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UTILIZATION OF NOVEL EST-SSR MARKERS TO EXPLORE THE GENETIC DIVERSITY AND POPULATION STRUCTURE OF *POLYGONUM CUSPIDATUM*

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Abstract

Polygonum cuspidatum, a medicinal plant renowned for its therapeutic properties, also poses ecological challenges as an invasive species in Asia and Europe. Despite extensive research on its pharmacological applications and management strategies, its genetic diversity is inadequately comprehended. This study involved an analysis of 52 wild individuals collected from four provinces in China (Hubei, Hunan, Guizhou, and Sichuan) to fill this knowledge gap. By utilizing 24 novel expressed sequence tag-simple sequence repeat (EST-SSR) markers, we determined 191 highly polymorphic alleles, revealing a rich and complex genetic landscape that underscores the potential for conservation and breeding efforts. This study provides an assessment of genetic variation using newly developed EST-SSR markers. It also demonstrates a foundation for sustainable utilization and genetic improvement of *P. cuspidatum*.

Key words: *Polygonum cuspidatum;* Genetic diversity; Germplasm resources; Population structure; EST-SSR

Introduction

Polygonum cuspidatum Sieb. et Zucc., commonly known as Japanese knotweed, is a medicinal plant of significant importance in traditional Chinese medicine. For millennia, people have utilized its rhizome to treat a range of ailments including inflammation, coughs, and infections, owing to their high content of bioactive compounds such as stilbenes, anthraquinones, flavonoids, and polyphenols(Vastano *et al*., 2000; Peng *et al*., 2013; Hong *et al*., 2016). Modern pharmacological research has further substantiated its therapeutic potential, highlighting antioxidant, anti-inflammatory, anticancer, antiviral, and hepatoprotective attributes (Arichi *et al*., 1982; Kim *et al*., 2005; Bralley *et al*., 2008; Shiyu *et al*., 2011). Owing to these attributes *P. cuspidatum* is now considered as a valuable source of resveratrol, a compound with diverse health benefits and substantial economic value(Sun *et al*., 2021). However, the overexploitation of wild populations to meet increasing demand has raised concerns about genetic erosion and habitat loss, underscoring the need for effective conservation strategies.

Despite extensive research on the pharmacological properties and ecological management of *P. cuspidatum*, its genetic diversity and population structure remain underexplored. Understanding these genetic parameters is critical for conserving wild germplasm resources, as genetic diversity provides the foundation for species adaptation, resilience to environmental stressors, and breeding initiatives aimed at improving medicinal compound content (Hartmann, 2007; Chen *et al*., 2014; Govindaraj *et al*., 2015; Ghorbani *et al*., 2020; Wang, 2020; Huang *et al*., 2021). Molecular markers such as random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and single nucleotide polymorphisms (SNPs) have been widely employed in plant genetic studies. Among these, simple sequence repeat (SSR) markers are especially valuable owing to their significant polymorphism, reproducibility, and cost-effectiveness (Varshney *et al*., 2005; Lindqvist *et al*., 2006). Nevertheless, traditional SSR indicators often fail to provide insights into the functional regions of the genome.

Expressed sequence tag-simple sequence repeat (EST-SSR) markers, originated from transcribed regions, overcome this limitation by offering greater transferability and direct relevance to expressed genes(Parthiban *et al*., 2018; Liu *et al*., 2021; Liu *et al*., 2022). These markers have shown promise in genetic studies of other medicinal plants but have not been extensively developed or utilized for *P. cuspidatum*. Addressing this gap, our study represents the first comprehensive effort to develop and apply novel EST-SSR markers for evaluating the genetic diversity and population structure of *P. cuspidatum*.

By analyzing 52 individuals from 10 wild populations across Hubei, Hunan, Guizhou, and Sichuan provinces in China, this study provides a detailed assessment of genetic variation using 24 newly developed EST-SSR markers. These markers, designed from full-length transcriptome sequences, enable the identification of alleles relevant to both conservation and breeding. Our findings highlight the genetic variety both within and between groups, reveal patterns of gene flow, and identify hotspots for in situ

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conservation. This research not only contributes to the preservation of a valuable medicinal resource but also establishes a foundation for sustainable utilization and genetic improvement of *P. cuspidatum*.

Material and Methods

Plant Materials and Extraction of DNA: Overall 52 *P. cuspidatum* individuals were sampled from 10 populations across four provinces in China: Hubei, Hunan, Guizhou, and Sichuan. Populations were designated using the first two letters of the province or city name, followed by the first two letters of the county name (e.g., QNWA for Wengan Country, Guizhou Province). Individual samples within populations were labeled sequentially (e.g., QNWA1, QNWA2) (Fig. 1). Collected plant materials were preserved in the medicinal plant resource nursery of Huazhong Agricultural University, Wuhan, China. The cetyltrimethylammonium bromide (CTAB) technique was used to recover genomic DNA from fresh young leaves (Huang *et al*., 2012; Zhang *et al*., 2018; Xia *et al*., 2019; Sihanat *et al*., 2020; Tilwari & Sharma, 2021). Isolated DNA was preserved at -20°C for further analysis.

