Integrated Phenotypic, Nutritional, and SSR Marker Analyses Reveal Genetic Diversity and Guide Germplasm Utilization in Lotus corniculatus

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Abstract

Lotus corniculatus is a perennial legume valued for its roles in forage production, soil and water conservation, and landscaping. However, the limited number of improved cultivars hampers its broader utilization. In this study, we characterized the genetic variation of 23 germplasm accessions from diverse geographic origins using 12 quantitative traits and 29 simple sequence repeat (SSR) markers. Quantitative trait analysis revealed substantial variation, particularly in plant height (CV = 31.76%), leaf area (30.09%), ether extract (38.53%), and crude fiber (31.79%). Significant correlations were observed between nutritional quality and morphological traits, indicating that phenotypic selection can indirectly improve forage quality. Cluster analysis based on phenotypic and nutritional data grouped the accessions into five categories, identifying germplasms with high crude protein and ether extract (Q1), superior leaf morphology (Q2), and high total sugar content with thick stems (Q5), each offering distinct breeding advantages. Genome-wide SSR mining identified 53,364 loci, dominated by dinucleotide repeats (52.5%), with (AT/AT)□ as the most frequent motif. Twenty-nine highly polymorphic SSR primers generated 299 alleles (mean = 10.17 per locus; PIC = 0.740). SSR-based clustering separated the accessions into three groups broadly aligned with geographic origin. Analysis of molecular variance (AMOVA) indicated that most genetic variation resided within populations, underscoring the potential for intra-population selection. These findings provide a germplasm classification framework that integrates phenotypic performance, nutritional quality, and genetic background, enabling breeders to select complementary parental combinations for developing *L. corniculatus* cultivars with improved yield, quality, and adaptability.

Introduction

Lotus corniculatus is a perennial, allopolyploid, and cross-pollinated legume species (Chen, 2023) widely cultivated for forage, soil and water conservation, and ornamental landscaping (Ünlüsoy et al., 2023). It is characterized by lush vegetative growth, high crude protein and tannin content, and stable nutritional quality across harvest periods (Shen et al., 1992), making it highly palatable to livestock without causing bloating in ruminants (Kostopoulou and Karatassiou, 2017). In addition to strong stress resistance and nitrogen fixation capacity, its extensive root system and creeping growth habit enable adaptation to poor, saline, and acidic soils, thereby preventing erosion and improving soil fertility (Hymes-Fecht et al., 2013; Wang et al., 2022; Zhao et al., 2023). With a long flowering period and abundant floral display, *L. corniculatus* also offers high ornamental value (Hao et al., 2024).

Elite cultivars are the cornerstone for the sustainable utilization of plant genetic resources, and comprehensive germplasm characterization is a prerequisite for targeted breeding programs (Zhao et al., 2024; Zhou et al., 2024). Despite its agronomic potential, the number of forage-type *L. corniculatus* cultivars remains limited, and existing varieties exhibit suboptimal yield and quality performance. Systematic assessments of genetic diversity and phenotypic variation across representative germplasm collections are therefore essential to establish the genetic foundation for cultivar improvement, facilitate the identification of elite parental lines, and enable the design of efficient hybridization strategies.

Forage legume germplasm has undergone long-term adaptation to diverse environments, resulting in rich genetic diversity that underpins population stability and evolutionary potential (Daniel et al., 2020). Traditional assessments using morphological and biochemical markers provide valuable but often environmentally influenced insights (Noohi et al., 2020). With advances in biotechnology, DNA-based molecular markers have become indispensable tools in genetic diversity research owing to their high efficiency, reproducibility, and independence from environmental variation (Ming et al., 2009; Noohi et al., 2020), and have been successfully applied across a wide range of plant species (Garcia-Mas et al., 2000; Luo et al., 2020). Among these, simple sequence repeats (SSRs) are particularly advantageous due to their high polymorphism, co-dominant inheritance, and robust reproducibility, making them suitable for resolving population structure, assessing gene flow, and elucidating genetic relationships (Calderón et al., 2019; Hu et al., 2024). Their broad applicability has been demonstrated in diverse taxa; for instance, SSRs outperformed ISSRs in assessing the genetic diversity of *Pandanus odorifer* (Noohi et al., 2020).

However, for *L. corniculatus*, integrative studies that combine morphological characterization, nutritional quality assessment, and SSR-based molecular analysis across a representative and diverse germplasm panel remain scarce. This gap in knowledge constrains our ability to identify trait–marker associations and to elucidate the genetic architecture underlying key agronomic traits. To address this, we evaluated 23 germplasm accessions of *L. corniculatus* using a combination of phenotypic characterization, nutritional quality analysis, and SSR-based genotyping. Our objectives were to (i) quantify genetic diversity within and among accessions, (ii) assess the relationships between morphological and nutritional traits, and (iii) identify high-performing germplasm with superior yield and forage quality traits. These results are intended to provide both the material basis and the theoretical framework for targeted breeding strategies to develop high-yield, high-quality *L. corniculatus* cultivars.

Materials and Methods

Plant Materials and Trait Assessment

A total of 23 germplasms of *Lotus corniculatus* L. were obtained from the National Medium-Term Forage Germplasm Bank, Inner Mongolia, China (Table 1). All *L. corniculatus* accessions were cultivated in the experimental greenhouse of the Department of Grassland Science, Guizhou University (Guiyang, China; 26°25'N, 106°40'E), which has a subtropical monsoon climate. The determination indexes of *Lotus corniculatus* include phenotypic traits and quality traits.

Phenotypic traits measured included plant height, stem diameter, branch number, and leaf morphology; nutritional quality traits included crude protein (CP), crude fat (EE), crude fiber (CF), dry matter (DM), and total sugar (TS) content. Plant height and stem diameter were recorded using a ruler and vernier caliper, respectively. Leaves were scanned with an Epson Perfection V800 photo scanner, and leaf area, circumference, length, and width were quantified using WinRHIZO software (Regent Instruments Inc., Quebec, Canada). Nutritional quality was determined according to Chinese national standards: CP (GB/T 6432 – 2018), EE (GB/T 6433 – 2006), CF (GB/T 6434 – 2006, filtration method), DM (GB/T 6435 – 1986), and TS (anthrone method).

