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ANALYSIS OF GENETIC DIVERSITY IN PURPLE LETTUCE (*LACTUCA SATIVA* L.) BY SSR MARKERS

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Abstract

In this study, we analysed the genetic diversity and relationships of the leaf lettuce germplasm by SSR markers. The HPLC and Colorimeter techniques were used to measure the anthocyanin contents and colour indices of 54 different coloured *Lactuca* cultivars. Five colour levels of lettuce can be established for leaf grading by our correlation analysis between the colour parameters and contents of anthocyanin. The purple part in the distribution of lettuce leaves can be classified into four categories. This study provides a measure of the leaf colour traits using the scientific method. We also analysed the genetic diversity and relationship among 39 purple leaf lettuce germplasms by SSR markers. Our findings showed that the average values of Nei's gene diversity and Shannon's information index for the 39 cultivars were 0.5635 and 1.0151, indicating a low degree of genetic diversity among the tested genotypes. Cluster analysis showed that all 39 varieties were clustered into two major groups with a similar coefficient of 1.2125. The group I was divided into two sub-groups: leaf fold lettuce and erect lettuce. The first sub-group of lettuce leaf colour was purple, and the anthocyanin contents were greater than those in the other sub-group Crisphead lettuce. The group II was further divided in two sub-groups: leaf fold lettuce and erect lettuce. There were three species in the same branch with a close genetic relationship. The 39 purple leaf lettuce varieties with different SSR fingerprints could serve as cultivar-specific patterns and as an important basis for varieties identification.

Key words: Purple lettuce, Classification, SSR, Phylogenetic relationship, Genetic diversity, Fingerprint.

Introduction:

Leaf lettuce (*Lactuca sativa* L.) is a leaf type of the family Asteraceae. It is one of the most important species of leaf vegetables in the world and as an important species in research. Genome Project (Vries, 1997; Wang, 2011). According to leaf colour, leaf lettuce can be divided into two categories: green and purple. Purple leaf lettuce is rich in anthocyanins, and purple foods containing anthocyanins have higher nutritional value than that in other lighter-coloured foods. Anthocyanins are important health-promoting pigments with strong oxidizing and anti-cancer effects. Their presence greatly enhances the nutritional value of fruits and vegetables and enriches the leaf colour of the vegetables. Purple leaf lettuce can resist hardening of the arteries, and it has good curative effect on patients with hypertension, heart disease and nephropathy, with an auxiliary curative effect on diseases such as mental retardation.

Anthocyanins are important flavonoids in angiosperms. It is an important material basis of plant flowers, leaves and fruit colour (Lin, 2007). There are 6 types of anthocyanins in purple fruits and vegetables, including geranium pigment (Pelargonidin), centaurea pigment (Cynadin), delphinium pigment (Delphinidin), peony pigment (Peonidin), morning glory pigment (Petunidin) and mallow pigment. Anthocyanin biosynthesis is a complex process. It is affected by a variety of physical factors, such as light, temperature, water, and mechanical damage. It is also affected by various mineral elements and biological factors, such as nitrogen deficiency stress and pathogen infection. Research has shown that the synthesis level of anthocyanin differs with growth stage. The synthesis of anthocyanin is also regulated by most plant growth hormones and some plant growth regulators. Various factors have diverse effects on the biosynthesis of anthocyanin (Gillian, 2001).

Epidermal cells of purple leaves lettuce accumulate significant anthocyanins that are adaxially purple. In terms of production, the colour of plant leaves is affected by many factors, such as the application of nitrogen fertilizer, which may enhance leaf colour of the leaves (Cheng *et al*., 2012). In addition, the colour changes in plant leaves after different growth periods also differed. Because the content of anthocyanins differs with distribution site, purple leaves show different colour characteristics (Ma *et al*., 2006). Vegetable leaf colour can be identified through direct observation with the naked eye or colour measurement instruments, which macroscopically observe a colour-coded card (Royal Horticultural Society Colour Chart, referred to as RHSCC) according to the standards of the Royal Horticultural Society (RHS). Leaf colour is divided into different classes for leaf lettuce classification, which is advantageous for screening high appreciation values and high-quality purple leaf lettuce. In our study, 54 purple leaf lettuce germplasms were used, as well as a chromatic metre and high-performance liquid chromatograph to analyse leaf lettuce leaf colour for the content of anthocyanins and to apply correlation analysis of the data, and establish the classification system.

Genetic diversity study is used to identify new elite genotypes (Shinwari *et al*., 2013; Jan *et al*., 2017; Shinwari *et al*., 2018; Jan *et al*., 2018). The genetic diversity of vegetables reflects the adaptability of a species to the environment and its potential for natural transformation and utilization. On the basis of evaluating the genetic diversity of plants, the origin and evolution of plants were discussed by comparing the similarities and differences among

different varieties, and systematic classification of plants has become an important part of plant resource research (Han *et al*., 2016). Molecular markers of DNA polymorphism have become a common method for evaluation of genetic diversity. SSR is widely used in the study of genetic diversity. By studying the sequence of conservative design primers at both ends of the gene, as well as PCR amplification, electrophoresis separation, detection and analysis of the polymorphisms of the plant microsatellite sequences to determine the sequence of genes and the phenotype of the gene, the target gene was successfully identified (Wang *et al*., 2008) (Fig. 1).

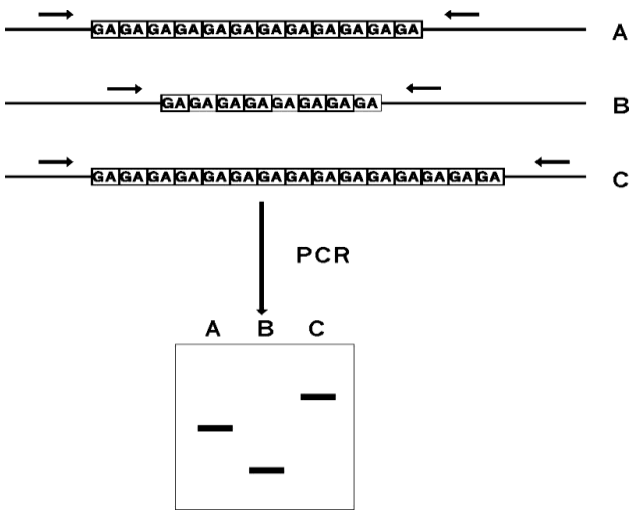

Fig. 1. The principle of SSR (Zhu, 2006). A, B and C represents different gene types.

SSR in groups usually have high polymorphism, which is a kind of good molecular marker, and because of its unique advantage, it is suitable for genetic mapping, diversity analysis and germplasm identification (Zhu *et al*., 2006; Rabbani *et al*., 2010; Turi *et al*., 2012; Shah *et al*., 2015). Molecular markers can detect genetic diversity within species and compare the genetic diversity of species. In recent years, SSR molecular markers have been applied to research on genetic diversity of crops such as soybean, wheat, maize and rice and have achieved great success in crop field (Wu, Tanksley, 1993; Senior, Henu, 2006).

The purple leaf lettuce is currently a new type of lettuce species, and the research on its genetic diversity is rare; however, there are far more reports on other herbs and other vegetables, such as lettuce and other close relatives. The genetic diversity of leaf lettuce resources was studied in other countries by the molecular marker method, and all kinds of labelling techniques were studied in the genus and proximal plants. Vande *et al*., (1999) developed 28 pairs of SSR primers in 1999, being the first development of the SSR lettuce primer. Dilpreet *et al*., (2011) found SSR markers in the EST sequences of wild lettuce and studied the genetic diversity of 22 wild lettuce varieties. Brigitte Uwimana *et al*., (2012) used SSR markers to analyse the population structure of European lettuce plant resources at that time and to determine the reason for the rapid growth and spread of wild lettuce in Europe according to the direction of hybridization.