Identification of EST-SSR and designing of primers: Comprehensive transcriptome sequencing data for roots, leaves, and stems were collected through Biomarker Technologies (Beijing, China). Microsatellite loci were identified from unigenes (length ≥ 1,000 bp) using the MISA software. With minimum repeat criteria of 10, 6, 5, 5, 5, and 5, correspondingly the search parameters comprised moni-, di-, tri-, tetra-, penta- and hexa-nucleotide motifs. Primer pairs were designed using Primer3 software with the following parameters: product size: 100–150 bp; primer length: 18–22 nucleotides; GC content: 45–55%; annealing temperature: 5°C lower than the melting temperature (Tm = 55-65°C). After synthesizing 227 primer pairs, 24 were chosen for their high polymorphism and stable amplification capabilities (Supplementary Table S1).

PCR Amplification: Amplification via polymerase chain reaction (PCR) was conducted using 25 µL reaction mixtures containing 1 µL (20 ng/µL) of genomic DNA, 12.5 µL of 2× Taq Master Mix (Vazyme, Nanjing, China), 1 µL (0.01 nmol/µL) of each primer, and 9.5 µL of ddH_2O. The following steps were part of amplification protocol: denaturation at 94°C for 2 minutes initially followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds and finally, a final extension at 72°C for 2 minutes. Products that were amplified were analyzed using the Fragment Analyzer 5300 system (Agilent, USA).

Data analysis

Genetic diversity metrics, such as, including major allele frequency (MAF), gene diversity (GD), and polymorphic information content (PIC), were computed using Power Marker v3.25 (Liu & Muse, 2005). Diversity measures including the number of distinct alleles (Na), effective number of alleles (Ne), Shannon's information index (I), expected heterozygosity (He), and unbiased expected heterozygosity (uHe), were calculated using GenAlEx v6.5 (Peakall & Smouse, 2012).

