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Assessment of genetic diversity and identification of core germplasm of *Pueraria* in Guangxi using SSR markers

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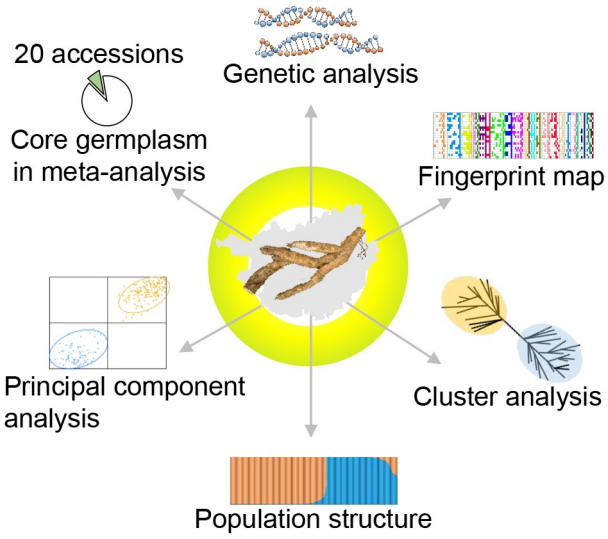
In Brief

Here, we used 23 pairs of simple sequence repeat primers to evaluate the genetic diversity and construct the core germplasm of the 272 individuals of *Pueraria* in Guangxi. 272 accessions were divided into two main clusters, which is consistent with the results based on principal coordinate analysis and STRUCTURE cluster analysis. The results also constructed a core collection of 20 *Pueraria* accessions. Our findings could provide valuable insights for further conservation and molecular breeding of *Pueraria* species.

Graphical abstract



Highlights

- 272 individuals of *Pueraria* species in Guangxi were divided into two main clusters in all analysis.
- 118 alleles were identified and 112 alleles were polymorphic.
- Overall genetic diversity was moderate.
- A core collection of 20 *Pueraria* accessions was constructed when the samples collected reached 7.35% (20/272).

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Assessment of genetic diversity and identification of core germplasm of *Pueraria* in Guangxi using SSR markers

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Abstract

Pueraria, extensively cultivated in Guangxi, China, stands as a pivotal commercial crop and a valuable supplement for human health. Despite its significance, the core germplasm and genetic diversity within Guangxi's *Pueraria* populations remain largely unexplored. This study delves into the genetic diversity of a comprehensive collection of 272 *Pueraria* germplasm accessions from Guangxi, utilizing 23 simple sequence repeat (SSR) markers. The average number of SSR alleles per locus was 5.13, ranging from 2 to 11, with four primers (PtSSR121, PtSSR196, PtSSR155, and PtSSR222) consistently producing at least two polymorphic bands, while PtSSR122 yielded an impressive 11 polymorphic bands. The analysis revealed 118 alleles, 112 of which were polymorphic. The average gene flow (N_m) was estimated at 1.7690, and the average predicted heterozygosity per location was 0.1841. Principal component and STRUCTURE cluster analyses corroborated the division of the 272 accessions into two main clusters. However, no significant statistical correlation was observed between geographic and genetic distances. The study identified a moderate level of genetic diversity. A core collection comprising 20 *Pueraria* accessions that encompass 105 alleles was proposed. These findings provide a theoretical basis for the strategic conservation of *Pueraria*'s genetic resources, laying the groundwork for future breeding programs.

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Introduction

Kudzu (*Pueraria montana* var. *lobata* (Ohwi) Maesen & S. M. Almeida) (2n = 2x = 22) is a semi-woody, perennial liana, that belongs to the Leguminosae family and is widely distributed throughout Asia, including China, Japan, Korea and other regions in Southeast Asia, as well as in North and South America. As an economic crop, it contains puerarin and other functional components and is used in the production of both pharmaceuticals and health foods. *Pueraria montana var. thomsonii* is another variety that shows higher starch content and thus is called starch kudzu. The roots of both *P. montana* var. *lobata* and *P. montana var. thomsonii* have been long used for treating fever, toxicosis, indigestion, and liver damage from alcohol abuse in traditional Chinese medicine[1], which was recorded in *The Divine Husbandman's Classic of Materia Medica* (*Shen Nong Ben Cao Jing*) compiled in the Eastern Han Dynasty (25–250 AD). China is probably the origin and distribution center of *Pueraria* species; however, for a long time, the identification and the breeding of germplasm resources has not received enough attention. Guangxi is a hotspot of *Pueraria* genetic resources in China. Kudzu is a traditional crop cultivated in Guangxi, with abundant germplasm resources at elevations of 100–199 m[2]. At present, the cultivation area of kudzu and starch kudzu in Guangxi accounts for 20% of the whole country[3]. However, the genetic diversity and core germplasm of the *Pueraria* species in Guangxi are not well understood.

With the development of urban society and excessive mining, many germplasm resources are facing the risk of loss or extinction. Genetic diversity provides a basis for the improvement of the crop for different desirable traits, evolutionary capability, species survival, management of germplasm collections, and breeding programs[4–6]. Therefore, it is necessary to fully understand the genetic diversity and genetic information of *Pueraria* core germplasm resources of the representative individuals, which can protect key genetic resources and shorten the breeding process[7,8]. Most recently, RAPD (random amplified polymorphic DNA), ISSR (inter-simple sequence repeat), SRAP (sequence-related amplified polymorphic), SCoT (start condon targeted polymorphism), and SSR (simple sequence repeats) markers have been used to analyze the genetic diversity in *Pueraria*[3,9–14]. Genic-SSRs have the most advantages among these five markers because of the more comprehensive genetic information in the genome[15,16]. Genic-SSRs were used to evaluate the diversity of *Pueraria*, however, the population is just 44 lines[17]. Although genetic analysis of *Pueraria* on some accessions of *Pueraria* or some germplasm resources in Guangxi has been done[3], the core germplasm resource and the overall evaluation on the genetic diversity has not yet been systematically evaluated.

Lack of systematic study of the genetic diversity and the core germplasm resource seriously restricts its efficient management, conservation and further utilization[5]. In the present study, 272 individuals of *Pueraria* collected in Guangxi were

used to estimate the extent of genetic diversity and construct the core germplasm. The findings of this study will be utilized for conservation and management of genetic resources in Guangxi, association mapping, and traits-based kudzu breeding.

Materials and methods

Plant material and DNA extraction

A total of 272 individuals of *Pueraria* were collected in Guangxi from September 2017 to April 2019 (Supplemental Table S1). Three to five fresh young leaves of each accession were collected and immediately frozen in liquid nitrogen and stored at −80 °C until DNA isolation.

Total genomic DNA was extracted from young leaf tissue of individual representative plants of each accession using a Plant DNA Isolation Reagent Kit (TaKaRa, Dalian, China). We measured the concentration and purity of the total DNA using both 1% agarose gel electrophoresis and a Nanodrop instrument (UV-2700). The total DNA extracts were stored at −20 °C until required for experiments.

Table 1. Amplification results and polymorphism information of 23 SSR primers.

