

Development of polymorphic SSR primers and fingerprint construction of *Ailanthus altissima* var. *erythrocarpa*

Manman Zhang

Agricultural University of Hebei

Conghui Zheng

Hebei Academy of Forestry and Grassland Sciences&Hebei Technical Innovation Center for Forest Improved Variety

Jida Li

Agricultural University of Hebei

Xueyong Wang

Hebei Academy of Forestry and Grassland Sciences&Hebei Technical Innovation Center for Forest Improved Variety

Chunpeng Liu

Hebei Academy of Forestry and Grassland Sciences&Hebei Technical Innovation Center for Forest Improved Variety

Xiangjun Li

Hebei Academy of Forestry and Grassland Sciences&Hebei Technical Innovation Center for Forest Improved Variety

Zhenhua Xu (✉ hblky303@126.com)

Hebei Academy of Forestry and Grassland Sciences&Hebei Technical Innovation Center for Forest Improved Variety

Kejiu Du

Agricultural University of Hebei

Article

Keywords:

Posted Date: April 12th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1508126/v1>

License:   This work is licensed under a Creative Commons Attribution 4.0 International License. [Read Full License](#)

Abstract

Ailanthus altissima var. *erythrocarpa* is one of *Ailanthus altissima* varieties with high economic, ecological and ornamental value. In this study, transcriptome analysis was used to develop SSR primers. Based on this, 34 Samara species with different redness were selected as research objects to screen polymorphic SSR primers and construct their fingerprints. Transcriptomic analysis revealed 10681 SSR loci, of which mononucleotide repeats were dominant (58.3%), followed by dinucleotide and trinucleotide repeats (16.6%, 15.1%), and pentanucleotide repeats were the lowest (0.2%). The PCR amplification system of est-ssr primers was (total volume 20 μ l), DNA 3 μ l (30ng/ μ l), 2 $\times$ Taq PCR Master Mix 10 μ l, upstream and downstream primers each 1 μ l (10 μ mol/ μ l), ddH₂O 5 μ l. Among 140 pairs of primers randomly selected, 13 pairs of core primers with good polymorphism were selected. The average number of alleles (Na), the average number of effective alleles (Ne), the average Shannon's diversity index (I), the average observed heterozygosity (Ho), the average expected heterozygosity (He), the fixed index (F) and the polymorphic information content (PIC) were as follows: 4.462, 3.067, 1.227, 0.402, 0.659, 0.391, 0.603. Among 13 pairs of primers, 4 pairs (P33, P69, P79, p95) could effectively distinguish and construct the fingerprint of the tested materials. This study is of great significance for the genetic structure, genetic diversity analysis, evolutionary history, germplasm conservation and germplasm innovation of *Ailanthus altissima* var. *erythrocarpa*.

Introduction

Ailanthus altissima (Mill) Swingle, belonging to Simaroubaceae and *Ailanthus*, is widely distributed in all continents except Antarctica, and is a pioneer tree of greening and barren mountain afforestation. It has high economic, medicinal, ecological and ornamental value¹⁻⁷. *Ailanthus altissima* var. *erythrocarpa* is a variant tree of *Ailanthus altissima* (Mill) Swingle, the fruiting six to nine months, due to its fruit red, crabapple period sustainable for months, with high ornamental value and high-profile⁸. There were significant differences in the appearance time, duration and chroma of fruit color (data have not been published yet). Genetic composition and environment can make plant morphology show certain stability and variability⁹, which required scientific identification and evaluation of germplasm of *Ailanthus altissima* var. *erythrocarpa*.

Express Sequence Tag-SSRs (EST-SSRs) is derived from mRNA transcription sequences, and is applicable to the construction of genetic linkage maps, genetic diversity analysis, population genetic structure research, species evolution and variation of plants with or without reference genomes. Compared with g-SSRs, EST-SSRs is more efficient, less time and cost. It has stronger transferability in species¹⁰⁻¹⁶ and has been widely used in plant variety identification, genetic structure analysis and fingerprint construction¹⁷⁻²². At present, due to the deficiency of genome and genetic information, there are few studies on genetic diversity of *Ailanthus altissima* (Mill) Swingle at home and abroad, mainly involving the development of nuclear microsatellite markers of *Ailanthus* from Mediterranean islands and eastern Austria; population structure and genetic diversity study of *Ailanthus altissima* (Mill) Swingle in North America; regional expansion of *Ailanthus altissima* (Mill) Swingle in Japan and patterns of genetic differentiation among plant populations; origin, expansion and genetic structure *sinensis* introduced from Austria; Developed 10 cpSSR markers and 13 EST-SSR markers, and analyzed the genetic diversity of 14 populations from different regions of China^{23-30,15}, which promoted the application of SSR markers in *Ailanthus altissima* (Mill) Swingle. There are few reports on the genetic diversity of *Ailanthus altissima* var. *erythrocarpa*, and there are no reports on the development of polymorphic SSR primers and the construction of fingerprint.

In this study, 34 wild winged fruits with different redness were collected and preserved of *Ailanthus altissima* var. *erythrocarpa* in central and southern Hebei province, and 13 pairs of rich polymorphic EST-SSR primers were screened out to distinguish and construct fingerprint of 34 wild type *Ailanthus altissima* var. *erythrocarpa*. It will provide technical support for resource collection and protection, germplasm bank establishment and management, germplasm identification and intellectual property protection in the future, and also lay a theoretical foundation for breeding and germplasm innovation of *Ailanthus altissima* var. *erythrocarpa* and *Ailanthus altissima* (Mill) Swingle.

Results

Number and distribution of EST-SSR loci of *Ailanthus altissima* var. *erythrocarpa*.

Based on Illumina Hi Seq TM high-throughput sequencing platform for the transcriptome sequencing of *Ailanthus altissima* var. *erythrocarpa*, SSR search of the spliced and assembled unigenes revealed 9828 accurate SSRs and 853 compound SSRs, totaling 10,681 SSR loci. The EST-SSR types of *Ailanthus altissima* var. *erythrocarpa* were abundant, and their distribution showed (Fig. 1a) that there were trinucleotides repeats, dinucleotide repeats and mononucleotide repeats in the largest number, among which 6228 mononucleotide accounted for the highest proportion (58.31%). 1777 dinucleotides, accounting for 16.64%; 1621 trinucleotides, accounting for 15.18%; tetra-hexanucleotide repeats were low, accounting for 0.99%, 0.24% and 0.66%, respectively. Other complex nucleotide repeats accounted for 7.99% of the total SSR, indicating that mono-trinucleotides were the main repeat motifs of EST-SSR of *Ailanthus altissima* var. *erythrocarpa*.