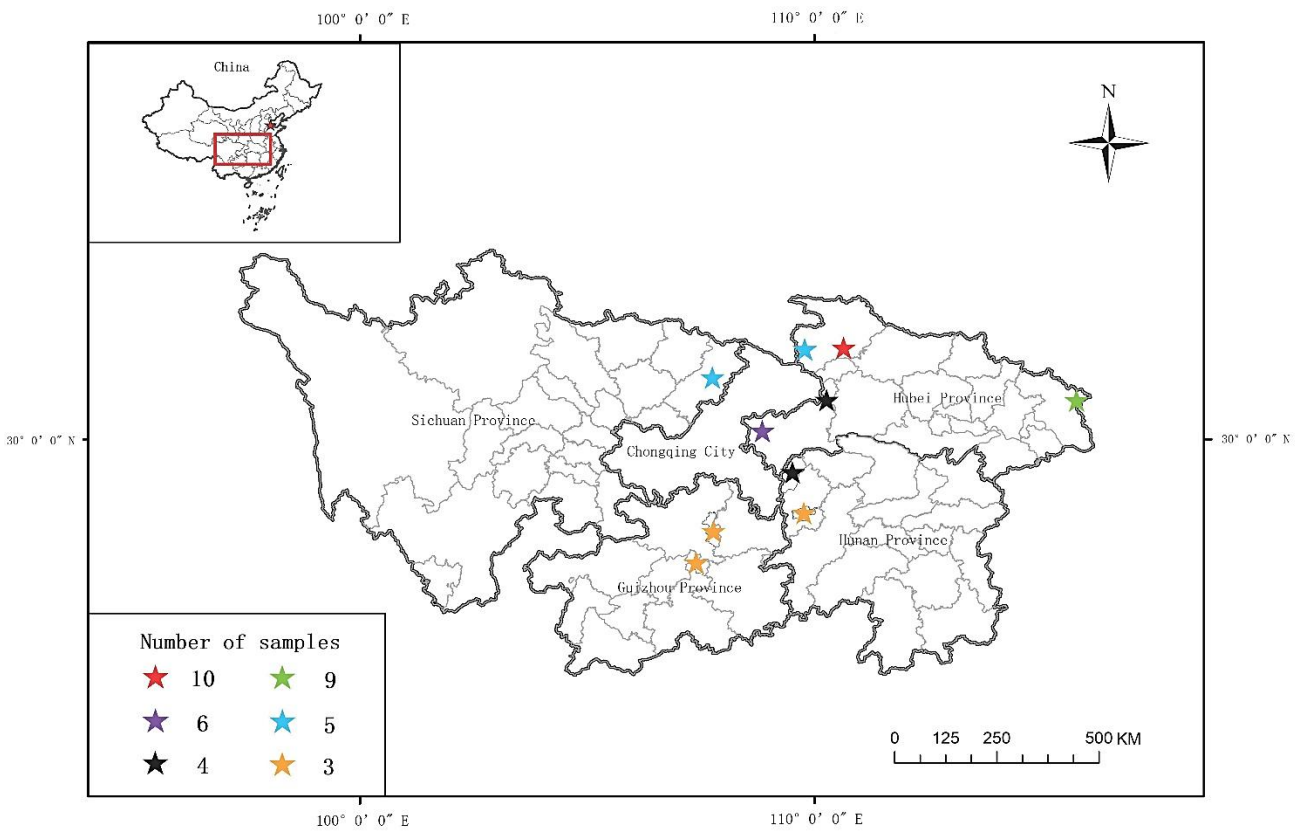

Fig. 1. The sampling locations of the *Polygonum cuspidatum*.
Note: Each star on the map represents a sampling point. Stars of different colors represent the number of samples taken at the sampling point.

Table S1. Genetic diversity parameters of the developed 24 EST-SSR markers across the 52 *P. cuspidatum* individuals.

Primer name	Primer sequence (5'-3')	SSR motif	Na	MAF	GD	PIC
3	F:CACAAAGCTTCCAAACAGCA R:TTTTGTCAGCAGTGTAGCAGG	(GAA)6	12	0.212	0.862	0.847
4	F:GTCTCACCACAAAGCAACCA R:TGCGAAGAACAGAGCAGAGA	(GA)6	16	0.231	0.845	0.828
17	F:CTCACGGAAACAACTCCCAT R:AAGTCGCCTTCTGTGGTGTT	(AGA)5	8	0.462	0.668	0.614
31	F:TGAGCGACACTCTTCCTTCA R:CTCGCACAAAACCTTCTTCC	(TAG)6	10	0.250	0.827	0.806
39	F:GAGAATACCCGACCGACGTA R:CACATGGGCACTTGGTACAC	(TCA)6	9	0.346	0.771	0.740
49	F:CTTGCCCTTCCTCTTCCTCT R:AAGATCCCGGTAAGGCTGAT	(TCA)5	6	0.404	0.686	0.633
59	F:CGTGACATTGGACCAACAAG R:AAACCCAACACACCCACAAT	(TGG)5	15	0.308	0.837	0.821
60	F:AACGAGCGTGTAACTGGAGG R:GTTGCAAGCTCACCATGAGA	(TC)7	7	0.385	0.734	0.692
86	F:CCTTGCTTGACCTTGAGGAG R:GGGGTTTTAGTCACCGGAAT	(TTAT)5	6	0.423	0.709	0.663
87	F:GGTTAACAGGCTTGTAGCCG R:GTGCTATCACGGAAACGGAT	(CGG)5	7	0.750	0.412	0.383
93	F:TGCCTGGATTTGTTAGTCACC R:CATCTTCAGCTTCACCGTCA	(GAC)6	6	0.365	0.707	0.652
105	F:TGCGCTGTAGAACACGAATC R:CCAAGAACACGTTCGTCAGA	(TAT)5	6	0.404	0.691	0.635
111	F:ATGAAGGAGAAGAGCTGGCA R:CATCTTCTGACGCTTGTCCA	(GAT)5	7	0.404	0.695	0.644
113	F:TGCCTGGATTTGTTAGTCACC R:CATCTTCAGCTTCACCGTCA	(GAC)6	7	0.346	0.709	0.655
114	F:GTGGATCAGTCCATGTCCCT R:CAGCAGAGGGAAGGAAAAAG	(AG)6	10	0.462	0.683	0.638
118	F:AATCTGGGCTTAGGGTTTCG R:ACCAGGATAACCGCCTTCTT	(GTG)5	7	0.731	0.444	0.420
123	F:AGAATCACCACCAGAATCCG R:GTGGCTAAATTGGTAGCCCA	(ATC)5	4	0.788	0.353	0.321
137	F:ATGGGTAACATTGGTGGTGG R:TGTTGTTGTGAGGCGTTCAT	(TGA)5	8	0.423	0.699	0.649
178	F:CTGTCATAAGAAAGGGGGCA R:TTATTCCTCAACAGCGGGAG	(CAA)5	8	0.510	0.675	0.638
179	F:TTGCAGAGATTGCGGTAGTG R:TCCAATTCTCCATCTCCGAC	(GA)6	8	0.577	0.621	0.589
191	F:AGAAGATGCTGGAAAGGCTG R:AAGGGGATCGTCTTCTTCGT	(CGA)5	6	0.692	0.490	0.459
205	F:AGATCAAATCGGCCATGCTA R:TGAAACTGCAATCACGAAGG	(TC)6	6	0.327	0.746	0.703
209	F:TGCCTGGATTTGTTAGTCACC R:CATCTTCAGCTTCACCGTCA	(GAC)6	6	0.385	0.725	0.679
224	F:GGTTTTACTGCCAGGGTTGA R:AACCAAGAATGAATGCAGGC	(TA)6	6	0.577	0.587	0.532
Mean			**7.958**	**0.453**	**0.674**	**0.635**