No.	Primer name	Sequences (5'-3')	Total number of lands	Number of polymorphic bands	Polymorphism rate (%)
1	PtSSR36	Fw: CTGAGTCTCTGCAAAGCCCA Rv: TGTCACTGTGCTCCAACTCC	10	10	100
2	PtSSR98	Fw: CATTCGGACCTCCATACCCG Rv: CCGCATCCAACCCTGATCAA	11	10	90.9
3	PtSSR99	Fw: GCTTTCCGCTGCTACCATTC Rv: GCAACCCCAATGCTTCACAG	7	7	100
4	PtSSR104	Fw: CACCCTCCCACCACTACAAC Rv: GCAATGTCCTCCTCAGCTGT	3	3	100
5	PtSSR108	Fw: AGCGTGCCCAACTCAGTTAA Rv: CGACGGAGAAGGAGGGAATG	3	3	100
6	PtSSR109	Fw: CAACCTGGCTTCTGTTGTGC Rv: CTCTGAAACGCTGGGCAATG	5	4	80
7	PtSSR121	Fw: ACACTCAACACTCCACCACC Rv: AGGGTTTCCACCTTGAACCG	3	2	66.67
8	PtSSR122	Fw: GGGGTTTCTTCTCGGCTGAA Rv: CACCCCCTTCACGCTTCATA	11	11	100
9	PtSSR130	Fw: ATCAGTGTCTACGTGGGGGA Rv: CACTGCAGCCACAACAACAT	5	4	80
10	PtSSR135	Fw: GATCCGCACCCTATCTGTGG Rv: CTGCGACAGCTCCGATCTTA	8	8	100
11	PtSSR144	Fw: TGTTGCTTTGAACACTAACATGCT Rv: TGCCCTTGTCAGACACAACA	3	3	100
12	PtSSR155	Fw: TTCAACATTCCCCCAACCCC Rv: AAGAAGAGGAACACCAGGCC	2	2	100
13	PtSSR168	Fw: GATCCCACCCACCACTTCTG Rv: GGCTCTAGTTCTGGTGCTGG	5	5	100
14	PtSSR172	Fw:TCTCCAAAACAAGAAGGAAACTCC Rv: TCTTTCCTCTTCTGGTATCCCA	4	3	75
15	PtSSR174	Fw: CAAAGAAGAAGCAGCCGCAG Rv: GTCAATCCCGAAGCACTTGC	6	6	100
16	PtSSR175	Fw: CTGAGTCTCTGCAAAGCCCA Rv: TGTCACTGTGCTCCAACTCC	7	7	100
17	PtSSR186	Fw: TGTTGCTTTGAACACTAACATGCT Rv: TGCCCTTGTCAGACACAACA	4	4	100
18	PtSSR187	Fw: TGTTGCTTTGAACACTAACATGCT Rv: TGCCCTTGTCAGACACAACA	4	4	100
19	PtSSR190	Fw: AACTGCAGGAGGAGCATGAC Rv: GAGCCTCCAGGTTCTTGTCC	5	5	100
20	PtSSR191	Fw: GGAAGCATTGCGGTTTGGTT Rv: TCACATCACATGCTGCCACT	3	3	100
21	PtSSR196	Fw: GCAAGAACCTGTGCTCCTCT Rv: TGCCAATGCCATTGTGGTTG	3	2	66.67
22	PtSSR201	Fw: GCCTCTTCCAGCGAGAACTT Rv: TGATCCTCCCCAACAAGCTG	4	4	100
23	PtSSR222	Fw: TGTGCAAGAAGGATGGGTGA Rv: GGTTGCATTCGGAAGCAACA	2	2	100
Total			118	112	
Avarage			5.13	4.87	94.91

SSR genotyping

The final concentration of DNA was adjusted to 50 ng/μl for PCR reaction. Based on the transcriptome of *P. montana* var. *lobata*, 28 SSR primers were designed and scored in six *Pueraria* collections from 229 SSRs[17]. Ultimately, 23 polymorphic markers were chosen for genetic diversity analysis (Table 1). SSR amplification was carried out in a thermal cycler by Bio-Rad (MyCycler TM), in a final volume of 20 μl containing: 100 ng of genomic DNA, 10 μl of Taq DNA polymerase mix (TaKaRa, Dalian, China), and 10 μM each, forward and reverse non-fluorescent primers. The program used for PCR amplification was as follows: initial denaturation at 94 °C for 5 min; 30 cycles of denaturation at 94 °C for the 30 s, annealing at 50 °C for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 10 min. Amplified products were separated in 6% non-denaturing polyacrylamide gel electrophoresis (PAGE). The SSR markers amplified at sizes between 100 and 400 bp were converted into '0' and '1' codes denoting 'absence' and 'presence', respectively.

Statistical analysis

For each SSR locus, Popgene32 version 1.32 was used to analyze the gene frequency, number of allele (N_a), effective number of alleles (N_e), polymorphic loci, Nei's genetic distance (D), Shannon–Weaver diversity index (I), Homogeneity test index (H) and gene flow (N_m).

Genetic structure was inferred by STRUCTURE version 2.3.1[18]. The number of genetic clusters (K) was set from 1 to 20 with a burn-in period of 50000 steps followed by a run with 100000 iterations. Twenty independent runs were undertaken for each K value. Later, three replicates of the analysis were implemented in CLUMPP software[19]. The mean posterior probabilities [Lnp(D)] values of each K were calculated according to Pritchard et al., along with ΔK[18,20] to explore the optimum number of clusters (K). The most likely number of clusters was determined using a structure harvester (http://taylor0.biology.ucla.edu/structureHarvester/)[21]. Cluster analysis by the unweighted pair group method with arithmetic mean (UPGMA) based on the jaccard method was also developed using the NTSYS-pc 2.10e software[22]. A principal component analysis (PCA) was performed using NTSYS 2.10.

The core collection was developed employing software Core hunter in R package[23]. To assess the core germplasm set, maximum Shannon's diversity index was estimated.

Results

SSR polymorphisms

Encoding binary digit format for genotyping sequence format to exploit the utility of potential core SSRs to fingerprint *Pueraria* accessions. The utilization efficiency and 23 primers information are shown in Table 1 & Supplemental Fig. S1. A total of 118 alleles were detected among 272 *Pueraria* individuals, leading to a mean number of alleles per locus of 5.13 (ranging from two for PsSSR155 and PtSSR222, to 11 for PtSSR98 and PtSSR122). A total of 112 polymorphic alleles (94.91%) was identified with an average of 4.87 effective alleles per locus. Among the 118 alleles, 11 (9.3%) were rare alleles with frequency less than 1% and four of them were found to be only once in one individual. The average of the observed number of alleles (N_a) and the effective number of alleles (N_e) were 1.9492 and 1.2841, respectively.

Population genetic diversity

The population-level genetic diversity of the *Pueraria* accessions under study is presented in Table 2. Nei's gene diversity ranged from 0 to 0.5 and Shannon's information index (I) ranged from 0 to 0.6931 across all 23 SSR loci with an average of 0.1778 and 0.2858, respectively. The average value of total expected heterozygosity (H_t) and N_m were recorded at 0.1841 and 1.7690, respectively.