According to EST-SSR repeat motifs type analysis, there were 166 exact SSR motifs, and the mononucleotide to hexanucleotides types were 2, 6, 30, 39, 23 and 66, respectively (Table 1). Mononucleotide were mainly A/T repeat type (6214), accounting for 58.18% of the total number of mononucleotides. Dinucleotides were mainly AT/AT (33.54%) and AG/CT (32.58%) repeat motifs, accounting for 5.58% and 5.42% of total SSR. The higher specific proportion of trinucleotides are AGA/TCT, AAG/CTT and CTT/AAG were 1.40%, 1.35% and 1.33%, respectively. AAAT/ATTT and AAAG/CTTT in the tetranucleotides accounted for 0.18% and 0.08%, respectively. The penta- to hexanucleotide repeat motifs appeared in many kinds, but the frequency was relatively low. Among all repeat types (Fig. 1b), the number of repeats of SSR loci motifs was 5 or more, and the frequency of distributed nucleotide repeats decreased gradually with the increase of number of repeats. The types with more than 10 repetitions accounted for 63.16% of the total, accounting for the highest proportion. Followed by 5 and 6, accounting for 9.90% and 9.14% of the total, respectively. The lowest proportion was 9 times, accounting for only 2.32%. The number of mononucleotide repeats was 10 or more; dinucleotides were 6 or more, and the highest repetition rate was 6 times. Trinucleotide frequency of repeated 5 times

was the highest when the nucleotide was 5 or more. The number of tetra- to hexanucleotides was mainly 5, which indicated that the polymorphism potential of *Ailanthus altissima* var. *erythrocarpa* was high.

Table 1 The type of SSR motifs in *Ailanthus altissima* var. *erythrocarpa*

Repeat motif length	Repeat motif and proportion (%)	Total
Mononucleotide	A/T+C/G58.31%	2
Dinucleotide	AC/GT0.99%AG/CT5.42%AT/AT5.58%CT/AG4.05%other0.59%	6
Trinucleotide	AAG/CTT1.35%AAT/ATT1.27%AGA/TCT1.40%CTT/AAG1.33%other9.83%	30
Tetranucleotide	AAAC/GTTT0.07%AAAG/CTTT0.08%AAAT/ATTT0.18%AAGA/TCTT0.07%ATAA/TTAT0.07%other0.52%	39
Pentanucleotide	AAATA+AAATC+AAGCC+ATCAA+ATGAG+ATTGG+CACTA+CCAAA+CCAGT+CCCCA+CGAAA+CTGAT+CTTTT/AAAAG+GAAAT+GAGAA+GTTGT+TAAAA+TCTCC+TCTTT+TGAGT+TGATG+TTTCT+TTTGA0.24%	23
Hexanucleotide	AAAAAG+AAAACA+AAAATA+AAGAAA+AAGAAC+AATCAA+ACCAAC+AGAAAA+AGAAAG+AGCAAG+AGCAGG+AGCCTG+ATAGTC+ATATTG+ATGAAT+ATGTAA+ATGTTT+ATTCGC+ATTTAC+ATTTTG+CAAACC+CAAATC+CAACAC+CAACAG+CAAGAA+CAGAGA+CATCTT+CATGGA+CCCAAT+CCTGAG+CCTTAA+CGCCAT+CTGAAC+CTTCAT+GAGAAG+GAGCAG+GGAGAA+GGCTCA+GGTCTC+GTGGGA+GTGGGC+GTTCTG+GTTGAA+GTTTTC+TAATAC+TATTGA+TCAGAT+TCATCC+TCCTCT+TCGGTA+TCTGGC+TGAAAA+TGACTG+TGCTGG+TGGGAC+TGGTGC+TGTGCG+GTTTCT+TGTTTT+TTCTGA+TTATGC+TTGGTT+TTGTTC+TTTGCC+TTTGTG+TTTTGG0.66%	66
Compound Nucleotide	7.99	

Screening and validation of EST-SSR primers for *Ailanthus altissima* var. *erythrocarpa*.

Based on transcriptome sequencing data analysis, SSR primer information was designed and obtained, and 140 pairs of primers were randomly selected to verify the effectiveness of SSR primers. By PCR amplification and agarose gel electrophoresis for screen at the beginning of 106 pairs of primer could amplified fragment length and is expected to conform to the size of the stripe, eventually to 34 *Ailanthus altissima* var. *erythrocarpa* material PCR amplification, DNA samples tested by polyacrylamide gel electrophoresis, the analysis results for 13 of polymorphic primer (table 2) clear and rich polymorphism, The effective amplification rate was 75.71% and primer polymorphism rate was 9.29%.The SSR markers developed in this study have high effective amplification rate, which is helpful for the analysis of genetic background and auxiliary breeding of *Ailanthus altissima* var. *erythrocarpa*.

Table 2 Information of polymorphic SSR primer

Primer No.	Repeat motif	Primer sequence (5'-3')	Expected size (bp)	Tm (°C)
p23	(ATA)5	TGGCGGAATAATGCTCCACT	250	59
		GCCTTCTGGGATTTCGACA		
p30	(TTC)7	TCAACAAATTGCGCCAAGGG	267	59
		GGGTTTCTGTGGTGTGGG		
p32	(AAAC)5	AGCCTCCATTGAAGAACACAGA	187	59
		TTGAGTGACCGGGTTGGAAG		
p33	(TTCT)5	CGCCAGCGTTCTCTTTCATC	220	59
		GCAGATTTCCGCCATCGAGA		
p46	(CACTA)5	TGCAATTGCGATGCCAACAA	231	59
		ATCCCAAAAGCTGCGACAGT		
p53	(GGAGAA)7	AGGAGAGAACGGGACCATGA	239	59
		CGATCTCGCAACTCCCTCTC		
p54	(CAAACC)5	ATTCCTTTACCGCAGAGGC	274	60
		GGTGGATGAAGGGCTGACTC		
p63	(T)10(A)11	CTCAGAACTGTCTCGCCGT	224	60
		TGGACGTGATTTGTAGGCACT		
p69	(T)11*(A)12	CACTGACACGCTCACCTTCT	181	60
		GGCAGATACCGACGTTAGGG		
p79	(A)10(AC)6*(A)10	TAAACCCCTACCGTGCGTTC	234	60
		GAACCTCTGCCAACACGCTG		
p95	(TGT)6	CTCCCCACGATTTGCCTGAT	274	60
		ATTTGTCCCTCGACGACTCG		
p125	(TG)9(TA)8	ATCAGACCAATGTGCAGGGG	185	60
		CTCTCTCGGTGTGCATGTGT		
p127	(T)13*(TC)6	ACAGAGTGCCCTTATCGTGT	218	58
		ATCCAATCGTTCCGGCCAT		
*: Compound SSR loci				

Primer polymorphism analysis

The 13 pairs of highly polymorphic primers were used for genetic diversity analysis (Table 3). 3 to 6 alleles (Na) were detected by 13 pairs of SSR primers, with an average of 4.46 alleles per pair of primers. The number of effective alleles (Ne) ranged from 2.217 to 4.433, with an average of 3.067. Na and Ne were the largest at p125, followed by p79 and p95. Shannon's diversity index (I) ranged from 0.925 to 1.638, with an average of 1.227. The observed heterozygosity (Ho) of different loci ranged from 0.118 to 1.000, with an average of 0.456. The expected heterozygosity (He) ranged from 0.549 to 0.774, with a mean of 0.659, with the lowest at p23 and the highest at p125. The fixed index (F) ranged from -0.596 to 0.799, with an average value of 0.391. PIC can measure the polymorphism of primers: PIC > 0.5 is highly polymorphic, 0.25 < PIC < 0.5 is moderately polymorphic, and PIC < 0.25 is low polymorphic. The PIC values of 13 loci ranged from 0.483 to 0.744, with an average of 0.603. The PIC values of p23 loci were 0.483, moderately polymorphic, and the PIC values of the other 12 loci were all higher than 0.5, which were highly polymorphic primers, indicating that the selected SSR primers could effectively reveal the genetic diversity of *Ailanthus altissima* var. *erythrocarpa*. The 13 SSR loci were tested for Hardy-Weinberg equilibrium, and all of them were in equilibrium state, indicating the genetic balance of this sample.

