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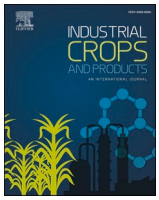

Integrative evaluation of traits and metabolomics in germplasm resources of the high-calcium crop *Primulina eburnea*

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ABSTRACT

Germplasm evaluation is the first step in the utilization and domestication of wild plant resources. Despite the increasing recognition of *Primulina eburnea* as a valuable plant-based resource for industrial calcium supplement production, systematic evaluation of its germplasm resources remains limited. This study assessed the diversity of 37 wild *P. eburnea* populations through common-garden experiments, analyzing 26 quality-related traits. Significant variation was found in phenotypic and nutrient traits, with mean coefficients of variation above 20 %, indicating potential for improvement. Phenotypic differentiation coefficient analysis showed that 54.52 % of the total variation was attributable to differences among populations. Principal component analysis and cluster analysis classified the populations into two distinct groups, corresponding to their eastern and western geographical distributions. Correlation analysis identified 95 significant trait relationships, enabling efficient germplasm selection. Environmental factor analysis showed that geographic isolation, which leads to differences in light, rainfall, and temperature, was a key driver of trait differentiation. The membership function models, outperforming PCA in balancing trait selection and genetic diversity, was used to establish a core germplasm bank of 13 populations. Metabolomic profiling further revealed significant differences between eastern and western populations, particularly in flavonoids and phenolic acids, which are potentially linked to bioactivity and medicinal properties. These findings support the robustness of the evaluation model and the reliability of the established core germplasm collection. Overall, these results provide a solid evaluation framework and a reliable core germplasm collection, supporting breeding, large-scale cultivation, and industrial development of *P. eburnea* as a high-calcium crop.

1. Introduction

Calcium is essential for human health, playing key roles in bone formation, nerve conduction, muscle contraction, and metabolic regulation. (Balk et al., 2017; Brini et al., 2013; Cormick and Belizán, 2019; Gao et al., 2005). Despite its importance, calcium deficiency remains a global concern, with average daily intake far below the Food and Agriculture Organization of the United Nations (FAO) recommended 800–1300 mg in many regions, especially developing countries. (Balk et al., 2017; Kranz et al., 2007; Williams et al., 2023). Calcium supplements play a key role in addressing dietary calcium deficiencies, but their high cost and limited accessibility remain major constraints. (Williams et al., 2023). Moreover, most commercial supplements are derived from inorganic or animal sources, which often show low

Abbreviation: HPLC, high performance liquid chromatography; MS/MS, tandem mass spectrometry; MS, mass spectrometry; ICP, inductively coupled plasma; MRM, multiple reaction monitoring; QQQ, triple quadrupole; RDA, redundancy analysis; PCA, principal component analysis; VIP, variable importance in projection scores; KEGG, Kyoto Encyclopedia of Genes and Genomes; AAT, annual average temperature; Lon, longitude; Lat, latitude; AAMinT, annual average minimum temperature; AAMaxT, annual average maximum temperature; AAR, annual average rainfall; ATSR, annual total solar radiation; ASD, annual sunshine duration; Con, soil electrical conductivity; SWCa, soil water-soluble calcium content.

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bioavailability and may cause adverse urinary effects (Kozyrakis et al., 2017). In contrast, plant-derived calcium typically occurs in organic forms, sometimes co-formulated with vitamins, supporting enhanced intestinal absorption. Previous studies indicate that plant-based calcium provides higher bioavailability, benefits bone health, and reduces kidney-stone risk (Craig et al., 2021; Zhang et al., 2021; Zarantonello and Brunori, 2023). In this context, plant-based calcium sources offer a promising solution for developing next-generation industrial calcium supplements.

Primulina, the largest genus within the Gesneriaceae family, is predominantly distributed in karst regions. (Xu et al., 2021). The calcium-rich limestone soils of these habitats have driven the evolution of specialized calcium adaptation mechanisms in many *Primulina* species. Many *Primulina* species are calciphilous plants, renowned for their remarkable calcium accumulation capacity (Hao et al., 2015; Qi et al., 2013). *P. eburnea*, a representative species of the genus, exhibits unique molecular adaptations to high-calcium environments (Yang et al., 2024). Unlike most plants, which predominantly accumulate inorganic calcium or calcium oxalate, *P. eburnea* accumulates higher proportions of bioavailable calcium forms such as water-soluble calcium and chelated calcium, which demonstrate superior absorption efficiency in humans (Shkembi and Huppertz, 2021; Zhang and Liu, 2022). This distinctive feature establishes *P. eburnea* as an exceptional plant-based calcium source. In addition to its nutritional value, *P. eburnea* has long been used in traditional medicine in southwestern China, where it is known as “Yan Bai Cai”. It is used to clear heat, detoxify, treat tuberculosis, relieve cough, replenish deficiency, and stop bleeding (Fang et al., 1986; Luo and Sun, 2013; Nanjing University of Chinese Medicine, 2006). Modern studies have confirmed its antimicrobial, antihypertensive, antioxidant, and antitumor activities, with active compounds including phenylethanoid glycosides, triterpenoids, and flavonoids (Chen et al., 2010a; 2010b; Wang et al., 2011; Wu et al., 2011; Yang et al., 2023). Notably, animal studies suggest that dietary supplementation with *P. eburnea* may enhance bone health and prevent calculus formation (unpublished data). These combined medicinal and nutritional attributes highlight *P. eburnea* as a promising plant-based resource for the development of safe, multifunctional calcium supplements.

Primulina eburnea is a promising calcium-rich plant species with significant potential for development. However, breeding efforts targeting its quality traits remain limited. To accelerate the utilization of this valuable resource, we initiated a targeted breeding program focused on enhancing calcium content, bioactive compound levels, biomass production, and overall nutritional quality, aiming to develop high-value plant-based calcium supplements. In the domestication of wild plants, germplasm evaluation based on specific breeding objectives is a critical foundational step (Huang et al., 2021). In this study, we conducted a comprehensive assessment of 37 wild *P. eburnea* populations under common garden conditions. Based on these evaluations, we developed a germplasm evaluation model and established a core germplasm collection. Furthermore, HPLC-based metabolomic profiling was conducted on representative eastern (LSFC141) and western (LSFC104) populations. The resulting metabolomic data not only validated the robustness of the evaluation model and the reliability of the core collection, but also revealed key metabolic pathways associated with breeding-relevant traits. This integrated evaluation provides essential support for the domestication, breeding, and industrial utilization of *P. eburnea* as a high-calcium crop with valuable medicinal properties.

2. Materials and methods

2.1. Plant material and sampling

All *P. eburnea* populations were collected by the Jiangxi Provincial Key Laboratory of ex Situ Plant Conservation and Utilization and planted at the breeding base located in the Lushan Botanical Garden, Chinese Academy of Science, Jiangxi Province, China (115.8382°E; 28.9112°N). To avoid the possibility of sampling and collecting clones of the selected accessions, each plant was sampled with an approximate spacing of 100 m. Fig. S1 and Table S1 show the voucher numbers and sources of the 37 *P. eburnea* populations. Among the 37 populations, 34 were from the Karst landform and 3 from the Danxia landform. To eliminate the impact of the environment and compare genetic variation between populations, a common garden experiment was used with clonal cuttings. In brief, uniformly sized and well-grown cuttings were selected and transplanted into pots filled with a mixture of peat soil and vermiculite in a 4:1 ratio (v:v). After transplant, all plants were cultivated in a greenhouse under consistent light and temperature conditions. Throughout the experiment, a general-purpose plant growth fertilizer (N: P: K = 20:20:20, micronutrient) was applied weekly. A 5 mmol/L calcium chloride solution was administered biweekly. After 10 months of cultivation, we selected four healthy and independent individuals from each population, resulting in a total of 148 plants. All quality traits were measured at the level of individual plants rather than pooled samples, allowing us to capture both within- and among-population variation. For each individual, the fourth pair of leaves was collected for subsequent analysis.

2.2. Determination of quality traits

2.2.1. Phenotypic traits

Six phenotypic traits were measured. The leaf fresh weight (FW) and dry weight (DW) were determined using an electronic microbalance with a precision of 0.01 g (BCE1202i-1CCN, Sartorius, Germany). Each leaf was photographed, and the leaf area (LA), maximum leaf length (MLL), and maximum leaf width (MLW) were measured using ImageJ software (v1.50). The leaf relative water content (RWC) was calculated using the following formula: $RWC = (FW - DW)/FW$.

2.2.2. Physiological traits

Seven physiological traits were measured. The leaf mass per area (LMA) was expressed as the ratio of leaf dry weight to leaf area. Flowering time (FT) was expressed as the logarithm base 2 of the number of days to flowering (Log_2day).

The chlorophyll and carotenoid contents were calculated using spectrophotometry (Xia et al., 2022). A 0.1-g leaf sample was placed in a centrifuge tube with 10 mL of 95 % ethanol and stored in the dark for 36 h. When the tissue was fully transparent, the absorbance was measured immediately using a spectrophotometer (BioSpEctrometer® basic, Eppendorf, Germany) at wavelengths of 665, 649, and 470 nm. The chlorophyll a (Chla), chlorophyll *b* (Chlb), and carotenoid (CAC) contents were calculated based on the absorbance values. The total chlorophyll content (TChl) was calculated as the sum of the chlorophyll a and chlorophyll *b* contents. The ratio of chlorophyll a to chlorophyll *b* content (Chla/Chlb) was also determined.

2.2.3. Flavor traits

A total of six flavor traits were measured. The cellulose content (CC) was determined using the anthrone–sulfuric acid method. A 0.5-g of leaf powder was weighed and placed in a tube, and 5 mL of 80 % H_2SO_4 was added. The mixture was incubated in a water bath at 80°C for 40 min. After cooling the sample to room temperature, it was centrifuged at 5000 ×g for 10 min, and the supernatant was discarded. Then, 5 mL of 60 % (v:v) H_2SO_4 was added to the tube, and the mixture was stored in a refrigerator at 4°C for 12 h. The sample was centrifuged at 5000 ×g for 15 min, and the supernatant was transferred to a 25-mL volumetric flask. The extraction process was repeated twice, and the final volume was adjusted to 25 mL with 60 % H_2SO_4. Subsequently, 0.1 mL of the extract was placed in a tube, and 0.9 mL of distilled water and 5 mL of anthrone–sulfuric acid were added and thoroughly mixed. The tubes were incubated in a water bath at 100°C for 10 min. After cooling to room temperature, the absorbance was recorded at 620 nm using a

spectrophotometer.

