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GENETIC DIVERSITY ANALYSIS AND SIMPLE SEQUENCE REPEAT FINGERPRINT CONSTRUCTION OF *Acer truncatum* BUNGE GERMPLASM FROM UDANTARA NATURAL RESERVE, CHINA

Zhiping WU[1, 2*], Xuefang WANG [1, 3, 4, 5*], Minghai HAN[2], Chi ZHANG[2], Jun WANG[1, 3, 4, 5], Hasengaowa BAO[2]

[1]State Key Laboratory of Tree Genetics and Breeding, Beijing Forestry University, Beijing, 100083, P. R. China.
[2]Tongliao Forestry and Grassland Research Institute, Tongliao, 028000, P. R. China.
[3]The National Facility Preservation Bank for Forestry and Grassland Germplasm Resources, Beijing Forestry University, Beijing, 100083, P. R. China
[4]National Engineering Research Center of Tree Genetics and Ecological Restoration, Beijing Forestry University, Beijing, 100083, P. R. China.
[5]College of Biological Sciences and Technology, Beijing Forestry University, Beijing 100083, P. R. China.
*Zhiping Wu and Xuefang Wang contributed to this work equally.

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Abstract

Acer truncatum Bunge is an ecologically and economically indigenous tree species in China, which is valued for ornamental, medicinal, and ecological contributions. As a major natural population of *A. truncatum* in China, the Udantara Natural Reserve harbors critical genetic resources for this species. To enhance genetic understanding and support conservation efforts, we analyzed the genetic diversity and population structure of 104 *A. truncatum* germplasm from Udantara using 20 pairs of simple sequence repeat (SSR) primers. Totally, 137 alleles were amplified, including 58.060 effective alleles. The average number of alleles (N_a), effective number of alleles (N_e), observed heterozygosity (H_o), expected heterozygosity (H_e), and Shannon's information index (I) were 6.850, 2.903, 0.424, 0.591, and 1.182, respectively, and the polymorphism information content (*PIC*) of the markers ranged between 0.293–0.839, with an average of 0.548, indicating moderate-to-high informativeness. The structure analysis partitioned the germplasm into two distinct genetic groups (28 and 76 individuals, respectively). Additionally, five SSR primers were identified as sufficient for generating unique genetic fingerprints for all 104 germplasm. Based on these findings, we propose a dual conservation strategy that integrates *in situ* protection of natural habitats within Udantara and *ex situ* preservation through a nationwide germplasm repository. Further recommendations include expanding the genetic representation by collecting germplasm across China and developing novel variants via hybridization and selective introduction. This study provides foundational genetic insights and actionable strategies to safeguard the biodiversity of *A. truncatum*, ensuring its ecological resilience and sustainable utilization in China.

Keywords: *Acer truncatum* Bunge. Fingerprint. Genetic diversity. Population structure. SSR marker.

Corresponding author:
Jun Wang
wangjun@bjfu.edu.cn
Hasengaowa Bao
hsgwdl@163.com

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1. Introduction

Acer truncatum Bunge is an important economic and horticultural species native to China, which is valued for ornamental morphology and striking autumn foliage (Wang 2019). Its seed oil, rich in unsaturated fatty acids (Le et al. 2023), has the potential to enhance mitochondrial metabolism, mitigate degenerative processes, and delay aging (Zhang et al. 2023c; Cheng et al. 2024). Notably, nervonic acid exhibits neuroprotective properties that contribute to improved cognitive function and reduced age-related neural decline (Li et al. 2019b; Qiao et al. 2019). In addition, *A. truncatum* demonstrates ecological resilience and thrives in diverse environments owing to its robust adaptability and stress tolerance (Wang 2019; Li et al. 2023).

This species is naturally distributed across ten provinces and autonomous regions in China, with the Inner Mongolia Autonomous Region harboring approximately 50% of the national germplasm resources, predominantly within the Horqin Sandy Land ecosystem (Wang 2019; Wang et al. 2024). Notably, the Udantara Natural Reserve in central Horqin supports one of China's largest *A. truncatum* populations, including individuals exceeding 500 years of age (Bao et al. 2019). However, extensive clonal propagation for meeting commercial demand, compounded by habitat fragmentation and unsustainable logging practices, has resulted in severe population decline and genetic erosion. These threats underscore the urgent need for conserving the unique germplasm of the Udantara Reserve, which is a critical genetic reservoir for Tongliao City, Inner Mongolia, and national biodiversity conservation efforts.