STRUCTURE v2.3.4 with Bayesian clustering was used to evaluate population structure (Pritchard *et al*., 2000) After a 100,000 burn in time, Markov Chain Monte Carlo (MCMC) parameters were set to 100,000 iterations. Optimal K values were determined using the ΔK method via STRUCTURE Harvester (Earl & VonHoldt, 2012). In GenA1Ex v6.5, principal coordinate analysis (PCoA) and analysis of molecular variance (AMOVA) were also carried out. ITOL was used to perform a cluster analysis based on Nei's genetic distance using the unweighted pair group method with arithmetic mean (UPGMA) (Letunic & Bork, 2021).

Results

Characterization of EST-SSR Loci: From 34,105 unigenes (≥1,000 bp) obtained via transcriptome sequencing, 12,335 EST-SSR loci were identified, of which 3.90% exhibited complex forms (Table 1). The most prevalent SSRs were mononucleotide motifs (52.30%) followed by trinucleotide (24.00%) and dinucleotide motifs (18.30%) (Table 1 and Fig. 2A). The most frequent repeat types were A/T for mononucleotides (51.56%) and AAG/CTT for trinucleotides (1.41%) (Fig. 2B). Using MISA and Primer3, 227 EST-SSR primer pairs were synthesized, of which 24 exhibited high polymorphism and stable amplification, confirming their suitability for genetic studies of *P. cuspidatum*. These markers represent a significant advancement over previously available SSR markers by targeting expressed genomic regions.

Genetic diversity analysis: On average, 7.96 alleles were found per locus among the 52 individuals studied using the 24 selected EST-SSR markers, for grant total of 191 alleles. The number of alleles per marker ranged from 4 (primer pair 123) to 16 (primer pair 4). The major allele frequency (MAF) varied from 0.212 to 0.788, and gene diversity (GD) ranged from 0.353 to 0.862, averaging 0.674. Polymorphic information content (PIC) values indicated high polymorphism across loci, with 83.33% of markers exhibiting PIC ≥ 0.5 (Supplementary Table S1). These results underscore the high discriminatory power of the developed EST-SSR markers.

At the population level, genetic diversity indices varied significantly. The *SYFX* population (Fangxian, Shiyan City) demonstrated the highest genetic diversity (Na = 4.25, He = 0.618, I = 1.178), while the *ZYFG* population showed the lowest values (Na = 2.167, He = 0.414, I = 0.648) (Table 2). These findings identify the *SYFX* population as a genetic diversity hotspot, making it a prime candidate for in situ conservation and future breeding programs.

Population structure: According to analysis of the molecular variance (Table 3), just 2% of genetic variation was caused by variations between populations, whereas 91% was found to be present within population. The genetic differentiation coefficient (FST) was 0.025, indicating minimal genetic differentiation among populations. Correspondingly, gene flow (Nm) was calculated as 9.908, suggesting extensive genetic exchange among populations. These findings align with the clonal reproductive nature of *P. cuspidatum*, where facultative sexual reproduction and pollen flow contribute to a great deal of variations within populations and relatively little variation between them.