In this study, we used the method of SSR molecular DNA markers of purple leaf lettuce to analyse the genetic relationship, genetic diversity, and genetic background to map the fingerprints. This study provides a theoretical basis for the identification, genetic breeding and variety improvement of purple lettuce, and it provides a scientific basis for the in-depth utilization of germplasm resources, guiding parental selection and improving breeding efficiency.

Materials and Methods

Plant materials, culture conditions and treatments: To determine the leaf colour phenotype of purple leaf lettuce, the seeds for this study were selected from the Beijing University Agriculture laboratory, with a total of 359 leaf lettuce germplasm resources, including 147 loose leaf types, 71 half-ball types and 141 ball types. On August 17, 2015, 100 seeds from 359 cultivars were sown in a 128-hole tray. Two peat types and vermiculite were mixed, and the average daily temperature was approximately 26 degrees Celsius, with an average daily humidity of approximately 85%. The seedlings were planted on September 14 in Changping District in the Beijing Seed Management Station No. 2 greenhouse. With ridge width 20 cm, ridge length 500 cm, ridge height 15 cm, and spacing 35 cm, the plants were arranged in a Z arrangement with drip irrigation. Mature plant leaves harvested on October 30 were used as test materials. Among the cultivars, 55 were purple leaf lettuce, and 54 different varieties with uniform growth rate and low specific plant rate, the representative purple leaf lettuces were selected as the research object. Three green commercial varieties (American Speed, LiSheng No. 2 and Italian lettuce) were selected as contrast (Table 1).

To analyse the relationship and diversity of purple leaf lettuce, we selected 39 purple leaf lettuces from 54 different varieties; the 39 selected materials are excellent cultivars that have been cultivated for many years and are proven to be stable and inherited, with uniform growth status and low specific rate. The species used for SSR marker analysis are shown in Table 2.

Sensory colour measurement: We investigated the leaf traits, leaf colour, leaf length, leaf width, leaf base shape, leaf margin, leaf cleavage, leaf folds, foliar gloss, and plant height. When using sensory colorimetry, the distance between the observer and the sample was 15-30 cm, and the viewing angle was maintained at 45-degree angle. The best colorimetric method was to place the sample to be measured and the standard colour sample under the same light source side by side and observe them simultaneously to judge the colour quality (Bai, 2007), as shown in the supplementary material.

Determination of colour parameters by colorimeter: The healthy plants were selected randomly for each variety. The leaves were measured randomly (from the 3-4 round of extrovert) using a CIELab Colour space (Bai *et al*., 2006) and WF32 type precision colorimeter from the Shenzhen Weifu Photoelectric Technology Co., Ltd. At the upper, middle and lower parts of the blade, the point position is selected randomly. Each part is established with 3 sets of repetition, reading and recording. The instrument light source is set to D65, and the light mode is SCI.

Table 1. List of tested varieties.

Lettuce type	No.	Breed name	Source
Purple loose leaf types	C1	W58	Beijing, China
	C2	W67	Beijing University of Agriculture
	C3	W93	Fujian Province, China
	C4	H2	Hebei Province, China
	C5	S55	America
	C6	Red Wrinkle	Beijing Green Oriental Agricultural Technology Institute
	C7	Luo Sheng No.1	BEIJING Vegetable Research Center
	C8	Purple Sha	Beijing Atlas Seeds Co.,Ltd.
	C9	S39	Beijing University of Agriculture
	C10	W65	Beijing University of Agriculture
	C11	Crimson Rosa	BEIJING Green Golden Blue Seed Co., Ltd
	C12	Violet	Fujian Province, China
	C13	Purple leaf lettuce	Liaoning Academy of Agricultural Sciences
	C14	171	Beijing University of Agriculture
	C15	American purple leaf lettuce	Hebei Qingfeng Seed Industry Co., Ltd
	C16	Purple lettuce	Beijing China Vegetable Seed Trchnology Co., Ltd
	C17	Hong Sheng No.1	Jingyan Yinong（Beijing）Seed Sci-Teach Co.,Ltd.
	C18	Italian purple leaf lettuce	Hebei Qingxian County earth Nursery Center
	C19	South Korea purple bald lettuce	Hebei Qingxian Wangzhen Seed Breeding Station
	C20	American purple lettuce	Hebei WANFENG SEED INDUSTRY Co., Ltd
	C21	W94	Hebei Province, China
	C22	H4	Beijing University of Agriculture
	C23	H5	Shandong Province,China
	C24	H10	Beijing University of Agriculture
	C25	WJ98	Beijing University of Agriculture
	C26	Acorn lettuce	Jingyan Yinong（Beijing）Seed Sci-Teach Co.,Ltd.
	C27	Acorn lettuce No.1	Jingyan Yinong（Beijing）Seed Sci-Teach Co.,Ltd.
	C28	Red salad bowl 1	Organic Wines in Canada
	C29	MERLOT	West Coast Seeds
	C30	Red salad bowl 2	West Coast Seeds
	C31	RED SAILS	West Coast Seeds
	C32	YN-E	BEIJING Yunong high quality agricultural products planting company
	C33	YN-F	BEIJING Yunong high quality agricultural products planting company
	C34	Fast growing purple leaf lettuce	BEIJING Jindi Yongfeng agricultural science and Technology Co., Ltd
	C35	Special red wrinkle	BEIJING Green Golden Blue Seed Co., Ltd
	C36	Hong Sheng	Beijing University of Agriculture
	C37	0004	BEIJING Happy Green Agricultural Technology Co., Ltd
	C38	00127	BEIJING Happy Green Agricultural Technology Co., Ltd
Purple (half) ball types	C39	P-J1	Beijing University of Agriculture
	C40	P-J2	Beijing University of Agriculture
	C41	704.0195	Beijing Academy of Agricultural and Forestry Sciences
	C42	Erect lettuce No.3	Jingyan Yinong (Beijing)Seed Sci-Teach Co.,Ltd.
	C43	Caesar Duo	Renee`s Garden
	C44	PRIZEHEAD	Lawn &Garden
	C45	RED DEER TONGUE	West Coast Seeds
	C46	Speckled Butterhead	West Coast Seeds
	C47	ROXY PELLETED	West Coast Seeds
	C48	CANASTA RRAT	BEIJING Yunong high quality agricultural products planting company
	C49	YN-B	BEIJING Yunong high quality agricultural products planting company
	C50	Purple Roma	BEIJING EASTEN GREEN SEEDS
	C51	0047	BEIJING Happy Green Agricultural Technology Co., Ltd
	C52	RED LETTUCE	BEIJING Happy Green Agricultural Technology Co., Ltd
	C53	L581	BEIJING Happy Green Agricultural Technology Co., Ltd
	C54	W31	BEIJING Happy Green Agricultural Technology Co., Ltd
Green loose leaf types	C55	American Speed	Jingyan Yinong（Beijing）Seed Sci-Teach Co.,Ltd.
Green(half) ball types	C56	Italian lettuce	The Yixian Formation in Liaoning Province Zhou Zhen Nongle seed
	C57	Erect lettuce No.2	BEIJING Vegetable Research Center

Table 2. List of tested varieties for SSR analysis.