Molecular markers have become indispensable tools for assessing genetic diversity and facilitating germplasm identification, phylogenetic analyses, and genomic mapping (Borges do val et al. 2020; Zhu et al. 2023). Previous studies on *Acer* species revealed substantial intraspecific variation. Chen et al. (2016) reported certain genetic variations among different provenances of *A. rubrum* based on 36 pairs of sequence-related amplified polymorphism (SRAP) primers. In a study of *A. monspessulanum* in Zagros forests, Iran, Motahari et al. (2021) detected high levels of genetic diversity among 19 provenances by using inter-simple sequence repeat (ISSR), start codon targeted polymorphism (SCoT), and simple sequence repeat (SSR) markers. Significant genetic variations among 93 varieties of *A. palmatum*, *A. shirasawanum*, *A. japonicum*, *A. rubrum*, *A. sieboldianum*, *A. freemanii*, *A. saccharinum*, *A. cappadocicum*, *A. saccharum*, and *A. elegantulum* have been identified based on 15 pairs of SSR primers (Lin et al. 2022). However, one of the largest populations of *A. truncatum* in China, the *A. truncatum* germplasm in the Udantara Natural Reserve has not yet been fully elucidated, and the genetic diversity of the population remains unknown.

This study addressed this knowledge gap by systematically evaluating 104 *A. truncatum* germplasm from the Udantara Natural Reserve. We used SSR markers to quantify population genetic diversity, elucidate population structure, and construct DNA fingerprints for individual identification.

2. Material and Methods

Plant materials

One hundred and four plus trees were selected from the Udantara Natural Reserve in Tongliao City, Inner Mongolia, based on growth traits, variations in autumn leaf color, and seed yields. The tender leaves of these excellent trees were collected, quickly frozen using liquid nitrogen, and stored in a -80℃ refrigerator (Thermo Fisher Scientific, Wilmington, DE, USA). To test the usability of SSR markers, two species of the genus *Acer*, *A. rubrum* L. and *A. platanoides* L., were used as outgroups (Long et al. 2024). The geographical locations of all test materials are listed in Table S1.

DNA extraction, amplification by polymerase chain reaction, and capillary electrophoresis

Genomic DNA was extracted from the leaf samples using a DNA Secure Plant Kit DP320 (Tiangen, Beijing, China) following the manufacturer's instructions. DNA quality and concentration were determined using a spectrophotometer NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE, USA). All DNA samples were diluted to 20 $ng \cdot \mu L^{-1}$ and stored at -20℃ for PCR.

The PCR reaction system (20 µL) comprised 20.0 ng DNA template, 10.0 µL 2 × Taq PCR Master Mix (Tiangen, Beijing, China), 0.4 pmol forward primer, 1.6 pmol reverse primer, and 1.6 pmol M13F primer (Long et al. 2024). The fluorescent markers that modified M13F were divided into FAM, HEX, ROX, and TAMRA to successfully detect more than one primer in each experiment. Information on the SSR primers based on Long et al (2024) is shown in Table 1.

The PCR reaction cycles were as follows: one cycle at 94℃ for 3 min; 30 cycles of denaturation at 94℃ for 30 s, annealing at 55℃ for 30 s, and extension at 72℃ for 1 min; then 10 cycles of denaturation at 94℃ for 30 s, annealing at 53℃ for 30 s, extension at 72℃ for 45 s; and final extension at 72℃ for 10 min. The products were sent to RuiBiotech Co., Ltd. (Beijing, China) for capillary electrophoresis, and the results were read and analyzed using GeneMarker v.2.2.0. Three biological and two technical replicates were used to ensure accuracy and reliability of the study.

Analysis of genetic diversity

GenAlex v.6.51 was used to calculate the number of alleles (N_a), effective number of alleles (N_e), observed heterozygosity (H_o), expected heterozygosity (H_e), and Shannon's information index (I), and to perform principal coordinate analysis (PCoA). PowerMarker v.3.25 was used to calculate polymorphic information content (*PIC*) and Nei's genetic distance. The unweighted pair-group method with arithmetic means (UPGMA) clustering was constructed using PowerMarker, based on the coancestry coefficients obtained from pairwise Nei's genetic distance matrices. MEGA-X and Adobe Illustrator were used to construct tree diagrams.

Analysis of population structure

The population structure of the 104 germplasm was analyzed using Structure v.2.3.4 (Evanno et al. 2005) with K = 1–7 under the set Run Length (Length of Burnin Period, 10000; Number of MCMC Reps after Burnin, 10000), and each K value was repeated five times. The optimal number of subpopulations was obtained using the Python package, Structure Harvester. The Q-matrix was merged using CLUMPP v.2.0, and a population structure diagram was constructed using R statistical environment v.4.3.3.