Table 1
Information of 23 *Lotus corniculatus* germplasms

Code	Collection Number	Species	Country of origin/source
1	00114	*Lotus corniculatus* L.	Wild, China
2	00115	*Lotus corniculatus* L.	Japan
3	00116	*Lotus corniculatus* L.	Guizhou, China
4	00420	*Lotus corniculatus* L.	Canada
5	01549	*Lotus corniculatus* L.	United States
6	01885	*Lotus corniculatus* L.	Canada
7	01886	*Lotus corniculatus* L.	Germany
8	01887	*Lotus corniculatus* L.	New Zealand
9	01888	*Lotus corniculatus* L.	United States
10	03046	*Lotus corniculatus* L.	Institute of Animal Science of CAAS, China
11	04670	*Lotus corniculatus subsp. frondosus Freyn*.	Tarbagatay Prefecture
12	04679	*Lotus corniculatus subsp. frondosus Freyn*	Tarbagatay Prefecture
13	05689	*Lotus corniculatus* L.	Xinjiang, China
14	05690	*Lotus corniculatus* L.	Gansu, China
15	07880	*Lotus corniculatus* L.	Stavropol Krai, Russian
16	08515	*Lotus corniculatus* L.	Meixian County, China
17	08516	*Lotus corniculatus* L.	Zhouzhi County, China
18	08517	*Lotus corniculatus* L.	Taibai County, China
19	08518	*Lotus corniculatus* L.	Fengxiang County, China
20	08519	*Lotus corniculatus* L.	Baoji County, China
21	08520	*Lotus corniculatus* L.	Long County, China
22	08521	*Lotus corniculatus* L.	Qianyang County, China
23	B08	*Lotus corniculatus* L.	Shibing County, China

DNA extraction and primer design

Genomic DNA was extracted from 100 mg of fresh leaf tissue using a Tiangen Plant Genomic DNA Kit (Tiangen Biotech Co., Beijing, China), following the manufacturer's instructions with minor modifications. Briefly, leaf samples were ground in liquid nitrogen, lysed with LP1 buffer and RNase A, followed by LP2 addition and centrifugation. The supernatant was mixed with LP3 buffer, transferred to an adsorption column (CB3), and washed twice. DNA was eluted with TE buffer after incubation at room temperature and quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) with TE buffer as the blank control. Samples with OD260/280 ratios between 1.7 and 1.9 were considered high-quality and

suitable for downstream analysis. DNA integrity was confirmed by 1% agarose gel electrophoresis, which showed bright, intact, and tail-free bands (Fig. S1).

SSR loci were identified from transcriptome sequencing data of a low phosphorus-tolerant accession (01549) and a low phosphorus-sensitive accession (08518) (Zhao et al., 2023). *Lotus japonicus* genome annotations were obtained from http://www.kazusa.or.jp/lotus/summary3.0.html. SSR detection was performed using MISA, and corresponding primers were designed using Primer3 (v1.1.4).

PCR Optimization and Primer Screening

One hundred primer pairs were synthesized (Shanghai Biological Engineering Co., Ltd., China) and optimized via an L16(4^5) orthogonal design to determine the optimal concentrations of Taq DNA polymerase, primers, Mg^{2+}, dNTPs, and template DNA (Table S1, Fig. S2). The optimized PCR system comprised 1.25 U Taq polymerase, 0.4 mM dNTPs, 0.2 μM primers, 3 mM Mg^{2+}, and 60 ng template DNA.

Four germplasms (00114, 00115, 01886, and 08518) with contrasting phenotypes were used for primer screening. PCR products were resolved on 1% agarose gels, and primers generating clear, polymorphic bands were selected. A total of 29 highly polymorphic primer pairs were retained for subsequent analysis (Table S2).

PCR Amplification and Capillary Electrophoresis

The 29 selected primer pairs were re-synthesized with fluorescent labels (FAM, HEX, ROX, and TAMRA) attached to the 5' end of the forward primers, while the reverse primers remained unlabeled (Fig. S3). All primers were synthesized by Shanghai Sangon Biotechnology Co., Ltd.

PCR reactions were carried out using the optimized reaction system described above. The thermal cycling profile consisted of an initial denaturation at 94 ℃ for 4 minutes, followed by 35 cycles of denaturation at 94 ℃ for 30 s, annealing at 58–61 ℃ for 30 s (depending on the primer), and extension at 72 ℃ for 20 s. A final extension was performed at 72°C for 10 min, and reactions were held at 4 ℃ until further analysis. PCR products were analyzed by capillary fluorescence electrophoresis using an ABI 3730XL automated DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

Phenotypic data were analyzed via ANOVA, Coefficients of variation and Shannon–Weaver diversity indices, correlation analysis, and principal component analysis (PCA) in SPSS 20.0, and visualized in Origin 2022. Shannon–Weaver diversity indices (H') were calculated as:

$$H' = -\sum P_i \ln P_i$$

where H' is Gene Diversity Index, P_i is the frequency of the i-th phenotypic category.

SSR allele sizes were scored using GeneMarker HID v2.9.0. Genetic diversity parameters—number of alleles (Na), observed heterozygosity (Ho), expected heterozygosity (He), and Shannon's information index (I)—were computed in GenAlEx 6.51b2. Polymorphic information content (PIC) was calculated in Cervus 3.0. Binary data matrices (presence/absence) were used for similarity coefficient calculation, UPGMA clustering, and genetic relationship analysis in NTSYSpc 2.11.

Results

Genetic variation in major phenotypic traits among L. corniculatus germplasm

A comprehensive statistical analysis of seven key agronomic traits across 23 *L. corniculatus* germplasms from diverse geographic origins revealed substantial phenotypic variability (Table 2; Table S3). Among the evaluated traits, plant height exhibited the highest coefficient of variation (CV = 31.76%), followed closely by leaf area (CV = 30.09%) and branch number (CV = 27.65%), indicating considerable morphological divergence among accessions. In contrast, leaf circumference (CV = 18.80%), leaf length (CV = 18.89%), and leaf width (CV = 18.50%) showed comparatively lower variability, suggesting more conserved leaf morphological proportions.

Table 2
Genetic variation in major morphological agronomic traits of *Lotus corniculatus*

Trait	Max	Min	Average	SD	CV(%)	GDI
Leaf area (cm^2)	3.33	0.60	1.77	0.53	30.09	1.13
Leaf circumference (cm)	13.04	5.12	9.24	1.74	18.80	1.07
Leaf length (cm)	2.14	0.84	1.54	0.29	18.89	1.06
Leaf width (cm)	3.22	1.21	2.22	0.41	18.50	1.11
Stem diameter (mm)	1.38	0.41	0.69	0.17	24.83	1.01
Number of branches	7.00	3.00	4.91	1.36	27.65	1.03
Plant height (cm)	23.01	4.45	11.92	3.78	31.76	1.05
Note: SD, Standard Deviation; CV, Coefficient of Variation; GDI, Gene Diversity Index.						

Across germplasms, the mean plant height was 11.92 cm, ranging from 6.403 cm in accession 04679 to 18.963 cm in accession 08516. Leaf area averaged 1.77 cm^2, with accession 01887 showing the largest mean leaf area (2.860 cm^2) and accession 03046 the smallest (0.617 cm^2). The number of branches varied between 3.333 in accession 01888 and 6.333 in accession 03046, while stem diameter ranged from 0.457 mm in accession 03046 to 1.117 mm in accession 08519. These findings highlight the presence of both high-performing and low-performing germplasms in specific traits, providing valuable material for targeted breeding programs.

Genetic variation in major nutritional quality traits among L. corniculatus germplasm

Analysis of five key nutritional quality traits revealed pronounced variation among the 23 *L. corniculatus* germplasms (Table 3; Table S4). Across traits, ether extract (EE) exhibited the greatest variability (CV = 38.53%), followed by crude fiber (CF, CV = 31.79%), total sugar (TS, CV = 14.81%), crude protein (CP, CV = 10.21%), and dry matter (DM, CV = 8.15%).