Lettuce type	No.	Name	Source
Loose leaf types	1.	Red Wrinkle	BEIJING EASTEN GREEN SEEDS
	2.	Luo Sheng No.1	BEIJING Vegetable Research Center
	3.	Purple Sha	BEIJING Atlas Seed Co. Ltd
	4.	S39	Beijing University of Agriculture
	5.	Crimson Rosa	BEIJING Green Golden Blue Seed Co., Ltd
	6.	Violet	Fujian Province, China
	7.	Purple leaf lettuce	Liaoning Academy of Agricultural Sciences
	8.	171	Beijing University of Agriculture
	9.	American purple leaf lettuce	Hebei Qingfeng Seed Industry Co., Ltd
	10.	Purple lettuce	BEIJING CHINA VEGETABLE SEED TRCHNOLOGY CO., Ltd
	11.	Red No.1	BEIJING Vegetable Research Center
	12.	Italian purple leaf lettuce	Hebei Qingxian County earth Nursery Center
	13.	American purple lettuce	Hebei WANFENG SEED INDUSTRY Co., Ltd
	14.	W94	Hebei Province,China
	15.	H4	Beijing University of Agriculture
	16.	H5	Shandong Province,China
	17.	H10	Beijing University of Agriculture
	18.	Acorn lettuce	BEIJING Vegetable Research Center
	19.	MERLOT	West Coast Seeds
	20.	Red salad bowl	West Coast Seeds
	21.	RED SAILS	West Coast Seeds
	22.	YN-F	BEIJINGYunong high quality agricultural products planting company
	23.	Fast growing purple leaf lettuce	BEIJING Jindi Yongfeng agricultural science and Technology Co., Ltd
	24.	Hong Sheng	Beijing University of Agriculture
	25.	00127	BEIJING Happy Green Agricultural Technology Co., Ltd
(Half) ball types	26.	P-J1	Beijing University of Agriculture
	27.	Erect lettuce No.3	Jingyan Yinong（Beijing）Seed Sci-Teach Co.,Ltd.
	28.	Caesar Duo	Renee`s Garden
	29.	PRIZEHEAD	Lawn & Garden
	30.	RED DEER TONGUE	West Coast Seeds
	31.	Speckled Butterhead	West Coast Seeds
	32.	ROXY PELLETED	West Coast Seeds
	33.	CANASTA RRAT	BEIJING Yunong high quality agricultural products planting company
	34.	YN-B	BEIJING Yunong high quality agricultural products planting company
	35.	Purple Roma	BEIJING EASTEN GREEN SEEDS
	36.	0047	BEIJING Happy Green Agricultural Technology Co., Ltd
	37.	RED LETTUCE	BEIJING Happy Green Agricultural Technology Co., Ltd
	38.	L581	BEIJING Happy Green Agricultural Technology Co., Ltd
	39.	W31	BEIJING Happy Green Agricultural Technology Co., Ltd

Chromatographic measurement of anthocyanin content: The purple leaf lettuce leaves were ground thoroughly in a mortar with liquid nitrogen for colour determination. Then, 5 g was added to a 4-mL centrifuge tube and 2 mL anthocyanin extract (formic acid: methanol: water=1: 80: 19) was added. An ultrasonic water bath was used to extract for 45 min at 45 degrees Celsius and centrifuged at 8000 rpm for 5 min, followed by filtering into 2-mL liquid phase vials using a 0.25-μm organic filter, with 3 repeats each. Anthocyanin content was determined using high-performance liquid chromatography; the wavelength was 520 nm, the column temperature was 25 degrees Celsius, the flow rate was 1 mL / min, the injection volume was 20 μl and the cycle time was 33 min. The mobile phase was 5% methanol and 10% acetonitrile，the volume ratio was 9 to 1.

DNA extraction from purple leaf lettuce: A DNA Kit (Beijing Ding Guo Biotechnology Co., Ltd.) was used to extract DNA; 800 μL of lysate was added to a 1.5 mL centrifuge tube and preheated at 65 degrees Celsius; then, β-mercaptoethanol was added to a final concentration of 0.1% and added to a centrifuge tube with 800 μL of pre-warmed lysate in a water bath at 65°C for 20-30 min. The sample was inverted several times and 12 μL RNase was added and incubated at room temperature for 5 min. Then, added 500 μL of chloroform and mixed thoroughly, centrifuged it at 12000 rpm for 10 min and aspirated the supernatant, added 700 μL of Binding buffer and mixed well, then the liquid was added to the spin column and centrifuged at 12000 rpm for 1 min. Then, 700 μL ethanol eluent A was added and centrifuged at 12000 rmp for 1 min, followed by waste disposal. Then, 700 μL ethanol containing eluent B was added and centrifuged at 12000 rpm for 1 min, followed by waste disposal and the step was repeated. The sample were centrifuged at 12000 rpm for 2 min and kept at room temperature for 10 min. Finally, 55 - 65 degrees Celsius preheated TE was added and left at natural room temperature for 5 min, followed by 12000 rpm centrifugation for 2 min. The centrifuge tube with the extracted DNA solution was stored at 2-8 degrees Celsius or at -20 degrees Celsius for long-term storage.

Table 3. Detail of SSR primers with arbitrary sequence.

No.	Primer name	Forward sequence	Reverse sequence	Repeat unit	Repeat times
1.	sml001	CCATGGATCCTGTGTGAAGA	CACCATGTTCCACTTCCACTT	CATGAT	6
2.	smll022	GGGCCTCAAATCCTCTCTG	TGTTCTTCCCCTCTTTGGAA	ATC	13
3.	ksl115	CATTGCACTCCGTCATCTCC	GGGTTGATTCCGAAAGTTCC	ATC	16
4.	ksl123	ATTGTAACTTCTGCGGGCCT	GCCTCACATGTTCTTCCCCT	ATC	13
5.	ksl173	ATAGTCACGACTCACGCCCA	CCATTTTCCTCTTTCTGCGA	CT	14
6.	sml013	TCCCATGATGGAGAGACTCA	CCCAAAAGGGAATAGCAACC	GAA/CTG	14/5
7.	ksl26	GGGCTTTCTCTCCTTTCCTTT	AATTTGGATCCTGTCGAGGG	TC	11
8.	ksl32	CGGGGAGCATTTAGTGTGTG	AATTTGGGGTCCGATTTGAG	CT	14
9.	ksl271	ACAAAGGCAAGATTGGGTCA	GCGGATATGCAGCCATAACA	ATG	12
10.	ksl51	CCCCTACCACCACCAAAGTC	TACCAAATGACATGCACCCC	ATG	10
11.	ksl97	CGCAGAAAAGGGATCAGACA	TCAGAGACACTGCAAAAGGGA	CT	11
12.	sh42	GCAAGCTAAAGGGCTTTTTGT	CAGCCTGGGAATATTTACTCTGA	ATT	27
13.	sh43	CTCTCGTTCCTTTTGTTGGTTT	TGGTAGTGGCTTCCTTGCTATT	AC	10
14.	sh66	GGTAGGGCAGTCAAGCAAGA	AATGATGATTTTGCCCTTGG	TC	28
15.	ksl92	GGTCTCTTTCCTCTGCCCTG	TCGCGTTCTGAAGTAGCCAT	CT	20
16.	sml015	TTGAGGAGGGCATTTACGTC	GAGGCGTATCTCCAAGGTGT	TGTTA	16
17.	sml026	GGGTTCTCATTGGCTGACAT	TGTCTTCCAACCAAAACATACA	GAA	11

SSR-PCR amplification and establishment of reaction system: SSR fluorescent primer system (total 20 µL): ddH2O 14.8 µL, dNTP 0.4 µL, Buffer 2 uL, Primer (F)0.3 µL (20 µM), Primer(R)0.3 µL (uM), DNA template 2 µL, Taq 0.2 µL. SSR PCR amplification procedure: denaturation at 94 degrees Celsius for 5 min; denaturation at 94 degrees Celsius for 30 s followed by annealing at 54°C (annealing temperature at 54°C) for 35 s, 72 degrees Celsius extension for 40S, 35 cycles; final extension for 72°C 3 min.