Construct SSR fingerprint

Core primer combinations were selected from 20 primer pairs listed in Table 1 in descending order of primer *PIC* values for effectively distinguishing 104 *A. truncatum* germplasm and editing the fingerprint codes, as previously described (Han et al. 2019). In brief, the SSR loci were marked as A, B, and C in sequence, and the allele marker configurations were recorded later (omit the unit "bp" and separate locus with ","). Both were combined with "-". The fingerprint code of each germplasm was formed by concatenating the combinations separated by "/". For example, the code "A-100, 100 / B-100, 110 / C-NA, NA" meant that the *A. truncatum* Bunge had two amplicons of 100 bp at locus A, two amplicons of 100 and 110 bp at B, but no amplicon at C. Finally, the detailed information (species and number) and fingerprint code were imported into the QR Code conversion tool (https://www.the-qrcode-generator.com/) for generating the fingerprint QR code for each germplasm.

3. Results

Genetic diversity of the 104 *A. truncatum* germplasm

A total of 137 alleles were amplified in the 104 germplasm (Table 1), ranging between 2–16, with an average of 6.850, including 58.060 effective alleles (ranging between 1.498–6.869, with an average of 2.903), and a polymorphism rate of 42.4%. The average values of H_o, H_e, and I were 0.424, 0.591, and 1.182, respectively, and the range of *PIC* was 0.293–0.839 with an average of 0.548. The *PIC*s of more than half of

the primers listed in Table 1 were > 0.5, indicating that polymorphisms of most primers were relatively high. Figure 1. shows the amplification plots of the primer Atr_6_19203 in six samples.

Table 1. Genetic diversity of 20 SSR markers.

SSR Locus	Forward Primer (5'-3')	Reverse Primer (5'-3')	N_a	N_e	H_o	H_e	*I*	*PIC*
Atr_1_23267	CCCAGTTACCAGGAAGACCA	CAACTTCCTTGAGCAAACCC	7	1.970	0.452	0.492	0.937	0.438
Atr_1_26457	TCGGAAAGAAGAAGAAAGGAAG	TTCTTTGCCGTCGATCTCTT	7	2.592	0.536	0.614	1.157	0.612
Atr_1_26582	TCTTTGGTAGCTCTGTAACCTCTG	CCCGAGTCCTGACTTTCTGT	7	2.556	0.577	0.609	1.137	0.538
Atr_1_8041	ATAACTCAAACGGGGTGCAG	CGGTTGATGTTGATGTGGAG	3	1.498	0.240	0.332	0.586	0.293
Atr_3_16029	TATGCAGCTGCTTTTGCTTG	CCAATCAGCGAGTTGGAAAG	4	1.876	0.385	0.467	0.838	0.409
Atr_3_44970	GCAAAACAATTAAGGGCAGA	AAGATAGGGGTAGGCCAACAA	9	5.257	0.261	0.810	1.883	0.821
Atr_3_5784	GGTTCTGTACCGGTTTCCAA	CGAGGACAATAGGGTGGAGA	8	2.965	0.650	0.663	1.268	0.610
Atr_3_7920	CTGTTGCCACGGACAGAATA	CCCTGGGCATTGATTAGAAA	11	5.414	0.210	0.815	1.930	0.809
Atr_4_17251	GAAAGCCCCTCACAACTCAA	TGTGCTCCACTAAAGAGGCA	6	3.032	0.452	0.670	1.364	0.633
Atr_5_13703	CGCATAAAACCACGTTATTTCC	GACCGAATCGAGTATTGACGA	2	1.995	0.337	0.499	0.692	0.374
Atr_6_12572	TTTTCCTGTTTGGCTTGCTT	GGGAGATCCAGAGGGAGAAC	5	2.595	0.375	0.615	1.091	0.536
Atr_6_14757	CATGTCACCACATAATTCGCA	CACCAATTATCCAACAGTAGCA	5	2.028	0.475	0.507	0.769	0.432
Atr_6_15889	TCACGTGACACTTGGTAATGC	GCATCCCGTGAGCAATTAGT	9	2.639	0.379	0.621	1.369	0.598
Atr_6_19203	TTTTCTTTCCTTTCCACCGA	GTGTCTCCCTGGTTTCTCCA	5	1.805	0.365	0.446	0.900	0.417
Atr_6_26096	GGTTTAGGTCGGTTTGGAGG	GATCGGAGGGAGAAATCACA	11	4.207	0.232	0.762	1.746	0.755
Atr_7_10821	AGCAATTCCCCTCACAGCTA	AAAAGACGCTGCCACGTAGT	6	2.076	0.587	0.518	0.982	0.459
Atr_8_12911	TCGTTAACCTATAGCCAAACACTAC	GGGTGGGGTCCAATCTAAGT	16	6.869	0.779	0.854	2.190	0.839
Atr_9_16774	CAAGCAGCAAATAGCAACGA	TGCTTCTTCATAAAGGTGGTG	7	1.599	0.320	0.375	0.819	0.369
Atr_9_9324	CAACGCTGAGAAGGAACCAT	TGCGATGATCCCAACCTAAT	7	3.204	0.422	0.688	1.318	0.646
Atr_12_26578	TCCCCTTGTTCTTCTTCTCC	GCTACAAGATGGCGTTGGTT	2	1.880	0.437	0.468	0.661	0.373
Mean			6.850	2.903	0.424	0.591	1.182	0.548

N_a, number of alleles; N_e, effective number of alleles; H_o, observed heterozygosity; H_e, expected heterozygosity; *I*, Shannon's information index; *PIC*, polymorphic information content.

