Pak. J. Bot., 58(3): 689-698, March 2026.

ARTICLE | DOI: http://dx.doi.org/10.30848/PJB2026-3(3)

GENETIC DIVERSITY IN TULIP CULTIVARS AND CHINESE WILD SPECIES BASED ON SSR MARKERS

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Abstract

Tulip (*Tulipa* L.) is one of the world-famous ornamental flowers, there are fewer studies on the genetic background of wild tulips and cultivars. The genetic structure, diversity and relationships of 5 tulip species native to China and 105 Dutch tulip cultivars were assessed by 12 highly polymorphic microsatellite markers. The mean values of Shannon's information index (I), Nei's diversity index (H) and polymorphism information content (PIC) were 0.91, 0.51 and 0.55, respectively, which indicated the high discriminatory power of the selected markers. One hundred accessions were genotyped using the selected makers and the total observed heterozygosity (Ho) was 0.49. Ho of species was the highest (0.62). Among cultivar groups, Ho of Fosteriana group was the lowest (0.35) while it was highest in Darwin hybrid group (0.55). The genetic distances among species and cultivar groups ranged from 0.0036 to 0.6946. Both the results of Unweighted pair-group method with arithmetic means (UPGMA) and Principal coordinates analysis (PCoA) presented that the 110 materials were separated into three main clusters: species, Fosteriana and *T. gesneriana* groups. A significant difference could be detected among three clusters (Fst= 0.231, $p<0.001$), which means 23% of the variance existed among clusters and 77% among species and cultivar groups. In conclusion, the above results indicated that the SSR markers selected in this research can be effectively used for the identification of wild tulips from China and Dutch cultivars. This study thus lays crucial groundwork for the conservation, characterization, and utilization of tulip genetic resources on a global scale.

Key words: Microsatellite, Phylogenetic relationships, Genetic structure, *Tulipa* L.

Introduction

Tulip (*Tulipa* L.) is an important bulbous ornamental plant which is favored by consumers because of its elegant, various, and showy flowers. Tulip is widely used as cut flowers, potted plants as well as garden plants. Tulips are found in the temperate regions of the northern hemisphere, Hoog (1973) proposed that the Pamir Alai and Tianshan mountains in central Asia are the primary gene diversity centers of wild tulips. The genus *Tulipa* has approximately 139 species in the world (Everett, 2013), in which 17 were found in China (Xing, 2017).

Tulip was introduced from Turkey to Europe in the sixteenth century (Hernández Bermejo & García Sánchez, 2009). Dutch breeders have worked on the development of tulip cultivars for over four hundred years and thousands of cultivars have been obtained. Nowadays, the widely grown tulip cultivars are almost all from the Netherlands, and they have high levels of heterozygosity. Majority of tulip breeders focused on breeding within *T. gesneriana* (van Tuyl & Creij, 2006). Therefore, the current commercial cultivars are mainly from *T. gesneriana*. Interspecific hybridization is an important approach for cultivar improvement. Although pre- and post-fertilization barriers exist, breeders have been trying to get hybrids through interspecific crossing to improve the tulip cultivars. Crossing barriers have been overcome using techniques including cut-style pollination, embryo rescue, and ovary culture. Van Eijk *et al*., (1991) and van Raamsdonk & de Vries (1995) found that tulip species of the same section can often be hybridized, for example, *T. gesneriana* is compatible with other species in the section *Tulipa*. Darwin hybrid is a widely grown group which was created by interspecific hybridization between *T. fosteriana* and *T. gesneriana* (Bryan, 2002). At present, tulip cultivars have been divided into fifteen divisions by the Royal General Bulb Growers' Association (KAVB) , which were classified based on blooming time and flower shape or defined by their species of origin (Kaufmanniana, Fosteriana and Greigii divisions) (Richard, 2006).

Wild tulips are important breeding materials for the improvement of cultivars because they have excellent genes such as resistance genes to diseases, drought, and cold (Richard, 2006). Also, they have unique ornamental characteristics that can be utilized, for instance, multiflora and fragrance. Knowing the taxonomic and evolutionary relationships between wild species and cultivars is the first step in assessing the breeding potential of wild species (Peralta *et al*., 2021). To improve modern cultivars through wild tulips, Xing *et al*., (2017) have collected wild tulip resources in China, and evaluated their ornamental value, utilization potential, and ecological adaptability. Qu *et al*., (2018) carried out the karyotype analysis of eight Chinese *Tulipa* species, providing basic cytological information that is very useful for further utilization of these species in breeding. Based on the previous research, interspecific

Received: 07-01-2025 | Revised: 13-08-2025 | Accepted: 20-08-2025 | Online: 05-12-2025

crossing of 5 tulip cultivars and 5 Chinese wild species was carried out and the results indicated that *T. altaica* was more closely genetically related to the selected cultivars (Xing *et al*., 2020). Tulip breeding is not only restricted by crossing barriers, but also impeded by its long juvenile period. Generally, tulips need 4-5 years to grow up from seed to a commercial bulb, and it takes more than 10 years to develop cultivars with the desirable characteristics. Therefore, the selection of parents is very important before starting to make crosses.