Genetic structure analysis

The clustering analyses using STRUCTURE under the admixture model suggested the optimum K was two by STRUCTURE HARVEST[21], which divided all sampled individuals into two groups. Correspondingly, the highest of adhoc measure (ΔK) analysis[20] gave a sharp peak at K = 2 (Fig. 1). Hence, the true number of groups were considered as two (Pop1 and Pop2). The accessions with a probability of more 80% were considered as pure and assigned to corresponding subgroups while less than 80% were categorized as admixture (Fig. 1). Among 272 genotypes, 259 were pure and 13 *Pueraria* accessions were admixture. With evidence for several admixtures within cluster I (code_collection number: 30_JCJ-30, 32_JCJ-32, 196_GL-32, 197_GL-33) or cluster II (code_collection number: 12_YZ-12, 26_LC-26, 27_LC-27, 28_HJ-28, 113_GP-21, 149_BS-13, 160_BS-24, 195_GL-31, 270_Y10), subpopulation P1 showed 152 pure (97.5%) and four admixed (2.5%) landraces, P2 had 107 pure (92.2%) and 9 (7.8%) admixed landraces. In addition, all of the 272 individuals could be clustered into one of four groups when K = 4 (Fig. 1). However, within each of the four closely related groups, a few individuals always contained an admixture of introgressed genetic material from another accession.

Cluster analysis and Principal Component Analyses (PCA)

Although there was no clear demarcation in the clustering pattern in the present study, the UPGMA dendrogram (Fig. 2) showed that all the accessions were divided into two main clusters at 0.378 similarity coefficient, which showed similar results to structure analysis. Furthermore, 272 accessions were divided into four main clusters at 0.684 similarity coefficient. The minimum similarity is 0.587 for most other accessions (Fig. 2). There was no distinctive trend of accessions in these two clusters according to their place of origin (Fig. 3). For instance, accessions from Longzhou county of Chongzuo (LZ-9 to LZ-13), were covered within these two clusters with no evident bias.

The PCA categorized all the accessions undertaken into two groups, which was in line with the results of UPGMA based phylogenetic tree and model-based STRUCTURE analysis. The first two axes of differentiation explained 89% of the total variation. The first coordinate explained 40% of the variation and the second coordinate explained 49% of the variation (Fig. 4). The results of PCA indicated that the genetic distance does not show a relationship with geographical distribution in this study.

Extraction of a core collection

One hundred and five SSR alleles found in this study could be represented by a core collection of 20 *Pueraria* accessions with 7.35% sampling proportion (Table 3, Supplemental Fig. S2). When the core selection capacity reached 20, the allele number was 105, so it captured close to 93.75% (105/112) of the total polymorphic loci. The average of the value of N_a, N_e, h, I was 1.8898, 1.3716, 0.2359, and 0.3727, respectively. Based on the dendrogram, the germplasm accessions could be divided into

Table 2. Genetic characteristics for 112 polymorphic microsatellite loci in 272 individuals of *Pueraria* species in the present study.

Locus	Sample size	N_a	N_e	h	I	H_t	H_s	G_{st}	N_m
36-1	272	2	1.0112	0.011	0.0344	0.0129	0.0128	0.0066	75.8301
36-2	272	2	1.0112	0.011	0.0344	0.0129	0.0128	0.0066	75.8301
36-3	272	2	1.0463	0.0443	0.1082	0.0517	0.0503	0.0273	17.8197
36-4	272	2	1.0074	0.0074	0.0244	0.0086	0.0086	0.0043	114.4978
36-5	272	2	1.1017	0.0923	0.1941	0.1063	0.1007	0.0531	8.9206
36-6	272	2	1.0843	0.0778	0.1696	0.0905	0.086	0.0499	9.5227
36-7	272	2	1.2654	0.2098	0.3650	0.2394	0.2025	0.1541	2.7446
36-8	272	2	1.9322	0.4824	0.6755	0.4915	0.4157	0.1543	2.7395
36-9	272	2	1.034	0.0329	0.0849	0.0309	0.0308	0.0032	156.1603
36-10	272	2	1.0074	0.0074	0.0244	0.0086	0.0086	0.0043	114.4978
98-1	272	2	1.0188	0.0184	0.0527	0.0216	0.0213	0.011	44.8945
98-2	272	2	1.1442	0.126	0.2473	0.145	0.1336	0.0786	5.8651
98-3	272	2	1.1499	0.1304	0.2539	0.1509	0.1374	0.0895	5.0838
98-4	272	2	1.0584	0.0552	0.1291	0.0634	0.0616	0.0275	17.6531
98-5	272	2	1.0383	0.0369	0.0933	0.0431	0.0421	0.0225	21.6887
98-6	272	2	1.0383	0.0369	0.0933	0.0431	0.0421	0.0225	21.6887
98-7	272	2	1.0671	0.0629	0.1433	0.0733	0.0704	0.0396	12.1273
98-8	272	2	1.0671	0.0629	0.1433	0.0733	0.0704	0.0396	12.1273
98-9	272	2	1.16	0.1379	0.2652	0.1595	0.1442	0.0957	4.7224
98-10	272	1	1.0000	0.0000	0.0000	0	0		
98-11	272	2	1.0149	0.0147	0.0436	0.0161	0.016	0.003	166.8934
99-1	272	2	1.0188	0.0184	0.0527	0.0216	0.0213	0.011	44.8945
99-2	272	2	1.0615	0.058	0.1343	0.0592	0.0592	0.0007	711.3620
99-3	272	2	1.288	0.2236	0.3830	0.2029	0.1857	0.0849	5.3899
99-4	272	2	1.2065	0.1712	0.3129	0.1855	0.1781	0.04	12.0065
99-5	272	2	1.14	0.1228	0.2424	0.1422	0.1304	0.0835	5.4886
99-6	272	2	1.3643	0.267	0.4375	0.2622	0.261	0.0043	114.6222
99-7	272	2	1.5399	0.3506	0.5353	0.3448	0.3421	0.0076	65.5416
104-1	272	2	1.034	0.0329	0.0850	0.0352	0.0351	0.0039	128.2046
104-2	272	2	1.9558	0.4887	0.6818	0.4964	0.3986	0.1969	2.0392
104-3	272	2	1.9992	0.4998	0.6929	0.4957	0.3738	0.2459	1.5335
108-1	272	2	1.6292	0.3862	0.5746	0.393	0.3882	0.0122	40.3512
108-2	272	2	1.4728	0.321	0.5016	0.3331	0.3234	0.0292	16.6414
108-3	272	2	1.6831	0.4058	0.5958	0.3972	0.3883	0.0223	21.9303
109-1	272	2	1.2934	0.2269	0.3872	0.2568	0.2167	0.1562	2.7010
109-2	272	2	1.0149	0.0147	0.0436	0.0161	0.016	0.003	166.8934
109-3	272	2	1.2934	0.2269	0.3872	0.2568	0.2167	0.1562	2.7010
109-4	272	2	1.0149	0.0147	0.0436	0.0161	0.016	0.003	166.8934
109-5	272	1	1	0	0.0000	0	0		
121-1	272	2	1.0753	0.07	0.1561	0.0805	0.0775	0.0374	12.8581
121-2	272	2	1.0753	0.07	0.1561	0.0805	0.0775	0.0374	12.8581
121-3	272	1	1	0	0.0000	0	0		
122-1	272	2	1.0304	0.0295	0.0778	0.0345	0.0339	0.0179	27.4911
122-2	272	2	1.1351	0.119	0.2367	0.1379	0.1268	0.0805	5.7097
122-3	272	2	1.0304	0.0295	0.0778	0.0345	0.0339	0.0179	27.4911
122-4	272	2	1.0887	0.0815	0.1760	0.0948	0.0898	0.0525	9.0192
122-5	272	2	1.0887	0.0815	0.1760	0.0948	0.0898	0.0525	9.0192
122-6	272	2	1.0671	0.0629	0.1433	0.0733	0.0704	0.0396	12.1273
122-7	272	2	1.0343	0.0332	0.0856	0.0388	0.038	0.0202	24.2677
122-8	272	2	1.4253	0.2984	0.4752	0.2929	0.2912	0.0058	85.4766
122-9	272	2	1.0037	0.0037	0.0134	0.0043	0.0043	0.0022	230.4989
122-10	272	2	1.5187	0.3415	0.5250	0.3673	0.3146	0.1435	2.9847
122-11	272	2	1.2973	0.2291	0.3902	0.2608	0.2152	0.1748	2.3604
130-1	272	2	1.2025	0.1684	0.3090	0.194	0.1703	0.1221	3.5945
130-2	272	2	1.2025	0.1684	0.3090	0.194	0.1703	0.1221	3.5945
130-3	272	2	1.0383	0.0369	0.0933	0.0431	0.0421	0.0225	21.6887
130-4	272	2	1.0383	0.0369	0.0933	0.0431	0.0421	0.0225	21.6887
130-5	272	1	1	0	0.0000	0	0		
135-1	272	2	1.0149	0.0147	0.0438	0.0172	0.0171	0.0088	56.4956
135-2	272	2	1.0587	0.0554	0.1296	0.0647	0.0624	0.0346	13.9494
135-3	272	2	1.3477	0.258	0.4264	0.2931	0.2295	0.2171	1.8032
135-4	272	2	1.0111	0.011	0.0343	0.0118	0.0118	0.0013	397.0141
135-5	272	2	1.3127	0.2382	0.4017	0.2716	0.219	0.1934	2.0859