Population structure of the 104 *A. truncatum* germplasm

According to Nei's (1983) genetic distances, the UPGMA method was used to cluster the 104 germplasm and two outgroups based on 20 pairs of SSR markers (Figure 2A). Thirty germplasm was classified as group 1, and the remaining 74 was assigned to group 2. Overall, the genetic relationships between the two aforementioned germplasm groups were similar. To further determine the optimal number of subpopulations, a line chart was drawn based on the Delta *K* value (Figure 2B), which showed that the optimal *K* was equal to 2. A population structure diagram was drawn according to the Q-value matrix (Figure 2C). Among the 104 germplasm, 28 belonged to Population 1 (Pop 1), and 76 belonged to Population 2 (Pop 2).

PCoA of the 104 *A. truncatum* germplasm

The results of PCoA revealed that the contribution rates of the first, second, and third coordinates were 6.88%, 6.60%, and 5.21%, respectively (Figure 3). A two-dimensional scatter plot based on the first and second coordinates showed that the 104 germplasm was evenly distributed in all directions without forming obvious clusters. In addition, the distribution of the 104 germplasm in the plot was roughly consistent with the results of the UPGMA cluster and population structure analysis (Figure 2).

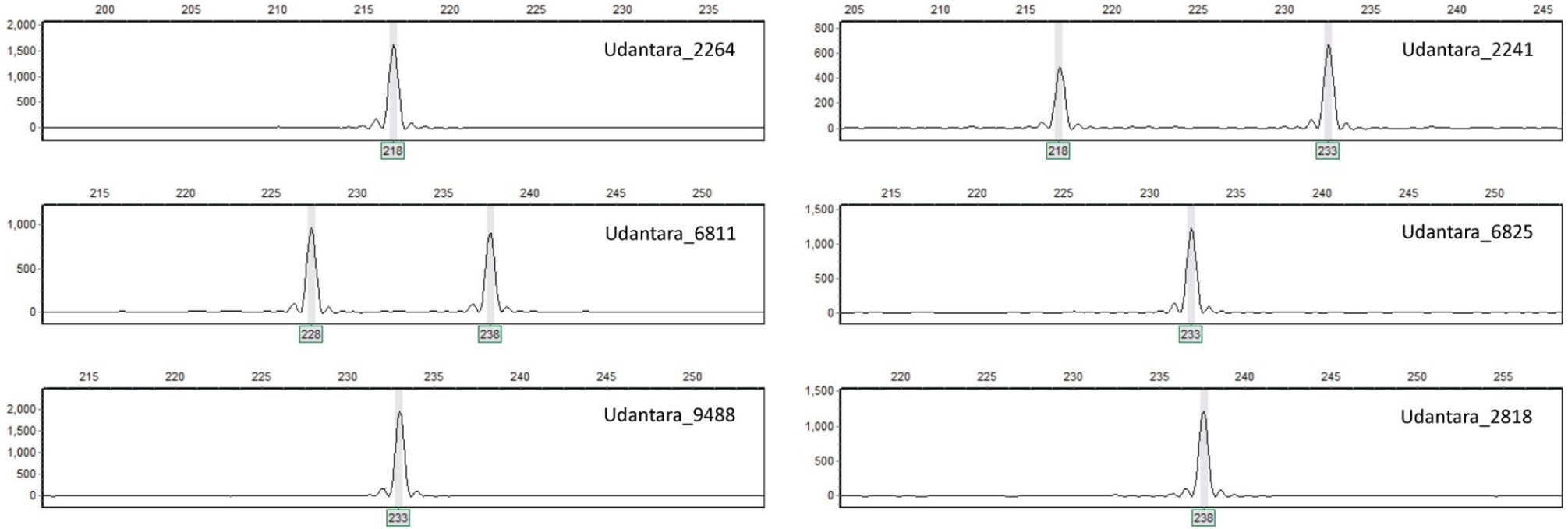

Figure 1. Capillary electrophoresis-mediated detection of six samples with the primer Atr_6_19203