Table 3
Genetic diversity of 13 pairs of primer of *Ailanthus altissima* var. *erythrocarpa*

locus	Na	Ne	I	Ho	He	F	PIC
p23	3.000	2.217	0.925	0.265	0.549	0.518	0.483
p30	4.000	2.232	1.019	0.294	0.552	0.467	0.500
p31	3.000	2.679	1.039	1.000	0.627	-0.596	0.554
p33	5.000	2.390	1.350	0.500	0.705	0.291	0.653
p46	4.000	2.418	1.022	0.118	0.587	0.799	0.510
p53	5.000	3.317	1.299	0.471	0.699	0.326	0.642
p54	5.000	3.120	1.304	0.265	0.679	0.610	0.628
p63	4.000	2.575	1.048	0.235	0.612	0.615	0.535
p69	4.000	3.456	1.300	0.676	0.711	0.048	0.657
p79	5.000	3.747	1.412	0.265	0.733	0.639	0.685
p95	5.000	3.711	1.396	0.647	0.731	0.114	0.681
p125	6.000	4.433	1.638	0.167	0.774	0.785	0.744
p127	5.000	2.577	1.202	0.324	0.612	0.471	0.571

Construction of DNA fingerprint of *Ailanthus auriculata*

According to the genotype statistics of 13 pairs of SSR primers, the primers with higher PIC and more genotypes were selected to distinguish 34 *Ailanthus altissima* var. *erythrocarpa*. According to the PIC value of SSR primers, the test materials were added successively from high to low and identified gradually until they were completely separated. It can be seen from Table 4, SSR primers p79 and p95 could distinguish 14.71% of the materials (5). SSR primers p79, p95, p69 could distinguish 55.88% of the materials (19). Four pairs of SSR primers, p79, p95, p69 and p33 could be used to distinguish 34 test materials. The amplified results of each material corresponding to the primer combinations (p79, p95, p69 and p33) were expressed as binary "0/1" codes, and the independent and unique codes of each sample were obtained, which constituted the finger map of 34 materials of *Ailanthus altissima* var. *erythrocarpa* (Table 5). In this study, 13 pairs of core primers were screened to completely separate 34 test samples of *Ailanthus altissima* var. *erythrocarpa*, and the obtained DNA fingerprints were used to evaluate and identify the genotypes of *Ailanthus altissima* var. *erythrocarpa* at the molecular level.

Table 4
Differentiation of 34 *Ailanthus altissima* var. *erythrocarpa* by adding SSR marker primer combinations one by one

Primer combination	The number of materials distinguished	Differentiation rate
p79 + p95	5	14.71
p79 + p95 + p69	19	55.88
p79 + p95 + p69 + p33	34	100

Table 5
SSR fingerprints of 34 *Ailanthus altissima* var. *erythrocarpa* constructed by 4 pairs of primers

No.	p79	p95	p69	p33
1	0111	1000	0011	1000
2	0110	0110	0100	1000
3	0110	0110	0001	1000
4	0100	0111	0001	0100
5	0100	1000	0001	0110
6	0100	0100	0001	0100
7	0100	0100	0011	0111
8	0010	1000	0101	0100
9	0010	0010	0011	0101
10	0001	0101	0011	0111
11	0010	0100	0101	0101
12	0001	0011	0010	0110
13	0001	0110	0101	0100
14	0001	0110	0001	0101
15	0100	0101	0011	0001
16	0010	0100	0101	0110
17	0100	0101	0001	0111
18	0010	0100	0011	0110
19	0100	0001	0001	0110
20	0011	0010	0101	0111
21	0110	0011	0100	0101
22	0110	0010	0011	1000
23	0111	1000	0101	0011
24	0110	0101	0100	1001
25	0110	0010	0110	0110
26	0101	0010	0100	0110
27	0010	0011	0110	0110
28	0010	0101	0100	0110
29	0110	0101	0100	0111
30	0100	0101	0100	0010
31	0101	0101	0100	1001
32	0110	0010	0110	0001
33	0011	1000	0100	0101
34	0101	0001	0110	1000

Individual cluster analysis and principal component analysis of *Ailanthus altissima* var. *erythrocarpa*

Thirteen pairs of primers were used for PCR amplification of 34 *Ailanthus altissima* var. *erythrocarpa*. The amplification data of 13 pairs of SSR primers were analyzed by GenAlEx6.502 software, and the genetic distance matrix was calculated and constructed for UPGMA clustering and principal component analysis (PCA). The validity of the developed primers was further verified. The results (Fig. 2) showed that 34 *Ailanthus altissima* var. *erythrocarpa* were 100% distinguishable and could be divided into 5 categories. Category 1 has only 1 material (H1);The second class has 4 materials, including H3, H4, H5, H16;The third category mainly includes P9, P8, H9, H10, H13, P3, H12;The fourth category includes the most materials, a total of 20 materials H2, P12, P15, P17, P4, P16, P10, P7, P11, P1, P14, H11, P2, H15, P13, H17, H6, H7, H8, H14;Category 5 includes only P6 and P5.

According to the results of principal component analysis (Fig. 3) of 34 tested materials, the contribution rates of the first and second principal components explained variation were 21.44% and 17.61%, respectively. The 34 *Ailanthus altissima* var. *erythrocarpa* could be divided into 5 groups by both genetic clustering and principal component analysis clustering. Materials from Handan city and Shijiazhuang city were not clearly differentiated, and there was strong gene exchange, which was related to the near location of sample collection, which further proved the effectiveness of the primers developed in this study and the accuracy of the narrow genetic background of the tested materials.

Discussion

In recent years, SSR molecular markers have been widely used in the study of genetic diversity of species due to the advantages of SSR primers, such as high species specificity and cross-related species transfer, and primer development is the primary condition. At present, only JoshatK. Saina et al.^{31,15} identified 219 SSR loci based on chloroplast genome of *Ailanthus altissima* (Mill.) Swingle in 2018. In 2021, 13 EST-SSRs were developed and 10 polymorphic chloroplast microsatellite (cpSSR) markers were constructed based on the transcriptome of *Ailanthus altissima* (Mill.) Swingle and 219 cpSSR markers. The distribution characteristics of cpSSR and EST-SSR were basically similar, but the difference was that cpSSR did not detect hexanucleotide and compound nucleotide repetition. In this study, 10681 potential microsatellite loci were obtained based on transcriptome sequencing results of tissue-cultured seedlings in young leaves of *Ailanthus altissima* var. *erythrocarpa*, which was far lower than that of JoshatK. Saina et al.¹⁵ (33084 potential SSR loci), and EST-SSR distribution characteristics were similar. This result may be related to the fact that it is a variety of *Ailanthus altissima* (Mill.) Swingle.