(to be continued)

Table 2. (continued)

Locus	Sample size	N_a	N_e	h	I	H_t	H_s	G_{st}	N_m
135-6	272	2	1.0149	0.0147	0.0436	0.0161	0.016	0.003	166.8934
135-7	272	2	1.7785	0.4377	0.6295	0.4706	0.1885	0.5994	0.3341
135-8	272	2	1.9656	0.4913	0.6844	0.4999	0.1558	0.6884	0.2263
144-1	272	2	1.0343	0.0332	0.0856	0.0388	0.038	0.0202	24.2677
144-2	272	2	1.9251	0.4805	0.6736	0.479	0.4776	0.0029	174.8282
144-3	272	2	1.6998	0.4117	0.6020	0.3877	0.3213	0.1713	2.4193
155-1	272	2	1.0698	0.0652	0.1475	0.0687	0.0684	0.0049	101.9746
155-2	272	2	1.2483	0.1989	0.3507	0.2281	0.1936	0.1512	2.8079
168-1	272	2	1.501	0.3338	0.5162	0.3124	0.2825	0.0956	4.7296
168-2	272	2	1.9254	0.4806	0.6736	0.4892	0.4321	0.1166	3.7876
168-3	272	2	1.5113	0.3383	0.5214	0.3181	0.2904	0.0869	5.2557
168-4	272	2	1.9569	0.489	0.6821	0.4954	0.4328	0.1262	3.4613
168-5	272	2	1.0074	0.0073	0.0243	0.0075	0.0075	0.0001	2000.0000
172-1	272	2	1.2368	0.1915	0.3407	0.2198	0.1882	0.1438	2.9771
172-2	272	2	1.0112	0.011	0.0344	0.0129	0.0128	0.0066	75.8301
172-3	272	1	1	0	0.0000	0	0		
172-4	272	2	1.0037	0.0037	0.0134	0.0032	0.0032	0.0016	310.4992
174-1	272	2	1.0074	0.0074	0.0244	0.0086	0.0086	0.0043	114.4978
174-2	272	2	1.074	0.0689	0.1542	0.0741	0.0734	0.0099	50.0991
174-3	272	2	1.1249	0.111	0.2241	0.1278	0.1192	0.0669	6.9725
174-4	272	2	1.0037	0.0037	0.0134	0.0043	0.0043	0.0022	230.4989
174-5	272	2	1.7565	0.4307	0.6221	0.4621	0.2446	0.4706	0.5625
174-6	272	2	1.9722	0.4929	0.6861	0.5	0.2054	0.5892	0.3486
175-1	272	2	1.0074	0.0074	0.0244	0.0086	0.0086	0.0043	114.4978
175-2	272	2	1.0932	0.0852	0.1823	0.0991	0.0937	0.0552	8.5594
175-3	272	2	1.0074	0.0074	0.0244	0.0086	0.0086	0.0043	114.4978
175-4	272	2	1.2069	0.1714	0.3132	0.1965	0.1735	0.117	3.7725
175-5	272	2	1.8504	0.4596	0.6522	0.4769	0.3654	0.2337	1.6394
175-6	272	2	1.7047	0.4134	0.6038	0.39	0.3252	0.1659	2.5132
175-7	272	2	1.0301	0.0292	0.0771	0.0298	0.0298	0.0003	1480.2742
186-1	272	2	1.0421	0.0404	0.1005	0.0462	0.0453	0.0181	27.1466
186-2	272	2	1.7726	0.4359	0.6276	0.4059	0.2739	0.3252	1.0373
186-3	272	2	1.7848	0.4397	0.6316	0.4698	0.2325	0.5051	0.4899
186-4	272	2	1.6977	0.411	0.6013	0.3808	0.2793	0.2664	1.3765
187-1	272	2	1.042	0.0403	0.1002	0.0449	0.0444	0.0122	40.4142
187-2	272	2	1.7641	0.4331	0.6247	0.4021	0.2655	0.3397	0.9718
187-3	272	2	1.875	0.4667	0.6594	0.4905	0.1571	0.6796	0.2357
187-4	272	2	1.6636	0.3989	0.5883	0.3687	0.2779	0.2465	1.5286
190-1	272	2	1.0993	0.0903	0.1908	0.0833	0.082	0.0164	29.9125
190-2	272	2	1.046	0.044	0.1076	0.0385	0.0377	0.02	24.4900
190-3	272	2	1.1535	0.1331	0.2580	0.1211	0.1166	0.037	13.0124
190-4	272	2	1.6024	0.3759	0.5634	0.4076	0.2996	0.2651	1.3862
190-5	272	2	1.9999	0.5	0.6931	0.4956	0.323	0.3483	0.9354
191-1	272	2	1.9284	0.4814	0.6745	0.498	0.1126	0.7738	0.1462
191-2	272	2	1.6789	0.4044	0.5942	0.3708	0.2541	0.3146	1.0893
191-3	272	2	1.1641	0.141	0.2696	0.1261	0.1192	0.0552	8.5656
196-1	272	2	1.2295	0.1867	0.3342	0.2136	0.1856	0.1312	3.3100
196-2	272	2	1.0074	0.0074	0.0244	0.0086	0.0086	0.0043	114.4978
196-3	272	1	1	0	0.0000	0	0		
201-1	272	2	1.0037	0.0037	0.0134	0.0043	0.0043	0.0022	230.4989
201-2	272	2	1.9978	0.4995	0.6926	0.4989	0.3531	0.2923	1.2103
201-3	272	2	1.404	0.2877	0.4625	0.3224	0.2509	0.2219	1.7536
201-4	272	2	1.4033	0.2874	0.4621	0.2798	0.2768	0.011	45.0326
222-1	272	2	1.3039	0.233	0.3951	0.2651	0.2176	0.1791	2.2910
222-2	272	2	1.7711	0.4354	0.6271	0.4617	0.3031	0.3435	0.9556
Mean	272	1.9492	1.2841	0.1778	0.2858	0.1841	0.1435	0.2204	1.7690
St. Dev		0.2206	0.3277	0.1741	0.2397	0.0305	0.0166		