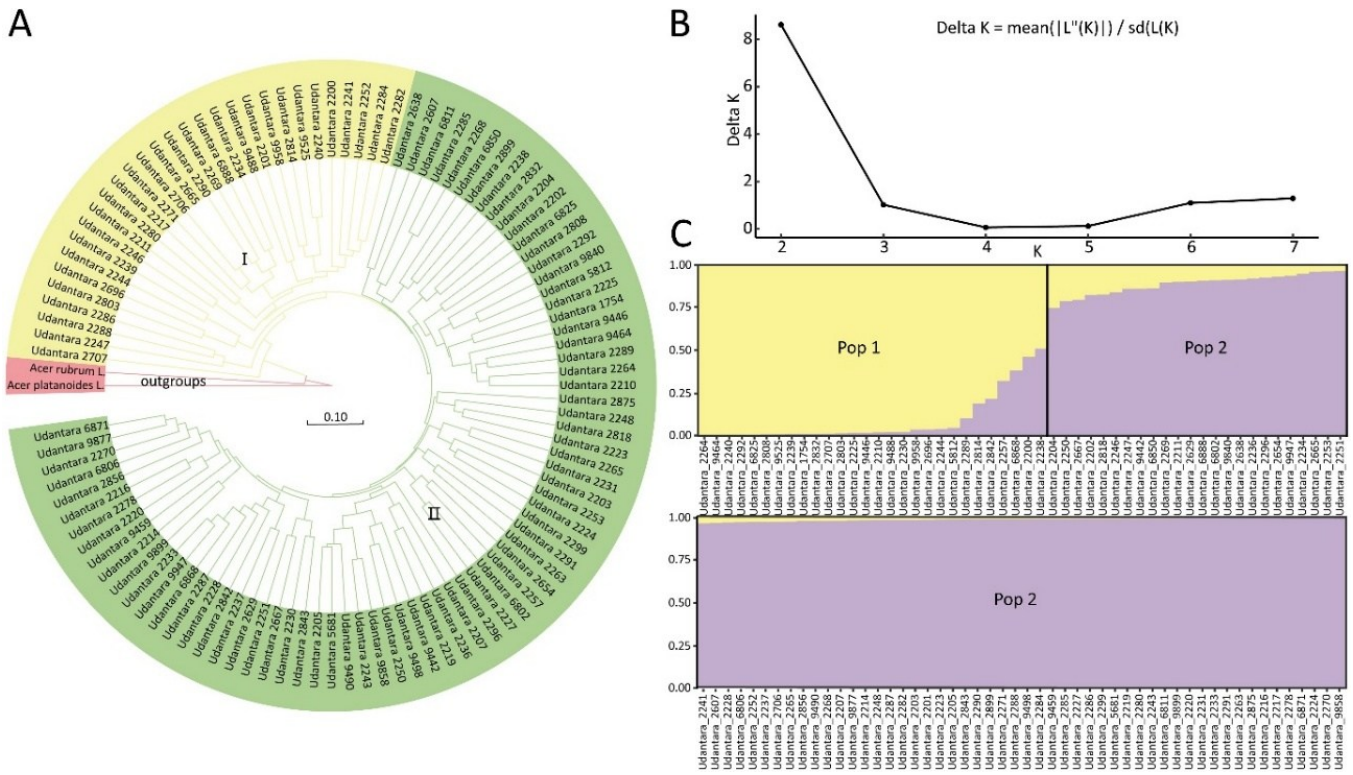

Figure 2. Population structure of *A. truncatum* germplasm. A - UPGMA dendrogram of 106 *Acer* plants based on the SSR markers; B - The line chart based on Delta *K* for inferring the optimal number of clusters; C - The population structure diagram of the 104 *A. truncatum* Bunge germplasm.

Construction of fingerprints

Polymorphic primers were arranged in descending order of the number of alleles, and the discrimination rates were analyzed under different primer combinations. When the number of primers reached five pairs (Atr_9_9324, Atr_6_26096, Atr_3_44970, Atr_3_7920, and Atr_8_12911), 104 germplasm was completely distinguished (Figure 4A); these five pairs of primers were used as the core primers for germplasm identification of *A. truncatum*. The N_es, H_es, and *PIC*s of the amplicons of these primers were higher than those of the others (3.204–6.869, 0.688–0.854, and 0.646–0.893, respectively), and their *I* values were also higher than those of most primers (1.318–2.190; Table 1). The molecular fingerprints of all 104 germplasm were generated according to the blocks with different colors marking different genotypes under the five markers, and the color combination in each row represented a unique genotype of each *A. truncatum* germplasm (Figure 4B).

These five pairs of primers were sequentially denoted by A–E, referring to the method of fingerprint code construction mentioned above, and the genotyping data of each germplasm was concatenated to construct a fingerprint QR code that was different from those of other germplasm. Table 2 provides examples of SSR fingerprint and QR codes for 10 *A. truncatum* germplasm.