Most plants use mono-nucleotide, di-nucleotide and tri-nucleotide as dominant repeat motifs, such as *Stephanandra incisa*²⁰, *Bougainvillea cultivars*¹⁸, *Cocos nucifera* L.³², *Ailanthus altissima* (Mill.) Swingle¹⁵, *Pinus bungeana*²¹, *Ulmus pumila* L.³³, The *Ailanthus altissima* var. *erythrocarpa* results of transcriptome analysis showed the same, accounting for 90.12% of total SSR. AG/CT was the most frequent di-nucleotide repeat motifs in most plants, and the homopurine-homopyrimidine extension was often found in the 5' untranslated region, which played an important role in gene expression and nucleic acid metabolism regulation in plants. AAG/CTT is the main nucleotide repeat type in dicotyledons^{15,20,22,31,34-39}. In this study, the di-nucleotide AT/AT repeat type had the highest frequency, which was consistent with the results of *pinus bungeana*^{21,40}, *Bougainvillea cultivars*¹⁸, and *Ailanthus altissima* (Mill.) Swingle cpSSR³¹. The majority of Tri-nucleotide repeats were AGA/TCT, followed by AAG/CTT. Among the 140 pairs of primers screened and synthesized, 106 pairs of primers obtained the amplification bands, and the effective amplification rate reached 75.71%, which was similar to that of *Ailanthus altissima* (Mill.) Swingle (80%)¹⁵ and *Cinnamomum chago* (70.59%)⁴¹. The polymorphism rate of primers was 9.29%, significantly lower than the results of JoshatK.Saina et al.¹⁵ (65%), which may be caused by more randomly selected primers, fewer samples, small genetic differences between samples and environmental selection pressure. Therefore, EST-SSR is still the most economical and effective way to develop a large number of primers at the same time using transcriptome data. The chloroplast genome of *A. altissima* is a circular molecule with a size of 160815 base pairs and has a tetrad structure. The chloroplast genome size was similar to that of *Arabidopsis thaliana* (L.) Heynh. (120-217kb) and *Populus trichocarpa* (155-159kb), so it was relatively easy to develop cpSSR markers for *A. altissima*. Compared with cpSSR, est-ssrs exist in the gene region of nuclear genome and are highly polymorphic^{15,42}. In this study, est-ssrs can be combined with the molecular markers of *Ailanthus altissima* var. *erythrocarpa* to study the mutation source points of *Ailanthus altissima* var. *erythrocarpa*, providing important research value for the subsequent germplasm resource protection and evolution research of *Ailanthus altissima* var. *erythrocarpa* population. Phenotypic traits combined with molecular markers could be used to analyze the correlation of traits, providing guidance for the utilization of germplasm resources and breeding of new varieties.

In this study, 13 pairs of clear polymorphic primers with the expected size were screened, and the average PIC value was 0.603, lower than the average PIC value of *A. altissima* (0.819)¹⁵. PIC > 0.5 indicated that the developed SSR locus had high genetic resolution, which was conducive to population genetic analysis, revealing that the studied *Ailanthus altissima* var. *erythrocarpa* had high genetic diversity. The result that PIC value of *Ailanthus altissima* var. *erythrocarpa* was lower than that of *A. altissima* may be related to the small sample size selected in this study, the close provenance and gene differences. Therefore, it is necessary to further study the genetic diversity and structure of this species on the basis of expanding the sample size. The clustering results of genetic relationship and principal component analysis showed that 34 individuals could be completely distinguished and divided into five categories, but the materials of the two sites were indistinguishable. There was no obvious correlation with geographical location, there was strong gene exchange, or it was related to the close proximity of the acquisition site. The range of strain selection could be expanded later. The polymorphism, genetic diversity, population genetic structure and homologous genes between *A. altissima* and its varieties were analyzed comprehensively and deeply.

DNA fingerprinting technology analyzes the genetic material itself, interferes with immune environmental factors, sample morphology, material source and other factors, and identifies the germplasm genotypes as indicators at the molecular level. It has been widely used in grain, oil, vegetable and fruit crops, flowers, shrubs, arbors and other plants^{13, 17-18,43}. DNA fingerprinting has high simple and stable genetic, variability, multiple loci, can distinguish the same family, belong to different species, different varieties, forms, and even the same strain and subtle variation between individuals DNA, is germplasm genetic relationship, genetic breeding, population genetic structure, ecology and evolution, classification and other valuable genetic marker methods⁴³⁻⁴⁵. However, there have been no reports on the fingerprint information of *Ailanthus altissima* var. *erythrocarpa*. In this study, four of 13 EST-SSR core primers (P79, P95, P69 and p33) were screened out to distinguish 34 tested *Ailanthus altissima* var. *erythrocarpa* materials and construct a unique molecular ID card. The establishment of fingerprinting of *Ailanthus altissima* var. *erythrocarpa* is of great significance for its subsequent breeding, which can identify varieties and excellent genes as well as their variation and transmission in offspring, and lay a foundation for the establishment and improvement of fingerprinting database of *Ailanthus altissima* var. *erythrocarpa* variety resources, genetic resource management and intellectual property protection. However, in this study, there were few test materials, the sample collection area was narrow, and the fingerprint background of "core germplasm" of *Ailanthus altissima* var. *erythrocarpa* was lack of in-depth research, which required further verification of the results' suitability.

Conclusion

In summary, 13 pairs of highly polymorphic SSR primers were developed in this study, and cluster analysis showed that 34 individuals were 100% distinguished. Furthermore, four pairs of SSR core primers were used to distinguish 34 *Ailanthus altissima* var. *erythrocarpa*. In order to provide referable SSR primer public resources and research theoretical basis for a deep understanding of the population genetic genetics, evolutionary history and functional genome research, germplasm resource identification, selection of dominant parents to cultivate new varieties, and intellectual property protection of *Ailanthus altissima* var. *erythrocarpa*.

Materials And Methods

Plant materials

A total of 34 wild *Ailanthus altissima* var. *erythrocarpa* materials with different samara redness were collected from the central and southern hebei regions of Hebei Province(Table 7, Fig. 4), and the collected materials were respectively high in the 3-year-old common *Ailanthus altissima* (Mill) Swingle. Materials H1 ~ H17 are from Handan City, Hebei Province, and materials P1 ~ P17 are from Shijiazhuang City, Hebei Province. See Table 6 for details.