The hemicellulose content (HCC) was determined using the 3,5-dinitrosalicylic acid (DNS) method (Soest, 1967). Briefly, 0.1 g of leaf powder was placed in a tube, and 10 mL of 80 % (v:v) calcium nitrate solution was added. The mixture was then incubated in a water bath at 100°C for 5 min. After cooling to room temperature, the sample was centrifuged at 8000 ×g for 10 min, and the precipitate was retained. The precipitate was washed 3 times with boiling water, and 10 mL of 2 mol/L hydrochloric acid was added to the tube and thoroughly mixed. The tubes were incubated in a boiling water bath for 45 min. After cooling to room temperature, the sample was centrifuged again at 8000 ×g for 10 min, and the supernatant was transferred to a 25-mL volumetric flask. The precipitate was washed three times with boiling water, and the washings were combined in a volumetric flask. One drop of phenolphthalein indicator was added to the volumetric flask, and the solution was neutralized with NaOH until a pink color appeared. The volume was adjusted to the mark on the flask and filtered into a beaker. A 2-mL aliquot of the filtrate was collected, and 1.5 mL of DNS reagent was added. The mixture was heated in a boiling water bath for 5 min. After cooling, the absorbance was measured at 540 nm. The hemicellulose content of the sample was calculated based on the standard curve.

The flavonoid content (FC) was determined using the aluminum chloride ($AlCl_3$) colorimetric method (Peinado *et al.*, 2009). A total of 5 g of leaf powder was placed in a conical flask containing 100 mL of 80 % (v:v) ethanol solution. Ultrasound-assisted extraction was performed using ultrasonic equipment (YS0410, Yunyi, China) at 40 kHz for 30 min at 60°C, followed by centrifugation at 10,000 ×g for 10 min at 25°C. The supernatant was collected for further analysis. Then, 1 mL of 80 % ethanol and 0.5 mL of 5 % $NaNO_2$ were added to the supernatant, mixed thoroughly, and allowed to stand for 6 min. Subsequently, 0.5 mL of 10 % $Al(NO_3)_3$ was added, mixed thoroughly, and allowed to stand for another 6 min. Finally, 2 mL of 4 % NaOH was added, mixed thoroughly, and allowed to stand for 15 min. The absorbance was measured at 510 nm. The total flavonoid content was calculated using rutin as the standard reference.

The total phenol content (TPC) was determined using the Folin–Ciocalteu colorimetric method (Aktumsek et al., 2013). A 1-g sample of leaf powder was placed in a conical flask, and 25 mL of 80 % (v:v) ethanol was added. The mixture was extracted at 55°C for 3 h and centrifuged at 10,000 rpm for 10 min, and the supernatant was collected. The supernatant was brought to a final volume of 50 mL with 80 % ethanol. An aliquot of 1 mL of the extract was transferred to a glass tube, followed by the addition of 0.3 mL of Folin–Ciocalteu reagent. After mixing, 1.5 mL of saturated sodium carbonate solution was added, and the volume was adjusted to 10 mL with distilled water. The mixture was left to stand at room temperature for 20 min, and the absorbance was measured at 760 nm. The phenol content of the sample was calculated based on the absorbance values and the standard calibration curve with gallic acid as the reference standard.

The lignin (LC) and pectin (PEC) contents were measured using commercial assay kits (BC4200 and BC1400, Solarbio, China) following the instructions from the manufacturer.

2.2.4. Nutrient traits

A total of seven nutrient traits were measured. The total acid content (TAC) was measured using acid-base titration according to the GB/T12456–2008 specifications for determining total acid in food, with citric acid as the standard. The total sugar content (TSC) was measured using the DNS method. Briefly, 0.1 g of leaf powder was placed in a glass tube with 1.5 mL distilled water and 1 mL of 6 mol/L HCl solution. The mixture was heated at 100°C in a water bath for 30 min. After complete hydrolysis, the extract was neutralized with a 10 % (v:v) NaOH solution, filtered, and diluted to 10 mL. The extract was then mixed with DNS solution and boiled for 5 min in a boiling water bath at 100°C. After cooling to an ambient temperature, the absorbance was measured at 540 nm using a spectrophotometer. The total sugar content was determined from the standard curve for glucose.

The starch content (SC) was determined using the iodine colorimetric method. A 0.5-g sample of leaf powder was mixed with 2 mL of distilled water and 3.2 mL of 60 % perchloric acid. The solution was collected and centrifuged at 7500 rpm for 6 min. Then, 0.5 mL of supernatant was obtained and mixed with 3 mL of distilled water and 2 mL of iodine reagent. The solution was analyzed at 660 nm using a spectrophotometer.

The vitamin B1 (VbC), vitamin C (VcC), and protein (PRC) contents were measured using three commercial kits (AKVI001C, Boxbio, China; AKVI005U, Boxbio, China; and BC3185 kit, Solarbio, China). All assays were performed following the manufacturers' instructions.

The extraction and quantification of water-soluble calcium were performed following the method described by Zhang (Zhang et al., 2025). Briefly, after drying, the leaves were ground into a fine powder and sieved. A 0.5-g sample was placed in a 15-mL centrifuge tube with 7 mL of distilled water and shaken at a constant temperature of 30°C for 18 h. The mixture was centrifuged at 10,000 rpm for 8 min, and the supernatant was filtered. This procedure was repeated twice, and the extracted solutions were combined and diluted to a final volume of 25 mL. For quality control, blank and standard samples were prepared with the test samples. The calcium content was subsequently measured using inductively coupled plasma mass spectrometry (ICP-MS; iCAP RQ, Thermo Scientific, Germany).

2.3. Metabolomic analysis

Select representative populations of *P. eburnea*, LSFC104 and LSFC14, with four plants randomly chosen from each population and four biological replicates for each. Leaves were collected for widely targeted metabolomic analysis using UPLC-MS/MS. The samples were placed in a vacuum freeze-dryer (Scientz-100F) for freeze-drying for 48 h. After freeze-drying, the samples were ground into powder using a grinding machine (MM 400, Retsch, Haan, Germany) at 50 Hz for 120 s. Then, 10 mg of the powder was weighed into a centrifuge tube, and 1.2 mL of 70 % methanol solution was added for ultrasonic extraction for 40 min. The extract was centrifuged at 1000 rpm for 10 min, and the supernatant was collected. A 0.22 μm microporous filter membrane (SCAA-104, ANPEL, China) was used for filtration, and the filtrate was stored in a chromatography vial.

The extracted samples were subjected to ultra-performance liquid chromatography-electrospray ionization tandem mass spectrometry (UPLC, Nexera X2, Shimadzu, Japan; MS, 4500 QTRAP, Applied Biosystems, USA). The liquid chromatography system was equipped with an Agilent SB-C18 column (1.8 μm, 2.1 mm × 100 mm). The mobile phase consisted of phase A (ultrapure water containing 0.1 % formic acid) and phase B (acetonitrile containing 0.1 % formic acid). The elution gradient started with 5 % phase B, linearly increased to 95 % in 9 min, maintained for 1 min, then decreased back to 5 % in 10 min, and held until 14 min for equilibration.

The mass spectrometry conditions were as follows: the analysis was performed using an electrospray ionization (ESI) source with a temperature of 550°C and ion spray voltages of 5500 V (positive ion mode) and −4500 V (negative ion mode). The pressures of ion source gas I (GSI), gas II (GSII), and curtain gas (CUR) were set at 50, 60, and 25 psi, respectively, with high collision-induced ionization parameters. The instrument was tuned and calibrated using 10 and 100 μmol/L polypropylene glycol solutions in triple quadrupole (QQQ) and linear ion trap (LIT) modes. The monitoring of multiple reaction monitoring (MRM) ion pairs was based on the MRM mode of QQQ scans, with collision gas (nitrogen) set to medium, and declustering voltage (DP) and collision energy (CE) optimized.

Metabolites were identified by comparing ion fragmentation patterns, retention times, and m/z values with the MWDB database (MetWare, China). Quantification of metabolites was performed using multiple reaction monitoring (MRM) mode on the triple quadrupole

mass spectrometer. The peak areas of all substances were integrated, and corrections were made for the same metabolite across different samples. Mass spectrometry data were processed using Analyst software (v1.6.3). Chromatographic peak integration and correction were performed using MultiQuant software (v3.0.2), with peak areas representing the relative content of the corresponding substances.

2.4. Environmental factors

Climate data obtained from the National Meteorological Information Center (https://data.cma.cn/) for the period 2000–2020 were used to calculate the climate information. The longitude (Lon) and latitude (Lat) of the sampling site were collected using a GPS locator. Table S1 summarizes the location and climate information for each sampling site.

2.5. Statistical analysis

The minimum value (MAX), maximum value (MIN), mean (AVE), standard deviation (SD), and coefficients of variation (CV) of each quality trait were calculated using Microsoft Office Excel 2019. The genetic diversity was measured using the Shannon–Wiener index (H'), which was calculated as follows:

$$H' = -\sum_{i=1}^{s} PilnPi$$

where s represents the total number of populations, and Pi is the proportion of the ith population relative to the total population.

Variance component analysis was performed based on a nested linear model using SPSS 26.0 (v26.0). The phenotypic differentiation coefficient (V_{st}) was calculated using the following formula:

$$V_{st}\ (\%) = [(\delta^2{}_{t/s}/(\delta^2{}_{t/s}+\delta^2{}_{s})] \times 100$$

where $\delta^2{}_{t/s}$ is the variance component between populations, and $\delta^2{}_{s}$ is the variance component within the population.

Principal component analysis (PCA) was performed using SPSS software (v26.0). The comprehensive evaluation value (Q) based on PCA was calculated using SPSS. Hierarchical clustering analysis was conducted using the factoextra package in R software (v4.2.3) based on the complete linkage method and Euclidean distance. In R software (v4.2.3), Pearson correlation analysis was performed using the corrplot package, and redundancy analysis (RDA) was conducted using the vegan package. Multiple linear regression was used to quantify the independent effects of environmental factors on trait variation. Analysis was conducted in R software (v4.2.3) using the *lm* function, with each trait treated as the response variable and selected environmental variables as predictors. To reduce multicollinearity, annual average temperature (AAT) was retained as the representative thermal index, while annual sunshine duration (ASD) was chosen to represent light availability.

The membership function values for each trait were calculated based on their measured values. The original data on quality traits were standardized. For traits positively correlated with breeding objectives, the membership function value (U) was calculated using the following formula: $U_{ij} = (X_{ij} - X_{jmin}) / (X_{jmax} - X_{jmin})$. For traits negatively correlated with breeding objectives, the membership function value was calculated using the inverse membership function formula, as follows:

$$U_{ij} = 1 - (X_{ij} - X_{jmin}) / (X_{jmax} - X_{jmin})$$

where U_{ij} is the membership function value of the jth trait of the ith population, X_{ij} is the measured value of the jth trait of the ith population, and X_{jmin} and X_{jmax} are the minimum and maximum values of the jth trait among populations, respectively. The membership function values for each trait within a population were summed to obtain the comprehensive evaluation value (U).

3. Results

3.1. Diversity of quality traits

The systematic evaluation of wild germplasm and the establishment of a core germplasm bank are fundamental to breeding and germplasm improvement (Huang et al., 2021). Leaf morphology analysis of 37 *P. eburnea* populations revealed significant variations in leaf phenotypes (Fig. 1). Frequency distribution histograms of the 26 quality traits were analyzed (Fig. S2), demonstrating wide data distributions across these traits. This indicates extensive genetic variation among the 37 *P. eburnea* populations at the phenotypic level.