Screening primers: Primer sequences were cited from Jee-Hwa Hong (Hong, Kong, 2015).Among the 21 primers used, 17 SSRs with high polymorphism and good reproducibility were screened for purple leaf lettuce (Table 3). The SSR primers were synthesized by the Shanghai Sangon Biotech Technology Co., Ltd.

Electrophoresis to check amplification products: Formamide and molecular weight internal standards were prepared from a 100: 1 volume ratio mix; 9 µL was added to the sample, and 1 µL of this mixture was diluted 10 times to give the PCR product. Capillary electrophoresis was performed using a sequencer. The original data obtained from the sequencer was analysed using the Fragment analysis software in Genemarker. The position of the internal standard molecular weight in each lane was compared to the peak position of each sample.

Statistical analysis of data: Using Excel and SPSS software statistics and analysis, we calculated the hue angle H, the hue ratio h and the saturation degree C using the formulas (1) - (3) (Wang, Dai, 2009). Anthocyanin content (Ant. content) was calculated from (4) (Hou, 2011), A is the area of the peak, V is the volume of the extract (mL) and m is the fresh weight of the sample.

$$H = tan\text{-}1\ (b / a) \quad (1)$$

$$h = a / b \quad (2)$$

$$C = (a2 + b2)\ ½ \quad (3)$$

$$Anthocyanin\ content\ (\mu g / g) = (0.003A + 0.3) / m\ R2 \quad (4)$$

The fragment size of each sample at each allele locus was entered into EXCEL according to the Convert 1.31 software and was then converted into the POPGENE software format. According to the electrophoresis results of the peak map readings, a peak is noted as 1, and no peak is recorded as 0. P (Percent of polymorphic loci), Ne (Effective number of alleles per loci), He (Expected Heterozygosity), Ho (Observed Heterozy-zygosity), and PIC (Gene frequency calculated by PIC software) were also recorded. I (Genetic Identity) and D (Genetic Distance) measure the size of genetic differentiation between various groups. Based on genetic distance, the UPGMA method was used to cluster various populations to construct the phylogenetic tree and analyse the genetic relationships among varieties. According to the original data matrix of the peak value, fingerprints of 39 purple lettuce leaves could be constructed using Excel.

Results

Leaf colour difference indicators: According to CIE Lab Color space (Fig. 2), the results showed that the overall L value of purple leaf lettuce was between 23.59 and 60.07; the value of a changed from green to red between -12.82 and 10.56, and the value of b changed from blue to yellow between 4.34 and 38.04. When leaf lettuce leaf colour changes from light purple to purple, the value of L gradually decreases, the value of a gradually increases and the value of b decreases gradually. In addition to the green varieties, the colour of W28 was lightest, and the colour of the leaves of W31 is the deepest, consistent with visual observation (Table 4).

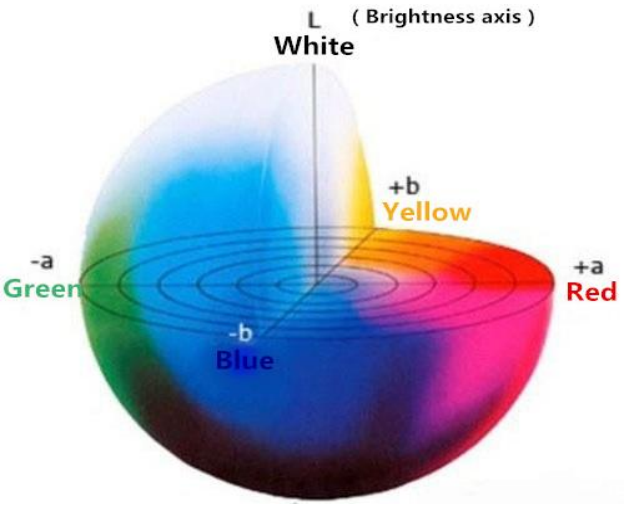

Fig. 2. CIELab Colour space. Three-dimensional colour space to L, a and b for the three-axis space Cartesian coordinate system; the L axis is the vertical axis, and the two orthogonal horizontal axes are a, b axis (Gonnet, 1993). The L value represents brightness: black when L = 0, white when L = 100, and grey between 0 and 100. Larger L values indicate higher brightness is, and the L value can better reflect different varieties with nuance (Cheng *et al*., 2012). The a value represents the degree of colour change between red and green; the value is positive, and the colour is reddish; larger values indicate darker red; negative values indicate greenish hue, and smaller values indicate darker green. The b value represents the degree of change in yellow compared to blue, with positive for yellow and negative for blue (Zhang *et al*., 2008).

The conversion of the hue angle H into an angle is generally from 0 to 180. When the value is 0, the colour is purple, and when the value is equal to 90, the colour is yellow, and when 180, it is green. When H >100, the larger the value of H is, the darker the green is. When H <50, the smaller the value of H, the darker the red colour. The h value is the colour ratio of the colour parameter. When h< 0, the smaller the value is, the darker the colour is. When h>0, the larger the value, the darker the colour of red. Saturation C indicates the vertical distance from the test colour to the L axis (Fig. 2). The greater the distance, the greater the saturation. According to the change of the numerical value, it can be inferred that the purple colour changes of the different leaf parts. The calculated H value of purple leaf lettuce varied from -0.37 to 1.49. The value of h varied between -0.37 and 2.79. The value of C varied between 7.35 and 40.95.

In comparison, all varieties according to the degree of purple can be divided into four categories: light purple, purple, dark purple and special purple. Among them, S39 was the lightest purple. When a < -7.5 and h < -0.34, the leaf colour of leaf lettuce was greenish and the purple was shallower; the values of H and h were negative, and the eligible leaf lettuce could be divided into light purple varieties, such as W28, W36, S39, Acorn lettuce, and Acorn lettuce No. 1. When a> 6.31 and h >0.76, the leaves are purple and can be classified into special purple varieties. The samples include Hongsheng No.1, W94, Red salad bowl, MERLOT, Hongsheng, 00127, 704.0195, Purple Roma, W31, and L581, including 10 varieties. The H value and h value are positive, and the purple covered the entire leaf. The sensory colour, colour parameters and anthocyanin content of various purple leaf lettuce leaves are shown in Table 4.

Correlation analysis of colour parameters and anthocyanin content: According to Table 4, the content of anthocyanins in the sample was between 0 and 36.89. With the increase of anthocyanin content, leaf colour darkened. Among them, the colour of W28 and W36 was lightest, the content of anthocyanin was lowest, the colour of W31 and L581 was deepest, and the content of anthocyanin was highest. Among the samples, 10 were special purple varieties, namely Hongsheng No. 1, W94, Red salad bowl 1, MERLOT, Hongsheng, 00127, 704.0195, Purple Roma, L581, and W31.

We analysed the correlation between the colour parameters and anthocyanin content of lettuce leaves of different colour grades. The results showed that deeper purple leaves indicated greater content of anthocyanin in leaves. The content of anthocyanins in leaves was negatively correlated with L and b values and was positively correlated with other colour parameters. The content of anthocyanins was significantly correlated with L, a, b and h values (Table 5).

Settings for purple leaf lettuce grading standards: The colour difference values and anthocyanin contents of the samples were comprehensively analysed, and the purple leaf lettuce was graded (Table 6) According to this standard, the content of anthocyanins in leaves can be roughly estimated from the range of sensory and colorimetry values.