The mean EE content was 3.019%, with the highest values observed in accessions 08518 and 01887, whereas accession 08515, had the lowest EE content. CF content averaged 15.238%, ranging from a maximum in accession 08519 to minima in 01887 and 00116. CP content averaged 242.462 g/kg, with accession 01886 showing the highest value; the lowest CP levels occurred in accessions 08521and 03046. DM content averaged 22.348%, with accession 08519 showing the highest proportion, while 08515 had the lowest. TS content averaged 15.801%, peaking in accession 08517 and reaching its minimum in 00116.

These patterns indicate that certain germplasms, such as 01886, 08517, and 08518, exhibit superior nutritional profiles in specific traits, making them valuable candidates for targeted breeding programs aimed at enhancing forage quality.

Table 3
Genetic variation in key quality traits of *Lotus corniculatus*

Trait	Max	Min	Average	SD	CV(%)	GDI
Ether extract(EE, %)	5.11	0.38	3.019	1.15	38.53	1.12
Crude fiber (CF, %)	30.47	10.21	15.238	4.84	31.79	0.75
Crude protein (CP, g/kg)	28.74	19.38	242.462	2.49	10.21	1.06
Dry matter (DM, %)	26.67	18.10	22.348	1.81	8.15	1.11
Total sugar (%)	22.18	11.86	15.801	2.34	14.81	1.04
Note: SD, Standard Deviation; CV, Coefficient of Variation; GDI, Gene Diversity Index.						

Principal component analysis based on quantitative traits

Correlation analysis among the 12 quantitative traits of the 23 *L. corniculatus* germplasms revealed several significant relationships (Fig. 1). The four leaf morphological traits—leaf circumference, leaf length, leaf width, and leaf area—were highly and positively correlated with each other. Plant height exhibited significant or highly significant positive correlations with leaf area, leaf circumference, leaf width, and stem thickness, but a significant negative correlation with branch number. Ether extract (EE) content was highly and negatively correlated with stem thickness. Crude protein (CP) showed a highly significant positive correlation with crude fiber (CF) and significant negative correlations with leaf width and stem thickness. Dry matter (DM) content was significantly negatively correlated with all four leaf morphological traits but positively correlated with CP. Total sugar (TS) content was significantly positively correlated with leaf area and leaf width, and significantly negatively correlated with CF, CP, and DM.

Principal component analysis (PCA) further indicated that all phenotypic and nutritional quality traits could be condensed into four principal components, collectively explaining 79.002% of the total variance (Table 4). PC1, representing a leaf morphology factor, was driven by leaf area, circumference, length, and width; PC2, associated with biological yield and nutritional quality, was dominated by DM and CP; PC3, representing a digestive limitation factor, was determined by CF and stem thickness; and PC4, corresponding to a yield composition factor, was mainly influenced by branch number. These patterns suggest that phenotypic variation in *L. corniculatus* is organized around distinct functional trait groups, including morphological, nutritional, and structural characteristics, which may be jointly targeted in breeding programs to optimize both forage quality and yield potential.

Table 4
Principal component analysis of quantitative traits in *Lotus corniculatus* germplasms

Trait	PC1	PC2	PC3	PC4
Leaf area	0.208	0.100	0.021	0.071
Leaf circumference	0.206	0.109	0.102	0.103
Leaf length	0.200	0.125	0.065	0.099
Leaf width	0.209	0.065	0.055	0.114
Stem thickness	-0.004	-0.241	0.399	0.167
Number of branches	-0.021	0.147	-0.143	0.660
Plant height	0.085	-0.144	0.293	-0.356
Ether extract(EE)	0.010	0.223	-0.346	-0.336
Crude fiber (CF)	-0.031	0.272	0.398	-0.093
Crude protein(CP)	0.099	-0.286	-0.221	0.150
Dry matter(DM)	-0.087	0.294	0.106	-0.068
Total sugar	-0.153	0.047	0.188	0.362
Eigenvalues	4.566	2.293	1.521	1.101
Contribution rate(%)	38.046	19.106	12.677	9.173
Cumulative contribution rate(%)	38.046	57.153	69.830	79.002

Genetic cluster analysis of L. corniculatus germplasm based on quantitative traits

UPGMA clustering based on seven morphological traits (plant height, leaf area, leaf circumference, leaf length, leaf width, stem diameter, and branch number) and five nutritional quality traits (ether extract, crude fiber, crude protein, dry matter, and total sugar) resolved the 23 *L. corniculatus* germplasms from diverse geographic origins into five distinct groups (Q1–Q5) at a dissimilarity coefficient of 10 (Fig. 2).

Cluster Q1 comprised 15 accessions, representing the largest group and encompassing germplasms with intermediate morphological and nutritional profiles. Q2 contained two accessions—08517 and 01887—characterized by the largest leaf area and circumference, coupled with the lowest CF content, making them promising candidates for improving forage nutritional quality. Q3 consisted solely of 08520, which exhibited high CF content, low CP content, and high DM content, indicating a need for targeted improvement in nutritional composition. Q4 included 08521, 00114, and 03046, displaying comparatively weaker leaf morphology and nutritional traits but with higher branch numbers, suggesting potential for yield enhancement through branching. Finally, Q5 grouped 04670 and 08519, which shared high TS content and greater stem thickness, traits that could contribute to improved energy content and structural robustness in forage.

Distribution of SSRs in the L. corniculatus genome

A total of 53,364 SSR loci were identified using the MISA software, with repeat motif lengths ranging from di- to hexa-nucleotides. Di-nucleotide repeats were the most abundant, comprising 28,063 loci (52.52%), followed by tri-nucleotide (29.42%) and tetra-nucleotide repeats (7.25%) (Fig. 3A). Among di-nucleotide motifs, (AT/AT)n was the most prevalent, representing 53.18% of all di-nucleotide repeats. For tri-nucleotide motif, (AAG/CTT)n was the dominant type (27.02%), whereas (AAAT/ATTT)n was the most frequent among tetra-nucleotide repeats, accounting for 41.24% of that category.

After removing mononucleotide repeats, the abundance of SSR loci showed a clear inverse relationship with repeat motif length, with longer motifs occurring less frequently. Additionally, PCR amplification artifacts, such as the propensity of Taq polymerase to add adenine residues to 3' termini, occasionally affected the discrimination of single-nucleotide differences in capillary electrophoresis profiles (Fig. 3B).

Genetic diversity analysis of SSR markers in L. corniculatus

To assess the molecular diversity of *L. corniculatus*, 69 accessions from 23 germplasm resources were genotyped using 29 highly polymorphic SSR loci (Table 5). A total of 299 alleles were detected, with an average of 10.31 alleles per locus, reflecting substantial allelic richness within the collection. Allele numbers varied markedly across loci, from only 2 at chr0_18003 to 24 at chr0_15876. The effective number of alleles (Ne) showed a similar pattern, averaging 10.168 and ranging from 2.471 (chr0_18003) to 23.534 (chr0_15876). The Shannon's information index (I) varied from 0.500 to 5.160 (mean = 3.167), indicating a high level of genetic complexity. Observed heterozygosity (Ho) ranged between 0 and 1.900 (mean = 1.064), while expected heterozygosity (He) ranged from 0.320 to 1.829 (mean = 1.425), suggesting moderate-to-high heterogeneity across loci.