N_a = Observed number of alleles; N_e = Effective number of alleles[56]; h = Nei's (1973) gene diversity; I = Shannon's Information index[57]; G_{st} = coefficient of gene differentiation; N_m = estimate of gene flow from Gst or Gcs. E.g., N_m = 0.5(1 - Gst)/Gst; H_t = Total expected heterozygosity; H_s = the average expected heterozygosity within subpopulations.

two main groups. The value of genetic similarity indices among 20 *Pueraria* germplasm accessions varied between 0.31 and 0.60, which indicates that there was a relatedly narrow genetic variation within the different *Pueraria* accessions belonging to the diverse geographic locations across the Guangxi region (Fig. 5). In addition, our COREFINDER analysis highlighted that

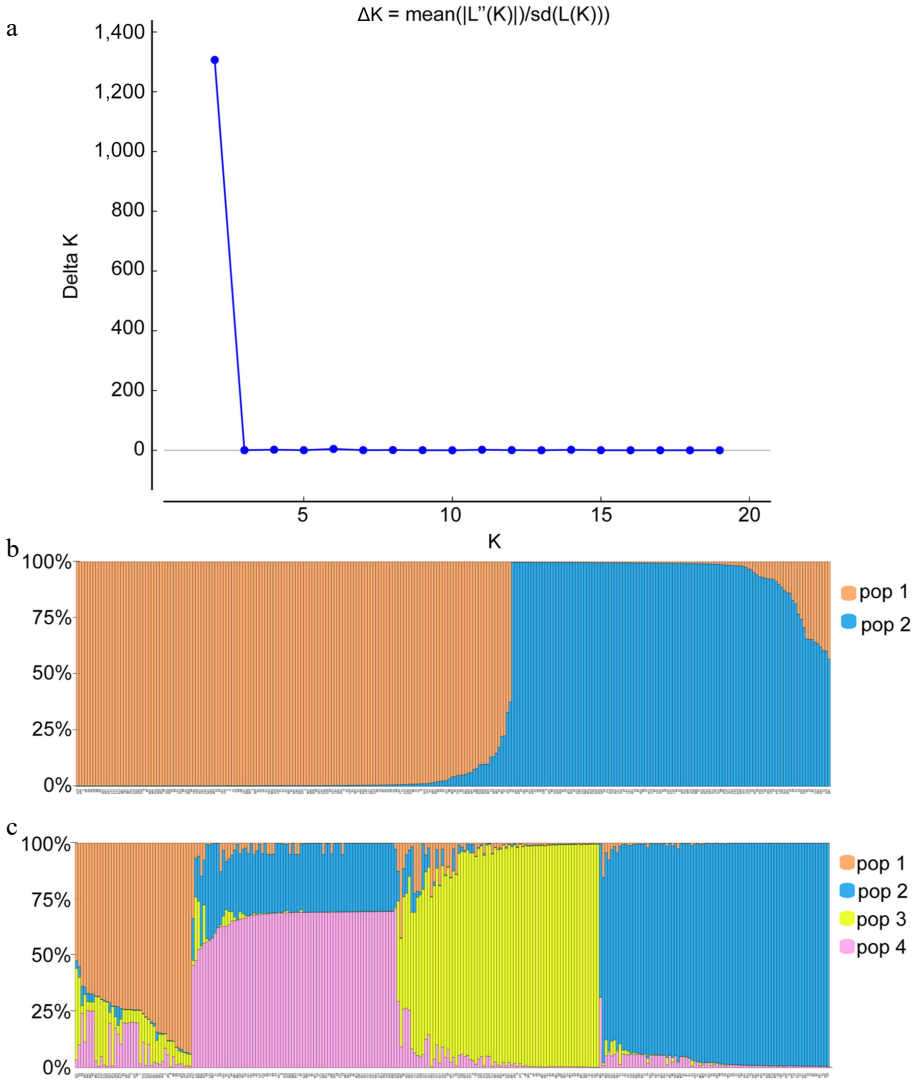

Fig. 1 Bar plots of all 272 individuals from Pueraria germplasm grouped into two or four genetic clusters with assignment probabilities obtained from STRUCTURE analyses of polymorphisms at 23 simple sequence repeat loci. (a) Distribution of delta K = 1–20. (b), (c) Histogram of the STRUCTURE assignment test when K = 2 or K = 4, respectively. The number represents the code in Supplemental Table S1.

10% of the entire core collection was represented by the *Pueraria* accessions grouped in Cluster I, while Cluster II contribute to the core collection at 90%.

Discussion

Polymorphism of newly developed SSR markers

We detected a total of 118 alleles with 23 SSRs segregating in the 272 *Pueraria* accessions in Guangxi, with an average of 5.13 alleles per locus. This value is higher than the number of alleles per SSR locus reported in a previous study with the 28 SSRs in the 44 *Pueraria* accessions from Guangxi[17]. This suggests that expanding the sample size is a powerful strategy for the analysis of genetic diversity in *Pueraria* germplasm in Guangxi. The number of effective alleles per locus (4.87) obtained in the Guangxi *Pueraria* accessions appears to be higher than the number of effective alleles per SSR locus found in 184 *Pueraria* accessions from Jiangxi (1.4503) and other crops, such as the value of 2.26 reported in rice[24], 3.17 in olive[25], but lower than the values of 5[26] or 7.2 in maize[27]. The results also showed that SSR allelic diversity of *Pueraria* germplasm was moderate (N_a = 1.9492, N_e = 1.2841, h = 0.1778). Zhou et al.[14] reported an average of N_e = 1.4503 and h = 0.2865 in a collection of 184 *Pueraria* accessions from Jiangxi. The number of markers and individuals, the sexual propagules and type of plant material, the population size may be responsible for the level of polymorphism and discrimination power.

