniana* formed one cluster. All *T. biflora* accessions were placed in cluster II. Moreover, accessions of *T. clusiana*, and *T. clusiana* var. *chrysantha* were grouped in one cluster. All these classifications are in accordance with morphological traits such as flower shape, flower color, and plant height. It should be noted that there is no information on genetic structuring in Iranian wild tulips.

AMOVA analysis describes variations within and between population based on partitioning of variation which may be influenced by certain factors such as crossing system (outbreeding vs. inbreeding) and habitat destruction (isolation). The *ΦPT* value of 0.824 and 0.927 ($P<0.0001$) which was found with EST-SSR genotyping and NBS profiling, respectively, verified the significant large molecular variation among tulip species and indicated that both molecular marker systems were potent to distinguish among tulip groups. In this research, high variation among populations within species (EST-SSR = 82% and NBS-LRR = 93%) was observed which indicates the low levels of genetic diversity among individuals within populations. Theoretically, higher variation among accessions comes from low genetic differentiation among individuals within each population which shows high levels of homogeneity in populations. It could be concluded that low intra-accession (inter individuals) variation is considered as a threat for conservation of these valuable wild tulip germplasms. Consequently, the conservation strategies (both *in-situ* and *ex-situ* methods) are needed to preserve and proliferate the existing patches of various tulip accessions to save them from extinction and prevent loss of these valuable wild germplasms. In order to conserve genetic resources in these regions, sampling from more distant locations in Iran might be

more applicable to reach the higher levels of genetic diversity.

Few molecular marker systems have been used by researchers to determine the phylogenetic relationships within and among species in the genus *Tulipa* (Yanagisawa *et al*., 2012; Christenhusz *et al*., 2013; Turktas *et al*., 2013). However, the phylogenies obtained with neutral markers (RAPD, ISSR, chloroplast DNA, ITS, etc.) cannot produce clear identification of diversity among the individuals of different accessions. Therefore, functional markers based on diversity in highly conserved regions of the genome might be more predictive.

Cluster analysis can be used to evaluate the discriminatory power of the molecular markers (Pineda-Martos *et al*., 2014). Genic microsatellites and NBS markers were used to cluster 27 Iranian wild tulips. Distance based cluster analysis estimates the relationships among individuals or populations which largely depend on similarity of the alleles generated by primers in individuals or populations (Tabin *et al*., 2016). Molecular phylogeny results show that UPGMA dendrogram derived from EST-SSRs data could divide all Iranian tulip populations into their related taxa. However, findings of this investigation demonstrated that the phylogenetic relationships among tulip populations revealed by NBS profiling data do not essentially resembled the cluster pattern observed with genic microsatellites. It can be assumed that it is a consequence of the nature of NBS domain in the genome. The NBS domain is located in the central region of NBS-LRR genes and possesses some conserved amino acid motifs. This domain is under no or less diversifying selection pressure (Parniske *et al*., 1997; McDowell, 1998; Meyers *et al*., 1998; Sun *et al*., 2001). Due to these reasons, NBS markers show different levels of polymorphism in comparison with SSRs.

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