Germplasm is the indispensable material basis for parental line selection in all plant breeding programs (Singh *et al*., 2022). It includes current cultivars, landraces, wild species, and elite breeding lines. Genetic diversity and the structure of the germplasm are useful to geneticists and breeders. Therefore, it is an important step to know this information before utilizing the germplasm. Genetic diversity study plays key role in the identification and characterization of unique genotypes for further improvement (Khan *et al*., 2019; Jan *et al.,* 2019; Malook *et al.,* 2019; Sardar *et al.,* 2021; Jan *et al.,* 2024). .Previously, genetic diversity of tulips was mainly analyzed using morphological characteristics (van Raamsdonk & de Vries, 1995; Van Raamsdonk & De Vries, 1996). With the fast development of molecular biology, molecular marker technique provided a new approach for plant breeding. The analysis of molecular variability can be used to clarify the genetic relationships among cultivars and species, which is very important in parental line selection in breeding programs. There were not many studies have been reported that can provide information on the genetic diversity of tulips. Luan *et al*. (2008) analyzed the genetic diversity and relationship of 10 cultivars and 4 species that are native to China using RAPD markers. ISSR markers were used to determine the genetic relationships between tulip species from Iran Kiani *et al*., (2012). In 2013, Tang *et al*., (2013) assessed the genetic diversity and structure of 72 tulip materials, comprising 17 breeding lines and 55 cultivars, using SNP markers. A recent study of genetic diversity and population structure has been carried out for 36 wild and 244 cultivated tulip accessions from Iran and the Netherlands using 15 polymorphic microsatellites (Pourkhaloee *et al*., 2018). Genetic diversity and population structure of 33 Iranian tulip subgenera were evaluated for the first time using the conserved DNA-derived polymorphism (CDDP) technique by Maryam Haerinasab *et al*., (2021), which showed that the selected materials had high polymorphism and could be clustered into two genetic groups, the group I included *T. biflora* accessions*,* whereas the group II comprised *T. humilis* and *T. sylvestris.* The genetic variation of the selected materials mainly within the population (81%). CDDP marker is a new type of target molecular marker technology based on single primer amplification of conserved sequences in plant functional genes or genomes. It tends to produce candidate functional markers with good polymorphism and can be used among different species (Collard & Mackill, 2009; Poczai *et al*., 2013). Tarikahya Hacioğlu *et al*., (2023) studied the genetic diversity and structure of 57 tulip materials from 17 taxa native to Turkey by ISSR markers, revealing for the first time the genetic diversity of Turkish tulips in all geographic distribution regions, which provides a certain reference value for plant taxonomists and ornamental plant breeders in future studies. Among various molecular markers, simple sequence repeats (SSRs) have the attributes that are favored by geneticists, which include multiple alleles, hypervariability, codominance, reproducibility, high abundance, and adaptability for high-throughput genotyping (Bhattarai *et al*., 2021). They are widely used in population genetics, taxonomic and phylogenetic studies, diversity and cultivar analysis, genetic mapping, functional genomics, hybridization, and marker assisted breeding (Adhikari *et al*., 2017). SSR markers are very suitable for diversity analysis and fingerprinting (Wang *et al*., 2022). EST-SSRs developed from expressed sequence tags (ESTs) detect variation in the expressed portion of the genome. Both EST-SSRs and genomic SSRs have their own advantages and disadvantages. They presented similar abilities in revealing diversity and discriminating varieties (Ramesh *et al*., 2020). However, EST-SSRs can be developed easily from the sequence data that is publicly available at a relatively low cost (Shirazi *et al*., 2023). And amplification of EST-SSRs across species is expected to be more successful compared to genomic SSRs (Li *et al*., 2004). Therefore, EST-SSRs have been successfully applied in analyzing the genetic diversity, population structure, and phylogeny of plants (Ellis & Burke, 2007).

This study aims to investigate the genetic diversity and relationships of 105 tulip cultivars from the Netherlands using SSR markers; and to clarify the phylogenetic relationships among Dutch cultivars and Chinese wild species.

Material and Methods

Plant materials and DNA isolation: Five tulip species native to China and 105 widely grown cultivars were used in this study (Table 1). The tulip species were collected in Xinjiang Province, China. The cultivars were imported from the Netherlands. All plant materials were obtained from the tulip germplasm of the Plateau Flower Research Center, Qinghai University. Young leaves from ten individuals of each species or cultivar were collected in the field, put into liquid nitrogen immediately, and then kept in -80°C until DNA isolation. Genomic DNA was isolated using EZ-10 Spin Column Plant Genomic DNA Purification Kit (Sangon Biotech, Shanghai, China). DNA was detected by 1% agarose gel electrophoresis, and concentration of DNA was detected by a microspectrophotometer (OSE-260) (TIANGEN Biotech, Beijing, China).

SSR amplification and fluorescence-based capillary electrophoresis: EST-SSRs were developed from tulip cultivars Kees Nelis and Cantata by Shahin. (2012). SSRs were selected according to the length of the repeat motif (Tang *et al*., 2015). Fifty-four SSR markers were selected in this study. The polymorphism of these markers was then tested in 5 *Tulipa* species and 30 randomly selected cultivars. Polymorphic SSR markers were screened, and all samples were genotyped using a fluorescence-based capillary electrophoresis (CE). The M13-tail method (M, 2000) was used for PCR amplification.

Table 1. Information of plant materials.