Genetic diversity of *Pueraria* germplasm

The overall clustering patterns generated by the STRUCTURE and PCA did not clearly distinguish the sampling areas, which is consistent with the previous results[10,13,17,28]. Few admixtures (13/272) were also detected due to shared ancestry during the breeding process, which is also observed in hybrid rice[29]. *Pueraria* resources have a low level of genetic differentiation (N_m = 1.7690). The degree of genetic differentiation among populations may decrease due to the existence of large gene flow (N_m > 1). The low genetic differentiation indicated that geographical isolation may not restrict gene exchange among *Pueraria* species populations in Guangxi. It is susceptible to external

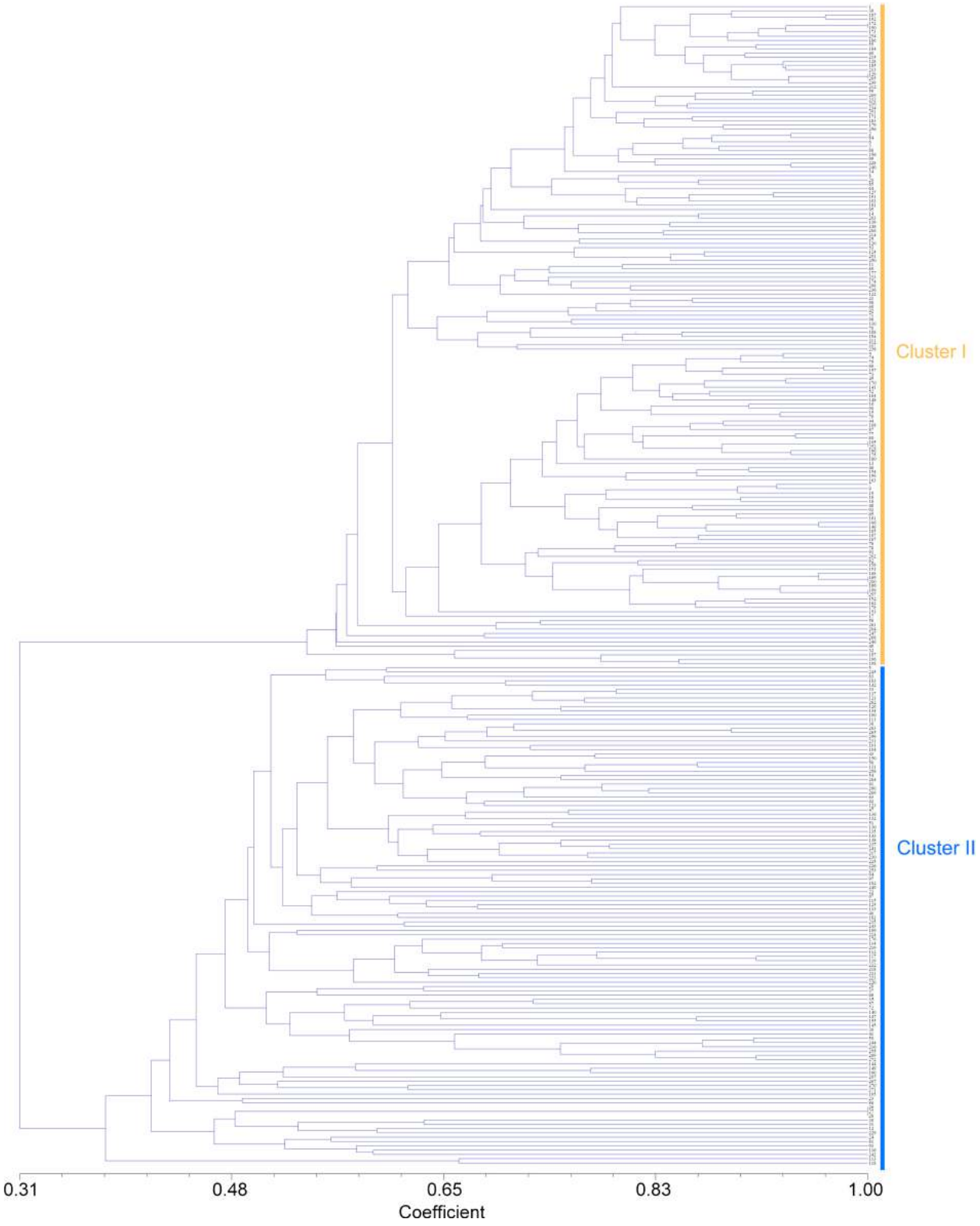

Fig. 2 Cluster diagram based on jaccard by UPGMA analysis calculated from alleles derived from 272 Pueraria accessions. The number represents the code in Supplemental Table S1.

factors even though there was a certain correlation between genetic variation and geographical distribution based on RAPD in several studies[12,30,31]. As a result, it is thought that *Pueraria* species has been cultivated and utilized for a long period in Guangxi since native cultivars of *Pueraria* still exist in the major regions, which is similar to *Perilla* in Korea[32]. The selection by humans could be responsible for this clustering pattern and moderate genetic diversity.

Our results revealed that *Pueraria* accessions display moderate genetic variation throughout Guangxi, while the

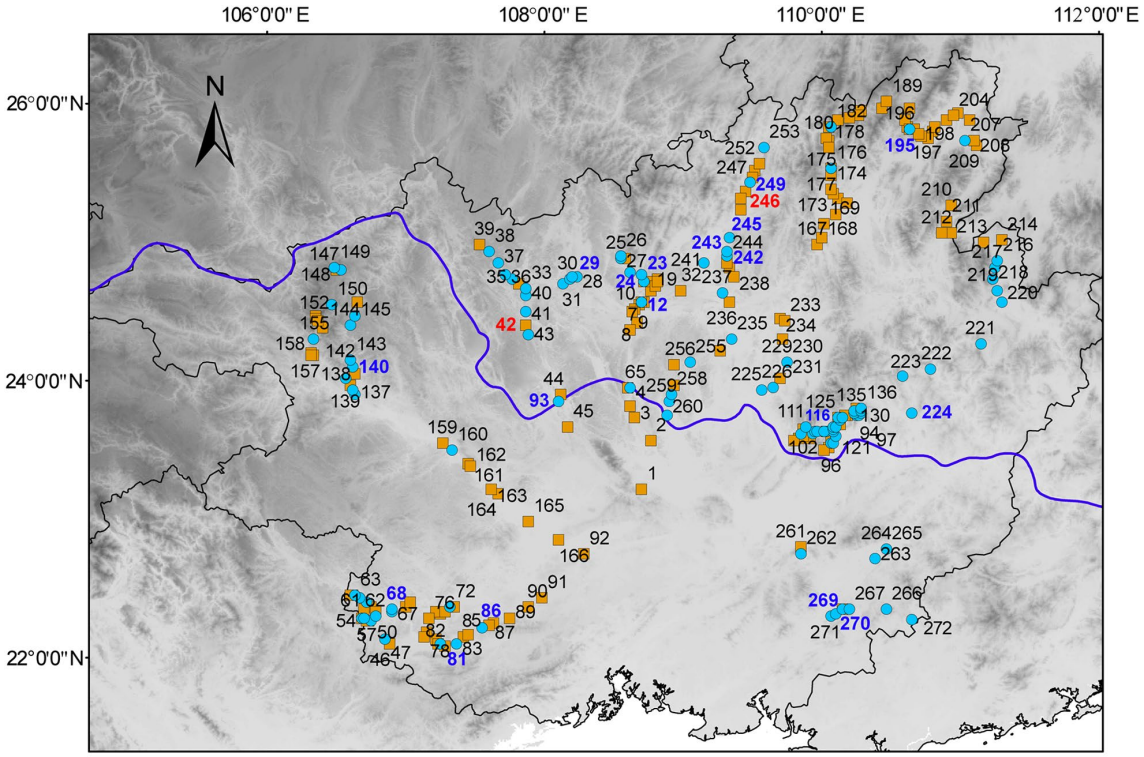

Fig. 3 Geographical distribution of the accessions collected in Guangxi. The number represents the code in Supplemental Table S1. The red and blue numbers represent two clusters of the 20 accessions of core germplasms. The orange squares represent the accessions of Cluster I and blue circles represent the accessions of Cluster II.