Table 6
Ailanthus altissima var. *erythrocarpa* germplasm

Individuals	No.	Variety	Orig(Hebei)
1	H1	<i>Ailanthus altissima</i> var. <i>erythrocarpa</i>	S202, Shexian, Handan City
2	H2	<i>Ailanthus altissima</i> var. <i>erythrocarpa</i>	X206, Shexian, Handan City
3	H3	<i>Ailanthus altissima</i> var. <i>erythrocarpa</i>	Qianyankou, Shexian, Handan City
4	H4	<i>Ailanthus altissima</i> var. <i>erythrocarpa</i>	East Wachi Village, Shexian County, Handan City
5	H5	<i>Ailanthus altissima</i> var. <i>erythrocarpa</i>	Songjia Village, Shexian County, Handan City
6	H6	<i>Ailanthus altissima</i> var. <i>erythrocarpa</i>	S224, Shexian County, Handan City
7	H7	<i>Ailanthus altissima</i> var. <i>erythrocarpa</i>	S224, Shexian County, Handan City
8	H8	<i>Ailanthus altissima</i> var. <i>erythrocarpa</i>	Shennanyu village, Wu 'an city
9	H9	<i>Ailanthus altissima</i> var. <i>erythrocarpa</i>	Nanping Village, Wu 'an city
10	H10	<i>Ailanthus altissima</i> var. <i>erythrocarpa</i>	Nanping Village, Wu 'an city
11	H11	<i>Ailanthus altissima</i> var. <i>erythrocarpa</i>	People's Government of Majiazhuang Township, Wu 'an city
12	H12	<i>Ailanthus altissima</i> var. <i>erythrocarpa</i>	Huangyan Bottom, Cixian County County, Handan City
13	H13	<i>Ailanthus altissima</i> var. <i>erythrocarpa</i>	Huangyan Bottom, Cixian County County, Handan City
14	H14	<i>Ailanthus altissima</i> var. <i>erythrocarpa</i>	North Yangcheng Village, Cixian County, Handan City
15	H15	<i>Ailanthus altissima</i> var. <i>erythrocarpa</i>	Zhabi Village, Cixian County, Handan City
16	H16	<i>Ailanthus altissima</i> var. <i>erythrocarpa</i>	Fengfeng mining area S222, Handan City
17	H17	<i>Ailanthus altissima</i> var. <i>erythrocarpa</i>	Daming County, Handan City
18	P1	<i>Ailanthus altissima</i> var. <i>erythrocarpa</i>	Gongku Village, Pingshan County, Shijiazhuang City
19	P2	<i>Ailanthus altissima</i> var. <i>erythrocarpa</i>	Liangcha, Pingshan County, Shijiazhuang City
20	P3	<i>Ailanthus altissima</i> var. <i>erythrocarpa</i>	Liangcha, Pingshan County, Shijiazhuang City
21	P4	<i>Ailanthus altissima</i> var. <i>erythrocarpa</i>	Liangcha, Pingshan County, Shijiazhuang City
22	P5	<i>Ailanthus altissima</i> var. <i>erythrocarpa</i>	Liangcha, Pingshan County, Shijiazhuang City
23	P6	<i>Ailanthus altissima</i> var. <i>erythrocarpa</i>	Liangcha, Pingshan County, Shijiazhuang City
24	P7	<i>Ailanthus altissima</i> var. <i>erythrocarpa</i>	Caishuwan, Pingshan County, Shijiazhuang City
25	P8	<i>Ailanthus altissima</i> var. <i>erythrocarpa</i>	G207, Pingshan County, Shijiazhuang City
26	P9	<i>Ailanthus altissima</i> var. <i>erythrocarpa</i>	Liujia Village, Pingshan County, Shijiazhuang City
27	P10	<i>Ailanthus altissima</i> var. <i>erythrocarpa</i>	Tangsix xian, Pingshan County, Shijiazhuang City
28	P11	<i>Ailanthus altissima</i> var. <i>erythrocarpa</i>	Mupanling, Pingshan County, Shijiazhuang City
29	P12	<i>Ailanthus altissima</i> var. <i>erythrocarpa</i>	Xiaomiyu Village, Pingshan County, Shijiazhuang City
30	P13	<i>Ailanthus altissima</i> var. <i>erythrocarpa</i>	Tuling Village, Pingshan County, Shijiazhuang City
31	P14	<i>Ailanthus altissima</i> var. <i>erythrocarpa</i>	Shuiyuhe Village, Pingshan County, Shijiazhuang City
32	P15	<i>Ailanthus altissima</i> var. <i>erythrocarpa</i>	Xibaipo, Pingshan County, Shijiazhuang City
33	P16	<i>Ailanthus altissima</i> var. <i>erythrocarpa</i>	Shangwendu Village, Pingshan County, Shijiazhuang City
34	P17	<i>Ailanthus altissima</i> var. <i>erythrocarpa</i>	Xibaipo Lake, Pingshan County, Shijiazhuang City

DNA extraction

Select young and tender *Ailanthus altissima* var. *erythrocarpa* leaves and bring them back in an ice box. Genomic DNA of *Ailanthus altissima* var. *erythrocarpa* was extracted by improved CTAB method and Omega kit. DNA concentration and quality were determined by microspectrophotometer (model: Nano-300). After qualified detection, DNA was diluted to 30ng/μL for each tube. A total of 5 tubes (150μL/ tube) were used as working liquid, The original liquid and working liquid should be placed in a refrigerator at -20°C for later use.

Development of SSR primers

Due to the lack of reference genome sequence, leaves of tissue-cultured plantlets were sent to Wuhan Matwell Biotechnology Co., Ltd. for transcriptome sequencing and primer design. SSR primer design data were provided by Professor Dukejiu of Hebei Agricultural University. 140 pairs of primers were randomly

selected from 10681 pairs of primers by Excel, and sequon Bioengineering (Shanghai) Co., Ltd. and TsingkeBiotechnologyCo.,Ltd. were commissioned to synthesize primers.

SSR primer screening and PCR amplification

Firstly, the mixed samples of 8 samples were used as templates to screen out primers with clear and bright bands that matched the size of the product. Secondly, primers with clear bands and good polymorphism were selected to amplify 6 samples. Finally, the core primers were screened out from 34 samples, which were verified with the primers of affination.

The optimized PCR reaction system were carried out in a volume of 20 µl: DNA template 3 µl (30 ng/µl), 2 ×Taq PCR Master Mix 10µl, upstream and downstream primers 1µl, respectively (10 µmol/µl), and ddH₂O 5 µl. The thermal cycle parameters used by the PCR procedure was 95°C pre-denaturation for 5min, followed by 33 cycles of 95°C for 1min, 55°C annealing 30s, and 72°C extension 45s, with a final elongation at 72°C for 8min. The samples were then stored at 4°C.

Initial screening conditions of polymorphism primers: 2% agarose gel electrophoresis, voltage 110V, 30min, real-time photography; Screening and verification conditions: 8% non-denaturized polyacrylamide gel electrophoresis, 200V, 100mA, 20W, 1h; 300V, 100mA, 20W, 2.5h, silver staining (AgNO₃), and photo storage for subsequent analysis.

data analysis

The results of 8% non-denaturized polyacrylamide gel electrophoresis of 34 samples were analyzed and the band reading statistics were carried out. The value of "1" was assigned to the band with the same shift position, and "0" was assigned to the band without the band, and each of the bands considered as an independent feature, irrespective of its strength²⁰. The 0/1 matrix was established in Excel. GenAlEx 6.502 software was used to calculate the average number of alleles (Na), average effective number of Allele (Ne), Shannon's information index (I), average Observed Heterozygosity (Ho), Expected Heterozygosity (He), and Fixation Index (F). Hardy-weinberg equilibrium tests were performed^{15,46}. Polymorphism information content (PIC) was calculated using Cervus 307 software^{15,46}. Finally, based on the verified polymorphic SSR primer-amplified electrophoretic atlas statistics, Then, using Excel 2007 software to transform it into binary data to construct DNA fingerprints of the 34 *Ailanthus altissima* var. *erythrocarpa* material^{17,20}.

statement

Permission to collect plant samples was obtained for this study; voucher specimens were identified by Alfred Rehder and deposited in the Chinese Field Herbarium (CFH); this study complies with local and national guidelines.