4. Discussion

In the present study, we successfully analyzed the genetic structure of the population of *A. truncatum* in the Udantara Natural Reserve in China using SSR markers and evaluated its genetic diversity. Specifically, 20 SSR markers were used to analyze the genetic diversity of *A. truncatum*, and a total of 137 alleles were amplified, including 58.060 effective alleles with an average *PIC* of 0.548. Previously, commonly used marker techniques for analyzing plant genetic variations have included restriction fragment length polymorphism, random amplified polymorphic DNA, amplified fragment length polymorphism, SRAP, SSR, and single nucleotide polymorphism (Amiteye 2021). As a molecular marker that detects DNA polymorphism by using

specific PCR primers, the SSR markers are more popular compared to other molecular markers because of their high polymorphism, excellent repeatability, codominance, and multi-allele variation, along with the advantage of their low cost (Babay et al. 2019; Hussein et al. 2023). This is a commonly used method in current studies on genetic diversity and construction of genetic maps (Rezk et al. 2024).

The fingerprints constructed using stable, efficient, and reliable DNA markers are important tools for germplasm identification and protection (Karihaloo 2015; Zhang et al. 2023a). It directly reflects the differences in plant genetic material at the molecular level without being affected by environmental factors, thereby providing theoretical support for germplasm identification and protection (Gade et al. 2021; Zhang et al. 2022). The construction of tree-characteristic fingerprints emphasizes the use of as few primers as possible for identifying as many varieties as possible, thereby achieving simple and economical procedures (Wang et al. 2019). Li et al. (2019a) selected 12 pairs of SSR markers from 25 markers based on the polymorphisms of primers and constructed fingerprint maps of *Robinia pseudoasia*. Zhang et al. (2023b) selected four pairs of expressed sequence tag-SSR primers from 19 pairs based on the *PIC* values of primers for constructing the fingerprints of *Ailanthus altissima* var. *erythocarpa*. High *PIC*s of primers indicate high polymorphisms and relatively high capability for distinguishing and identifying specific germplasm, making them core primers for constructing DNA fingerprints (Akash et al. 2023). In our study, the *PIC*s of the primers were sorted in descending order, and the five top pairs of primers with the highest *PIC*s (0.646–0.893) were selected. The 104 *A. truncatum* germplasm was completely distinguished using the five primers. Establishing fingerprints for *A. truncatum* holds significant value for protecting excellent germplasm and supporting future breeding efforts, thereby laying the groundwork for managing genetic resources and safeguarding intellectual property rights related to *A. truncatum*.