No.	Name	Species/ Cultivar group	Origin
1.	*T. thianschanica*	Species	Xinjiang, China
2.	*T. biflora* Pallas	Species	Xinjiang, China
3.	*T. iliensis*	Species	Xinjiang, China
4.	*T. altaica*	Species	Xinjiang, China
5.	*T. patens*	Species	Xinjiang, China
6.	Christmas Marvel	Single early	the Netherlands
7.	Purple Prince	Single early	the Netherlands
8.	Sweet Prince	Single early	the Netherlands
9.	Diamond	Single early	the Netherlands
10.	Avignon	Single late	the Netherlands
11.	Holland Beauty	Single late	the Netherlands
12.	King Blood	Single late	the Netherlands
13.	Menton	Single late	the Netherlands
14.	Blushing Lady	Single late	the Netherlands
15.	Big Smile	Single late	the Netherlands
16.	Cape Holland	Single late	the Netherlands
17.	Queen of Night	single late	the Netherlands
18.	Muscadet	Single late	the Netherlands
19.	Cum laude	Single late	the Netherlands
20.	Strong Gold	Triumph	the Netherlands
21.	Inzell	Triumph	the Netherlands
22.	Leen Van der Mark	Triumph	the Netherlands
23.	Royal Ten	Triumph	the Netherlands
24.	White Dream	Triumph	the Netherlands
25.	Barcelona	Triumph	the Netherlands
26.	Negrita	Triumph	the Netherlands
27.	Yokohama	Triumph	the Netherlands
28.	Dynasty	Triumph	the Netherlands
29.	Kung Fu	Triumph	the Netherlands
30.	Happy Generation	Triumph	the Netherlands
31.	Jan Van Ness	Triumph	the Netherlands
32.	Sweet Rosy	Triumph	the Netherlands
33.	Avenue	Triumph	the Netherlands
34.	Cheers	Triumph	the Netherlands
35.	Carnaval de Rio	Triumph	the Netherlands
36.	Shirley	Triumph	the Netherlands
37.	Salmon Dynasty	Triumph	the Netherlands
38.	Verandi	Triumph	the Netherlands
39.	Red Power	Triumph	the Netherlands
40.	Carola	Triumph	the Netherlands
41.	Peking	Triumph	the Netherlands
42.	Pallada	Triumph	the Netherlands
43.	Escape	Triumph	the Netherlands
44.	Mistress	Triumph	the Netherlands
45.	Orleans	Triumph	the Netherlands
46.	Eskimo Chief	Triumph	the Netherlands
47.	Bolroyal Dream	Triumph	the Netherlands
48.	Purple Cloud	Triumph	the Netherlands
49.	Jumbo Pink	Triumph	the Netherlands
50.	Royal Virgin	Triumph	the Netherlands
51.	Synaeda Amor	Triumph	the Netherlands
52.	Judith Leyster	Triumph	the Netherlands
53.	Purple Flag	Triumph	the Netherlands
54.	First Class	Triumph	the Netherlands
55.	Dow Jones	Triumph	the Netherlands
56.	Ollioules	Darwin hybrid	the Netherlands
57.	American Dream	Darwin hybrid	the Netherlands
58.	World's Favorite	Darwin hybrid	the Netherlands
59.	Oxford	Darwin hybrid	the Netherlands
60.	Van Eijk	Darwin hybrid	the Netherlands
61.	Golden Oxford	Darwin hybrid	the Netherlands
62.	Golden Parade	Darwin hybrid	the Netherlands
63.	Ad Rem	Darwin hybrid	the Netherlands
64.	Parade Design	Darwin hybrid	the Netherlands
65.	Jaap Groot	Darwin hybrid	the Netherlands
66.	Parade	Darwin hybrid	the Netherlands
67.	Golden Apeldoorn	Darwin hybrid	the Netherlands
68.	Heart of Poland	Darwin hybrid	the Netherlands
69.	Pink Impression	Darwin hybrid	the Netherlands
70.	Roze Pink	Darwin hybrid	the Netherlands
71.	Oxford's Elite	Darwin hybrid	the Netherlands
72.	Red Impression	Darwin hybrid	the Netherlands
73.	Daydream	Darwin hybrid	the Netherlands
74.	Salmon Impression	Darwin hybrid	the Netherlands
75.	Banja Luka	Darwin hybrid	the Netherlands
76.	Darwidesign	Darwin hybrid	the Netherlands
77.	Apeldorn Elite	Darwin hybrid	the Netherlands
78.	World's Fire	Darwin hybrid	the Netherlands
79.	Sahara Rally	Darwin hybrid	the Netherlands
80.	Design Impression	Darwin hybrid	the Netherlands
81.	Hakuun	Darwin hybrid	the Netherlands
82.	Apeldoorn	Darwin hybrid	the Netherlands
83.	Viking	Double early	the Netherlands
84.	Flash Point	Double early	the Netherlands
85.	Abba	Double early	the Netherlands
86.	Red Baby Doll	Double early	the Netherlands
87.	Yellow Pompenette	Double late	the Netherlands
88.	Mount Tacoma	Double late	the Netherlands
89.	Angelique	Double late	the Netherlands
90.	Black Hero	Double late	the Netherlands
91.	Blue Diamond	Double late	the Netherlands
92.	Miranda	Double late	the Netherlands
93.	Madame Lefeber	*T. fosteriana*	the Netherlands
94.	Purissima	*T. fosteriana*	the Netherlands
95.	Wit White	*T. fosteriana*	the Netherlands
96.	Yellow Purissima	*T. fosteriana*	the Netherlands
97.	Maja	Fringed	the Netherlands
98.	Fringed Family	Fringed	the Netherlands
99.	Crispa Fabio	Fringed	the Netherlands
100.	Crystal Star	Fringed	the Netherlands
101.	Crystal Beauty	Fringed	the Netherlands
102.	Aladdin	Lily flowering	the Netherlands
103.	Royal Gift	Lily flowering	the Netherlands
104.	Purple Dream	Lily flowering	the Netherlands
105.	Flaming Parrot	Parrot	the Netherlands
106.	Bright Parrot	Parrot	the Netherlands
107.	Black Parrot	Parrot	the Netherlands
108.	Flaming Spring Green	Viridiflora	the Netherlands
109.	Yellow Spring Green	Viridiflora	the Netherlands
110.	Spring Green	Viridiflora	the Netherlands

Table 2. Sequence of 12 primer combinations.

No.	Locus	Forward primer (5'-3')	Reverse primer (5'-3')	Product size (bp)	Repeat motif	Annealing temperature (°C)
1.	Co_5373	TCGCTAGATCCAATTGTTCT	GATTTCTCTCCAACAACCAA	143	(TGTATT)5	56
2.	Co_8126	GCTAGAATTGTTGCATCCAT	TAGATGGGGTAGAACACGAT	146	(CGC)6	57
3.	CA_8508	AGAATTTGTCTTGCGACAGT	TAGGGGTACCAATTTGTGTT	325	(GTT)10	56
4.	KN_21967	GAGAGGGGGAGTAAGTTGTC	ACATTTTCAGCCATTAGCAT	326	(GATGAA)5	58
5.	KN_16442	GAAGGGTGTAATTACCTCCC	ACATTGGCATTCTCAATTTC	200	(CAC)7	57
6.	KN_47	CTAGTGCAACATTTGTCGAA	AACATCGTTAGAGGGTAGCA	290	(CATA)5	57
7.	KN_5851	ACATGATAGATCCGTTTTGG	GTCTATGCCTTACCACTTCG	313	(AATG)5	58
8.	KN_30291	TGTTCAAACAGAACAGTTGG	GTAGGATGGTGTTGGAAAAA	348	(AAAT)5	56
9.	KN_19222	TCCTTCAATCTTTTGCATTT	ACCAAAACAGGGTGATACAG	375	(TTACC)5	55
10.	KN_1177	GTTGTTTGGGAGTGAAGTGT	ACCCGGAGCTTTAAAGATAC	391	(GGAGAA)4	56
11.	KN_16427	CTGATGGGTCAGTTTCAAAT	ATGTTACTGCCAATCATTCC	451	(CAACAG)5	56
12.	KN_28578	AGACCTTAAAGAGAGGGCAC	GAGTGGTATCGGGATTGTAA	285	(TAC)9	59