Table 1 presents the mean value, minimum value, maximum value, standard deviation, CV, and Shannon-Wiener index of the 26 traits across the 37 *P. eburnea* populations. The CVs for the 26 traits ranged from 1.12 % to 46.44 %, with leaf fresh weight exhibiting the highest CV and flowering time the lowest. Among the 26 traits, 9, namely cellulose content, flavonoid content, water-soluble calcium content, maximum leaf width, vitamin B1 content, leaf dry weight, starch content, leaf area, and leaf fresh weight, had CVs exceeding 20 %, indicating substantial genetic diversity in these traits. The 7 phenotypic traits showed the highest average CV, at 27.87 %. Within this category, the relative leaf water content had the lowest CV (1.72 %). For physiological traits, the chlorophyll a/b ratio demonstrated the greatest variability (CV=19.51 %). Regarding flavor traits, cellulose content displayed the highest CV (24.26 %), while lignin content was the lowest (CV=10.77 %). Nutritional traits varied most in starch content (CV=41.29 %) and least in vitamin C content (CV=14.54 %). The Shannon–Wiener diversity index (H'), which reflects both the richness and evenness of trait variation, ranged from 1.53 to 2.11 for the 26 traits, indicating a diverse and relatively evenly distributed trait variation. Among the traits, the total acid and carotenoid contents had H' values exceeding 2.0, further emphasizing the high diversity within these traits.

Variance analysis and phenotypic differentiation coefficient analysis were also conducted for the 26 quality traits (Table 1 and S2). The F-values within the populations ranged from 0.06 to 7.93, with significance tests indicating that all traits except leaf relative water content exhibited significant variation. The between-population F-values ranged from 1.10 to 20.12, with 24 traits showing significant differences.

The phenotypic differentiation coefficient reflects the degree of trait differentiation among populations. Among the 26 traits, the leaf fresh weight exhibited the highest *Vst* value (89.39 %), while the chlorophyll a content had the lowest *V*st value (0.01 %). The mean *Vst* values for different trait categories were ranked as follows: phenotypic traits (71.76 %) > nutritional traits (62.27 %) > flavor traits (52.12 %) > physiological traits (34.05 %). Five of the six phenotypic traits (namely leaf fresh weight, maximum leaf length, maximum leaf width, leaf area, and leaf dry weight) had *Vst* values exceeding 70 %, indicating significant variation in biomass potential across populations. Among the physiological traits, the chlorophyll a and total chlorophyll contents had relatively low *Vst* values, while the chlorophyll *b* content exhibited a *Vst* value of 66.67 %. The mean *Vst* value for the 26 quality traits was 54.52 %, indicating that most of the variation in *P. eburnea* traits was due to differences among populations.

3.2. PCA and cluster analysis

PCA was performed on 26 quality traits across 37 *P. eburnea* populations (Table 2). Six principal components (PCs) with eigenvalues greater than 1 were extracted, collectively explaining 80.53 % of the total variation, indicating these components captured the majority of variability in the 26 traits.PC1 (34.22 % contribution) showed positive correlations with starch, protein, cellulose, and flavonoid contents, while exhibiting negative correlations with fresh leaf weight, leaf area, total sugar content, flowering time, and maximum leaf width. PC2

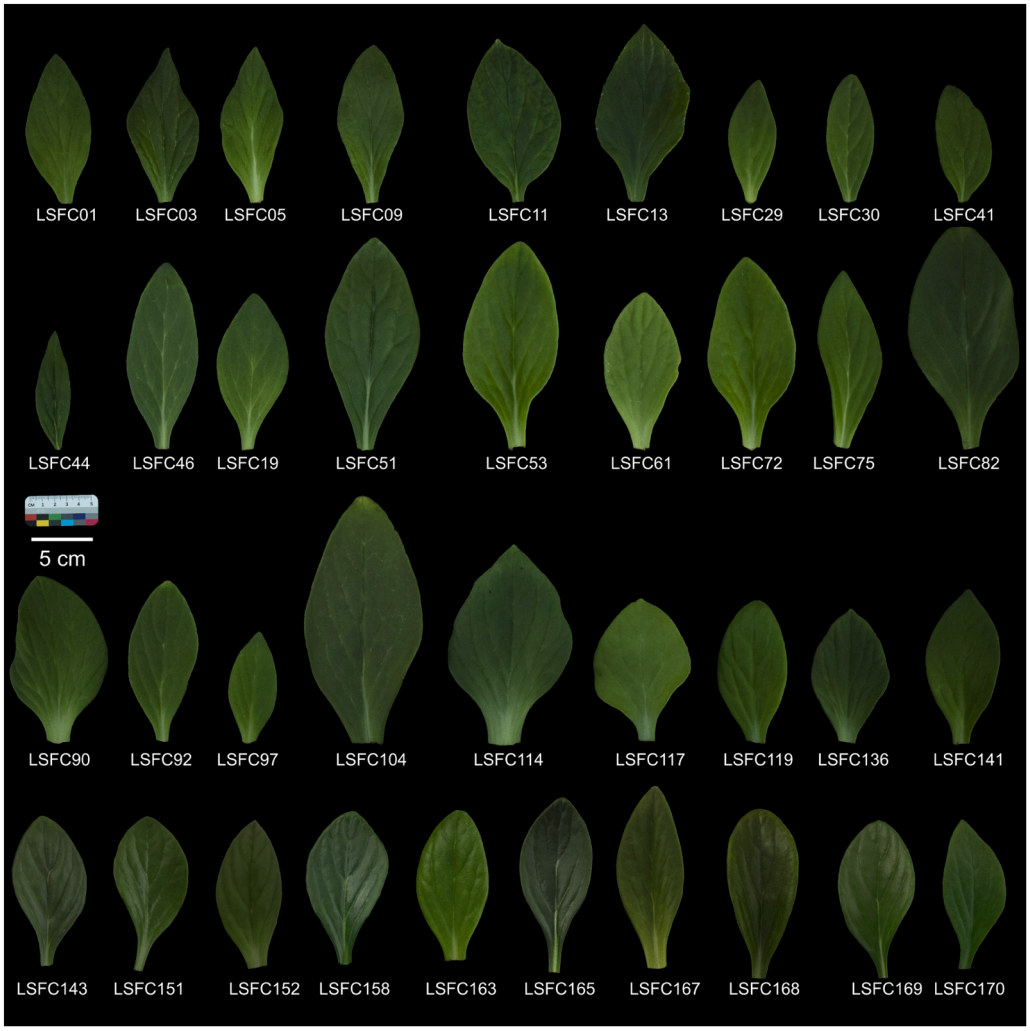

Fig. 1. Leaf morphology of 37 *Primulina eburnea* populations. Scale bar = 5 cm.

demonstrated high loadings for water-soluble calcium content, specific leaf weight, total chlorophyll content, maximum leaf width, chlorophyll *b* content, and flavonoid content. PC3 was primarily associated with maximum leaf length, leaf dry weight, and specific leaf weight. PC4 showed significant loadings for chlorophyll a content, chlorophyll *b* content, and chlorophyll a/b ratio, indicating its role in explaining chlorophyll-related variation. PC5 and PC6 were mainly associated with lignin and carotenoid contents, respectively. Based on PCA results, a score plot was generated using PC1 and PC2 (Fig. 2). Thirteen populations (LSFC49, LSFC136, LSFC13, LSFC60, LSFC11, LSFC05, LSFC09, LSFC141, LSFC01, LSFC03, LSFC30, LSFC29, and LSFC41) clustered into Group A, while four populations (LSFC51, LSFC53, LSFC44, and LSFC46) showed intermediate distribution, and the remaining 20 populations formed Group B.

Cluster analysis of the 37 *P. eburnea* populations using the 26 quality traits (Fig. 3) revealed two distinct Clusters at a Euclidean distance of 18.37. Cluster A comprised populations LSFC136, LSFC49, LSFC30, LSFC29, LSFC41, LSFC61, LSFC03, LSFC01, LSFC141, LSFC09, LSFC05, LSFC13, LSFC11, LSFC46, and LSFC44, while the remaining 22 populations formed Cluster B. This classification was consistent with the PCA results. Further geographical analysis indicated that Cluster A primarily consisted of populations from eastern regions, whereas Cluster B was predominantly composed of western populations (Fig. 3 and Fig. S1).

3.3. Correlation analysis of quality traits

Pearson correlation analysis was performed on the 26 quality traits, followed by hierarchical clustering based on the results (Fig. 4). A total of 95 significant correlations were identified. Leaf area, maximum leaf length, maximum leaf width, leaf dry weight, and fresh leaf weight showed significant positive correlations with each other, indicating that leaf expansion is crucial for increasing leaf biomass. The chlorophyll *b* content was significantly negatively correlated with leaf mass per area and positively correlated with leaf area, but other chlorophyll traits (chlorophyll a content, total chlorophyll content, and chlorophyll a/b ratio) did not show significant correlations. This indicates that the chlorophyll *b* content is more important for leaf area expansion and growth. Flowering time exhibited significant positive correlations with both leaf area and fresh leaf weight, suggesting that prolonged vegetative growth under rapid growth conditions delays bolting and enhances biomass yield in *P. eburnea*. Notably, flowering time also correlated positively with water-soluble calcium content, total sugars, pectin, and total acids, implying that extended vegetative growth can improve calcium extractability. However, flowering time and water-soluble calcium content showed significant negative correlations with vitamin C, flavonoids, and protein content and forming a distinct cluster, it is unfavorable for linkage-based breeding and trait improvement. Moreover, starch, vitamin C, cellulose, and flavonoid contents were positively correlated with each other.

To further analyze the quality traits, PCA was performed, extracting

Table 1
Statistical analysis of 26 quality traits across 37 *Primulina eburnea* populations.