Polymorphism information content (PIC) values spanned from 0.178 to 0.934, with a mean of 0.740, confirming that the selected SSR markers possess high discriminatory power for genetic diversity assessment. Notably, chr0_15876 exhibited the highest allele number, Ne, and PIC, making it a particularly informative locus for population genetic studies. These results collectively highlight the broad genetic base of the tested *L. corniculatus* germplasm and provide robust molecular tools for future breeding, conservation, and association mapping efforts.

Table 5
Summary of diversity statistics based on 29 SSR markers in 69 samples from 23 *Lotus corniculatus*

Primer name	Number of alleles(Na)	Effective number of alleles(Ne)	Shannon Diversity Information Index(I)	Observation of heterozygosity(Ho)	Expected heterozygosity(He)	Polymorphism Information Content(PIC)
chr0_14344	16	18.418	4.603	0.929	1.775	0.912
chr5_724	21	15.500	4.671	1.608	1.742	0.871
chr5_3797	21	19.379	4.816	1.324	1.790	0.908
chr0_17962	11	14.091	3.946	1.900	1.690	0.878
chr0_17429	7	6.950	2.626	0.667	1.383	0.751
chr0_15414	15	9.499	3.788	1.518	1.572	0.822
chr0_4872	17	17.728	4.638	1.571	1.773	0.904
chr2_4161	12	11.409	3.629	0.582	1.608	0.865
chr0_15876	24	23.534	5.160	1.857	1.829	0.934
chr3_2976	9	8.575	3.304	1.800	1.522	0.752
chr0_7339	18	18.093	4.573	1.600	1.779	0.908
chr0_18003	2	2.471	0.500	0.000	0.320	0.178
chr4_868	3	4.488	1.674	0.286	1.094	0.486
chr5_3453	16	16.052	4.533	1.590	1.748	0.875
chr0_9069	9	10.777	3.638	1.429	1.617	0.808
chr3_3963	2	2.600	0.562	0.000	0.375	0.365
chr0_6664	9	10.542	3.550	1.750	1.620	0.810
chr6_1459	4	4.119	1.666	0.286	1.028	0.511
chr0_14998	8	8.609	3.207	1.714	1.528	0.750
chr4_4327	7	10.003	3.427	1.786	1.597	0.780
chr5_2279	9	7.092	2.938	0.736	1.429	0.755
chr6_130	4	4.712	1.955	1.467	1.150	0.523
chr4_2109	6	4.375	1.767	0.881	0.877	0.368
chr1_2455	11	9.067	3.357	0.686	1.556	0.859
chr5_3017	6	6.013	2.553	1.095	1.334	0.697
chr1_83	14	12.00	3.699	0.833	1.653	0.907
chr0_12234	5	5.133	1.916	0.200	1.144	0.701
chr4_2393	6	6.509	2.543	0.250	1.378	0.789
chr5_3457	7	7.121	2.590	0.500	1.401	0.798

Population structure of L. corniculatus based on SSR markers

The population genetic structure of *L. corniculatus* germplasm was inferred using 299 allelic loci generated from 29 highly polymorphic SSR markers. Genetic similarity coefficients, calculated with NTSYSpc 2.11, ranged from 0.276 to 0.983, with an average of 0.773, indicating a broad spectrum of genetic relatedness among accessions. UPGMA cluster analysis yielded a high cophenetic correlation coefficient (r = 0.98355), confirming the robustness of the clustering results. As illustrated in Fig. 4, at a genetic similarity threshold of 0.65, the 23 germplasms were resolved into three major clusters (Class I–III). Class I and Class III each comprised a single, genetically distinct accession (00114 and 01886, respectively), suggesting unique genetic backgrounds. Class II contained the majority of accessions (n = 21) and represented the core genetic group within the panel. Further subdivision of Class II at a similarity coefficient of 0.85 revealed two subgroups corresponding to geographical origin: group a, consisting primarily of Chinese accessions, and group b, containing accessions from other countries. This geographic stratification indicates a degree of regional differentiation within *L. corniculatus* germplasm, which may reflect historical selection pressures, adaptation to local environments, or breeding history.

Principal coordinate analysis (PCoA) based on SSR marker data was performed in GenAlEx 6.51b2 to visualize patterns of genetic differentiation among the 69 *L. corniculatus* accessions. The first three principal coordinates explained 25.65%, 14.42%, and 11.88% of the total genetic variation, respectively, accounting for a cumulative variance of 51.95% (Table 6). In the PCoA plot (Fig. 5), each point represents an individual accession, and points of the same color indicate samples belonging to the same group identified by UPGMA clustering. Spatial proximity among points reflects genetic similarity, whereas greater separation indicates stronger genetic divergence. Notably, most group a accessions (domestic germplasm) clustered predominantly in the fourth quadrant, whereas group b accessions (foreign germplasm) were distributed mainly across the second and third quadrants, highlighting marked genetic differentiation between Chinese and non-Chinese germplasm.

Table 6
Contribution rates of principal components derived from SSR marker analysis in *Lotus corniculatus*

Ingredients	PC1	PC2	PC3
Contribution rate	25.65	14.42	11.88
Cumulative contribution rate	25.65	40.07	51.95

Analysis of molecular variance (AMOVA) further quantified the genetic partitioning, revealing that 56% of the total variation resided within germplasm, while 44% was attributable to among-germplasm differences (Table 7).

Table 7
Analysis of molecular variance (AMOVA) within the *Lotus corniculatus* germplasm population

Source of variation	df	Sum of squares	Variance components	Percentage of variation(%)	*P* Value
Among germplasms	22	340.804	4.962	44	<0.001
Within germplasms	46	145.000	6.304	56	<0.001
Total	68	485.804	11.266	100	

Discussion

Genetic diversity of phenotypic and nutritional quality traits in L. corniculatus germplasm

Genetic diversity within germplasm resources constitutes a critical reservoir for the improvement of agronomic traits, enabling the introduction of favorable alleles to enhance yield, quality, and stress resistance through hybridization or introgression lines (Angessa et al., 2016). In the present study, coefficients of variation (CV) for phenotypic traits revealed

substantial heterogeneity, with plant height and leaf area exhibiting the highest variability (31.76% and 30.09%, respectively), followed by branch number, stem diameter, and leaf morphology traits. Such levels of variation are indicative of broad selection potential, particularly for traits directly linked to forage biomass and harvest index. Stems and leaves are central determinants of forage yield, and previous studies have highlighted the significance of plant height, stem thickness, leaf area, and branching density in determining productivity in *L. corniculatus* and related forage legumes (Zhang et al., 2024). Accessions such as 08516 and 01887, which exhibit superior plant height and leaf area, respectively, represent promising parental candidates for breeding programs targeting these yield-associated traits.