Declarations

Data availability

All data generated or analysed during this study are included in this published article.

Acknowledgements

This work was supported by Hebei Provincial Innovation Ability Promotion Program (20567648H); Shijiazhuang Science and Technology Research and Development Program (201520322A); Hebei Provincial High-level Talents Funding Project (A202001039), We thank Mr. Jianfeng Dai for his help during the experiment.

Author contributions

Z.X. and K.D. designed the experiments and proofread the article; M.Z. collected the plant materials, performed the experiments, conducted the data analysis, and wrote the manuscript; C.Z. collected part of the plant materials and performed part of the experiments; J.L. extracted the DNA; X.W., C.L. and X.L. collected the plant materials

Competing interests

The authors declare that they have no conflict of interest.

References

1. Gürbüz,R.&Kahramanolu,I.Possibility of using leaf extracts of tree-of-heaven (*Ailanthus altissima* (Mill.) Swingle) for the postharvest quality preservation of fresh apricot fruits. *PHYSIOL MOL PLANT P* **113**,101594.<https://doi.org/10.1016/j.pmpp.2020.101594>(2020).
2. Shu,M.et al.Physiological response of main greening tree species to dust pollution in northwest liaoning province.*Acta Bot.Bor-Occid.Sin.***40**,1740–1750(2020).

3. Liu,L.J.,Leng,P.S.,Hu,Z.H.,Zhang,C.L.&Yang,Y.Influences of growth media mixed from municipal raw sludge and construction waste on growth and heavy metal transferring of *Ailanthus altissima*.Chinese Journal of Applied & Environmental Biology **24**,1390–1397.<https://doi.org/10.19675/j.cnki.1006-687x.2018.02031>(2018).
4. Tan,Q.W.,Ni,J.C.,Shi,J.T.,Zhu,J.X.&Chen,Q.J.Two novel quassinoid glycosides with antiviral activity from the samara of *ailanthus altissima*.*Molecules*.**25**,5679(2020)..
5. Yan,Z.Y.et al.Terpenylated coumarins from the root bark of *Ailanthus altissima* (Mill.) Swingle. *Phytochemistry* **175**,112361.<https://doi.org/10.1016/j.phytochem.2020.112361>(2020).
6. Zhu,X.Q.,Zhang,P.A.,Chen,J.Y.&Zhao,T.B.Ornamental types of *Ailanthus* Desf. in Henan.Journal of Henan Forestry Science and Technology **20**,10–12 + 15(2000).
7. Kowarik,I.&Säumel,I.Biological flora of centralEurope: *Ailanthus altissima* (Mill.) swingle. *Perspect.Plant Ecol.Evol.Syst.***8**,207–237 <https://doi.org/10.1016/j.ppees.2007.03.002>(2007).
8. Xu,H.Study on the characteristics of morphology and physiological of *Red Fruit Ailanthus altissima*.*Liaocheng University*(2015).
9. Coleman,J.S.,McConnaughay,K.D.&Ackerly,D.D.Interpreting phenotypic variation in plants. *Trends Ecol.Evol.***9**,187 – 91.[https://doi.org/10.1016/0169-5347\(94\)90087-6](https://doi.org/10.1016/0169-5347(94)90087-6)(1994).
10. Wang,X.et al.Development of EST-SSR markers and their application in an analysis of the genetic diversity of the endangered species *Magnolia sinostellata*. *Mol.Genet.Genom.***294**,135–147.<https://doi.org/10.1007/s00438-018-1493-7>(2019).
11. Zhang,J.,Liu,T.&Rui,F.Development of EST-SSR markers derived from transcriptome of *Saccharina japonica* and their application in genetic diversity analysis.*J.Appl.Phycol.***30**,2101–2109.<https://doi.org/10.1007/s10811-017-1354-5>(2017).
12. Vašek,J.et al.New EST-SSR Markers for Individual Genotyping of Opium Poppy Cultivars (*Papaver somniferum* L.). *Plants*.**9**,10.<https://doi.org/10.3390/plants9010010>(2019).
13. Gao,P.,Ma,H.Y.,Luan,F.S.&Song,H.B.DNA fingerprinting of Chinese melon provides evidentiary support of seed quality appraisal. *PLoS ONE*.**7**,e52431.<https://doi.org/10.1371/journal.pone.0052431>(2012).
14. Kumar,N.et al. SSR-Based DNA Fingerprinting and Diversity Assessment Among Indian Germplasm of *Euryale ferox*: an Aquatic Underutilized and Neglected Food Crop. *Appl.Biochem.Biotechnol.***185**,34–41.<https://doi.org/10.1007/s12010-017-2643-9>(2018)
15. Saina,J.K.,Li,Z.Z.,Mekbib,Y.,Gichira,A.W.&Liao,Y.Y.Transcriptome sequencing and microsatellite marker discovery in *Ailanthus altissima* (Mill.) Swingle (Simaroubaceae). *Mol.Biol.Rep.***48**,2007–2023.<https://doi.org/10.1007/s11033-020-05402-w>(2021).
16. Kim,J.M.et al. Analysis of genetic diversity and relationships of *Perilla frutescens* using novel EST-SSR markers derived from transcriptome between wild-type and mutant *Perilla*. *Mol.Biol.Rep.***48**,6387–6400.<https://doi.org/10.1007/s11033-021-06639-9>(2021).
17. Li,Q.et al. Construction of fingerprint and genetic diversity analysis of 61 high-oil soybean varieties (lines).*J.Henan Agric.Sci.***50**,53–62.<https://doi.org/10.15933/j.cnki.1004-3268.2021.09.007>(2021).
18. Pavan Kumar,P.,Janakiram,T.&Bhat,K.V. Microsatellite based DNA fingerprinting and assessment of genetic diversity in *bougainvillea* cultivars. *Gene* **30**,753:144794.<https://doi.org/10.1016/j.gene.2020.144794>(2020).
19. Ma,S.et al. Fingerprint identification of white clover cultivars based on SSR molecular markers. *Mol.Biol.Rep.***47**,8513–8521.<https://doi.org/10.1007/s11033-020-05893-7>(2020).
20. Zhang,C.et al. De novo transcriptomic analysis and identification of EST-SSR markers in *Stephanandra incisa*. *Sci.Rep.***11**,1059.<https://doi.org/10.1038/s41598-020-80329-7>(2021).
21. Yang,X.et al. Development and application of EST-SSR molecular markers in *Pinus bungeana*.*J.Beijing Forestry Univ.***43**,1–11(2021).
22. Wu,Q.et al. Full-length transcriptome sequencing analysis and development of EST-SSR markers for the endangered species *Populus wulianensis*. *Sci.Rep.***10**,16249.<https://doi.org/10.1038/s41598-020-73289-5>(2020).
23. Dallas,J.F.,Leitch,M.J.&Hulme,P.E. Microsatellites for tree of heaven (*Ailanthus altissima*). *Mol.Ecol.Res.***5**,340–342.<https://doi.org/10.1111/j.1471-8286.2005.00920.x>(2005).
24. Neophytou,C.et al. Analysis of microsatellite loci in tree of heaven (*Ailanthus altissima* (Mill.) Swingle) using SSR-GBS. *Tree.Genet.Genom.***14**,82.<https://doi.org/10.1007/s11295-018-1295-4>(2018).
25. Aldrich,P.R.et al. Genetic structure of the invasive tree *Ailanthus altissima* in eastern United States cities. *J Bot* **1–9**.<https://doi.org/10.1155/2010/795735>(2010).
26. Brusa,A.&Holzapfel,C. Population structure of *Ailanthus altissima* (Simaroubaceae): The role of land-use history and management1. *The J Torrey Botan Soc* **145**,55–69.<https://doi.org/10.3159/TORREY-D-17-00012.1>(2018).
27. Chuman,M.,Kurokochi,H.,Saito,Y.&Ide,Y. Expansion of an invasive species, *Ailanthus altissima*, at a regional scale in Japan. *J Ecol Environ* **38**,47–56(2015).
28. Kurokochi,H.,Saito,Y.,Chuman,M.&Ide,Y. Low chloroplast diversity despite phylogenetically divergent haplotypes in Japanese populations of *Ailanthus altissima* (Simaroubaceae). *Botany* **91**,148–154 <https://doi.org/10.1139/cjb-2012-0159>(2012).
29. Kurokochi,H.,Saito,Y.&Ide,Y. Genetic structure of the introduced heaven tree (*Ailanthus altissima*) in Japan: evidence for two distinct origins with limited admixture. *Botany* **93**,133–139(2014)
30. Neophytou,C.,Pötzelsberger,E.,Curto,M.,Meimberg,H.&Hasenauer,H. Population bottlenecks have shaped the genetic variation of *Ailanthus altissima* (Mill.) Swingle in an area of early introduction. *Forestry*.<https://doi.org/10.1093/forestry/cpz019>(2019).