Trait category	Trait	Abbreviation	MAX	MIN	AVE	SD	CV (%)	H'	V_{st}
Phenotypic trait	Leaf fresh weight (g)	FW	13.09	1.55	5.73	2.66	46.44	1.75	89.39
Phenotypic trait	Leaf dry weight (g)	DW	1.07	0.18	0.58	0.24	40.57	1.53	76.56
Phenotypic trait	Leaf relative water content (*100 %)	RWC	0.92	0.87	0.9	0.02	1.72	1.56	2.44
Phenotypic trait	Leaf area (cm^2)	LA	117.7	15.76	53.13	23.52	44.27	1.68	89.09
Phenotypic trait	Maximum leaf length (cm)	MLL	16.35	7.06	11.35	2.19	19.34	1.94	85.48
Phenotypic trait	Maximum leaf width (cm)	MLW	10.05	3.26	6.48	1.77	27.35	1.88	87.6
Physiological trait	Flowering time (Log_2day)	FT	8.4	8.05	8.21	0.09	1.12	1.82	79.56
Physiological trait	Leaf mass per area	LMA	0.02	0.01	0.01	0	15.43	1.91	35.59
Physiological trait	Chlorophyll a content (mg/g)	Chla	0.91	0.63	0.77	0.07	9.15	1.7	0.01
Physiological trait	Chlorophyll b content (mg/g)	Chlb	0.42	0.23	0.32	0.05	14.63	1.94	66.67
Physiological trait	Chlorophyll a/b ratio	Chla/Chlb	3.96	1.69	2.51	0.49	19.51	1.74	46.44
Physiological trait	Total chlorophyll content (mg/g)	TChl	1.22	0.93	1.09	0.08	7.51	1.86	9.09
Physiological trait	Carotenoid content (mg/g)	CAC	0.25	0.18	0.22	0.02	9.98	2.11	50.64
Flavor trait	Cellulose content (mg/g)	CC	20.37	7.69	13.86	3.36	24.26	1.87	40.73
Flavor trait	Hemicellulose content (mg/g)	HCC	5.74	3.35	4.36	0.63	14.33	1.61	30.69
Flavor trait	Lignin content (mg/g)	LC	19.3	11.97	14.96	1.61	10.77	1.59	79.97
Flavor trait	Pectin content (μmol/g)	PEC	40.02	17.96	25.1	4.97	19.81	1.7	46.77
Flavor trait	Total phenol content (mg/g)	TPC	9.8	5.23	7.32	1.32	17.98	1.84	56.45
Flavor trait	Flavonoid content (mg/g)	FC	11.9	4.21	8.1	1.99	24.61	1.89	77.04
Nutrient trait	Total sugar content (mg/g)	TSC	74.25	39.86	56.64	11	19.43	1.96	0.99
Nutrient trait	Total acid content (mg/g)	TAC	1.86	1.13	1.43	0.22	15.28	2.03	37.39
Nutrient trait	Starch content (mg/g)	SC	7.88	1.2	5.06	2.09	41.29	1.86	57.8
Nutrient trait	Protein content (mg/g)	PRC	24.31	13.08	18.59	2.96	15.94	1.77	52.9
Nutrient trait	Vitamin B1 content (mg/g)	VbC	268.76	62.68	155.88	51.61	33.11	1.98	89.21
Nutrient trait	Vitamin C content (mg/g)	VcC	265.54	165.98	205.96	29.94	14.54	1.67	63.93
Nutrient trait	Water-soluble calcium content (mg/kg)	WcaC	152.95	78.4	107.91	17.23	15.97	1.57	65.12
Mean									54.52

Table 2
Principal component loading matrix for 26 quality traits.

	PC1	PC2	PC3	PC4	PC5	PC6
Eigenvalue (λ)	8.898	3.684	2.888	2.223	1.754	7.491
Contribution rate (%)	34.224	14.168	11.107	8.55	6.746	5.734
Cumulative contribution rate (%)	34.224	48.391	59.499	68.049	74.795	80.529
FW	−0.799	0.339	0.446	0.069	−0.101	0.014
DW	−0.683	0.276	0.616	0.189	−0.025	−0.118
RWC	−0.527	0.247	−0.426	−0.224	−0.212	0.416
LA	−0.787	0.456	0.325	0.147	−0.02	−0.131
MLL	−0.633	0.439	0.535	0.197	−0.018	−0.107
MLW	−0.726	0.52	0.172	0.163	0.004	−0.218
FT	−0.739	−0.294	−0.083	−0.118	0.041	−0.287
SLA	0.277	−0.525	0.648	0.082	0.02	0.031
Chla	−0.129	0.256	−0.472	0.756	0.207	0.194
Chlb	−0.452	0.523	−0.135	−0.559	0.34	0.152
Chla/Chlb	0.253	−0.319	−0.132	0.859	−0.172	−0.041
TChl	−0.367	0.516	−0.479	0.327	0.37	0.252
CC	0.753	0.306	0.327	0.101	0.205	−0.13
HCC	−0.022	−0.245	0.412	−0.099	0.316	0.452
LC	0.457	−0.247	0.397	−0.157	0.49	0.264
PEC	−0.629	−0.223	−0.092	−0.284	0.012	−0.017
TSC	−0.781	−0.35	−0.233	−0.049	−0.057	0.144
TAC	−0.657	−0.336	0.15	−0.085	−0.016	0.037
SC	0.783	0.226	0.21	0.025	−0.125	0.305
CAC	−0.551	0.07	0.044	0.057	−0.385	0.552
PRC	0.77	0.435	0.006	0.032	−0.028	−0.127
TPC	0.372	0.462	−0.228	−0.156	0.47	−0.225
FC	0.707	0.541	0.093	−0.079	−0.021	0.071
VbC	0.186	0.212	−0.327	−0.325	−0.484	−0.383
VcC	0.667	0.101	0.123	−0.024	−0.493	0.156
WcaC	−0.29	−0.622	−0.207	0.139	0.35	−0.256

six PCs with eigenvalues greater than 1. A three-dimensional score plot was constructed using PC1, PC2, and PC3 (Fig. S3). The results demonstrated that leaf area, maximum leaf width, fresh leaf weight, maximum leaf length, dry leaf weight, and leaf relative water content clustered together, indicating these phenotypic traits were closely associated with leaf biomass. Another distinct cluster included flowering time, water-soluble calcium content, total sugar content, total acid content, and pectin content. Meanwhile, vitamin C, vitamin B_1, flavonoids, total phenolics, and protein content formed a separate cluster. The PCA results were consistent with Pearson correlation analysis, further validating the reliability of our findings.

3.4. Relationships between environmental factors and quality traits

To evaluate the effects of environmental factors on quality traits, we combined correlation analysis, redundancy analysis, and multiple regression modeling. Pearson correlation analysis revealed significant relationships between environmental factors and traits (Fig. 5A). Latitude, longitude, annual sunshine duration, and annual total solar radiation (ATSR) were negatively correlated with flowering time, pectin, total sugars, and water-soluble calcium, but positively with flavonoids, proteins, and vitamin C. These consistent patterns suggest that light regulation is crucial for improving specific compounds and scaling cultivation. Annual average rainfall (AAR) correlated positively with fresh leaf weight, flowering time, pectin, total sugars, and water-soluble calcium, but negatively with total phenols, flavonoids, and protein content, indicating that drought stress constrains growth and calcium accumulation. Among soil factors, pH was correlated with four traits, while soil electrical conductivity (Con) and soil water-soluble calcium content (SWCa) each correlated with nine traits; notably, soil-soluble calcium was positively associated with leaf calcium, reflecting efficient uptake and storage.

RDA confirmed that environmental factors explained substantial trait variation. Model validation showed the axis length of DCA1 was 2.49 (<3.0), supporting the suitability of RDA. RDA1 and RDA2 explained 40.7 % and 17.4 % of the variation, respectively (Fig. 5B). Monte Carlo tests indicated that, except for annual average minimum temperature (AAMinT), annual average maximum temperature (AAMaxT), and pH, most factors significantly affected trait variation (Table S3, $P < 0.05$). Annual average rainfall mainly influenced flowering time, pectin, cellulose, proteins, and sugars, whereas annual sunshine duration and annual total solar radiation promoted starch, protein, flavonoid, and vitamin C accumulation.

Multiple regression analysis further quantified the independent

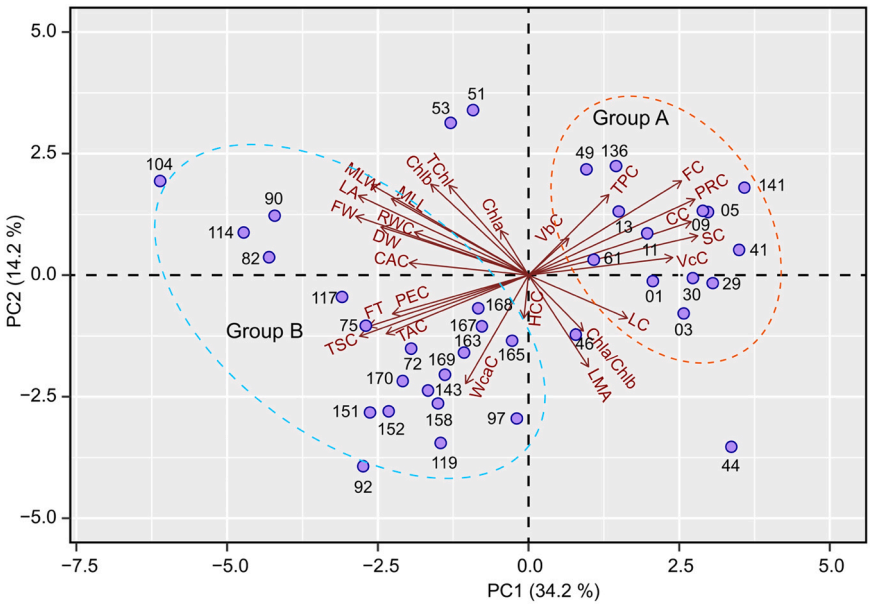

Fig. 2. PCA score plot for 37 *Primulina eburnea* populations based on 26 quality traits.