Nutritional quality traits displayed equally notable diversity, particularly ether extract (EE) and crude fiber (CF), which recorded CV values of 38.53% and 31.79%, respectively. EE is not only a concentrated energy source but also provides essential fatty acids such as linoleic, α-linolenic, and arachidonic acids that are critical for growth, reproduction, and immune function in livestock (Valentina et al., 2016; Jančík et al., 2022). However, excessive EE (> 5% of dry matter) can impair rumen microbial activity and digestive efficiency in ruminants (Vahmani et al., 2020), underscoring the importance of balanced selection. In contrast, high CF can limit digestibility, making the concurrent increase of crude protein (CP) and reduction of CF a key breeding objective for enhancing forage nutritional value (Powell et al., 2003). In this context, 08518 and 01887 emerged as elite germplasm with high EE and low CF, while 01886 possessed the highest CP, making these accessions valuable for quality-oriented improvement.

Correlation analyses revealed significant associations between phenotypic and nutritional traits, consistent with findings in wild oats (*Avena fatua*) by Onyśk and Boczkowska (2017). For example, CP was negatively correlated with leaf width and stem diameter, suggesting possible trade-offs between structural and biochemical traits. Such correlations imply that unidirectional selection for a single trait may inadvertently affect others, reinforcing the necessity of multi-trait selection strategies in *L. corniculatus* breeding.

Principal component analysis (PCA), as a multivariate statistical tool, offers an effective means of integrating complex phenotypic and nutritional data, thereby enhancing the precision of parent selection in multi-objective breeding programs (Liu et al., 2022). In this study, PCA revealed that the measured traits could be grouped into four distinct functional dimensions: leaf morphology, biological yield and nutritional quality, digestive constraints, and yield composition. This dimensionality not only clarifies the underlying structure of trait variation but also provides a practical framework for matching complementary parental types in breeding plans. Compared with earlier findings (Drobná, 2010), which emphasized stem and internode traits, the present results highlight a stronger influence of leaf-related and nutritional factors, likely due to the broader geographical origins and higher genetic diversity of the germplasm set. These insights reinforce the need for breeding objectives that simultaneously address both morphological and nutritional targets, ensuring balanced improvements in yield potential, feed quality, and adaptability.

The phenotypic clustering of *L. corniculatus* germplasm revealed distinct groupings based on combinations of morphological and nutritional quality traits, yet the substantial within-group heterogeneity highlights that cluster membership does not necessarily equate to uniform breeding potential. This suggests that phenotypic clustering should serve as an initial germplasm organization tool, with final selection guided by trait-specific performance and genetic background. Q1, the largest group (15 accessions), encompassed both high-CP/low-CF accessions suitable for improving forage quality and morphologically superior but nutritionally average types, indicating its value as a broad donor pool rather than a coherent breeding block. Q2 demonstrated the pitfalls of interpreting cluster means in breeding contexts: while both members grouped together, 01887 combined the largest leaf area with the lowest CF, whereas 08517 exhibited higher CF and average leaf morphology, underscoring the need for accession-level evaluation within clusters. Q3 (08520) exemplified a biomass–digestibility trade-off, with high dry matter and CF but low CP, while Q4's consistently high branch number suggests potential for yield enhancement through increased tiller density when integrated with high-nutrition backgrounds. Q5's elevated total sugar and stem thickness could contribute to energy-rich forage but may require balancing against digestibility constraints. Similar patterns of within-group variability have been reported in *Medicago*

sativa and *Coffea arabica*, where phenotypic clustering captured broad adaptation types but failed to fully resolve functional trait variation critical for breeding (Annicchiarico et al., 2015; Andini et al., 2025). Therefore, phenotypic grouping should be combined with molecular data to identify complementary crosses and avoid overlooking high-value outliers.

Genetic diversity of phenotypic traits in L. corniculatus germplasm revealed by SSR markers

Molecular markers, particularly Simple Sequence Repeats (SSRs), have proven to be indispensable tools in the characterization of genetic diversity across a wide range of plant species (Yin et al., 2023). Due to their codominant inheritance, high polymorphism, and genome-wide distribution, SSRs are especially well suited for applications such as genetic diversity assessment, QTL mapping, DNA fingerprinting, and marker-assisted selection (Misiukevičius et al., 2023; Tyagi et al., 2021). Over the past decade, their utility has been demonstrated in both model and non-model species. For example, Carvalho et al. (2020) employed SSR markers to assess the genetic diversity of common bean (Phaseolus vulgaris L.), identifying 172 allelic variants, while Hemasai et al. (2024) characterized 13 miRNA-SSR markers and their corresponding target gene SSRs derived from yield-responsive miRNAs in rice

In the present study, analysis with GeneMarker HID V2.9.0 revealed a total of 299 alleles across 29 SSR loci, with an average of 10.310 alleles per locus—substantially exceeding the 4.8 alleles per locus reported in white clover (*Trifolium repens* L.) by Kölliker et al. (2001). The polymorphism information content (PIC) ranged from 0.178 to 0.934, with a mean of 0.740, which is notably higher than the values reported for *L. corniculatus* by Daudi et al. (2021) (0.34) and Belalia et al. (2019) (0.575). While PIC variation can be influenced by the marker set and genotypes under investigation, the consistently high PIC values observed here suggest substantial allelic richness and differentiation, indicating that the tested germplasm harbors broad genetic variation suitable for breeding improvement.

Furthermore, the average effective number of alleles (Ne) was 10.168, with a total Ne of 294.859, reflecting a high degree of allelic evenness. Diversity indices also supported this conclusion: the mean Shannon information index (I) was 3.167, and observed (Ho) and expected heterozygosities (He) averaged 1.064 and 1.521, respectively. These values exceed those reported in analogous SSR-based studies of *Spinacia oleracea* (Göl et al., 2017), *Papaver somniferum* (György et al., 2022), and guava (*Psidium guajava*) (Ma et al., 2020), further reinforcing the premise that *L. corniculatus* germplasm exhibits a higher-than-average reservoir of genetic diversity. Such diversity not only reflects the wide geographical origins of the accessions but also signals a rich pool of alleles that can be strategically combined to enhance both yield and forage quality.

Cluster analysis remains a cornerstone method for elucidating genetic relationships among crop germplasm resources (Bakayoko et al., 2021; Souza et al., 2023; Chikh-Rouhou et al., 2021). In the present study, UPGMA clustering based on SSR data at a genetic similarity coefficient threshold of 0.65 partitioned the 23 *L. corniculatus* accessions into three major groups (Groups I–III). Within Group II, a clear subdivision was observed that largely corresponded to geographical origin: subgroup a comprised predominantly Chinese germplasm, whereas subgroup b was enriched in accessions from other countries. This geographic pattern is consistent with the separation trends detected by principal coordinate analysis (PCoA), suggesting that domestically adapted *L. corniculatus* lines in China have diverged from foreign counterparts through prolonged selection under distinct agroecological conditions.