31. Saina,J.K.,Li,Z.Z.,Gichira,A.W.&Liao,Y.Y. The Complete Chloroplast Genome Sequence of Tree of Heaven (*Ailanthus altissima* (Mill.) (Sapindales: Simaroubaceae), an Important Pantropical Tree. *Int J Mol Sci* **19**,929.<https://doi.org/10.3390/ijms19040929>(2018).
32. Preethi,Pet al. Development of EST-SSR markers for genetic diversity analysis in coconut (*Cocos nucifera* L.). *Mol Biol Rep* **47**,9385–9397.<https://doi.org/10.1007/s11033-020-05981-8>(2020).
33. Zuo,L.H.,Zhang,S.H.,Liang,H.Y.,Wang,J.M.&Yang,M.S. Development of the elm transcriptome with EST-SSR primers and genetic diversity analysis.*Journal of Plant Genetic Resources* **19**,57–166.<https://doi.org/10.13430/j.cnki.jpgr.2018.01.018>(2018).
34. Sharma,H.et al. Development of polymorphic EST-SSR markers and their applicability in genetic diversity evaluation in *Rhododendron arboreum*. *Mol Biol Rep* **47**,2447–2457.<https://doi.org/10.1007/s11033-020-05300-1>(2020).
35. Zhou,Q.,Zhou,P.Y.,Zou,W.T.&Li,Y.G. EST-SSR marker development based on transcriptome sequencing and genetic analyses of *Phoebe bournei* (Lauraceae). *Mol Biol Rep* **48**,2201–2208.<https://doi.org/10.1007/s11033-021-06228-w>(2021).
36. Yin,Y.et al. SSR information in transcriptome and development of molecular markers in *Lycium ruthenicum*.*Journal of Zhejiang A & F University* **36**,422–428(2019).
37. Xiao,Y.S.,Chen,X.Q.,Li,H.C.,Wang,K.L.&Song,L.Y. Development and identification of SSR molecular markers based on transcriptome sequencing of *Wedelia trilobata*.*Molecular Plant Breeding* **19**,2293–2299.<https://doi.org/10.13271/j.mpb.019.002293>(2021).
38. Morgante,M.,Hanafey,M.&Powell,W. Microsatellites are preferentially associated with nonrepetitive DNA in plant genomes. *Nat Genet* **30**,194–200.<https://doi.org/10.1038/ng822>(2002).
39. Martienssen,&R.A. DNA methylation and epigenetic inheritance in plants and filamentous fungi. *Science* **293**,1070–1074.<https://doi.org/10.1126/science.293.5532.1070>(2001).
40. Duan,D.,Jia,Y.,Yang,J.&Li,Z.H. Comparative Transcriptome Analysis of Male and Female Conelets and Development of Microsatellite Markers in *Pinus bungeana*, an Endemic Conifer in China. *Genes (Basel)***8**,393.<https://doi.org/10.3390/genes8120393>(2017).
41. Zhang,X.,Zhang,Y.,Wang,Y.H.&Shen,S.K.(2018) Transcriptome Analysis of *Cinnamomum chago*: A Revelation of Candidate Genes for Abiotic Stress Response and Terpenoid and Fatty Acid Biosyntheses. *Front Gene* **9**,505.<https://doi.org/10.3389/fgene.2018.00505>(2018).
42. Thiel,T.,Michalek,W.,Varshney,R.K.&Graner,A. Exploiting EST databases for the development and characterization of gene-derived SSR-markers in barley (*Hordeum vulgare* L.). *Theor Appl Gene* **106**,411 – 22.<https://doi.org/10.1007/s00122-002-1031-0>(2003).
43. Sarimana,U.et al. Analysis of genetic diversity and discrimination of Oil Palm DxP populations based on the origins of pisifera elite parents. *Breed Sci* **71**,134–143.<https://doi.org/10.1270/jsbbs.20043>(2021)
44. Jiang,F.DNA fingerprint technology and its application in Chinese medicine.*Strait Pharmaceutical Journal* **17**,183–185.<https://doi.org/10.3969/j.issn.1006-3765.2005.04.111>(2005).
45. Chen,J.,Zheng,G.C.,Jing,Y.H.,Ma,Z.Q.&Zheng Y The analysis of DNA fingerprints in the four series maize inbred from different source.*Journal of Inner Mongolia Minzu University(Natural Sciences)* 289–292.<https://doi.org/10.14045/j.cnki.15-1220.2007.03.015>(2007).
46. Shi,Y.T.,Lai,B.W.,Wang,X.J.,Qin,H.Z.&Chai,S.F. Development EST-SSR markers based on transcriptome sequences in *Keteleeria calcarea*.*Molecular Plant Breeding* 1–18,<http://kns.cnki.net/kcms/detail/46.1068.S.20210906.1107.002.html>(2022)