UPGMA dendrogram showed that 272 accessions were divided into two main clusters with 37.8% genetic similarity, four main clusters with 68.4% genetic similarity. However, previous studies revealed that *Pueraria* accessions or species possessed from moderate to the high level of genetic diversity with high clonal reproduction and perennial[3,14,17,28,30,33–37]. The inconsistencies observed, except for various taxon sampling and markers, could have originated from the following: 1) the populations were found by sexual propagules could contribute to the maintenance of high genetic variation in clonal populations regardless of recruitment of sexual offspring[38]; 2) introductions from across its multiple native populations into novel habitats from seed stock[37]; 3) clonal populations with fewer genotypes still maintain higher genetic diversity at each locus[39].

Moreover, *Pueraria* species, as strictly self-pollinating and clonally persisting clumps plants, have considered heterozygosity (Table 2), like many clonal plants, e.g. *Castanea dentata*[40] and *Musa balbisiana*[41]. Our results showed that relatively low H_t (0.1841) and H_s (0.1435), which suggest that accessions were inbred due to little outcrossing during maintenance[42]. Moreover, we could not rule out a case that the existence of ancient clonality and the somatic mutation, which accumulates genetic variation within clonally persisting clumps may account for some of the heterozygosity, especially given rapid mutation of SSR fingerprints.

Implications for utilization of core germplasm resources in Guangxi

Core germplasm plays a key role in the conservation, management, and utilization of germplasm resources, which is critical for the development of plant breeding. Individuals reflecting genetic information can be selected to build the core germplasm resources. China is the center of distribution of *Pueraria*, with a long history of growing *Pueraria* species. However, fewer excellent *Pueraria* germplasm have been established due to artificial over-mining, lack of conservation, and management of resources. Previous researchers have shown that a sampling proportion between 5% and 30% is enough to include at least 80% of the alleles representing the genetic diversity of the entire collection[43,44]. According to dynamic extracted results, our results revealed that when the samples collected reached 7.35% (20/272) of *Pueraria* accessions accounted for 105 alleles, accounting for approximately 93.75% of all alleles loci. Interestingly, the retention value of *Pueraria* core collection genetic diversity was lower than the allele retention values of 100%, 100%, and 97.5% in rosewood, licorice, and eggplant, with sampling ratios reaching 12.4%[45], 16.84%[46] and 12.03%[47], respectively. *Pueraria* species are abundant in Guangxi, especially in Tengxian and Wuzhou[2]. The most likely reason was that the breeding of a majority of *Pueraria* accessions in Guangxi was still from layering breeding and self-crossing, and lacked extensive gene exchanges from cross-breeding, which led to a decrease in the ratio of the core collection. Our findings will be useful in breeding programs for the introgression of noble alleles into modern cultivars by exploiting natural genetic variation existing in *Pueraria* genetic resources. Combined with the analysis of phenotypic diversity (e.g. puerarin, starches) of *Pueraria* species, we may detect the important polymorphic loci associated with the traits based on correlation analysis, which could provide a foundation for developing the molecular marker-assisted breeding or detection of target genes soon[7].

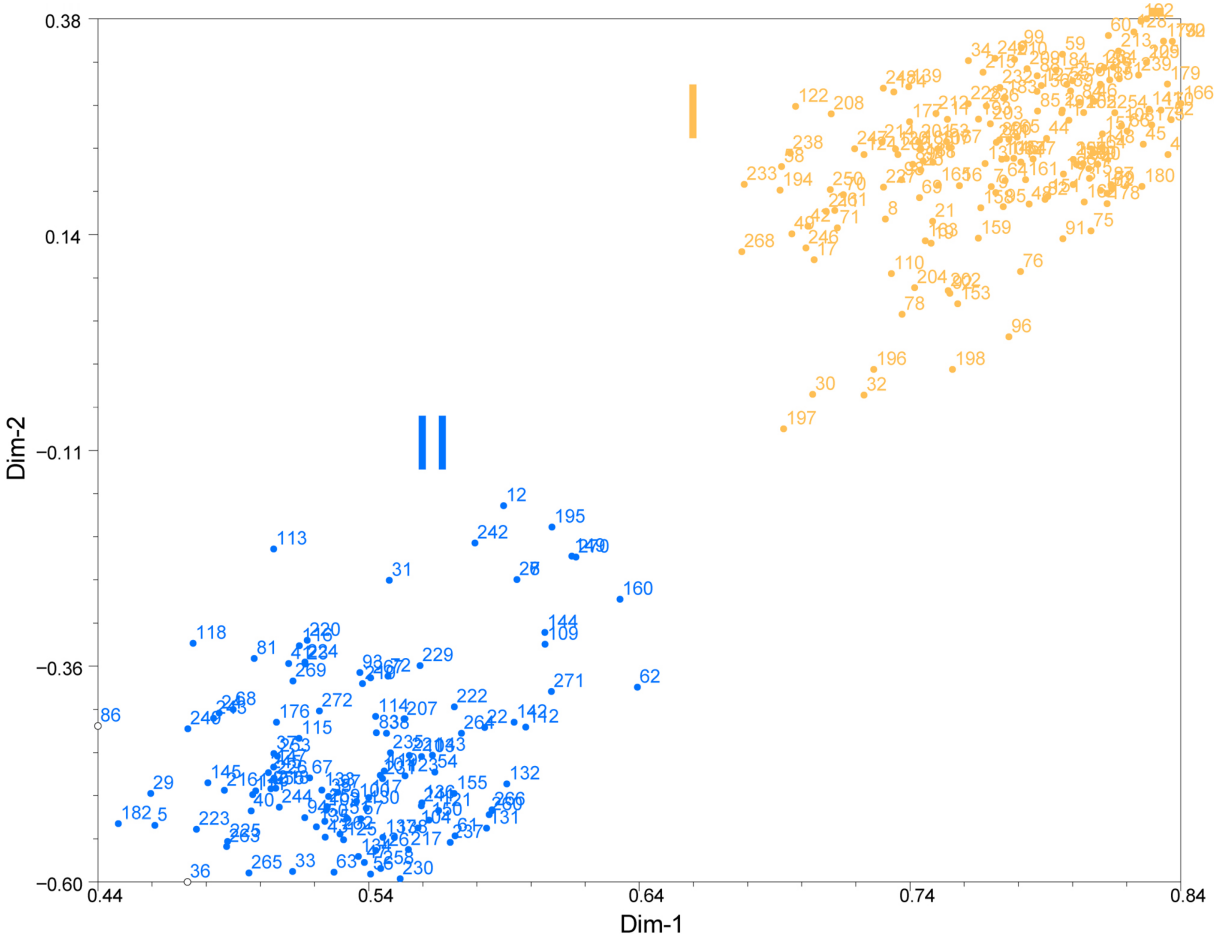

Fig. 4 PCA of *Pueraria* accessions based on dissimilarity matrix (Jaccard). The number represents the code in Supplemental Table S1. The number represent 272 accessions of *Pueraria*. The orange circles represent the accessions of Cluster I and blue circles represent the accessions of Cluster II.

Meanwhile, the genetic clusters were not consistent with species delimitation and geographic distribution. For instance, accession number 140 and 68 classified as *P. montana* var. *montana*, shares a close relationship with three numbers *P. montana* var. *lobata* accessions (29, 243, and 245). *Pueraria* plants were introduced from different regions, which may result in a certain degree of inconsistency between actual germplasm sources and clustering results[17]. Furthermore, this also implies the complex evolutionary history with the human process blur the relationship among these species.