When compared with the clustering based on quantitative phenotypic traits, both approaches could broadly distinguish germplasm within China from that outside China, yet the detailed grouping patterns were not entirely congruent. Certain accessions that were phenotypically aligned with germplasm within China clustered genetically with foreign accessions, and vice versa. Such inconsistencies have been reported in other species, including *Camellia oleifera* (Zhu et al., 2022) and Indian Mustard (Sharma et al., 2022), and may arise from several factors: (i) phenotypic traits are often under polygenic control and strongly influenced by environmental conditions; (ii) SSR markers capture genome-wide polymorphisms, including variation in non-coding regions, which may not directly translate into morphological differences.

These factors together highlight the complementary value of integrating phenotypic and molecular data in germplasm evaluation.

From a breeding perspective, this integrated clustering framework serves as a roadmap for parental selection: genetically divergent accessions with complementary phenotypic and nutritional traits can be used to maximize heterosis and generate novel trait combinations, whereas genetically similar accessions with stable performance can be employed to fix desirable traits in uniform cultivars. Such a combined strategy is particularly pertinent to *L. corniculatus*, where the simultaneous improvement of yield, nutritional quality, and environmental adaptation remains a core breeding objective.

Molecular analysis of variance (AMOVA) indicated that most of the genetic variation in *L. corniculatus* occurs within populations rather than between them, a pattern also reported for other predominantly outcrossing forage legumes such as alfalfa and white clover (Annicchiarico et al., 2020; Kölliker et al., 2001). This high intra-population diversity is likely a consequence of its predominantly outcrossing reproductive system, which promotes heterozygosity and maintains a broad genetic base through pollen-mediated gene flow. The relatively low genetic differentiation among populations may reflect both historical and ongoing germplasm exchange across regions, facilitated by formal breeding programs and informal farmer-to-farmer seed exchange (Loskutov et al., 2011; Ustariz et al., 2022). For breeders, this means that a large proportion of exploitable genetic variability is accessible within existing local germplasm, reducing reliance solely on exotic introductions. Nonetheless, targeted crosses between phenotypically divergent and genetically distinct accessions remain the most effective route to expand adaptive potential and achieve multi-trait improvement.

Conclusions

By integrating phenotypic, nutritional, and SSR marker data, we comprehensively assessed the genetic diversity of 23 *Lotus corniculatus* germplasm accessions. The large variation in key agronomic and nutritional traits, together with high SSR polymorphism (PIC = 0.740), revealed substantial diversity at both morphological and molecular levels. Differences between phenotypic and molecular clustering underscored the value of multi-dimensional analyses for germplasm characterization. Importantly, several accessions combined superior forage quality with distinct genetic backgrounds, making them promising parents for breeding programs targeting yield, quality, and adaptability. These findings provide a practical framework for germplasm classification and the strategic selection of complementary parental combinations, thereby facilitating the genetic improvement and sustainable utilization of *L. corniculatus*.

Declarations

Acknowledgments

Not applicable.

Author contribution statement

LLZ and PCW conceived and designed research. WWZ and YW conducted experiments. LLZ, WWZ and JYZ analysed the data. LLZ, JYZ and PCW wrote the manuscript. All authors read and approved the manuscript.

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Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

There are no ethical issues involved in this paper.

Consent for publication

The manuscript is approved by all authors for publication.

Availability of data and materials

All data supporting the findings of this study are available within the paper and its supplementary information. simple sequence repeat (SSR) primer sequences are provided in supplementary Table 2.

Competing interests

The authors declare no competing interests.

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Figures

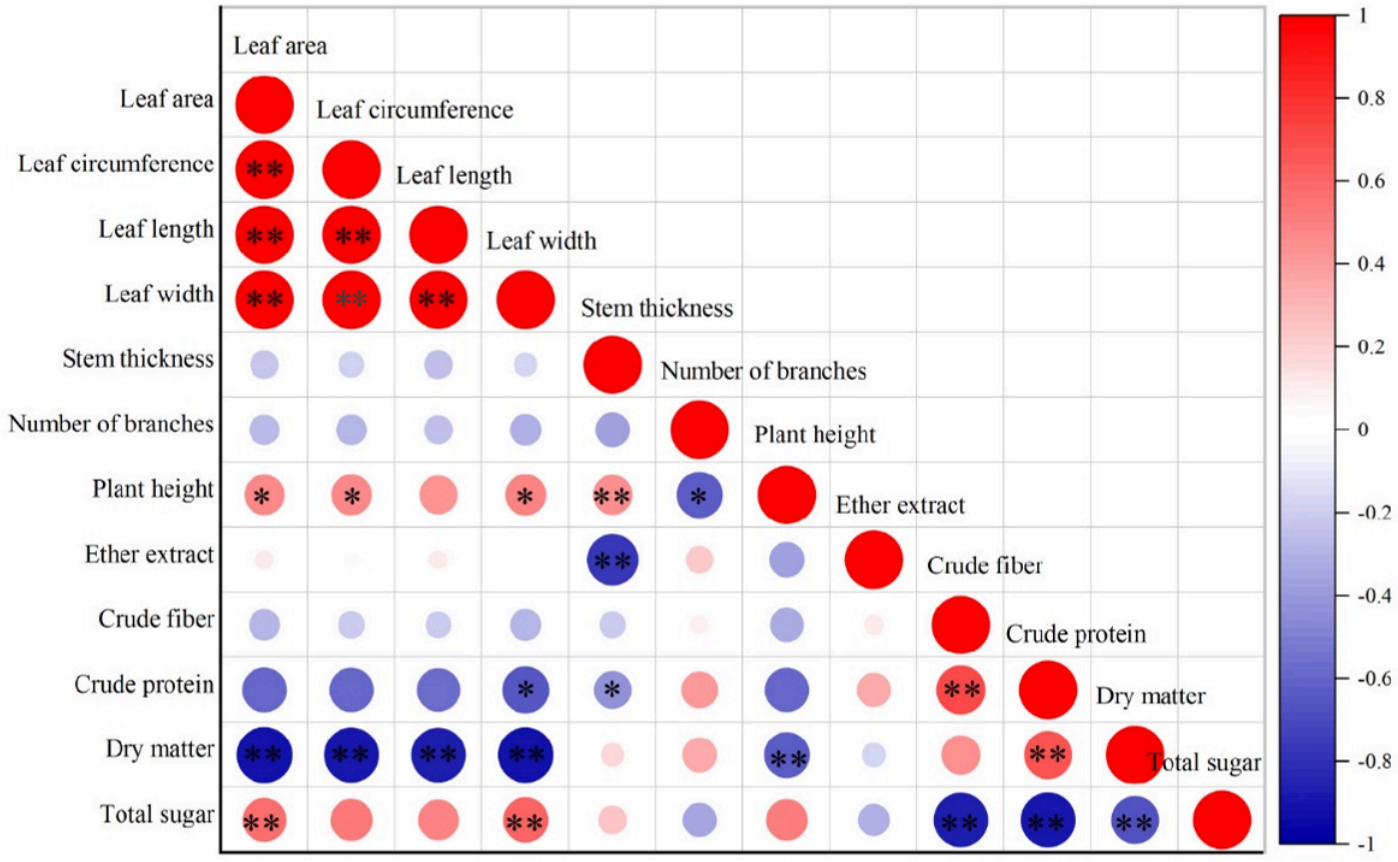

Figure 1

Correlation matrix of agronomic traits in *Lotus corniculatus* germplasm resources
Note: P < 0.01 (**), *P* < 0.05 (*).