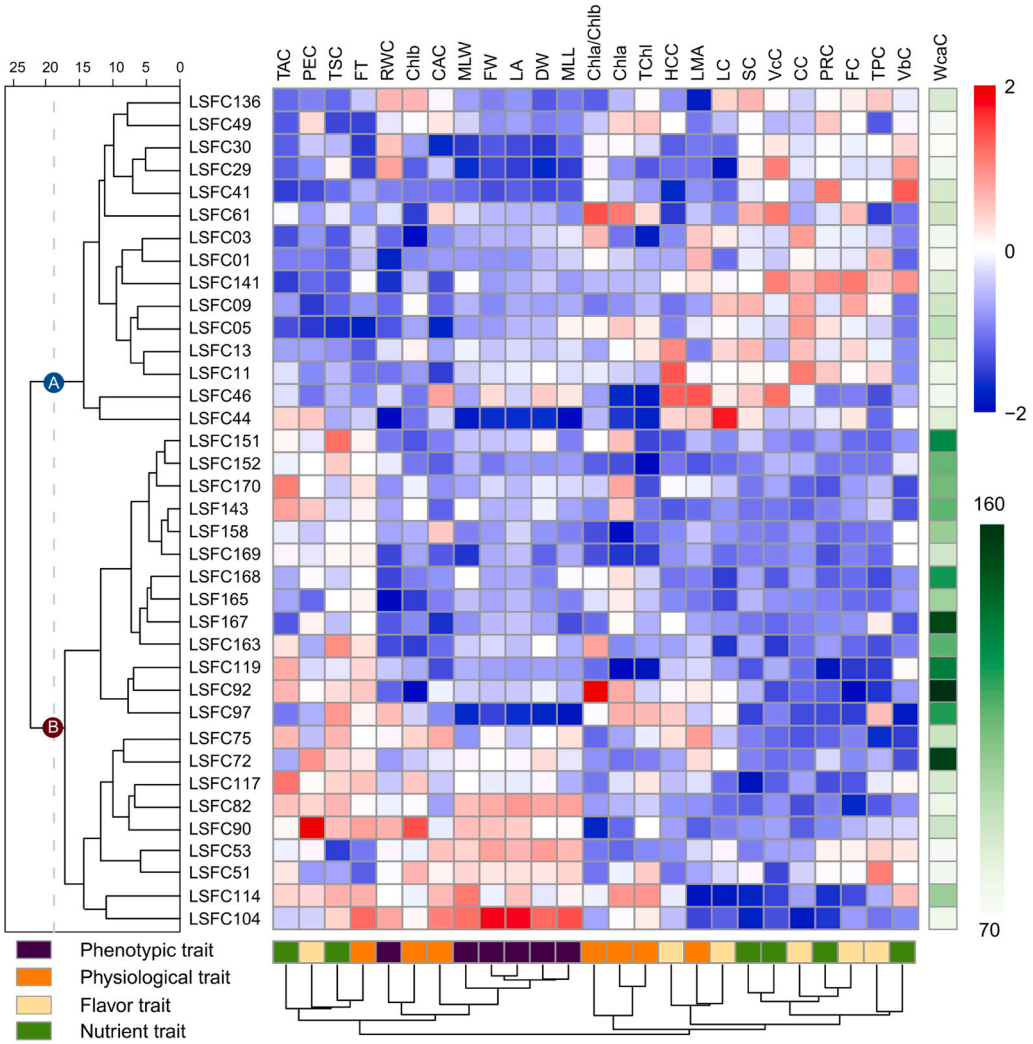

Fig. 3. Cluster analysis of 37 *Primulina eburnea* populations based on quality traits. The color indicates the value of traits, from low (blue) to high (red). The green bar represents the water-soluble calcium content in leaves.

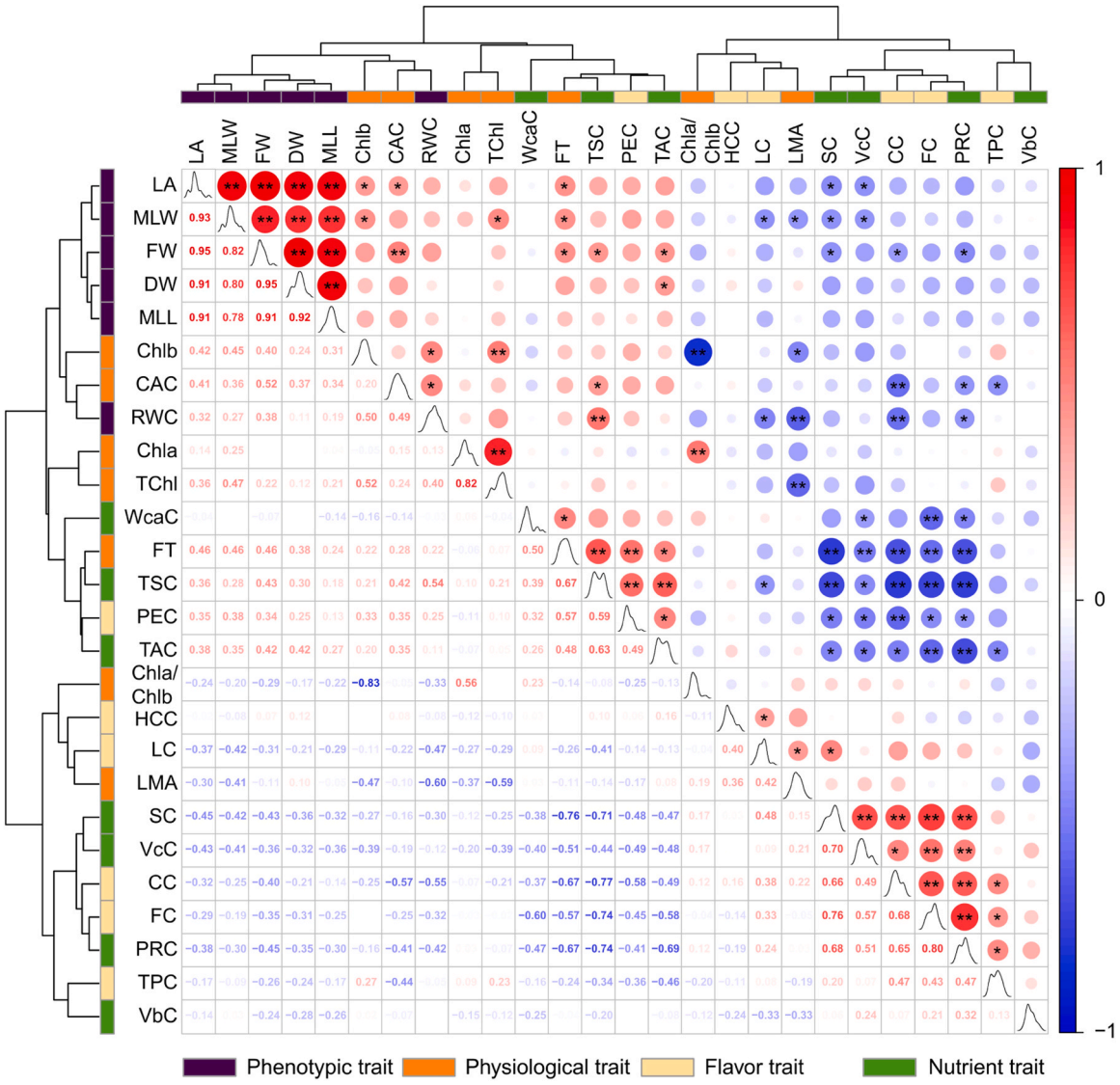

Fig. 4. Pearson correlation analysis between 26 quality traits. Significant correlations are marked with * ($P < 0.05$) and ** ($P < 0.01$).

contributions of environmental factors (Table S4). Annual sunshine duration had a significant negative effect on flowering time and total sugar content, but positively influenced flavonoids, starch, and vitamin C. Annual average rainfall enhanced fresh weight, while geographical coordinates were strongly associated with both growth traits (fresh weight, leaf area, and maximum leaf length) and metabolites (flavonoids, cellulose, and proteins). Importantly, annual average temperature also emerged as a key driver, significantly affecting fresh weight, starch, vitamin B, and protein contents. Taken together, the three analyses showed that light, water, and temperature are the main environmental factors affecting trait variation in *P. eburnea*.

3.5. Establishment of core germplasm collection

Comprehensive quality trait data serve as the foundation for constructing a core germplasm collection. In this study, we established a core germplasm collection for *P. eburnea* based on 26 quality traits. Two established methods, PCA and membership function analysis were employed for germplasm evaluation. Since the first six PCs explained the majority of trait variation, we calculated component scores using corresponding coefficients and standardized data matrices. By integrating PC factor scores weighted by their respective variance contribution rates, we developed a comprehensive evaluation model for *P. eburnea* germplasm as follows:

$$Q = 0.3422P1 + 0.1417P2 + 0.1111P3 + 0.8550P4 + 0.6746P5 + 0.5734P6$$

where P1-P6 represent the scores of the first six PCs.

The *Q*-value reflects the comprehensive performance of germplasm, with higher values indicating superior overall trait performance. Population LSFC104 achieved the highest composite score (Fig. 6; Table S5), demonstrating optimal overall trait expression. Other high-scoring populations included LSFC82, LSFC114, LSFC90, LSFC51, LSFC53, and LSFC75. Among the top 10 ranked populations, LSFC51, LSFC53, LSFC72, LSFC75, LSFC82, LSFC90, LSFC104, LSFC114, and LSFC117 exhibited superior performance in biomass-related traits including fresh leaf weight, dry leaf weight, leaf area, maximum leaf length, and maximum leaf width. However, these populations showed relatively lower values in nutritional and medicinal traits such as vitamin C, protein, flavonoid, and total phenol content (Fig. 3 and Fig. 6).

Because PCA places greater weight on traits with large variance, biomass-related traits dominated the ranking results. To better align with breeding objectives, we further applied the membership function method, which allows flexible trait selection and integrates both positive and negative contributions of traits. Guided by their relevance to

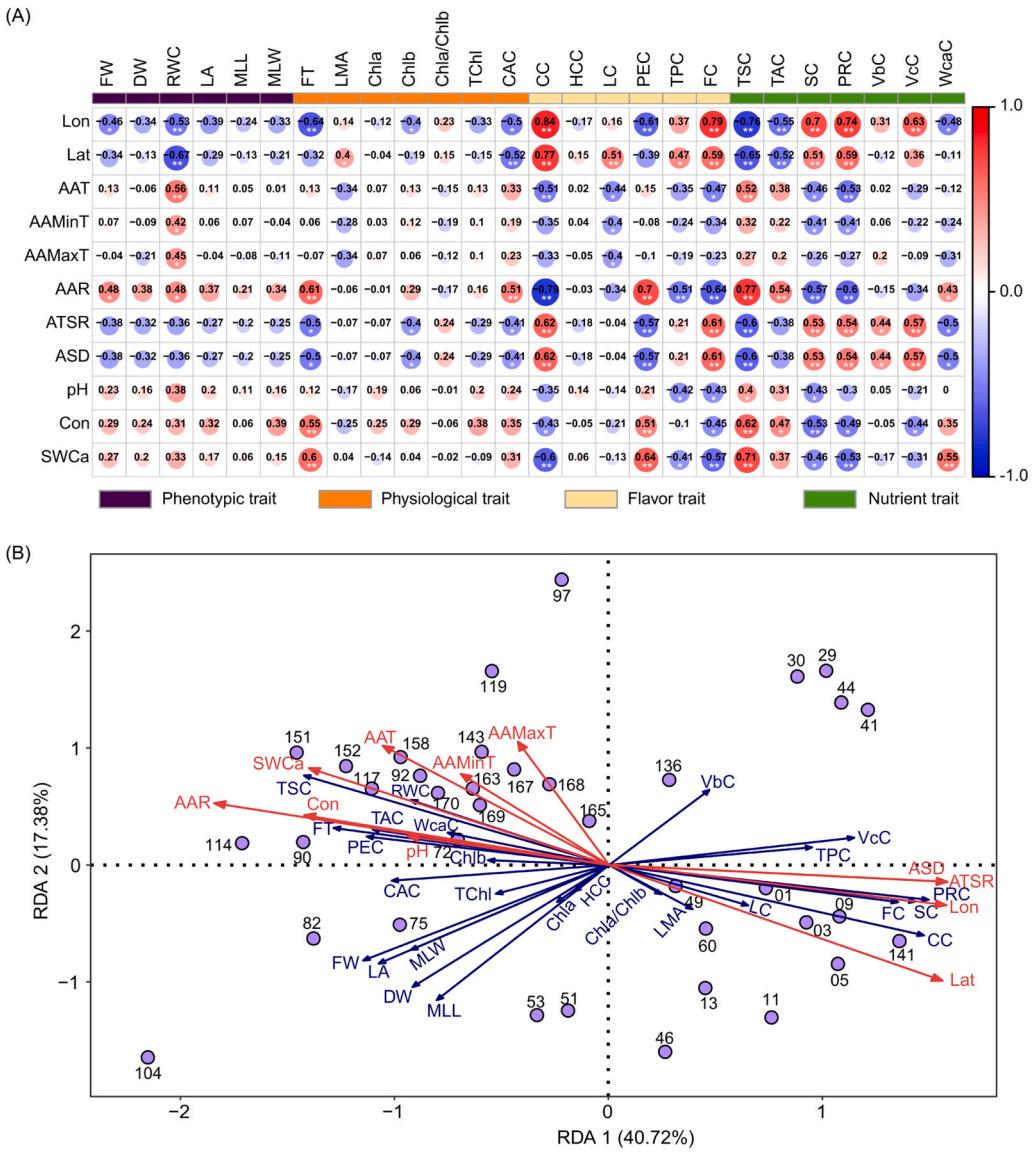

Fig. 5. Influence of environmental factors on quality traits. (A) Correlation analysis between environmental factors and quality traits. The color indicates the r value, from −1.0 (blue) to 1.0 (red). (B) Redundancy analysis of environmental factors and quality traits.

breeding targets (high biomass, calcium enrichment, nutritional quality, and bioactive compounds) and trait correlation clusters (Fig. 4), we narrowed the analysis to 12 representative traits from the original 26 (Table S6). For instance, traits such as cellulose content, vitamin C content, protein content, and starch content clustered tightly with multiple other traits (Fig. 4), meaning they can effectively represent correlated trait groups while avoiding redundancy.