Purple leaf lettuce classification according to purple distribution of leaves: According to the colour differences in different parts of leaves and the distribution of purple parts in leaves, purple leaf lettuce can be divided into four categories, which are very helpful to develop new varieties. Edge purple and the purple area accounted for less than 30% of the total leaf area; the purple was obviously concentrated at the leaf edge, most commonly in loose leaf lettuce. Gradient purple and purple area were more than 30% of the total leaf area, from the leaf margin to the leaf base; leaf colour from the purple to green gradient and the gradient purple type are the most common and have the largest proportion, which is common in the half-ball varieties. Full-leaved purple and the purple area comprised more than 60% of the total leaf area or purple that covers the whole leaf (when the purple area is more than 60% of the whole leaf, the overall visual effect of the plant is the same as that of the whole leaf purple); and piebald purple, purple spots or patches evenly distributed in the blade surface. This classification applies to all purple leaf lettuce, which provides a basis for breeding new varieties.

Among the 54 varieties of purple leaf lettuce, 14 varieties can be classified as the edge purple type and 30 varieties can be classified as the gradient purple type, including Hongsheng No.1, W94, Red salad bowl 1, MERLOT, Hongsheng, and 00127. A total of 10 varieties can be classified as full-leaved purple type, and only Speckled Butterhead is a type of piebald purple. Most loose-leaf lettuce belongs to the edge purple type; most of the half-ball varieties can be classified as the gradient purple type, of which 704.0195, Violet, W31 and L581 can be classified as the full-leaved purple type. This classification facilitates the screening of purple leaf lettuce with different colour traits (Table 7).

Table 4. The anthocyanin contents and color indices of different colored lactuca varities.

No.	Name	L	a	B	H	h	C	Ant. Content	Color
1.	W58	44.37	4.79	24.10	1.39	0.19	24.79	1.31	P
2.	W67	44.06	1.53	24.00	0.43	0.10	24.23	3.73	P
3.	W93	40.79	5.51	18.24	1.22	0.39	19.68	8.33	P
4.	H2	44.75	1.81	23.41	1.49	0.08	23.49	8.21	P
5.	S55	42.74	6.01	21.54	1.17	0.53	23.32	6.19	Dp
6.	Red Wrinkle	40.28	6.47	15.72	1.18	0.41	16.998123	7.63	P
7.	Luo Sheng No.1	43.97	3.17	19.09	1.41	0.17	19.36	7.26	P
8.	Purple Sha	40.53	4.67	18.92	1.33	0.25	19.49	15.65	P
9.	S39	47.02	-7.51	28.53	-1.24	-0.34	23.13	0.41	Lp
10.	W65	45.03	-4.65	29.57	-0.43	-0.10	30.55	8.99	P
11.	Crimson Rosa	45.37	-4.65	29.57	-1.41	-0.16	29.93	2.12	P
12.	Violet	43.33	-2.89	28.50	-0.50	-0.02	29.43	3.59	P
13.	Purple leaf lettuce	43.01	-2.83	27.63	-0.47	-0.06	28.43	0.82	P
14.	171	43.03	-3.29	28.71	-0.44	-0.09	29.04	1.37	P
15.	American purple leaf lettuce	45.31	1.26	30.40	0.46	0.07	30.65	1.56	P
16.	Purple lettuce	45.43	2.83	22.91	1.45	0.12	23.09	2.99	P
17.	Red No.1	31.12	8.98	12.08	0.81	1.23	15.89	25.25	Sp
18.	Italian purple leaf lettuce	43.12	0.03	27.45	-0.57	0.05	27.66	0.57	P
19.	South Korea purple bald lettuce	43.72	0.76	29.96	0.44	0.08	30.63	0.51	P
20.	American purple lettuce	43.33	2.67	23.00	0.37	0.16	23.36	3.11	P
21.	W94	31.83	7.62	11.09	0.86	1.07	13.63	20.69	Sp
22.	H4	44.59	0.44	26.12	0.45	0.08	26.78	2.44	P
23.	H5	40.83	4.40	21.47	0.26	0.28	22.50	9.07	Sp
24.	H10	42.31	1.15	20.34	1.51	0.06	20.37	1.36	P
25.	WJ98	44.27	0.72	23.19	-0.52	0.00	23.53	1.61	P
26.	Acorn lettuce	46.88	-10.77	29.47	-1.22	-0.37	31.38	0.30	Lp
27.	Acorn lettuce No.1	45.17	-9.67	28.79	-1.25	-0.33	30.39	0.26	Lp
28.	Red salad bowl	34.43	7.77	8.78	0.83	0.93	11.79	21.52	Sp
29.	MERLOT	33.18	7.62	14.92	0.84	1.29	17.74	36.83	Sp
30.	Red salad bowl	46.09	-1.74	26.93	-1.51	-0.06	26.98	3.86	P
31.	RED SAILS	43.69	-1.02	24.23	-1.53	-0.04	24.25	1.77	P
32.	YN-E	43.15	5.33	21.57	1.37	0.21	25.21	14.26	Dp
33.	YN-F	38.29	4.50	15.91	1.21	0.39	16.79	8.62	Dp
34.	Fast growing purple leaf lettuce	44.71	1.08	25.93	0.48	0.05	26.01	1.31	P
35.	Super red wrinkle	37.18	6.05	15.73	1.13	0.52	17.76	10.26	Dp
36.	Hong Sheng	35.05	10.20	12.31	0.85	0.93	16.26	22.33	Sp
37.	0004	41.44	5.01	20.78	1.30	0.28	21.73	13.31	Dp
38.	00127	36.70	8.64	11.92	0.93	0.76	14.77	26.86	Sp
39.	P-J1	51.66	-10.14	39.09	-1.32	-0.26	40.39	0.06	Lp
40.	P-J2	53.36	-8.44	40.07	-1.36	-0.21	40.95	0.06	Lp
41.	704.0195	26.79	6.31	4.80	0.67	1.68	7.45	21.78	Sp
42.	Erect lettuce No.3	44.19	-3.35	21.96	-0.46	-0.06	22.75	2.13	P
43.	Caesar Duo	43.20	-2.05	22.04	-1.48	-0.09	22.14	2.26	P
44.	PRIZEHEAD	44.89	-3.20	27.54	-1.46	-0.12	27.73	1.97	P
45.	RED DEER TONGUE	42.89	-2.39	14.30	-1.40	-0.17	14.50	2.16	P
46.	Speckled Butterhead	43.10	1.66	23.83	1.49	0.08	23.92	0.53	P
47.	ROXY PELLETED	38.08	1.83	19.24	1.48	0.09	19.32	2.14	P
48.	CANASTA RRAT	35.70	2.02	13.37	1.42	0.15	13.52	1.92	P
49.	YN-B	42.86	4.56	23.93	1.38	0.19	24.36	13.89	P
50.	Purple Roma	23.54	6.92	4.34	0.62	1.40	7.35	23.29	Sp
51.	0047	42.45	-3.06	22.04	-0.42	-0.10	22.45	1.77	P
52.	RED LETTUCE	40.48	-0.48	17.66	-0.50	-0.03	17.68	2.05	P
53.	L581	35.15	7.43	13.85	0.90	2.03	18.13	36.89	Sp
54.	W31	28.27	10.56	12.06	0.68	2.79	19.29	33.41	Sp
55.	Fast growing in America	60.07	-11.89	38.04	-1.27	-0.31	39.86	0	G
56.	Erect lettuce No.2	58.78	-12.26	37.35	-1.25	-0.33	39.31	0	G
57.	Italian lettuce	57.11	-12.82	37.63	-1.25	-0.34	39.76	0	G

Note: Lp = Light purple; P = Purple; Dp = Dark purple; Sp = Special purple; G = Green

Table 5. Correlation between the color parameters and anthocyanin contents.