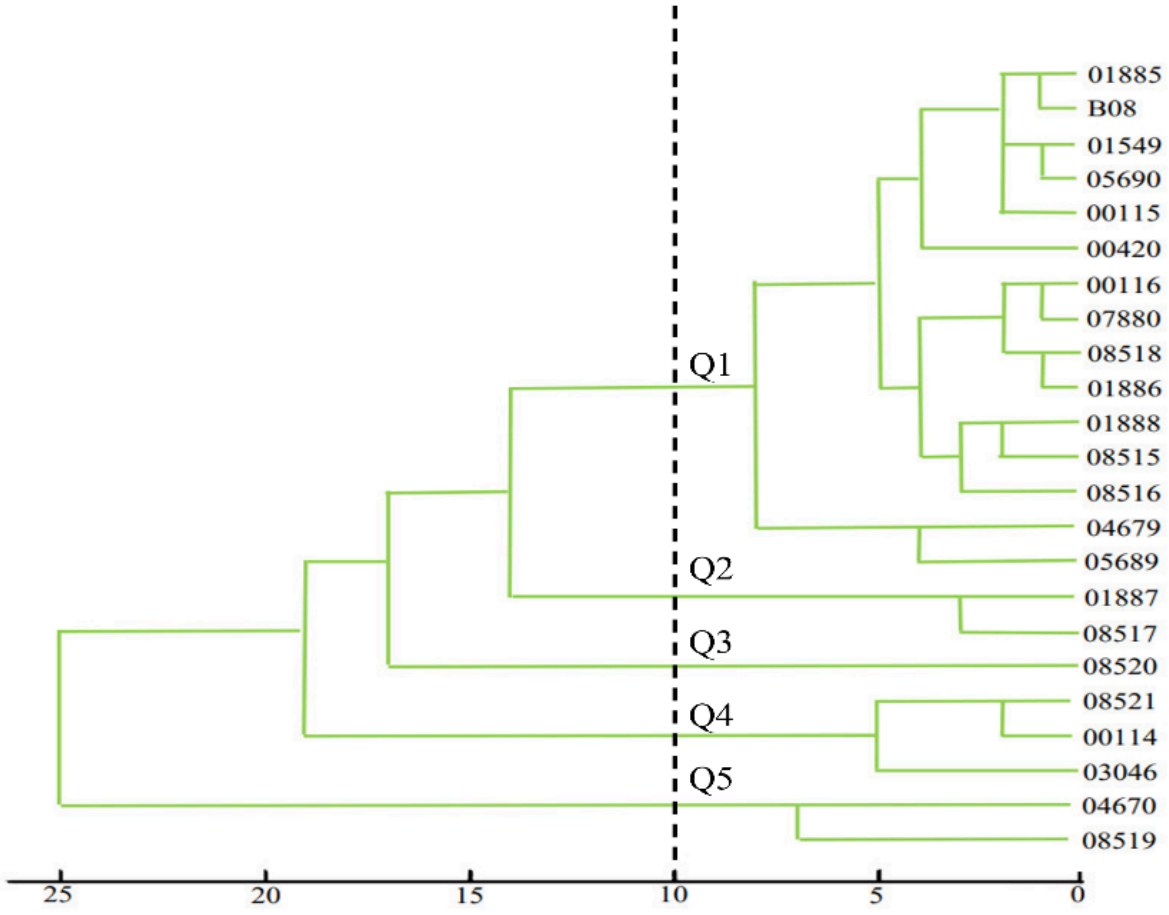

Figure 2

Cluster dendrogram of 23 *Lotus corniculatus*germplasms based on quantitative trait data

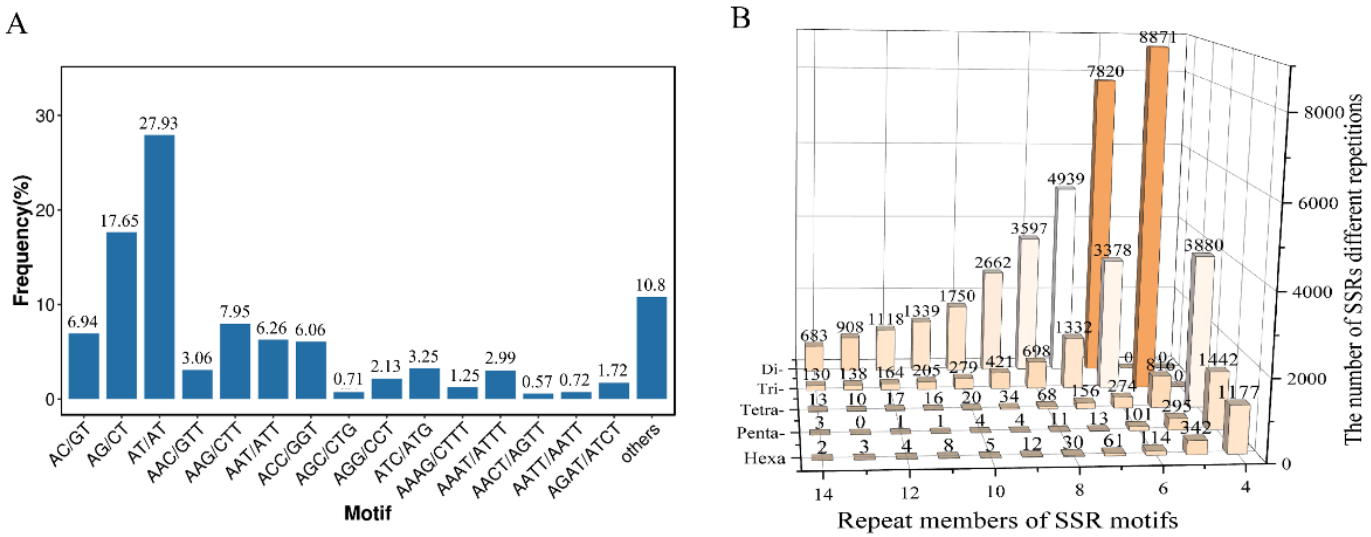

Figure 3

(A) The percentage of different SSR repeat motifs in *L. corniculatus*; (B) The distribution of repeat motif occurrences in SSR loci within the genome.