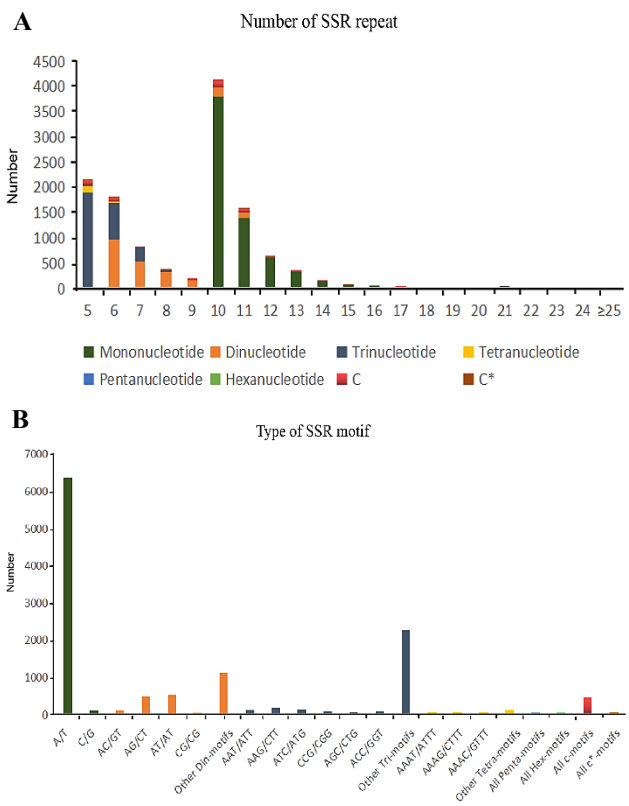

Fig. 2. Distribution of simple sequence repeat (SSR) repeat numbers and SSR motif types in *Polygonum cuspidatum.* A Number of SSR repeat. B Type of SSR motif.

Table 1. Occurrence of simple sequence repeats (SSRs) in the transcripts of the *Polygonum cuspidatum.*

Type of SSR	Number	Ratio in all SSRs (%)	Mean distance (bp)	Length (bp)
Mononucleotide	6454	52.30	98.69	10-23
Dinucleotide	2263	18.30	231.23	12-62
Trinucleotide	2943	24.00	116.92	15-33
Tetranucleotide	157	1.20	86.92	20-24
Pentanucleotide	17	0.10	198.31	25-30
Hexanucleotide	14	0.10	218.63	30-36
C	472	3.8	713.79	20-184
C*	15	0.1	247.2	21-41
Total	12335			

Note: C stands for exactly consistent tandem repeats (e.g. $(GA)_{15}$). C* means that there is mutation in the core repeat region (such as $(G^{**}G^{**})_3(GA)_{12}$)

Table 2. Genetic diversity of ten *Polygonum cuspidatum* populations.

Population	Na	Ne	I	He	uHe	PPL	F
SYFX	4.250	3.071	1.178	0.618	0.650	100.00%	0.941
ESLC	3.083	2.440	0.906	0.508	0.554	95.83%	0.946
SYZX	3.208	2.792	1.032	0.594	0.660	95.83%	0.947
SCDZ	3.208	2.897	1.038	0.598	0.665	95.83%	0.943
XXLS	2.958	2.712	0.997	0.592	0.677	95.83%	0.940
XXJS	2.292	2.192	0.733	0.465	0.558	83.33%	0.931
ZYFG	2.167	2.058	0.648	0.414	0.497	79.17%	0.937
QNWA	2.125	1.992	0.656	0.428	0.514	83.33%	0.931
ESBD	2.417	2.089	0.743	0.461	0.527	91.67%	0.934
HGYS	3.667	2.696	1.040	0.566	0.599	95.83%	0.947
Mean	2.938	2.494	0.897	0.525	0.590	91.67%	0.940

Note: Na: Number of different alleles; Ne: Number of effective alleles; I: Shannon's information index; He: Expected heterozygosity; uHe: Unbiased expected heterozygosity; PPL: Percentage of polymorphic loci; F: Fixation index

Table 3. Analysis of the molecular variance (AMOVA) of the 52 *Polygonum cuspidatum* individuals.

Source	DF	SS	MS	Est. Var.	%	FST
Among population	9	159.070	17.674	0.203	2%	0.025
Among individual	42	655.536	15.608	7.549	91%	
Within individual	52	26.500	0.510	0.510	6%	
Total	**103**	**841.106**		**8.262**	**100%**	

According to the ΔK approach, STRUCTURE analysis found that two (K=2) genetic clusters were the most probable (Fig. 3A and 3B). Bayesian clustering grouped individuals into two distinct genetic clusters, with some populations exhibiting admixture (Fig. 3C). Principal coordinate analysis (PCoA) corroborated this result (Fig. 4), with the first two axes explaining 15.92% and 8.71% of genetic variance, respectively. The UPGMA dendrogram, based on Nei's genetic distance, also supported this subdivision (Fig. 5). Interestingly, the genetic clusters did not align with geographical proximity, suggesting that factors other than physical distance, such as human-mediated dispersal or ecological adaptation, influenced population structure.

Discussion

This study utilizes newly designed expressed sequence tag-simple sequence repeat (EST-SSR) markers to gain a better understanding of the genetic diversity and population structure of *P. cuspidatum.* These markers, derived from transcribed regions, provide higher polymorphism, better transferability, and more direct biological relevance than traditional SSR markers. By analyzing 52 individuals from 10 wild populations across four provinces in China, we offer a comprehensive genetic assessment that fills a critical gap in the conservation and sustainable utilization of this important medicinal plant. The research reveals substantial genetic diversity within *P. cuspidatum* populations, with an average of 7.96 alleles per locus and high polymorphism across most markers (average PIC = 0.635). Notably, the *SYFX* population in Fangxian, Shiyan City, demonstrated the highest genetic diversity (Na = 4.25, He = 0.618, I = 1.178). High genetic diversity indicates better adaptability to environmental changes and stresses, making the *SYFX* population a vital resource for in situ conservation and genetic improvement(Booy *et al*., 2000). In contrast, populations such as *ZYFG* exhibited lower diversity, warranting targeted conservation strategies to prevent genetic erosion.