Color parameters	Ant. content	L	a	b	h
L	-0.739**	1			
a	0.721**	-0.854**	1		
b	-0.771**	0.940**	-0.889**	1	
h	0.881**	-0.832**	0.763**	-0.815**	1

Table 6. The grading criteria for lettuce in purple leaf.

Grade	Color	L	a	B	h	Ant. content(CA)
0	G	L≥55.24	a≤-11.33	b≥38.71	h＜-0.35	CA≤0.20
1	Lp	55.24＞L≥45.24	-11.33＜a≤-6.08	38.71＞b≥29.47	-0.35＜h≤-0.16	0.20＜CA≤0.47
2	P	45.24＞L≥42.88	-6.08＜a≤4.60	29.47＞b≥22.38	-0.16＜h≤0.2	0.47＜CA≤8.59
3	Dp	42.88＞L≥36.94	4.60＜a≤6.47	22.38＞b≥14.79	0.2＜h≤0.65	8.59＜CA≤18.17
4	Sp	L＜36.94	a＞6.47	b＜14.79	h＞0.65	CA＞18.17

Table 7. Four categories of purple lettuce by purple part in distribution of leaf.

Sort	Quantity of breeds	Number	Name
Edge purple	14	C1、C2、C3、C4、C6、C7、C8、C10、C11、C32、C37、C49、C39、C40	W58、W67、W93、H2、Red Wrinkle、Luo Sheng No.1、Purple Sha、W65、Crimson Rosa、YN-E、0004、P-J1、P-J2、YN-B
Gradient purple	30	C5、C9、C12、C13、C14、C15、C16、C18、C19、C20、C22、C23、C24、C25、C26、C27、C30、C31、C33、C34、C35、C42、C43、C44、C45、C46、C47、C48、C51、C52	S55、S39、Violet、Purple leaf lettuce、171、American purple leaf lettuce、Purple lettuce、Italian purple leaf lettuce、South Korea purple bald lettuce、American purple lettuce、H4、H5、H10、WJ98、Acorn lettuce、Acorn lettuce No.1、Red salad bowl 2、RED SAILS、YN-F、Fast growing purple leaf lettuce、Super red Wrinkle、Erect lettuce No.3、Caesar Duo、PRIZEHEAD、RED DEER TONGUE、ROXY PELLETED、CANASTA RRAT、YN-B、0047、RED LETTUCE
Full-leaved purple	10	C17、C21、C28、C29、C36、C38、C41、C50、C53、C54	Red No.1、W94、Red salad bowl 1、MERLOT、Hong Sheng、00127、704.0195、Purple Roma、L581、W31
Piebald purple	1	C35	Speckled Butterhead

Purple leaf lettuce DNA detection and primer amplification results: A clear DNA band was detected in 39 samples of purple leaf lettuce varieties (Fig. 3). Although some of the DNA was slightly degraded, the amount of DNA used by the SSR was small and could be used for subsequent experiments.

A total of 17 pairs of primers and 23 lettuce samples were subjected to capillary electrophoresis using a 3730XL sequencer, and the amplified fragment size of the primer was obtained according to the peak position of each sample. Tables 8 and 9 show the amplification range of the primers.

Statistical analysis of each primer locus data: The analysis of the PIC value represents the polymorphic information content for the primer (locus). Among the 17 primer pairs, the highest value of the primer ksl173 was 0.707, and the lowest value of the primer sml026 was 0.1516, as shown in Table 10.

The number of observed alleles (Na), effective number of alleles (Ne), observed heterozygosity (Ho), expected heterozygosity (He), and Shannon information index (I) at each locus reflect the population genetic diversity. Common indicators were used to measure the size of genetic variation. Statistics for genetic variation and the heterozygosity of all loci are shown in Table 11.

The results of statistical analysis (Table 11) showed that the average number of alleles (Na) in each pair of primers ranged from 2 to 6 between 17 pairs of primers. Primer 7 was highest; primer 9 and 17 were lowest. The average was 3.8235; the average number of effective alleles (Ne) was between 1.198 and 3.9774. The highest was primer 5, the lowest was primer 17, and the average was 2.4739. The range of variation (Ho) was 0.4783 - 1 and the average of primer 13 was 0.964. The range of expected heterozygosity (He) was 0.1691 - 0.7652, the highest was for primer 5 and the lowest was primer 17, and the average was 0.5765. The Shannon Information Index (I) ranged from 0.3046 to 1.4744, with the highest primer 5 and the lowest primer 17 and an average of 1.0151. The Nei's gene diversity index ranged from 0.1653 to 0.7486, with an average of 0.5635.

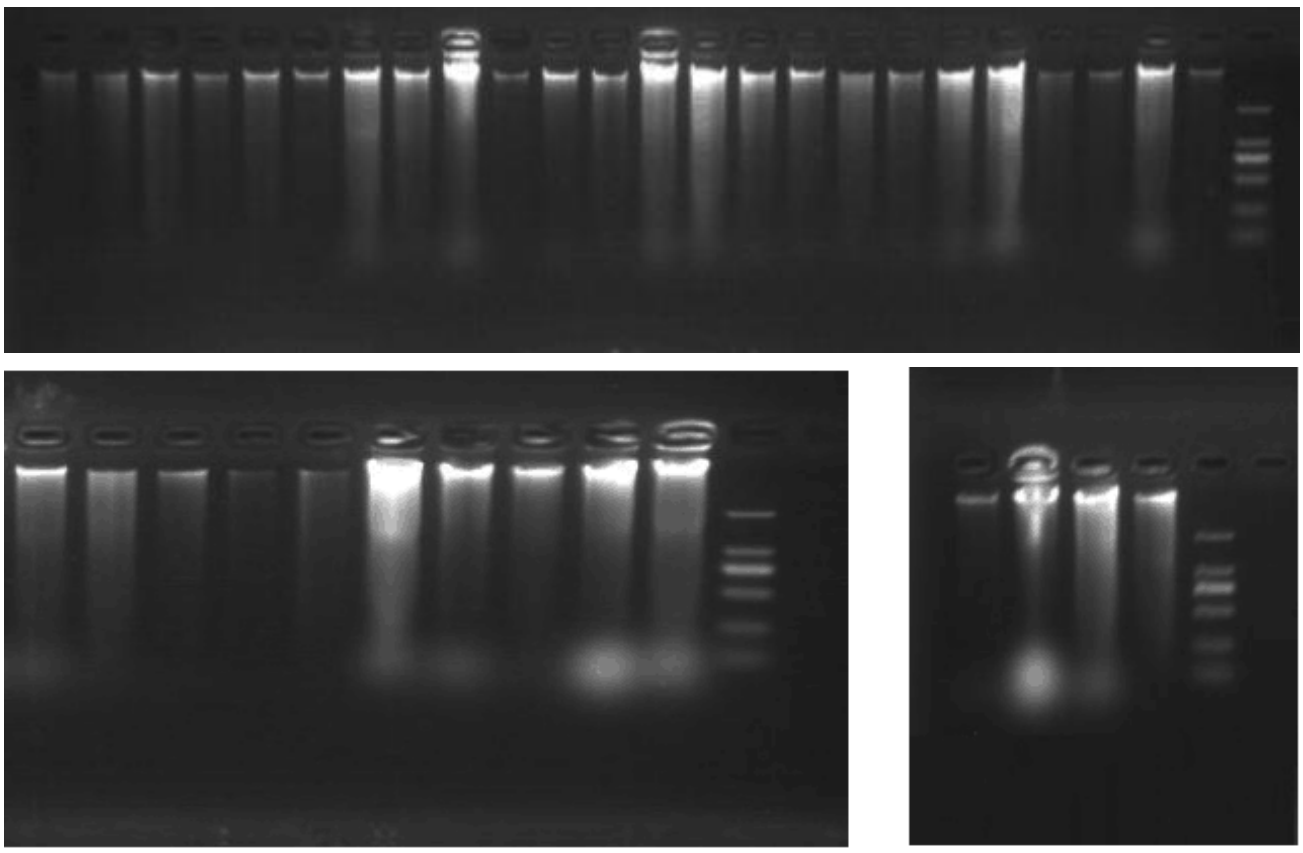

Fig. 3. Detection results for DNA. Extracted DNA was verified by electrophoresis on a 1.5% agarose gel.Clear DNA bands were detected in 39 purple leaf lettuce samples.