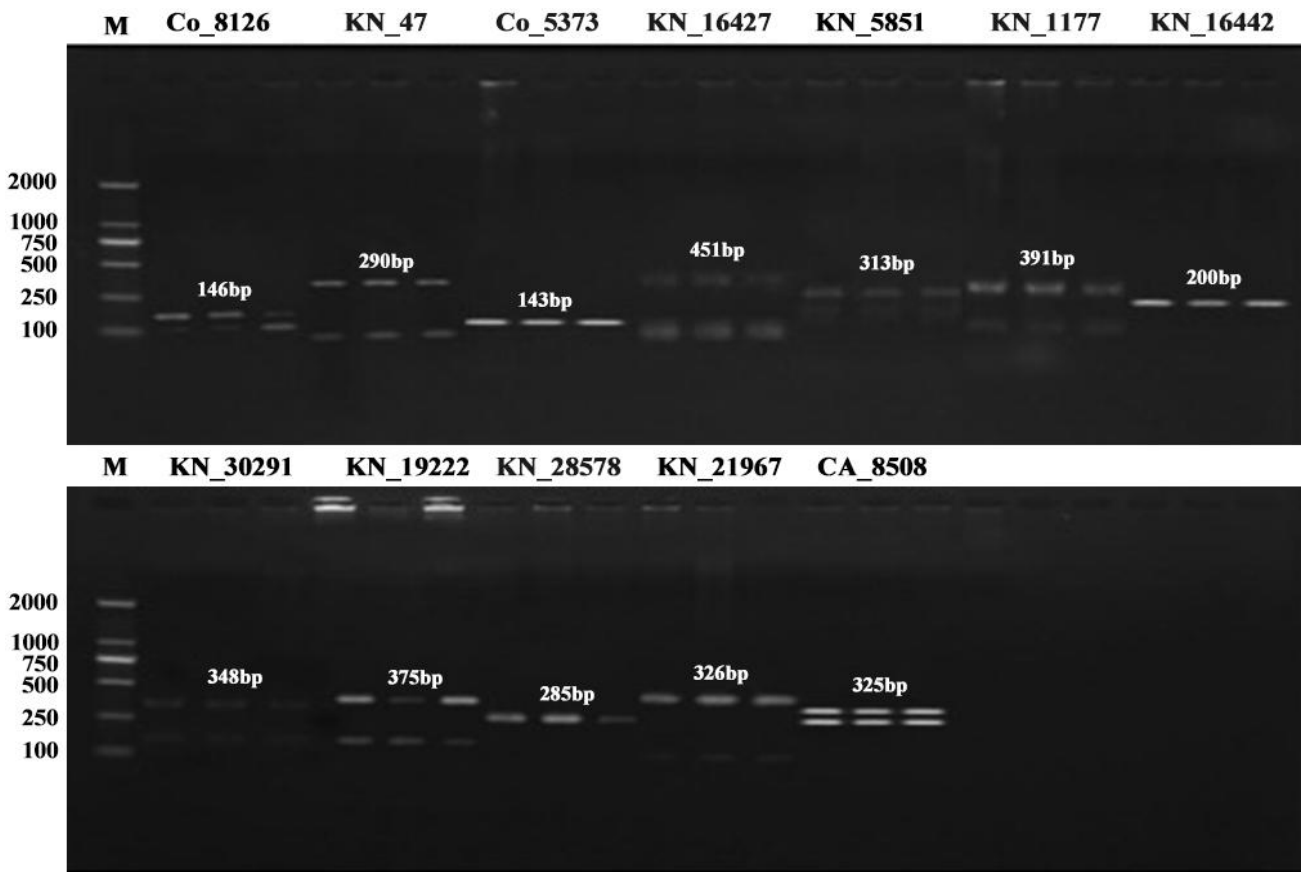

Fig. 1. Amplification of 12 pairs of primers.
Note: M, DL2000 DNA marker

The PCR reaction was performed in a 20 μL reaction mixture consisting of 2 μL genomic DNA (5 ng/μL), 0.8 μL forward primer with M13 tail (4 μM), 2.0 μL reverse primer (10 μM) and 1.2 μL universal FAM labeled M13 primer (6 μM), 2 μL 10×PCR Buffer (Mg^{2+} free), 1.6 μL Mg^{2+} (2.0 mM), 6 μL dNTPs (0.75 mM), and 0.06 μL *Taq* polymerase (2.5 U/μL).

The PCR amplification was programmed in the following conditions: 94°C for 3 min, 32 cycles of 94°C for 30 s, annealing for 45 s (annealing temperature of each primer combination can be found in Table 2), 72°C for 60 s and a final extension step of 72°C for 10 min, holding at 4°C. PCR products were sent to Genwiz (Suzhou, China) for capillary electrophoresis. SSR alleles were resolved using an ABI PRISM 3730xl Genetic Analyzer.

Genetic diversity analysis and population structure: The original data from capillary electrophoresis were checked using GeneMapper v4.0. The allele frequencies per locus, Ho, He, and I, were obtained using Popgene32 (Lewontin, 1972; Mohammadi *et al*., 2010; Nei, 1973; Yao *et al*., 2009). PIC was calculated, and UPGMA cluster analysis was based on genetic consistency using PowerMaker (Liu & Muse, 2005). The genetic structure was analyzed using the Structure 2.3.4 software.