Evaluation of all 37 *P. eburnea* populations using the membership function method revealed that among the top 13 performers: seven populations (LSFC09, LSFC29, LSFC30, LSFC41, LSFC61, LSFC136, and LSFC141) belonged to Cluster A, while six (LSFC51, LSFC53, LSFC82, LSFC90, LSFC104, and LSFC114) originated from Cluster B (Fig. 3 and Fig. 6). The seven Cluster A populations exhibited superior performance in nutritional and medicinal traits (vitamin C, protein content, flavonoid content, and total phenol content), whereas the six Cluster B populations showed significant advantages in phenotypic traits, particularly in fresh leaf weight, dry leaf weight, leaf area, maximum leaf length, and maximum leaf width.

3.6. Metabolomic analysis of representative populations from clusters A and B

To investigate the metabolic basis underlying trait differentiation between eastern and western *P. eburnea* populations and to further validate the robustness of our germplasm evaluation model, we conducted a widely targeted metabolomic analysis using UPLC-MS/MS. Leaves from representative populations LSFC141 (Cluster A) and LSFC104 (Cluster B) were subjected to metabolic profiling. PCA of normalized mass spectrometry data demonstrated clear separation among LSFC141 and LSFC104, with tight clustering of intra-group data points (Fig. 7A). Furthermore, high within-group sample correlations ($R > 0.80$) confirmed the reliability of the metabolomic dataset (Fig. 7B). The distinct metabolic profiles observed between LSFC141 and LSFC104 leaves provide robust validation for the scientific validity of our comprehensive evaluation model.

A total of 1045 metabolites were identified in *P. eburnea* leaves, which were classified into 11 major categories: flavonoids (249), phenolic acids (179), lipids, organic acids, amino acids and derivatives, alkaloids (54), terpenoids (53), nucleotides and derivatives, lignans and coumarins, quinones, and others (Fig. 7C and D; Table S7-11). The

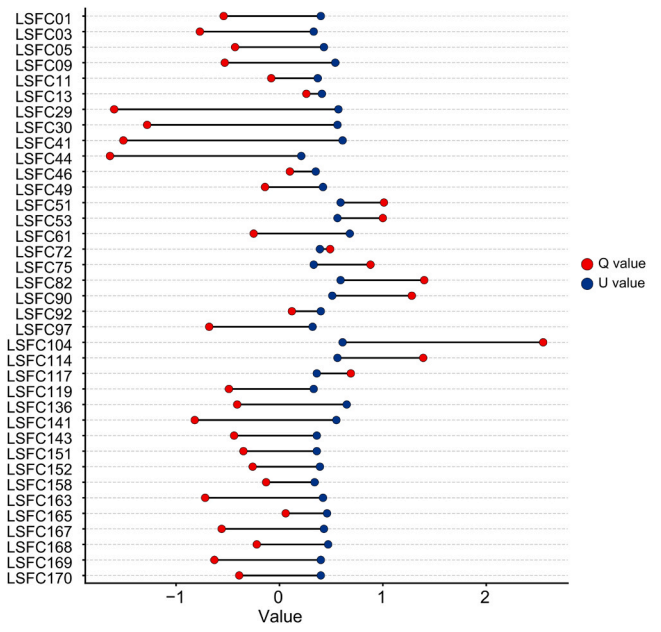

Fig. 6. Dumbbell chart of comprehensive evaluation value for 37 populations.

predominance of flavonoids and phenolic acids, along with substantial quantities of alkaloids and terpenoids, demonstrates the rich repertoire of pharmacologically active compounds. This metabolic profile enables simultaneous calcium supplementation and intake of antioxidant/anti-inflammatory substances, thereby reducing disease risks (Fig. 7C and Fig. 9). In addition, several vitamins associated with calcium absorption were detected, including direct absorption promoters such as menatetrenone (vitamin K2) and cholecalciferol (vitamin D3) (Capozzi et al., 2020), as well as absorption facilitators like 4-oxoretinol (vitamin A), dehydroascorbic acid (vitamin C), and menadiol diacetate (vitamin K4) (Table S12) (Haxhiu et al., 2014; Morcos et al., 1976).

To identify metabolites that significantly contribute to sample discrimination, we performed supervised OPLS-DA analysis. This analysis effectively distinguished the samples, indicating significant inter-group differences in metabolite profiles between LSFC141 and LSFC104 (Fig. 8A). The OPLS-DA model showed high reliability ($R^2X = 0.787$, $R^2Y = 0.999$) and strong predictive power ($Q^2 = 0.993$), supporting the robustness of our germplasm evaluation model (Fig. S4). Metabolites with VIP > 1 are generally considered key contributors to sample differentiation (Fig. 8B). Visualization of the top 20 metabolites with the highest VIP values revealed that flavonoids and phenolic acids were the predominant classes driving inter-group discrimination, followed by compounds classified as "others" and lipids (Fig. S5 and Fig. S6; Table S13). This suggests that *P. eburnea* may employ flavonoids and phenolic acids as adaptive strategies to cope with environmental differences between eastern and western habitats. Differential metabolite analysis identified 242 differentially accumulated metabolites (DAMs) between LSFC141 and LSFC104, comprising 176 upregulated and 66 downregulated metabolites (Fig. 8C; Table S14). The upregulated metabolites were predominantly flavonoids (56), phenolic acids

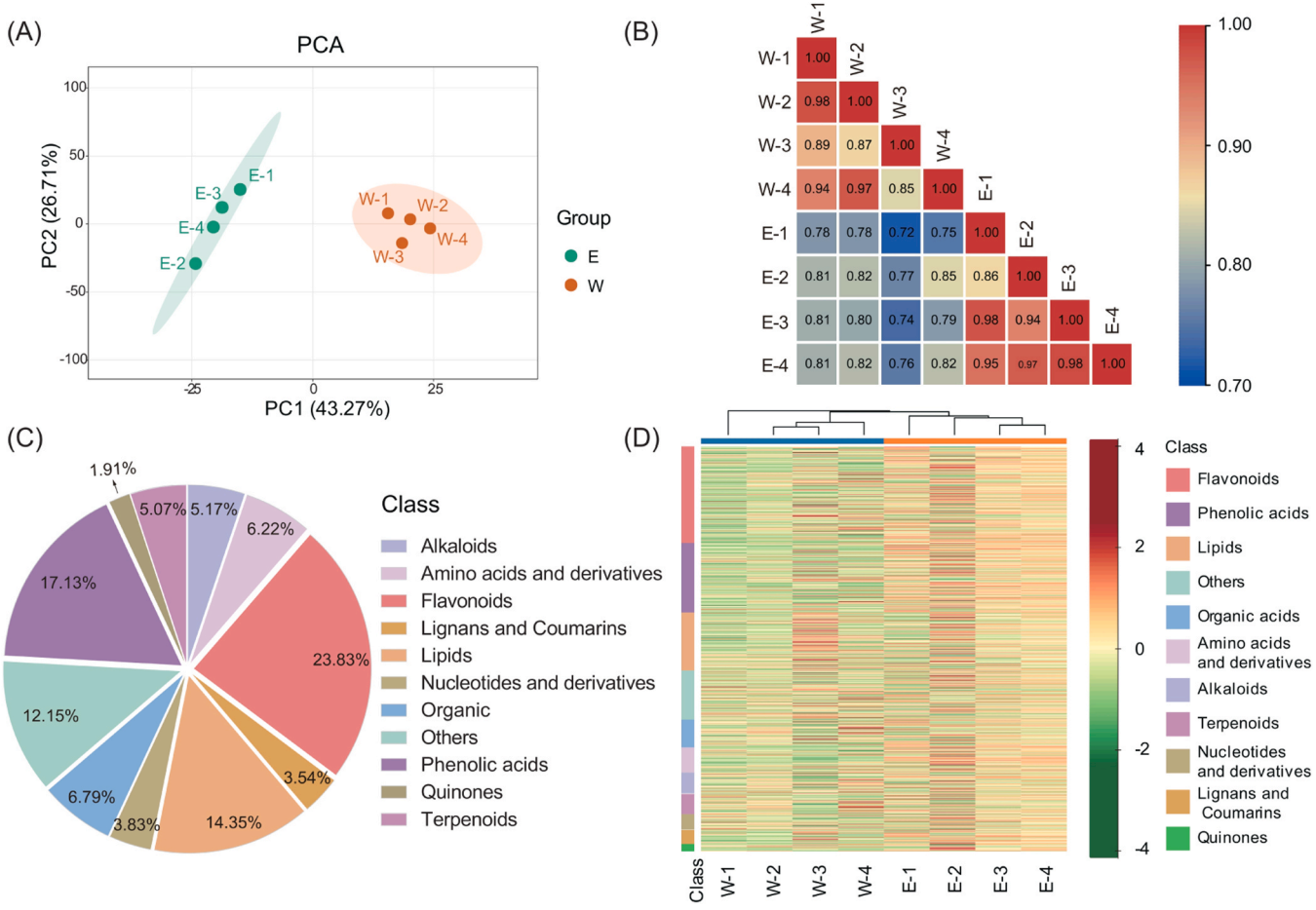

Fig. 7. Overview of metabolomic profiles in *P. eburnea*. (A) PCA score plot of mass spectrometry data (E: eastern populations; W: western populations; the same hereafter). (B) Correlation heatmap among samples. (C) Statistical analysis of different metabolite classes. (D) Cluster analysis of all identified metabolites in *P. eburnea*.