The He value of 17 pairs of primers, except for primer 13, were higher than the value of Ho, indicating that the primer heterozygous level has reached the required balance ratio, and the 13 primers' lack of heterozygosity failed to meet the balance of the required ratio. The order of the number of alleles (Ne) is 5>2>7>14>4>15>6>8>12>10>13>1>11>3>16>9>17.
The order of the degree of heterozygosity (He) is 5>2>7>14>4>15>6>8>12>10>13>1>11>3>16>9> 17; according to the Shannon information. The index (I) is sorted by the size of the indicator. The order is 5>7>2>15>14>4>12>6>8>1>10>13>11>3>16>9>17.

According to the F statistics and gene flow (Table 12), among the 17 primers, the average rate of inbreeding in the population is -1, the average rate of selfing in the population is 0.9369, and the average rate of population differentiation is 0.9685, of which the lowest is 0.5519. "Sh43" has the lowest rate of population differentiation and the highest gene flow values.

Genetic distance and genetic similarity coefficient: Genetic distance is a quantitative measure of the degree of kinship among groups. It uses genetic frequency as a function of genetic differences between populations. To analyse the degree of genetic differentiation among tested cultivars, we calculated the genetic similarity coefficient I and the genetic distance D (Appendix 2). The genetic identity was between 0.06 and 1, and the genetic distance ranged from 0 to 2.8132. Further comparative analysis showed that Luosheng No.1, Purple Sha, and purple lettuce had the highest genetic identity, lowest genetic distance and highest genetic similarity. The genetic identity between Red Lettuce and Fast-growing purple leaf lettuce was lowest, and the genetic distance was highest, with the highest degree of genetic differentiation. UPGMA cluster analysis was performed to obtain the clustering map of 39 samples of purple leaf lettuce to infer the genetic relationship between varieties (Fig. 4).

The results of our cluster analysis showed that purple leaf lettuce included leaf fold and the erect type; leaf colour was darker and anthocyanin content was obviously higher than other branches in the first subgroup of Group I; the second subtypes included the types for leaf fold, ball and the erect lettuce. Most are "Acron lettuce" with a light purple colour and low anthocyanin content. Group II consisted of the leaf fold type and erect lettuce. The leaf shape, leaf folds, leaf tips, leaf margin and other characteristics of the leaves were similar, and the phylogenetic relationships were also closer. As seen from the Fig. 4, Luosheng No.1, Purple Sha, and purple lettuce, three samples were located on the same clade, indicating that the three have the most recent kinship.

Construction of fingerprints of purple leaf lettuce varieties: A total of 17 pairs of primer combinations were used for capillary electrophoresis experiments. According to the peak value of the electrophoresis result, the peak count was 1, and the peak free count was 0, which formed the original data matrix. According to the data, the fingerprints of 39 purple leaf lettuce cultivars can be constructed with Excel (Table 13).

Table 8. The results of Primer amplification (1).

S. No.	sml001		smll022		ksl115		ksl123		ksl173		sml013		ksl26		ksl32	
1.	231	231	182	182	227	227	299	299	183	183	277	277	192	192	264	264
2.	231	231	182	182	227	227	299	299	183	183	277	277	192	192	264	264
3.	231	231	182	182	227	227	299	299	183	183	277	277	192	192	264	264
4.	231	231	198	198	221	221	271	271	183	189	269	269	190	190	264	264
5.	231	231	182	182	227	227	—	—	183	183	277	277	192	192	254	254
6.	240	240	182	182	227	227	293	293	183	187	277	277	196	196	254	254
7.	231	240	201	201	227	227	299	299	187	187	277	277	194	194	264	264
8.	231	231	182	182	229	229	293	293	183	189	277	277	192	192	264	264
9.	231	231	182	182	227	227	293	293	187	187	267	267	192	192	264	264
10.	231	231	182	182	227	227	299	299	183	183	277	277	192	192	264	264
11.	231	231	182	182	227	227	299	299	183	187	277	277	185	185	264	264
12.	231	231	182	182	227	227	293	293	183	187	267	267	192	192	264	264
13.	231	231	182	182	227	227	—	—	187	187	267	267	192	192	254	254
14.	240	240	201	201	227	227	299	299	183	189	269	269	194	194	264	264
15.	231	231	198	198	229	229	277	277	183	189	269	269	194	194	264	264
16.	231	231	198	198	229	229	299	299	183	183	269	269	194	194	254	254
17.	231	231	198	198	229	229	—	—	183	189	269	269	194	194	254	254
18.	231	231	198	198	227	227	293	293	183	189	269	269	194	194	254	254
19.	231	231	182	182	227	227	299	299	183	187	277	277	192	192	264	264
20.	231	231	182	182	227	227	290	290	183	187	277	277	196	196	264	264
21.	240	240	182	182	229	229	293	293	—	—	277	277	192	192	254	254
22.	231	231	198	198	229	229	299	299	183	189	277	277	192	192	264	264
23.	231	231	182	182	227	227	293	293	187	187	275	275	196	196	254	254
24.	197	197	328	328	208	208	350	350	154	154	261	261	306	306	209	209
25.	175	175	335	335	206	206	355	355	149	149	273	273	—	—	—	—
26.	175	175	331	331	208	208	353	353	149	149	273	273	315	315	207	207
27.	175	175	331	331	208	208	353	353	149	149	273	273	315	315	207	207
28.	175	175	321	321	206	206	336	336	162	162	264	264	319	319	207	207
29.	175	175	328	328	208	208	350	350	149	149	261	261	306	306	207	207
30.	197	197	328	328	206	206	350	350	157	157	273	273	298	298	207	207
31.	181	181	328	328	208	208	350	350	154	154	264	264	306	306	207	207
32.	181	181	312	312	208	208	336	336	162	162	273	273	298	298	215	215
33.	175	175	328	328	208	208	350	350	157	157	273	273	306	306	207	207
34.	192	192	328	328	206	206	350	350	154	154	261	261	310	310	207	207
35.	175	175	312	312	208	208	353	353	162	162	273	273	298	298	215	215
36.	175	175	331	331	208	208	353	353	149	149	173	173	315	315	207	207
37.	197	197	321	321	208	208	336	336	162	162	273	273	306	306	215	215
38.	—	—	—	—	208	208	350	350	154	154	261	261	306	306	209	209
39.	197	197	328	328	208	208	350	350	154	154	261	261	298	298	209	209

Table 9. The results of Primer amplification (2).