The identification of genetic diversity hotspots not only advances foundational knowledge but also offers actionable insights for resource management. Prioritizing populations with high diversity, such as *SYFX*, for conservation and breeding programs could enhance the medicinal and ecological value of *P. cuspidatum*, supporting its sustainable utilization (Nachimuthu *et al*., 2015). The low genetic differentiation among populations (FST = 0.025) and high gene flow (Nm = 9.908) highlight extensive genetic connectivity (Wang & Zhang, 1996). The structure, PCoA, and UPGMA analyses consistently identified two genetic clusters that were not aligned with geographical proximity, further underscoring the influence of gene flow and clonal reproduction in shaping genetic structure.

These findings contribute to broader theories of population genetics, particularly in clonal and outcrossing plants. While clonal reproduction often reduces genetic diversity, *P. cuspidatum* appears to maintain high variability through facultative sexual reproduction, supporting theories that clonal plants can achieve genetic diversity through alternative reproductive strategies (Menken *et al*., 1995; Kirsten *et al*., 1998). By identifying key populations and alleles with high breeding potential, this study lays the groundwork for marker-assisted selection aimed at enhancing the medicinal value of *P. cuspidatum*.

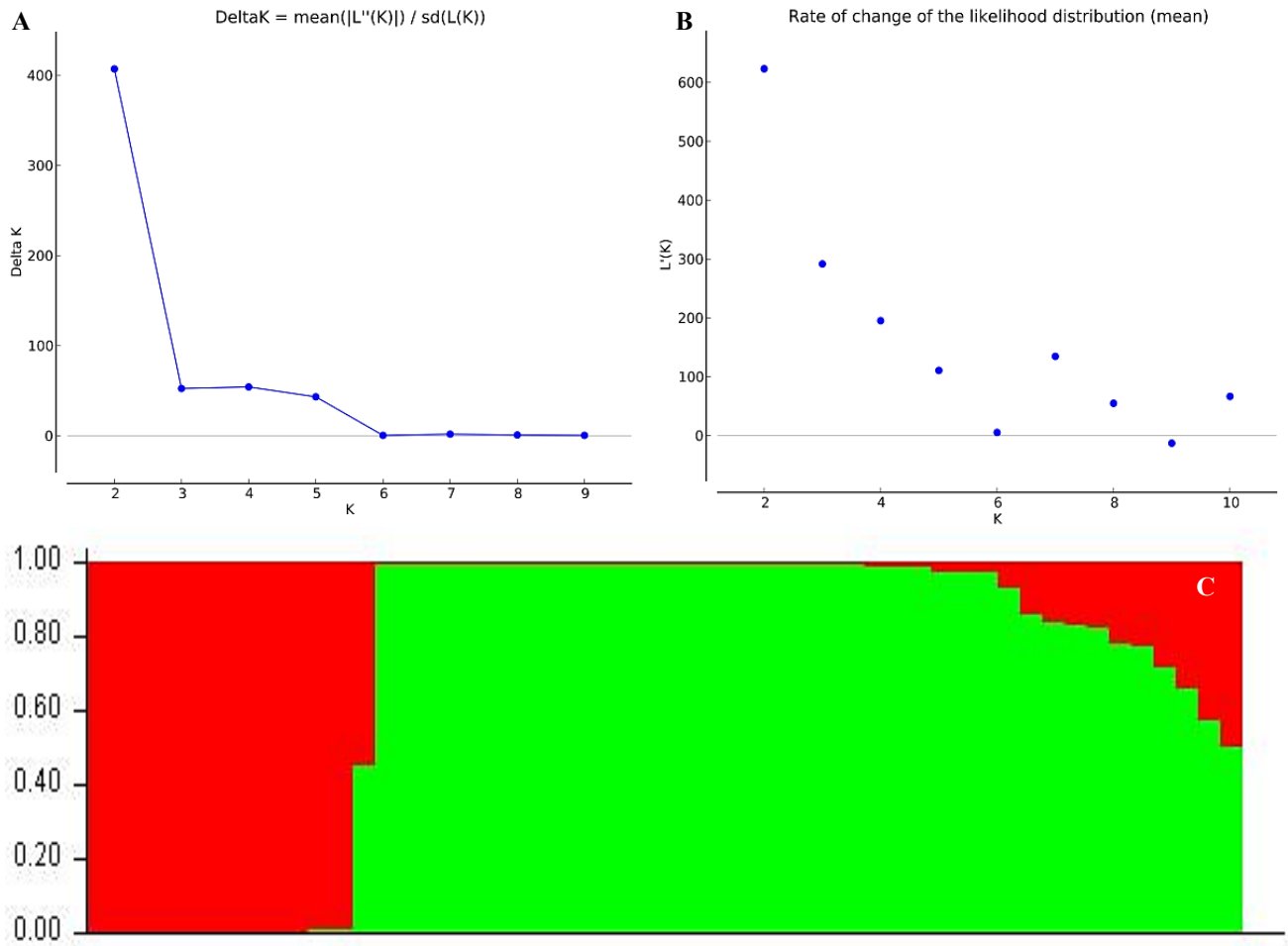

Fig. 3. Bayesian clustering results of the STRUCTURE analysis for *Polygonum cuspidatum* assessed with 24 microsatellite loci. A Estimates of ΔK for K. B Plot of the probability of the data (LnP(D)) values. C Genetic group structure with K = 2.

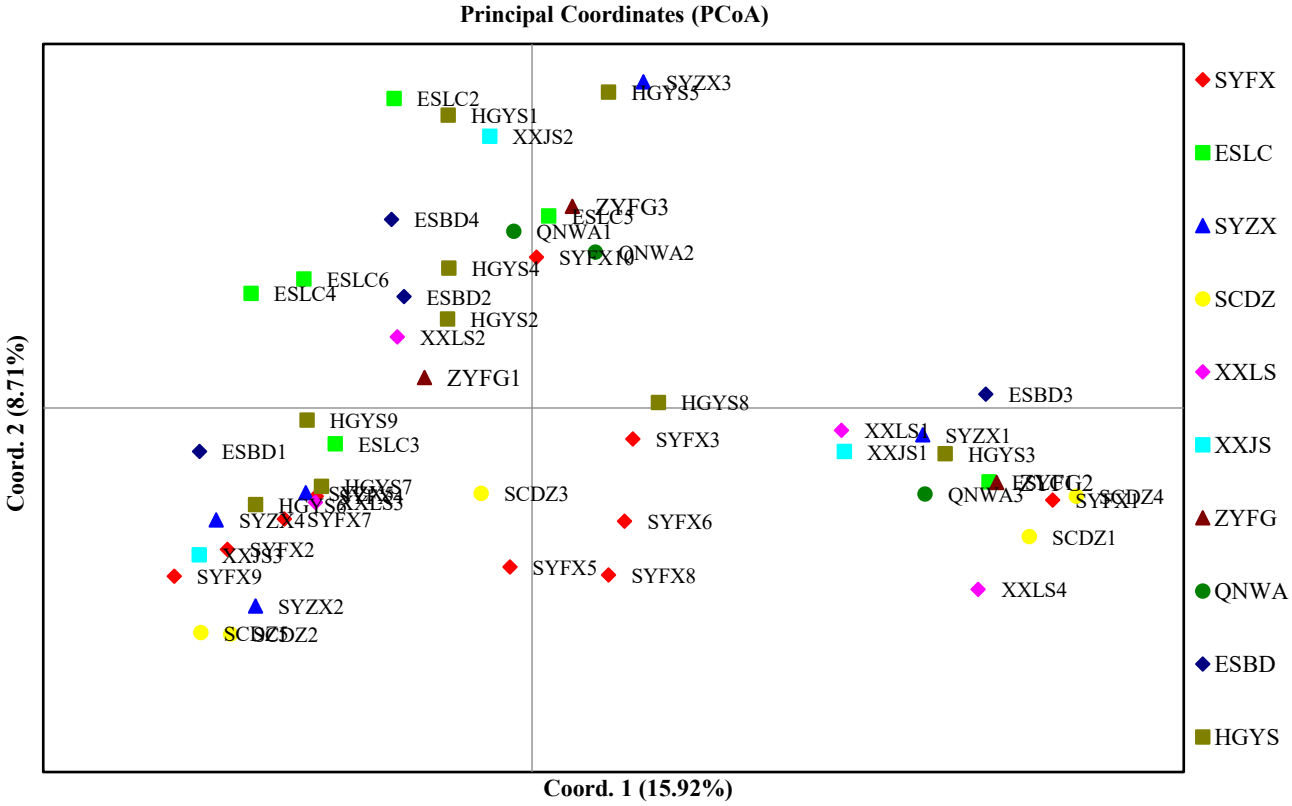

Fig. 4. A principal coordinate analysis (PCoA) plot for the ten *Polygonum cuspidatum* populations showing the separation into two main clusters.