Complex genetic relationships among *Pueraria* species

Molecular marker based on SSR can help exploiting and utilizing plant variety resources reliably without the appraiser and environmental factors[48]. The present results include new clues in genetic relationships among *Pueraria* species based on SSR markers, that is moderate genetic variation and low genetic differentiation play a key role in the species delimitation of *Pueraria*. *Pueraria* DC. (Fabaceae, Phaseoleae) comprises ca. 20 species, occurring in tropical and East Asia. Eight species and two varieties have been recorded in China[49], with four groups or three sections as infrageneric classification based on morphological traits[50,51]. However, molecular studies have revealed that *Pueraria* is not a monophyletic group[52,53]. For example, taxonomically kudzu (*P. montana* var. *lobata*) is placed under the genus *Pueraria*. *Pueraria montana* var. *thomsonii* and *P. montana* var. *lobata* were treated as varieties for *P. montana* in flora of China. However, the phylogenetic relationship and classification among these three species are still confused based on various molecular markers and sampling taxon[54,55]. Thus, molecular markers for germplasm identification of kudzu or even *Pueraria* species may be limited. A wider taxon sampling with higher resolution genetic markers would increase confidence for the phylogenetic relationship among *Pueraria* species, efforts that are currently underway.

Conclusions

In this study, we used 23 pairs of simple sequence repeat primers to evaluate the genetic diversity and construct core germplasm of the 272 individuals of *Pueraria* species in Guangxi. Our results revealed that *Pueraria* accessions display moderate genetic variation throughout Guangxi. There was a non-significant relationship between genetic distance and geographical distance. The results could provide the basis for the breeding program of *Pueraria*. We consider the SSR markers to be a useful tool for both genetic diversity and the core germplasm of *Pueraria*.

Table 3. Summary of the extraction of a core collection.

Sampling proportion	Sample number	N_a	N_e	h	I	Number of polymorphic loci	Percentage of polymorphic loci	Percentage of total loci
5%	14	1.8644 ± 0.3438	1.3839 ± 0.3116	0.2413 ± 0.1639	0.3778 ± 0.2243	102	91.07%	86.44%
7.00%	19	1.8644 ± 0.3438	1.3779 ± 0.3123	0.2381 ± 0.1635	0.3736 ± 0.2242	102	91.07%	86.44%
7.35%	**20**	**1.8898 ± 0.3144**	**1.3716 ± 0.3084**	**0.2359 ± 0.1598**	**0.3727 ± 0.2170**	**105**	**93.75%**	**88.98%**
7.70%	21	1.8983 ± 0.3035	1.3658 ± 0.3063	0.2333 ± 0.1580	0.3702 ± 0.2139	106	94.64%	89.83%
8%	22	1.8983 ± 0.3035	1.3655 ± 0.3049	0.2333 ± 0.1581	0.3699 ± 0.2145	106	94.64%	89.83%
10%	27	1.8983 ± 0.3035	1.3577 ± 0.2991	0.2297 ± 0.1575	0.3648 ± 0.2154	106	94.64%	89.83%
15%	41	1.9068 ± 0.2920	1.3451 ± 0.3055	0.2208 ± 0.1613	0.3518 ± 0.2203	107	95.54%	90.68%
20%	54	1.9237 ± 0.2666	1.3384 ± 0.3106	0.2161 ± 0.1621	0.3459 ± 0.2195	109	97.32%	92.37%
30%	82	1.9322 ± 0.2525	1.3204 ± 0.3052	0.2060 ± 0.1625	0.3314 ± 0.2217	110	98.21%	93.22%
40%	109	1.9407 ± 0.2372	1.3180 ± 0.3158	0.2024 ± 0.1664	0.3249 ± 0.2272	111	99.11%	94.07%
50%	136	1.9322 ± 0.2525	1.3146 ± 0.3273	0.1980 ± 0.1703	0.3171 ± 0.2327	110	98.21%	93.22%
100%	272	1.9492 ± 0.2206	1.2841 ± 0.3277	0.1778 ± 0.1741	0.2858 ± 0.2397	112		94.92%

N_a = Observed number of alleles; N_e = Effective number of alleles[56]; h = Nei's (1973) gene diversity; I = Shannon's Information index[57].

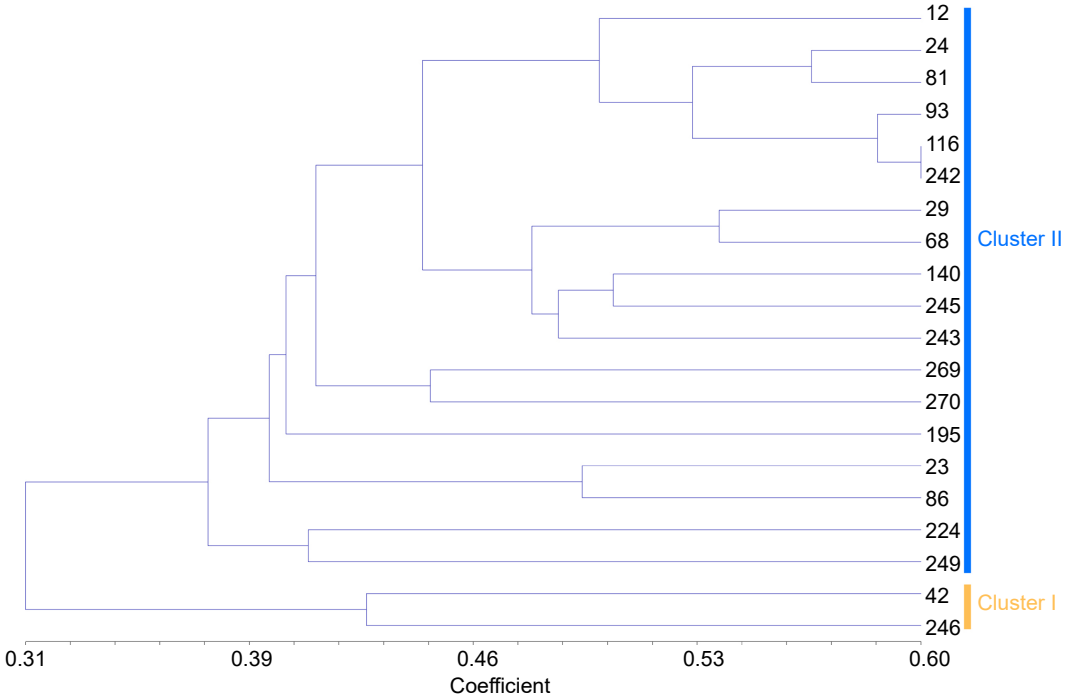

Fig. 5 Cluster diagram based on jaccard by UPGMA analysis calculated from alleles derived from 20 Pueraria accessions of core germplasm. The number represents the code in Supplemental Table S1.

Author contributions

The authors confirm contribution to the paper as follows: study conception and design: Yan H; data collection: Cao S, Zeng W, Wu Z; analysis and interpretation of results: Shi P, Zhou Y, Shang X; draft manuscript preparation: Xiao L, Zhou Y. All authors reviewed the results and approved the final version of the manuscript.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Acknowledgments

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Conflict of interest

The authors declare that they have no conflict of interest.

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