Analysis of molecular variance: PCoA was performed by GenAlEx6.503 (Peakall & Smouse, 2012). The presence of genetic structure at different levels was assessed using the AMOVA by GenAlEx 6.503 (Peakall & Smouse, 2012).

Results

Polymorphism of SSR markers: A total of 54 SSR primer pairs were tested in the pre-screening experiment, in which 12 pairs were detected as being highly polymorphic (Fig. 1). The number of alleles produced in a locus indicates the level of heterozygosity, locus abundance, and microsatellite polymorphism. In total, 44 alleles were detected among 110 tulip materials, and the observed number of alleles (Na) ranged from (KN_30291) to 5 (Co_8126) with an average of 3.67 per locus. The effective number of alleles (Ne) ranged from 1.11 (KN_5851) to 3.54 (KN_1177), with an average of 2.27 per locus. A high level of heterozygosity was observed for the 12 SSR loci. The lowest and the highest He values were 0.10 and 0.72 for KN_5851 and KN_1177, respectively, with an average of 0.51. The lowest Ho was 0.02 for KN_5851 while the highest value 0.92 was for KN_28578, with an average of 0.50. The average PIC and I value of the selected 12 markers were 0.55 and 0.91 (Table 3), respectively, indicating the primers were highly polymorphic.

Genetic diversity analysis: In this study, the lowest percentage of polymorphic loci (PPL) was observed in the cultivar group in Fosteriana (41.67%) as the highest was found in Darwin hybrid (100%). Ho of the 110 tulip accessions was 0.49. Comparing the genetic diversity among different groups, it was found that Ho ranged from 0.35 (Fosteriana) to 0.62 (species) with an average value of 0.49. Meanwhile, the Shannon Information Index (I) ranged from 0.32 (Fosteriana) to 0.88 (Darwin hybrid). The Nei's Gene Diversity Index (H) was between 0.21 (Fosteriana) and 0.52 (species) (Table 4).

Table 3. Polymorphism of 12 primer pairs.

Locus	Na	Ne	I	Ho	He	H	PIC	Fst
Co_5373	4	2.68	1.13	0.71	0.63	0.63	0.61	0.06
Co_8126	5	1.49	0.72	0.38	0.33	0.33	0.45	0.13
CA_8508	4	2.30	1.05	0.55	0.57	0.56	0.60	0.33
KN_21967	3	1.66	0.72	0.39	0.40	0.40	0.42	0.16
KN_16442	3	2.53	1.01	0.35	0.61	0.61	0.65	0.42
KN_47	4	1.59	0.65	0.34	0.37	0.37	0.57	0.15
KN_5851	3	1.11	0.23	0.02	0.10	0.10	0.15	0.80
KN_30291	2	1.95	0.68	0.75	0.49	0.49	0.44	0.12
KN_19222	4	2.41	1.05	0.48	0.59	0.58	0.54	0.19
KN_1177	4	3.54	1.32	0.54	0.72	0.72	0.67	0.24
KN_16427	4	2.89	1.12	0.57	0.66	0.65	0.80	0.39
KN_28578	4	3.06	1.23	0.92	0.67	0.67	0.69	0.14
Mean	3.67	2.27	0.91	0.50	0.51	0.51	0.55	0.23

Note: Na: Observed number of alleles; Ne: Effective number of alleles; I: Shannon's information index; Ho: Observed heterozygosity; He: Expected heterozygosity; H: Nei's diversity Index; PIC: Polymorphism information content; Fst: Genetic differentiation index

Table 4. Genetic diversity of different tulip groups.

Group	No. individual	PL	PPL	Ho	He	I	H
Species	5	10	83.33%	0.62	0.58	0.85	0.52
Single early	4	9	75.00%	0.45	0.46	0.62	0.40
Single late	10	11	91.67%	0.53	0.47	0.75	0.44
Triumph	36	11	91.67%	0.47	0.49	0.85	0.48
Darwin hybrid	27	12	100.00%	0.55	0.51	0.88	0.50
Double early	4	11	91.67%	0.50	0.57	0.72	0.46
Double late	6	11	91.67%	0.47	0.52	0.84	0.49
Fosteriana	4	5	41.67%	0.35	0.24	0.32	0.21
Fringed	5	9	75.00%	0.43	0.40	0.59	0.36
Lily flowering	3	10	83.33%	0.53	0.44	0.57	0.37
Parrot	3	10	83.33%	0.42	0.43	0.58	0.36
Viridiflora	3	8	66.67%	0.53	0.44	0.47	0.31
Mean	12	9.75	81.25%	0.49	0.46	0.67	0.41

Note: PL: No. polymorphic loci; PPL: Percentage of polymorphic loci; I: Shannon's information index; Ho: The observed heterozygosity; He: Expected heterozygosity; H: Nei's gene diversity index

Table 5. Genetic distance among tulip groups.

pop ID	Species	Single early	Single late	Triumph	Darwin hybrid	Double early	Double late	Fosteriana	Fringed	Lily flowering	Parrot	Viridiflora
species	-											
Single early	0.3242	-										
Single late	0.4283	0.1269	-									
Triumph	0.4351	0.1259	0.0315	-								
Darwin hybrid	0.4339	0.2217	0.0805	0.0650	-							
Double early	0.4296	0.1607	0.1199	0.1016	0.1305	-						
Double late	0.4155	0.1365	0.0410	0.0218	0.0542	0.0711	-					
Fosteriana	0.7339	0.6186	0.3590	0.3373	0.1884	0.5107	0.3516	-				
Fringed	0.5775	0.2271	0.0554	0.0481	0.0791	0.1634	0.0812	0.3399	-			
Lily flowering	0.6627	0.2132	0.0879	0.0886	0.1694	0.2417	0.1195	0.4926	0.0917	-		
Parrot	0.5992	0.2253	0.1039	0.0650	0.1585	0.1817	0.0912	0.4607	0.0882	0.0913	-	
Viridiflora	0.5782	0.2208	0.1072	0.0583	0.1320	0.1887	0.0960	0.5192	0.0455	0.1184	0.0870	-

Table 6. Analysis of molecular variance (AMOVA) for three clusters of the 110 tulips obtained from PCoA.