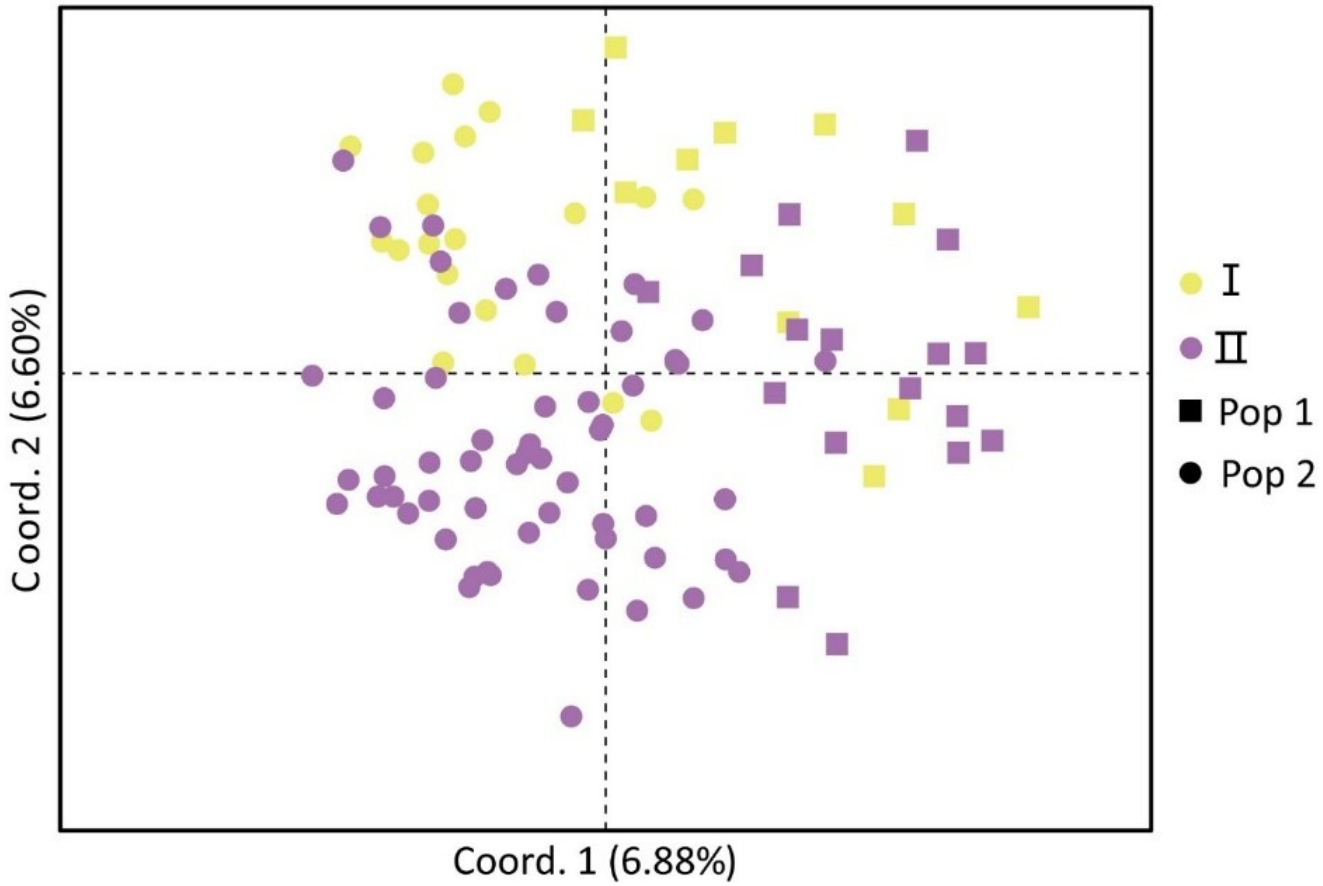

Figure 3. The plot of PCoA for the 104 *A. truncatum* Bunge germplasm is based on the 20 SSR markers. Different colors represent different groups in the UPGMA dendrogram, and different shapes represent different subpopulations in the analysis of population structure.

Genetic diversity is a prerequisite for the survival, adaptation, development, and evolution of species (Cisternas-Fuentes et al. 2022; Li et al. 2022; Wan et al. 2022). Luo et al. (2022) analyzed the genetic diversity

of the endangered plant *Acer yangjuechi* using SSR markers and found that their H_e and *I* values reached 0.873 and 2.319, respectively. In our study, the *I* value of *A. truncatum* was only 1.182, and average H_o (0.424) was clearly lower than that of H_e (0.591), indicating a relatively low level of genetic diversity within the population. A possible reason for this is that the population size of *A. truncatum* in the Udantara Natural Reserve is limited, and its distribution is relatively concentrated, far away from the surrounding populations, resulting in restricted gene flow between populations. Qiao et al. (2022) also reported similar results. Therefore, we speculate that the population probably experienced years of inbreeding or genetic drift. The common bisexual flowers of *A. truncatum* (Zhang et al. 2014) may also inhibit genetic diversity because of the possibility of self-pollination. Analysis of the genetic structure further divided the germplasm into two groups, reinforcing the hypothesis of restricted gene flow.

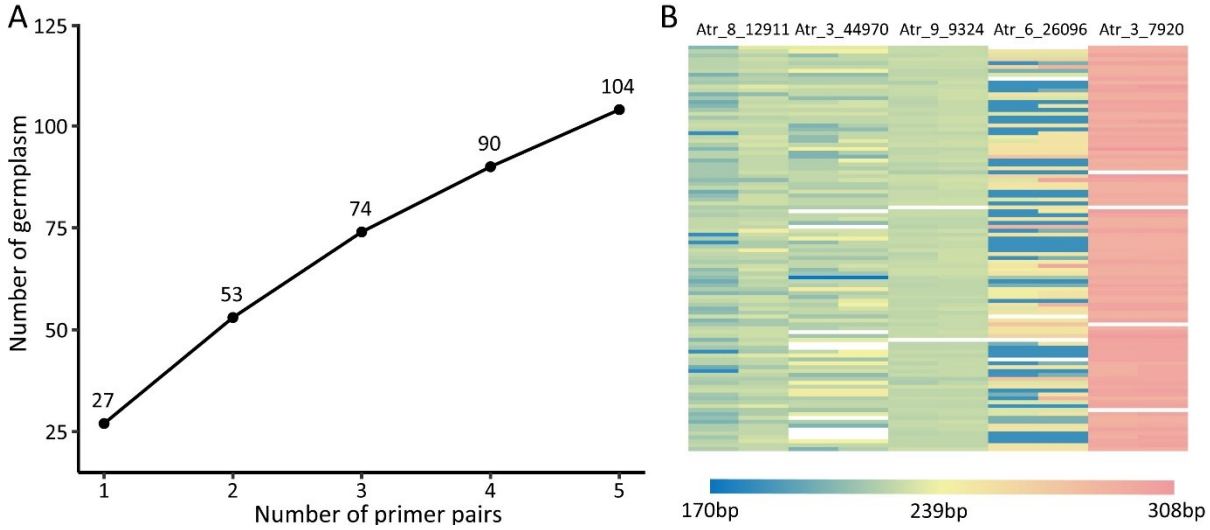

Figure 4. Screening core primers for constructing fingerprints of the 104 *A. truncatum* germplasm. A - The number of *A. truncatum* germplasm distinguished by different combinations of SSR primer pairs; B - The molecular fingerprint of the 104 *A. truncatum* germplasm. Different colors represent different amplicon sizes, and the blocks with no color represent that no amplicon was detected in the samples.