Tables

Table 7 is available in the Supplementary Files section

Figures

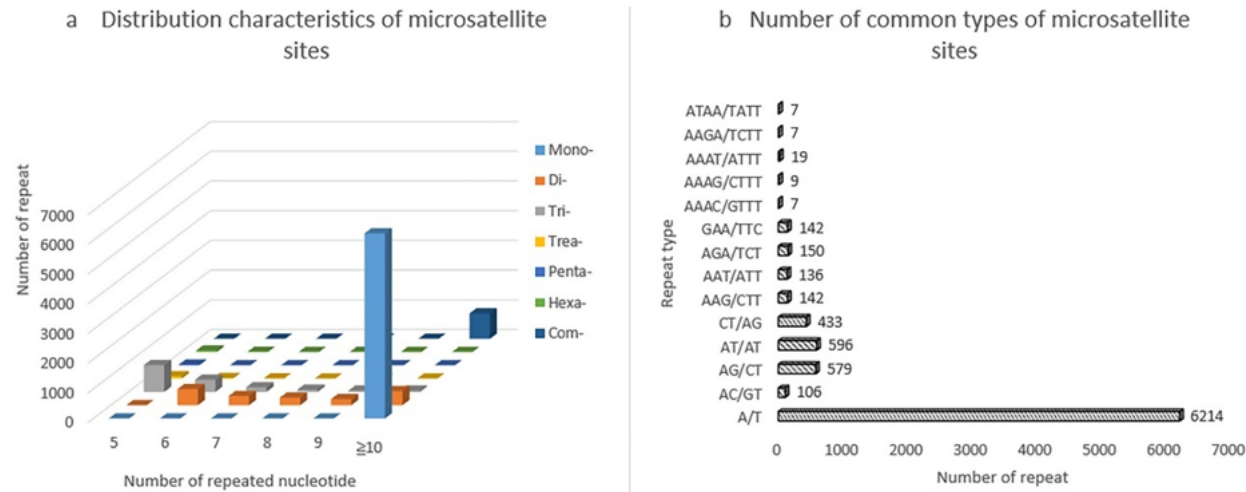


Figure 1

Mono-, Mononucleotide repeat motif; Di-, Dinucleotide repeat motif; Tri-, Trinucleotide repeat motif; Tetra-, Tetranucleotide repeat motif; Penta-, Pentanucleotide repeat motif; Hexa-, Hexanucleotide repeat motif; Com-, Compound nucleotide repeat motif.

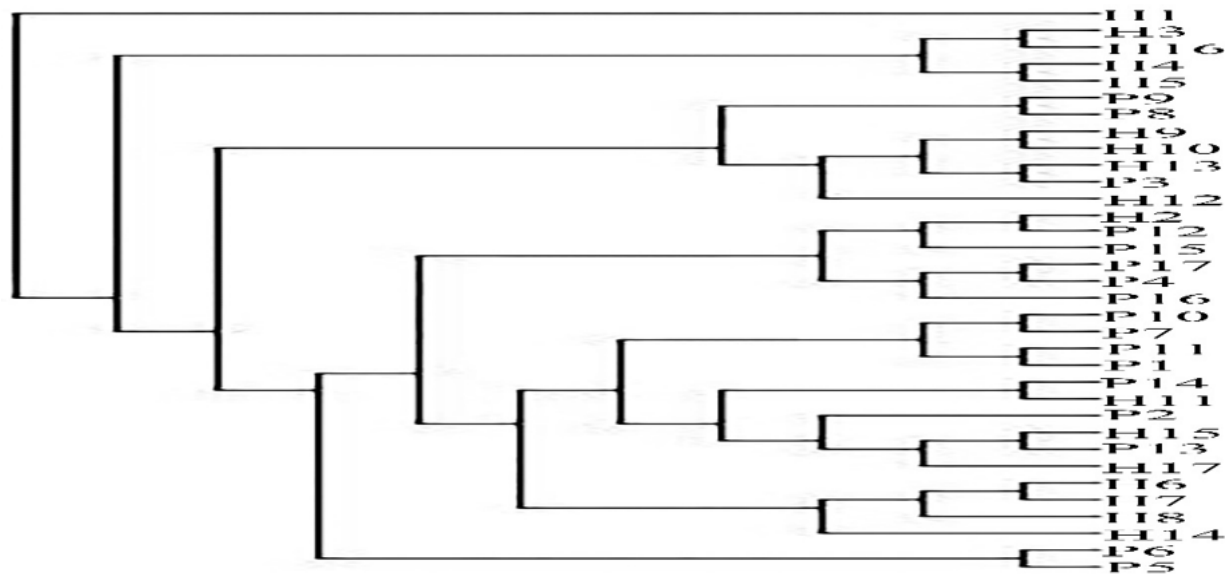


Figure 2
Clustering results of 34 *Ailanthus altissima* var. *erythrocarpa*

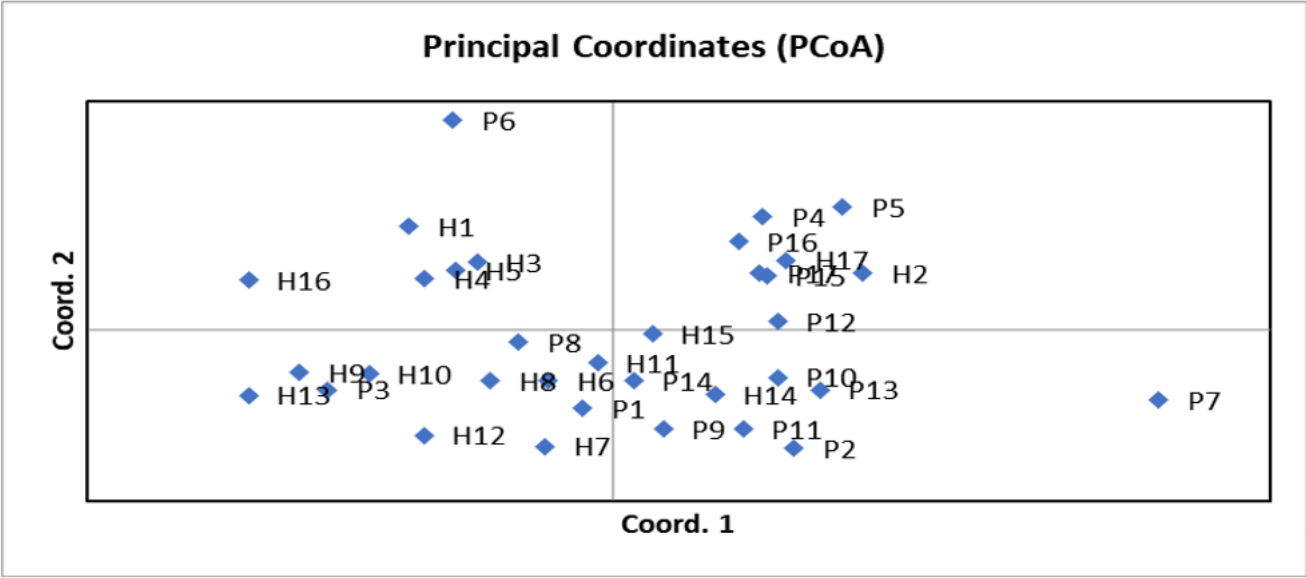


Figure 3
Clustering results of 34 *Ailanthus altissima* var. *erythrocarpa* based on principal component analysis. Coord.1: first principal component; Coord.2: second principal component



Figure 4

Comparison figure of samara color of some *Ailanthus altissima* var. *erythrocarpa* material in the same period (in the field)

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Table7.docx](#)