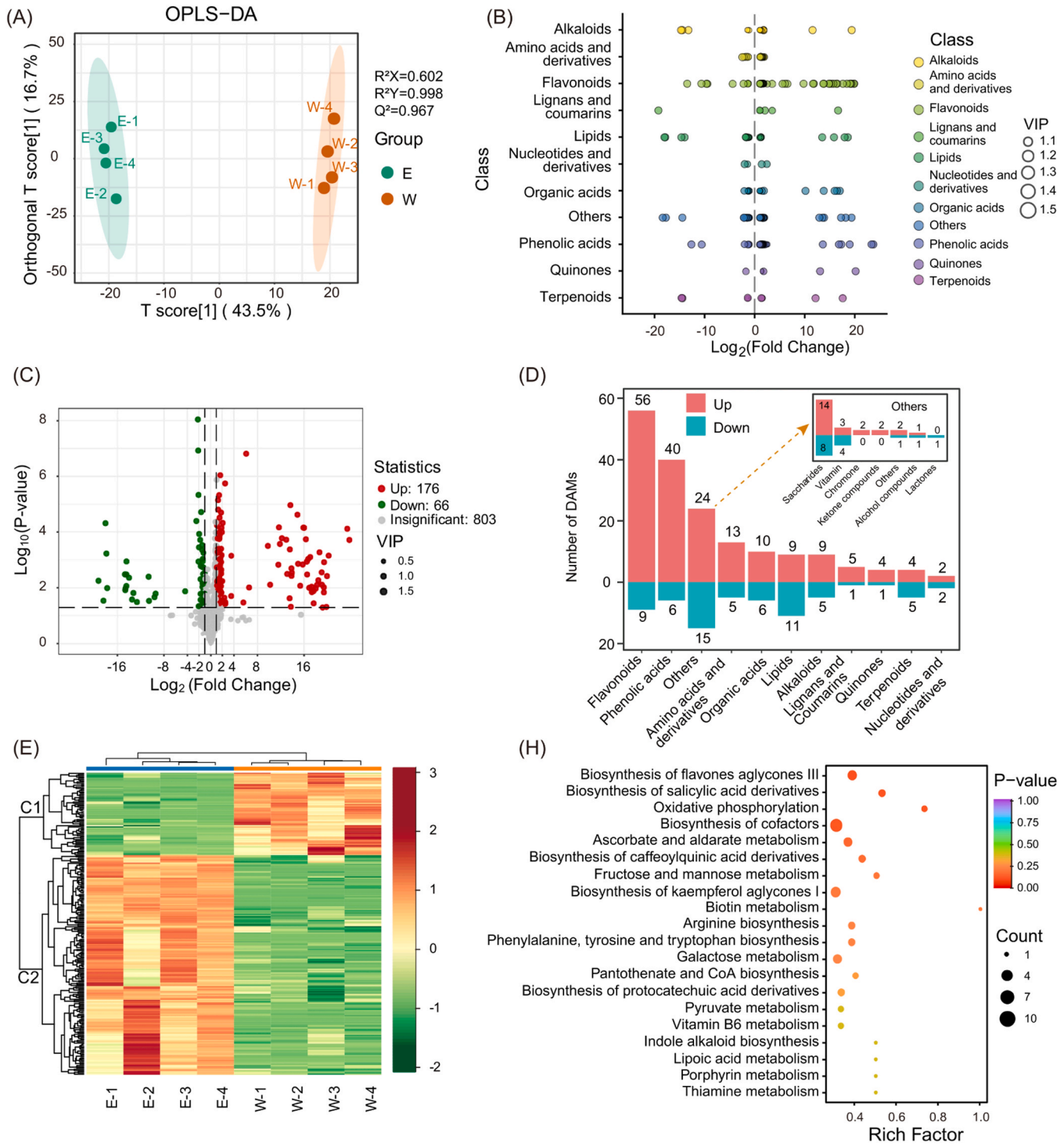

Fig. 8. Differential metabolite analysis between eastern and western populations of *P. eburnea* (A) OPLS-DA score plot. (B) Scatter plot of DAMs (E_vs_W). (C) Volcano plot of DAMs (E_vs_W). (D) Statistical analysis of DAMs. (E) Hierarchical clustering analysis of DAMs (E_vs_W). (F) KEGG enrichment analysis of DAMs (E_vs_W).

(40), and compounds classified as "others" (24), while the down-regulated metabolites were primarily lipids (11), "others" (15), and flavonoids (9) (Fig. 8D; Table S15).

The results demonstrate that while the eastern population exhibits higher concentrations of flavonoids and phenolic acids, it is noteworthy that certain bioactive lipids, flavonoids, and saccharides show significantly elevated levels or uniqueiy in the western population. These include Gingerglycolipid C, 12-oxo-phytodienoic acid, Negundin A, tangeretin, oleanolic acid, 3-hydroxydammara-21-oic acid 21,23-lactone, Alangionoside L, and D-trehalose, These compounds exhibit antioxidant, anti-inflammatory and other bioactive properties (Ashrafizadeh et al., 2020; Ayeleso et al., 2017; Chaitanya et al., 2021; Rao et al., 2022) (Fig. 8E; Tables S16 and S17).

KEGG pathway enrichment analysis was performed on the DAMs, with the top 20 pathways ranked by *P*-value being selected for visualization (Fig. 8H). The results demonstrated significant enrichment in several key metabolic pathways: biosynthesis of flavones aglycones III, biosynthesis of salicylic acid derivatives, oxidative phosphorylation,

biosynthesis of cofactors, ascorbate and aldarate metabolism, and biosynthesis of caffeoylquinic acid derivatives. Notably, both the flavones aglycones III biosynthesis and salicylic acid derivatives biosynthesis pathways are associated with phenylpropanoid metabolism, indicating the crucial role of phenylpropanoid pathway in *P. eburnea* adaptation to environmental differences between eastern and western regions. Further metabolic pathway analysis was conducted focusing on pharmacologically active compounds including flavonoids, phenolic acids, alkaloids and terpenoids with established bioactivities (Fig. 9). The results showed significant differences in the accumulation of phenylpropanoid metabolites between the LSFC141 and LSFC104 populations. Notably, the content of phenylalanine, the upstream precursor of the phenylpropanoid pathway, was higher in the eastern population. In contrast, two important intermediate metabolites, p-coumaric acid and chalcones, exhibited higher levels in the western population, suggesting divergent metabolic flux regulation between the two groups. Interestingly, despite lower chalcone content in LSFC141, downstream flavonoid subclasses, including flavanones, flavonols, and flavones, were significantly more abundant in the eastern population. These results demonstrate that the eastern population exhibits higher conversion efficiency toward bioactive flavonoid biosynthesis. Similarly, 5 out of 7 phenolic acids showed eastern population-specific accumulation patterns. For the 6 alkaloids and 4 terpenoids analyzed, 3 alkaloids were more abundant in eastern populations while 3 terpenoids were preferentially accumulated in western populations. These findings suggest that eastern populations possess superior medicinal properties including enhanced antioxidant, anti-inflammatory, antimicrobial, antitumor, immunomodulatory and cardioprotective effects (Billowria et al., 2024; Ge et al., 2022; Deng et al., 2024; Sehrawat et al., 2022; Zheng et al., 2022). The predominance of flavonoids pathway-associated bioactive compounds in eastern populations provides valuable guidance for future breeding strategies aimed at optimizing these beneficial traits.

4. Discussion

As a promising breeding material for developing premium calcium preparations, comprehensive assessment of quality trait diversity in *P. eburnea* holds significant importance. This study conducted a comprehensive evaluation across 37 *P. eburnea* populations covering all known distribution areas in China, and successfully established a core germplasm collection. These findings offer valuable guidance for future breeding programs, particularly in enhancing the medicinal and nutritional value of calcium-enriched products derived from *P. eburnea*.

4.1. Diversity of quality traits

A comprehensive evaluation of 37 *P. eburnea* populations was conducted based on specific breeding objectives. Phenotypic differentiation coefficient analysis revealed significant variation among traits, with fresh leaf weight exhibiting the highest *Vst* value (89.39 %), reflecting substantial biomass differences between populations. Furthermore, the mean *Vst* value for phenotypic traits was highest (71.76 %), indicating rich inter-population variation and significant potential for genetic improvement. This high phenotypic differentiation likely reflects adaptive responses to environmental pressures such as light intensity and water availability, suggesting rapid evolutionary rates under both natural and artificial selection (Opedal et al., 2023). In contrast, physiological traits typically exhibit stronger genetic constraints and play central roles in plant adaptive evolution (Thoen et al., 2017). The lower mean *Vst* value for physiological traits (34.05 %) in this study demonstrates their greater genetic stability across populations and weaker association with environmental factors, a conclusion further supported by fewer significant correlations between physiological traits and environmental variables in Pearson analysis. The overall mean *Vst* value of 54.52 % across all 26 traits confirms that *P. eburnea* possesses readily utilizable natural variation for breeding programs through selective hybridization, enabling enhanced genetic variation and improved breeding efficiency.

PCA extracted six PCs with eigenvalues greater than 1, cumulatively explaining 80.53 % of the total trait variation, demonstrating effective capture of the overall phenotypic diversity (Table 2). The PCA score plot revealed distinct separation of the 37 populations into two major

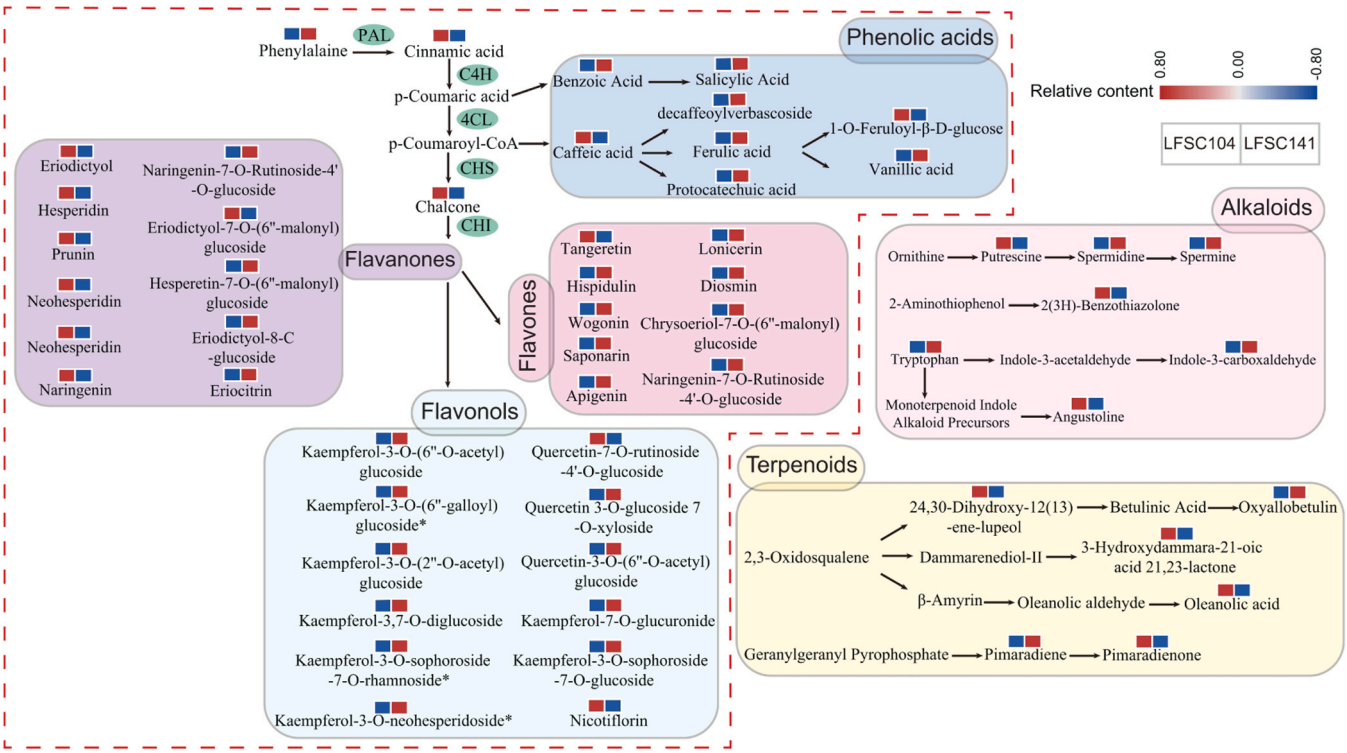

Fig. 9. Metabolic pathway analysis of flavonoids, phenolic acids, alkaloids, and terpenoids with demonstrated pharmacological activities in *P. eburnea*.