Source	df	SS	MS	Est. Var.	%
Among clusters	2	41.552	20.776	1.028	23%
Within clusters	217	742.039	3.420	3.420	77%
Total	219	783.591		4.447	100%
Fixation index	Fst=0.231	p<0.001			

Note: df: Degree of freedom; SS: Sum of squared observations; MS: Mean of squared observations; EV: Estimated variance

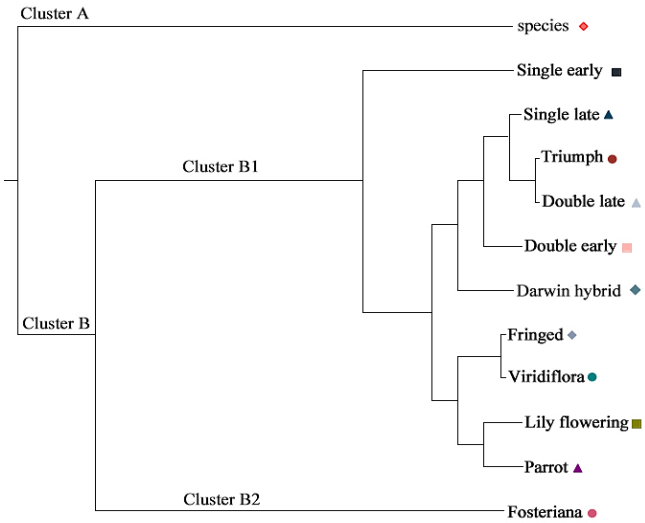

Fig. 2. UPGMA cluster analysis of 12 tulip groups.

Genetic structure and relationship: The genetic distance among 12 tulip groups was calculated to elucidate the relationship between tulip cultivars and species. As expected, species presented the largest genetic distances to cultivar groups. Genetic distances between species and Fosteriana group were largest (0.6946), while it was lowest between species and group Single early group (0.2700). The smallest genetic distance was found between groups Double late and Triumph (0.0036). Among 11 cultivar groups, Fosteriana which comes from *T. fosteriana* showed large genetic distances to cultivars that from *T. gesneriana* (Single early, Single late, Triumph, Double early, Double late, Fringed, Lily flowering, Parrot and Viridiflora). The largest genetic distance was found between Fosteriana and Single early (0.5857), followed by Viridiflora (0.4889). As the interspecific hybrid descendant of *T. fosteriana* and *T. gesneriana,* Darwin hybrid was much closer to Fosteriana (0.1742) rather than cultivar groups of *T. gesneriana*. Further analysis of genetic relationship among cultivar groups from *T. gesneriana* showed that the genetic distance was relatively close with each other (0.0036 to 0.1695) (Table 5).

According to the UPGMA clustering result (Fig. 2), 12 groups were classified into 2 clusters. First of all, wild species (cluster A) were separated from cultivars (cluster B). Among the cultivar groups, Fosteriana was separated from *T. gesneriana* cultivar groups and Darwin hybrids. Cluster BI was further divided into 2 sub-clusters, in which Fringed, Viridiflora, Lily flowering and Parrot (Sub-cluster 2) were separated from group Darwin hybrid, Single late, Triumph, Double late and Double early.

Result of UPGMA clustering presented that 110 tulip accessions were divided into two clusters (Fig. 3), in which 5 wild species were grouped in cluster A. Cultivars were grouped in cluster B. Cluster B can be further divided into three sub-clusters in which sub-cluster B1 included 13 cultivars, most of which belonged to group Fosteriana and Darwin hybrid. Sub-cluster B2 included 67 cultivars belonging to cultivar group Triumph, Darwin hybrid, Double late, Fringed, Lily flowering, Parrot, and Viridiflora. Sub-cluster B3 contains 23 cultivars, most of which belong to Triumph group.