Notably, such situations could be exacerbated by severe climate change, historical geographic isolation, and active jamming (Liao et al. 2024). In addition, the population of *A. truncatum* in the Udantara Natural Reserve is suffering from aging; therefore, maintaining natural regeneration is difficult, which leads to a sharp decline in resources (Bao et al. 2019). The low genetic diversity of *A. truncatum* in the Udantara Natural Reserve poses an extinction risk (Schmidt et al. 2023). Effective breeding methods can generate high-quality varieties of *A. truncatum*, and the collection and protection of germplasm is vital for efficient breeding (Badri and Ludidi 2022; Singh et al. 2022). Therefore, protecting the *A. truncatum* germplasm in the Udantara Natural Reserve is necessary. *In-situ* conservation is widely used to protect wild plant resources, which promotes the evolution of species and protects ecosystem biodiversity (Heywood 2019; Morcia et al. 2023). However, with continuous intensification of global climate change, *in-situ* conservation strategies for wild plant resources are being challenging, whereas *ex-situ* preservation and facility protection of excellent germplasm are gradually gaining recognition (Engels and Ebert 2021; Liu et al. 2024). We suggest establishing a germplasm resource bank based on *in-situ* protection of the 104 excellent germplasm of *A. truncatum* selected in this study for improving the effectiveness and safety of germplasm resource protection. In addition, more than 10 different natural populations of *A. truncatum* in China are facing long-term geographical isolation with the population of Udantara, including the Yongji population in Shanxi and Jiuzhaigou population in Sichuan (Qiao et al. 2022). In the future, we should actively introduce excellent germplasm from these regions and obtain highly distinctive and new germplasm through hybridization with local varieties, which is important for improving the genetic diversity of *A. truncatum* in Udantara and broadening the genetic basis of *A. truncatum* nationwide. The protection and utilization of these excellent germplasm resources will highly promote the development of *A. truncatum* breeding in China.

Table 2. SSR fingerprint codes and QR codes of 10 *Acer truncatum* Bunge germplasm.

No.	Germplasm	SSR fingerprint code	Fingerprint QR code
1	Udantara_2200	A-221, 227 / B-239, 239 / C-225, 225 / D-250, 250 / E-290, 290	
2	Udantara_2201	A-215, 221 / B-209, 224 / C-223, 225 / D-232, 232 / E-300, 300	
3	Udantara_2202	A-221, 227 / B-224, 224 / C-227, 227 / D-262, 262 / E-288, 290	
4	Udantara_2203	A-221, 224 / B-219, 219 / C-225, 225 / D-184, 202 / E-300, 300	
5	Udantara_2204	A-221, 224 / B-224, 224 / C-225, 225 / D-262, 286 / E-290, 290	
6	Udantara_2205	A-224, 227 / B-239, 239 / C-223, 223 / D-202, 202 / E-288, 290	
7	Udantara_9877	A-215, 227 / B-NA, NA / C-223, 223 / D-184, 184 / E-290, 300	
8	Udantara_9899	A-221, 230 / B-239, 239 / C-223, 223 / D-184, 184 / E-298, 298	
9	Udantara_9947	A-224, 224 / B-224, 224 / C-223, 223 / D-250, 250 / E-296, 300	
10	Udantara_9958	A-218, 224 / B-204, 219 / C-227, 227 / D-250, 250 / E-290, 290	

5. Conclusions

In this study, we found relatively low genetic diversity of *A. truncatum* resources in the Udantara Natural Reserve, China, using SSR marker analysis. one hundred and four germplasm accessions were divided into two groups. The fingerprints were successfully constructed based on the allelic information of five pairs of SSR primers with the highest *PIC*s, which laid an important foundation for reservation and breeding of *A. truncatum* germplasm resources. Furthermore, a protection strategy for *A. truncatum* germplasm was proposed, which combines *in situ* conservation with *ex situ* conservation by establishing a germplasm resource bank. This study enhances our awareness of the importance of protecting the germplasm resources of *A. truncatum* in China.

Authors' Contributions: WU, Z.: acquisition of data, analysis and interpretation of data, and drafting the article; WANG, X.: acquisition of data, analysis and interpretation of data, drafting the article, and critical review of important intellectual content; HAN, M.: acquisition of data; ZHANG, C.: analysis and interpretation of data; BAO, H.: conception and design, drafting the article, and critical review of important intellectual content; WANG, J.: conception and design, drafting the article, and critical review of important intellectual content.

Conflicts of Interest: The authors declare no conflicts of interest.

Ethics Approval: Not applicable.

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