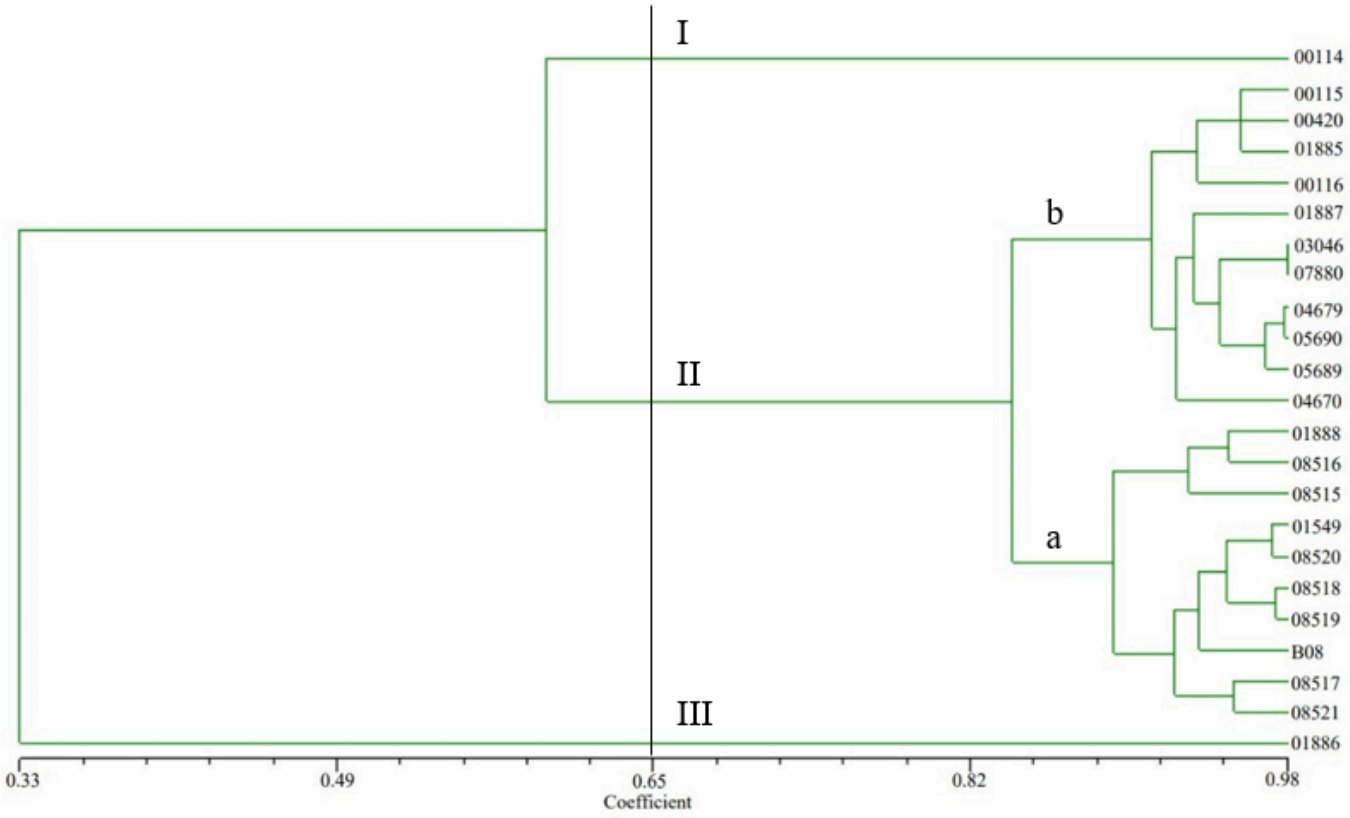

Figure 4

Cluster analysis of 23 *Lotus corniculatus*germplasms based on SSR marker profiles

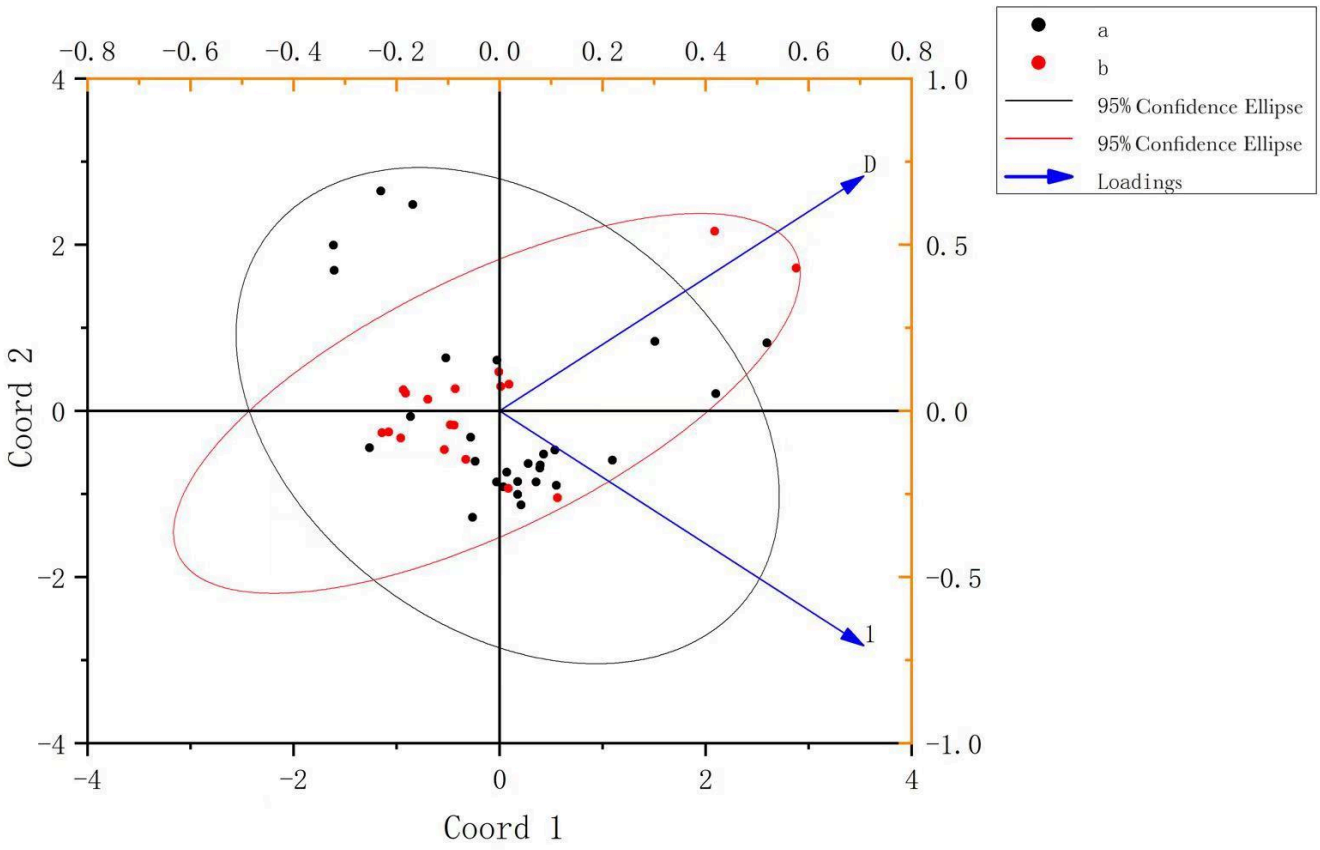

Figure 5

Principal coordinates analysis (PCoA) of 23 *Lotus corniculatus* germplasms based on SSR marker data

Supplementary Files

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