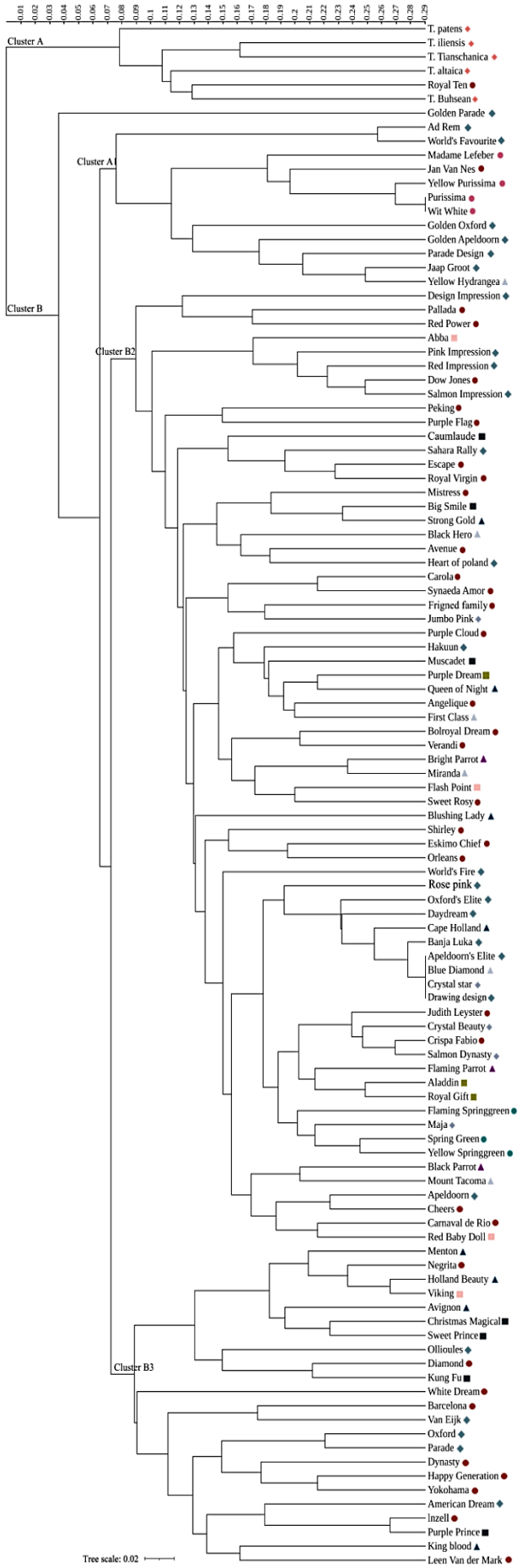

Fig. 3. UPGMA cluster analysis of 110 tulip materials.

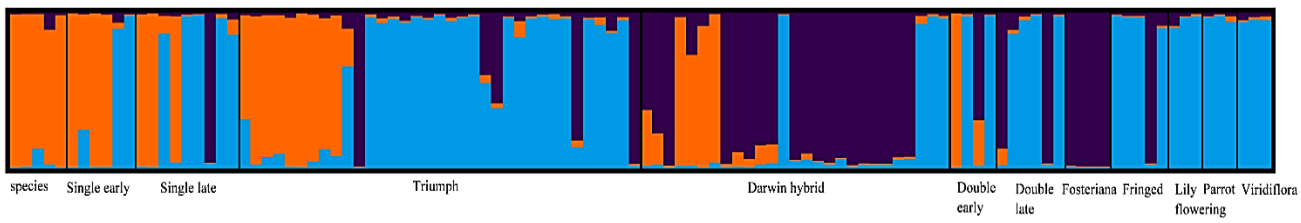

Fig. 4. Inferred genetic structure of tulips based on posterior probability by STRUCTURE (K=3).

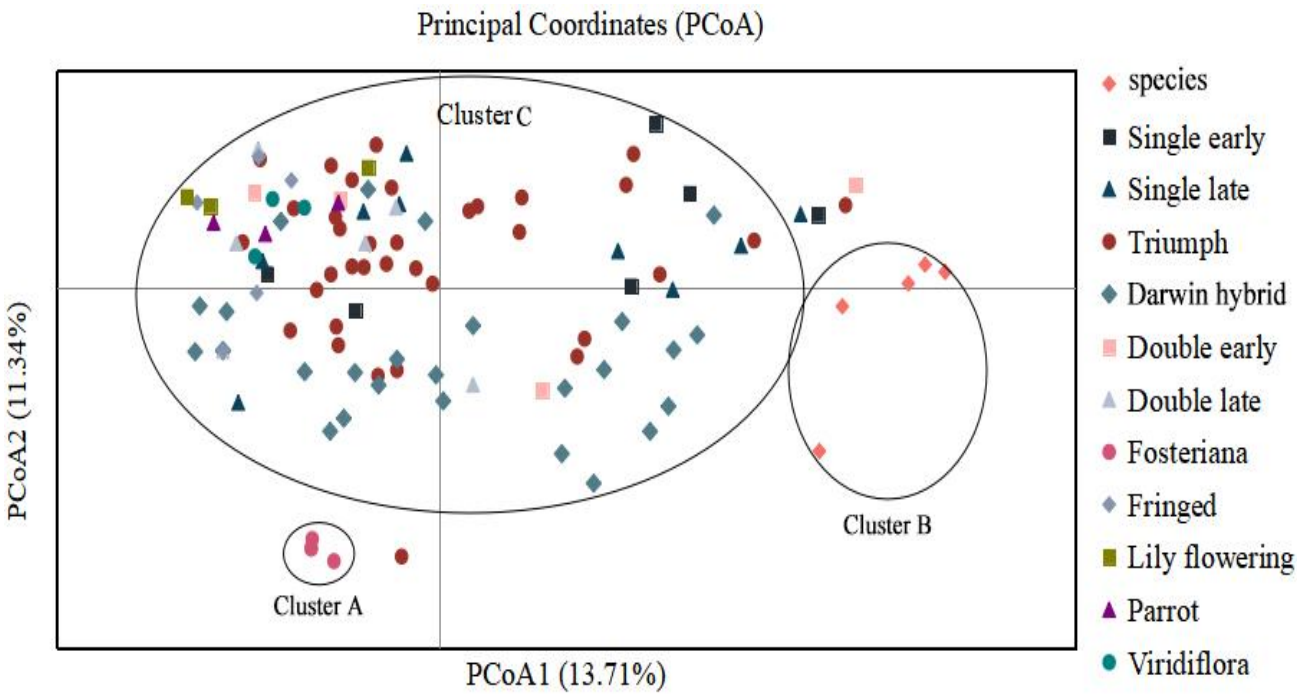

Fig. 5. Distribution of *Tulipa* L. accessions between factors PC1 (x-axis) and PC2 (y-axis), calculated by PCoA of 12 EST–SSR loci.

STRUCTURE analysis was carried out across the 110 tulip materials based on the distribution of 12 SSR markers. The maximum ΔK was presented at K=3, suggesting that the 110 tulip materials could be divided into three distinct clusters (Fig. 4). Cluster A involved 5 wild species and 21 *T. gesneriana* cultivars mainly in 2 cultivar groups (Single early and Triumph). In the bar plot, 50 *T. gesneriana* cultivars in 4 cultivar groups (Triumph, Lily flowering, Parrot, Viridiflora) were assigned into cluster B. *T. fosteriana* cultivars and Darwin hybrid were assigned into cluster C. Thus *T. fosteriana* cultivars contributed more to the genetic background of Darwin hybrid cultivars. As expected, no substructure was found within-group through subsequent STRUCTURE analysis.