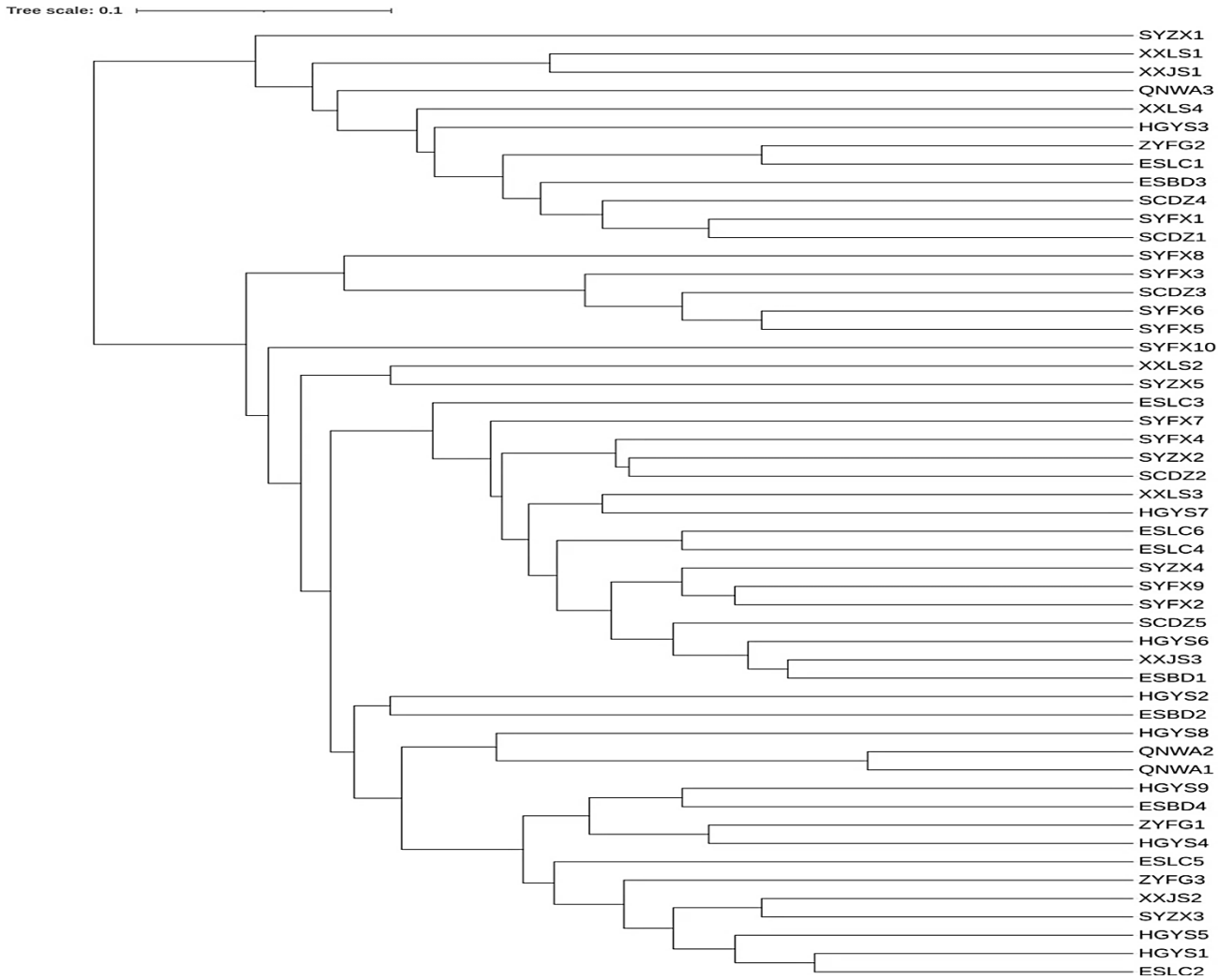

Fig. 5. A unweighted pair group method with arithmetic mean (UPGMA) dendrogram of the 52 *Polygonum cuspidatum* individuals.

Conclusion

In conclusion, this study provides a robust genetic framework for *P. cuspidatum*, addressing key knowledge gaps in its genetic diversity and population structure. The novel EST-SSR markers developed herein offer precise, high-resolution insights into allelic variation, enabling targeted conservation and breeding strategies. The identification of conservation hotspots, such as the SYFX population, and the evidence of extensive gene flow among populations highlight the resilience and adaptability of this species. Beyond advancing the field of *P. cuspidatum* research, this work exemplifies how molecular genetic tools can perform conservation and resource management for other economically and ecologically important plants. Future studies should expand to populations in additional regions and integrate phenotypic analyses with genetic data to further enhance the sustainable utilization and preservation of this valuable medicinal resource.

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