groups, Similarly, in the cluster analysis, the populations were divided into two branches. Both analytical approaches consistently showed significant population differentiation based on quality traits (Fig. 2 and Fig. 3), with clear geographical correspondence Cluster A populations predominantly distributed in eastern regions and Cluster B in western areas. This geographical patterning aligns with prior phylogenetic and population structure analyses using molecular markers (Wang et al., 2017). Genetic divergence and gene flow dynamics suggest that geographical isolation and restricted gene flow serve as primary evolutionary forces driving population differentiation (Gao et al., 2015; Ke et al., 2022). Consequently, the quality trait-based clustering provides valuable guidance for identifying unique germplasm resources and designing strategic crossing schemes for breeding programs, enabling targeted utilization of the observed geographical differentiation in trait expression.

The influence of environmental factors on quality traits was evaluated using correlation analysis, RDA, and regression modeling (Fig. 5). Both correlation analysis and RDA consistently showed that longitude, latitude, annual average rainfall, annual sunshine duration, and annual total solar radiation were strongly associated with metabolite accumulation and growth performance. In contrast to these environmentally responsive metabolites, pigment-related traits (chlorophyll a and chlorophyll *b*) displayed limited variation among populations and weak sensitivity to environmental factors. This stability is consistent with observations in other karst and medicinal plants (Liu et al., 2021; Ma et al., 2025), where photosynthetic stability is highly conserved to ensure efficient primary metabolism. Soil water-soluble calcium was significantly positively correlated with leaf calcium content, indicating efficient uptake and suggesting that appropriate calcium fertilization could further enhance calcium accumulation in *P. eburnea*. Regression analysis further quantified these effects, identifying annual average temperature as an additional key driver of growth- and quality-related traits. Given the geographic differentiation revealed by PCA and clustering, the strong associations of longitude and latitude with multiple traits highlight the role of spatial isolation in shaping trait divergence. Collectively, ecological heterogeneity in light, water, and temperature associated with geographical isolation in karst habitats highlights their dominant roles in regulating biomass production, calcium accumulation, and bioactive compound formation (Hatfield and Dold, 2019; Lawson and Vialet-Chabrand, 2019). These findings not only explain natural population differentiation but also provide practical guidance for cultivation and breeding. In particular, developing efficient cultivation systems with optimized light, water, and temperature management will be essential to support large-scale factory-based production.

4.2. Comparison of comprehensive evaluation models

PCA and membership function based germplasm evaluation methods are commonly used approaches for assessing crop germplasm. The PCA model has been successfully applied for comprehensive germplasm evaluation and selection in crops such as pear (Zhang et al., 2022), Chinese cherry (Zhou et al., 2023), and lettuce (Tian et al., 2022). Similarly, studies on wild *Leymus chinensis* germplasm showed that PCA is particularly effective for agronomic traits, while the membership function approach is more suitable for comprehensive evaluation of agronomic and quality traits (Chang, 2020). These findings underscore that different methods should be chosen depending on breeding objectives and trait categories.

The present study employed both PCA and membership function methods. PCA identifies axes of maximum variance and thus heavily weighted biomass-related traits in our dataset (FW, DW, LA, MLL, and MLW), which showed the greatest inter-population variability. As a result, PCA favored populations with large biomass, but these often exhibited poorer performance in nutritional and medicinal traits. Moreover, nine of the top ten PCA-ranked accessions belonged to Cluster B, suggesting limited genetic representation. This highlights the limitation of PCA-based selection for breeding programs that require multi-objective improvement. In contrast, the membership function method avoids such bias by allowing flexible trait inclusion based on breeding priorities rather than variance magnitude. To better align with the four breeding objectives, we deliberately narrowed the analysis to 12 representative traits from the original 26. This strategy ensured that all four breeding goals were incorporated into the evaluation. As a result, the membership function approach identified accessions excelling not only in biomass (Cluster B) but also in nutritional and medicinal traits (Cluster A), producing a more balanced and diverse outcome. Importantly, this method enabled multi-objective optimization, directly linking germplasm evaluation to breeding goals rather than variance-driven weighting. Consequently, we adopted the membership function method as the primary evaluation framework and established a core collection comprising 13 accessions with U values greater than 0.50. This core set effectively integrates the complementary strengths of both eastern and western populations, ensuring genetic diversity while maximizing the potential for developing superior cultivars with combined desirable traits.

4.3. Integrated breeding analysis based on metabolomics and quality traits

Metabolomics has emerged as a powerful tool to enhance breeding efficiency and precision, significantly accelerating plant breeding programs (Enfissi et al., 2021). In this study, we employed UPLC-MS/MS based widely targeted metabolomics to profile metabolites in representative eastern (LSFC141) and western (LSFC104) populations of *P. eburnea*. Among the 1045 metabolites detected, significant differences were observed between eastern and western populations, consistent with results from cluster and PCA analyses (Fig S7), thereby validating the rationality of our comprehensive evaluation model for germplasm assessment. Notably, flavonoids (249) and phenolic acids (179) constituted the most abundant metabolite classes (Fig. 7D). The rich diversity of these compounds underlies notable antimicrobial, anti-inflammatory, and antiviral properties of *P. eburnea*, reinforcing its ethnopharmacological value (Billowria et al., 2024; Calis et al., 2020; Wu et al., 2017). Beyond common flavonoids and phenolic acids, *P. eburnea* also produces phenylethanoid glycosides unique to the genus *Primulina*, particularly Chiritoside A, Chiritoside B, and Conandroside. Rare in other plants, these compounds exhibit important pharmacological activities such as antioxidant, anti-inflammatory, and hepatoprotective effects (Chen et al., 2010b; Damtoft and Jensen, 1994; Nanjala et al., 2022; Shen et al., 2025), highlighting new opportunities for developing calcium supplements with added medicinal benefits. Moreover, in the context of developing plant-based calcium supplements, *P. eburnea* offers a distinct advantage: it naturally contains absorption-promoting components such as vitamin K2, vitamin D3, glutamic acid, and aspartic acid (Table S12), which enhance calcium bioavailability (Capozzi et al., 2020). Although these components did not differ significantly between eastern and western populations, their inherent presence in *P. eburnea* reduces the need for external supplementation. From a manufacturing perspective, this feature can significantly lower production costs compared with formulations based on other calcium sources.

We focused on three key attributes of *P. eburnea* as a raw material for calcium preparations: calcium content, medicinal compounds, and biomass. Metabolomic analysis revealed clear population-specific patterns: eastern populations accumulated significantly higher levels of flavonoids and phenolic acids (Fig. S8, S9; Table S18, S19), compounds with well-documented antioxidant and pharmacological activities (Li et al., 2018). In contrast, western populations exhibited higher biomass and calcium content (Fig. 3), supported by larger leaf size, greater fresh and dry weight, and enriched levels of sugars such as D-glucuronic acid, D-mannose, and D-trehalose that are associated with cell wall synthesis and stress-tolerant biomass accumulation (Caffall and Mohnen, 2009;

Fernandez et al., 2010; Gu and Bar-Peled, 2004; Mounet-Gilbert et al., 2016). Pathway analysis indicated that phenylalanine supply was not the limiting factor for flavonoid biosynthesis. Instead, the higher flavonoid levels in eastern populations likely result from stronger regulation at downstream steps, particularly flavanone 3-hydroxylase, flavonol synthase, and flavone synthase as well as enhanced glycosylation capacity by UDP-glycosyltransferases. These processes favor the accumulation of stable flavonol/flavone glycosides and represent priority targets for future breeding and metabolic improvement (Moose and Mumm, 2008), as demonstrated in Carthamus tinctorius (Liu et al., 2016). Taken together, these findings suggest a complementary distribution of traits: eastern populations excel in medicinal compounds, while western populations provide superior biomass and calcium enrichment. Hybrid breeding that integrates both advantages could yield elite cultivars with balanced agronomic and pharmacological traits (Lin et al., 2020; Sacco et al., 2013), establishing *P. eburnea* as a unique resource for producing cost-effective, high-bioavailability calcium supplements with added bioactive health benefits.

5. Conclusions

This study conducted a comprehensive germplasm evaluation of 37 *P. eburnea* populations and identified substantial inter-population variation in biomass-related and medicinal-related traits. Environmental analysis indicated that differences in light, temperature, and precipitation were major drivers of population divergence. PCA and cluster analysis revealed clear geographical differentiation, classifying populations into two distinct clusters. The membership function model proved superior to PCA for core collection construction by effectively balancing ideal trait selection with genetic diversity. Metabolomic profiling further highlighted significant differences in flavonoids and phenolic acids between eastern and western groups, reinforcing their complementary breeding value and supporting the robustness of our comprehensive evaluation model. Overall, these findings establish *P. eburnea* as a promising high-calcium industrial crop and provide valuable resources for breeding programs.

CRediT authorship contribution statement

Tianjiao Jia: Investigation, Formal analysis. **Jie Zhang:** Methodology, Investigation. **Yi Zhang:** Writing – review & editing, Writing – original draft, Software, Methodology, Investigation, Data curation, Conceptualization. **Chen Feng:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization. **Xue Qiu:** Writing – review & editing, Writing – original draft, Software, Methodology, Investigation, Data curation, Conceptualization. **Endian Yang:** Writing – review & editing, Methodology, Data curation. **Minjun Zha:** Methodology, Investigation, Data curation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.indcrop.2026.122750.

Data Availability

Data will be made available on request.

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