The differentiation of 105 tulip cultivars and 5 species was further investigated by a principal coordinate analysis (Fig. 5). Results showed that the first and second coordinates accounted for 13.71% and 11.34% of the total genetic variation, in respective. All individuals were divided into 3 clusters, which was consistent with the result of UPGMA. Cluster A included five species. Cluster B included all cultivars belonging to group Fosteriana, while all *T. gesneriana* cultivars were included in cluster C. AMOVA analysis was conducted to confirm the results of UPGMA and PCoA. The differences among clusters were significant ($p<0.001$) and explained 23% of the total genetic variation (Table 6).

Discussion

SSR makers: Among various molecular markers, SSR is an excellent marker in the application of population genetics due to the advantages of large amount, high polymorphic, codominant and providing abundant genetic information. In this study, a total of 12 SSR markers were selected to analyze the genetic diversity of 110 tulip accessions and 44 alleles were detected. The average Shannon's information index (I) was 0.91, which was similar to the results of Pourkhaloee (2018). Comparing with other molecular markers, such as RAPD (Luan *et al*., 2008) and ISSR makers (Ju *et al*., 2019). PIC value of SSR markers selected in this research ranged from 0.15 to 0.80, with an average value of 0.55. High polymorphism markers (PIC＞0.5) accounted for 67% (8 markers). The polymorphism and universality of the selected SSR markers in this study were higher than SRAP markers used by Qin (2024) and ISSR makers used by Kritskaya (2021). Moreover, the SSR markers chosen in this study have the above characteristics compared to SSR markers developed for other species, such as Garlic (PIC=0.37, H=0.25) (Li *et al*., 2022) and Tree Peony (PIC=0.54, Ho=0.37) (Cheng *et al.*, 2021).

Genetic diversity and structure: Genetic diversity is a crucial indicator in assessing availability of breeding of

plants and their adaptability to the environment. The higher of the genetic diversity, the adapt ability of the plant population to the environment variation is stronger. Evaluation of genetic diversity in plants is very important in screening of core collection and germplasm protection (Wen *et al*., 2010). China is one of the distribution centers of tulip in the world. Wild species is important breeding material since they have high disease resistance, stress resistance and tolerance to barren (Chan *et al*., 2022). It was found that the genetic diversity of group Darwin hybrid (PPL = 100.00%, Ho = 0.55, He = 0.51) was higher than other cultivar groups, while the diversity of group Fosteriana was lowest (PPL= 41.67%, Ho = 0.35, He = 0.24). Darwin hybrid presented complex genetic background since it was created by interspecific hybridization between *T. fosteriana* and *T. gesneriana* tulips (Marasek-Ciolakowska *et al*., 2006).

Genetic relationship of 110 tulip materials showed that 5 wild species originated from China were clearly separated from 105 Dutch cultivars. Among the 105 cultivars, as expected, cultivars belonging to Fosteriana group were separated from *T. gesneriana* cultivars. Similar results were found by Luan (2008), Tang (2013) and Ju (2019). It was verified that the selected 12 SSR markers could be used in distinguishing species, cultivars belonging to Fosteriana group and *T. gesneriana* cultivars.

It was found that cultivars belonging to the group Fosteriana were grouped in one cluster with Darwin hybrid cultivars. Integrate the result of STRUCTURE analysis, it was deduced that cultivars of Fosteriana groups contributed more to the genetic background of Darwin hybrid than *T. gesneriana* cultivars (Orlikowska *et al*., 2018). Population structure obtained from STRUCTURE analysis showed that 110 materials were clustered into three clusters, however the division of species and cultivars was not consistent with the result of UPGMA and PCoA. Pritchard *et al*., (2000) found that there may not always be a clear biological explanation for the results of K-value calculations in simulated data using Bayesian modeling. The study pointed out that the posterior distribution of K in Bayesian modeling-based clustering tends to be very dependent on the uniform prior and modeling assumptions. Meanwhile, the accuracy of sample allocation could be affected by multiple factors, such as sample size, the number of loci, the number of samples with mixed origins and differences in allele frequencies among populations.

Genetic differentiation: The genetic differentiation index (Fst) is usually used to determine the magnitude of genetic variation between populations (Wright, 1978). AMOVA analysis of this study presented a high genetic differentiation across species, Fosteriana and *T. gesneriana* cultivar groups, since the Fst value was 0.231 ($p<0.001$), which means 23% of the variance existed among clusters and 77% was found among species and cultivar groups. Pourkhaloee *et al*., (2018) performed a molecular variance analysis for 6 wild tulip species and 9 cultivars using SSR makers, which showed that 65% of the molecular variation existed among 27 populations of 6 wild species. For cultivars, 51% of the variation existed among cultivar groups. Tang *et al*., (2013) analyzed the genetic diversity and population structure of 72 tulip cultivars. It was found that 79.2% of total variance existed within clusters *T. fosteriana*, *T. gesneriana* and GF hybrids (hybrids obtained between *T. gesneriana* and *T. fosteriana*). Both the results of this research and previous report indicated that genetic variation mainly existed among tulip cultivar groups.

Conclusion

In this study, the genetic diversity and relationship of 5 tulip species native to China and 105 Dutch tulip cultivars were assessed by 12 polymorphic SSR markers. Great genetic differences were found among species and cultivar groups. Meanwhile, different genetic diversity was found among different cultivar groups. Based on the SSR genotyping data, 110 materials could be classified into three main clusters: species, Fosteriana and *T. gesneriana* groups. In this study, the selected SSR markers are useful in revealing the genetic diversity and differentiation among tulips. It highlighted the unique genetic identity of Chinese wild tulip species and provided important insights for reveal genetic structure and relationships among Dutch tulip cultivars. Results of this study provided a valuable molecular basis for tulip germplasm conservation, core collection construction and breeding.

Acknowledgements

We are thankful for the support from the Scientific and Technological Achievements Commercialization Project funded by Science and Technology Department of Qinghai Province (2023-NK-151), Foundation Kunlun Talents·Innovative and Career-creating Talents, China.

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