S. No.	ksl271		ksl51		ksl97		sh42		sh43		sh66		ksl92		sml015		sml026	
1.	231	231	182	182	227	227	299	299	183	183	277	277	192	192	264	264	-	-
2.	231	231	182	182	227	227	299	299	183	183	277	277	192	192	264	264	196	196
3.	231	231	182	182	227	227	299	299	183	183	277	277	192	192	264	264	196	196
4.	231	231	198	198	221	221	271	271	183	189	269	269	190	190	264	264	196	196
5.	231	231	182	182	227	227	—	—	183	183	277	277	192	192	254	254	196	196
6.	240	240	182	182	227	227	293	293	183	187	277	277	196	196	254	254	169	169
7.	231	240	201	201	227	227	299	299	187	187	277	277	194	194	264	264	196	196
8.	231	231	182	182	229	229	293	293	183	189	277	277	192	192	264	264	196	196
9.	231	231	182	182	227	227	293	293	187	187	267	267	192	192	264	264	196	196
10.	231	231	182	182	227	227	299	299	183	183	277	277	192	192	264	264	196	196
11.	231	231	182	182	227	227	299	299	183	187	277	277	185	185	264	264	196	196
12.	231	231	182	182	227	227	293	293	183	187	267	267	192	192	264	264	196	196
13.	231	231	182	182	227	227	—	—	187	187	267	267	192	192	254	254	196	196
14.	240	240	201	201	227	227	299	299	183	189	269	269	194	194	264	264	196	196
15.	231	231	198	198	229	229	277	277	183	189	269	269	194	194	264	264	196	196
16.	231	231	198	198	229	229	299	299	183	183	269	269	194	194	254	254	196	196
17.	231	231	198	198	229	229	—	—	183	189	269	269	194	194	254	254	196	196
18.	231	231	198	198	227	227	293	293	183	189	269	269	194	194	254	254	196	196
19.	231	231	182	182	227	227	299	299	183	187	277	277	192	192	264	264	—	—
20.	231	231	182	182	227	227	290	290	183	187	277	277	196	196	264	264	196	196
21.	240	240	182	182	229	229	293	293	—	—	277	277	192	192	254	254	196	196
22.	231	231	198	198	229	229	299	299	183	189	277	277	192	192	264	264	196	196
23.	231	231	182	182	227	227	293	293	187	187	275	275	196	196	254	254	169	169
24.	231	231	182	182	229	229	299	299	183	183	277	277	192	192	264	264	196	196
25.	240	240	201	201	227	227	299	299	183	189	269	269	194	194	264	264	196	196
26.	240	240	201	201	221	221	274	274	189	189	269	269	192	192	250	250	196	196
27.	231	231	198	198	221	221	274	274	189	189	269	269	192	192	250	250	196	196
28.	231	231	182	182	227	227	293	293	187	187	277	277	198	198	254	254	196	196
29.	231	231	182	182	221	221	274	274	189	189	275	275	192	192	250	250	196	196
30.	240	240	198	198	229	229	274	274	189	189	279	279	194	194	245	245	182	182
31.	231	231	198	198	229	229	293	293	189	189	277	277	194	194	264	264	196	196
32.	231	231	198	198	229	229	293	293	183	189	267	267	192	192	264	264	196	196
33.	240	240	198	198	229	229	299	299	183	189	275	275	194	194	264	264	196	196
34.	231	231	201	201	229	229	299	299	183	183	275	275	194	194	254	254	196	196
35.	231	231	198	198	229	229	293	293	183	189	267	267	192	192	250	250	196	196
36.	240	240	198	198	221	221	274	274	183	189	269	269	192	192	250	250	196	196
37.	231	231	182	182	229	229	293	293	183	189	277	277	192	192	254	254	196	196
38.	231	231	182	182	221	221	299	299	183	183	277	277	194	194	264	264	196	196
39.	231	231	182	182	227	227	—	—	183	183	277	277	192	192	264	264	196	196

Table 10. PIC value of 17 different SSR primers.

S. No.	Locus	Number of alleles	PIC value
1.	sml001	4	0.4952
2.	smll022	5	0.6419
3.	ksl115	3	0.4259
4.	ksl123	4	0.5804
5.	ksl173	5	0.707
6.	sml013	4	0.5388
7.	ksl26	6	0.6393
8.	ksl32	4	0.5106
9.	ksl271	2	0.2653
10.	ksl51	3	0.5153
11.	ksl97	3	0.4791
12.	sh42	5	0.5261
13.	sh43	3	0.5153
14.	sh66	4	0.5956
15.	ksl92	5	0.5757
16.	sml015	3	0.3994
17.	sml026	2	0.1516

DNA fingerprinting data: primers sml001: 175, 181, 192, 197; smll022: 312, 321, 328, 331, 335; ksl115: 206, 208; ksl123: 336, 350, 353, 355; ksl173: 149, 154, 157, 162, 164; sml013: 173, 261, 264, 273; ksl26: 296, 298, 306, 310, 315, 317, 319; ksl32: 195, 207, 209, 215; ksl271: 231, 240; ksl51: 182, 198, 201; ksl97: 221, 227, 229; sh42: 271, 274, 277, 290, 293, 296, 299; sh43: 183, 187, 189; sh66: 267, 269, 275, 277, 279; ksl92: 185, 190, 192, 194, 196, 198; sml015: 245, 250, 254, 264; sml026: 169, 182, 196 (Table 13).

Except for Luosheng No.1, Purple Sha, and Purple lettuce, the three cultivars were closely related, and all parameters were the same; the fingerprints identified 36 germplasm resources. Genetic fingerprinting can be used for purple leaf lettuce authenticity identification and control fingerprints can distinguish species; thus, species protection and rights protection have great significance.

Discussion

The CIELab method was used to analyse the colour parameters and changes in lettuce leaves in purple leaves, and the correlation between colour parameters and anthocyanin content was analysed. The leaf anthocyanin content and leaf colour have a certain connection. (Zhang *et al*., 2008), Different levels of anthocyanins affect different CIELab colour parameters (Pang *et al*., 2008). Then, a grading standard and classification system were established (Liang, Han & He, 2014). Based on the cluster parameters and anthocyanin content, the purple leaf lettuce can be divided into five grades: green, light purple, medium purple, dark purple and special purple (Table 6). Based on this, a leaf colour grading standard was established; purple leaf lettuce could be divided into edge purple, gradient purple, full-leaved purple and piebald purple 4 categories (Table 7). The leaf colour of vegetable products is an important appearance quality, and leaf colour characteristics are a key feature in this breeding work. This study provides a scientific method for measuring and evaluating the colour traits of leaves and helps to cultivate and screen new varieties with a good appearance.

In this study, SSR molecular markers were used in 39 of the purple leaf lettuce varieties tested, with fingerprinting, genetic diversity and genetic relationship analysis, and the result shows that the genetic similarity coefficients ranged from 0.0537 to 2.8132, except for Luosheng No.1, Purple Sha and purple lettuce, which belonged to the same clade; the Nei's gene diversity index ranged from 0.1653 to 0.7486 with an average of 0.5635. The Shannon's index ranged from 0.3004 to 1.4744, with an average of 1.0151 (Table 11). From the cluster analysis map, 39 germplasms showed a narrow genetic basis, a close genetic relationship and low genetic diversity, but there was still high genetic variation among germplasm. Similar morphological varieties of leaves are closely related and tend to group together. Red Wrinkle, Purple Sha, Crimson Rosa, and Red No. 1, belonged to the same group, with close kinship. Because the seed companies are in Beijing, we inferred that geographical area had a greater impact on germplasm resources. To a large extent, the cultivars produced in the same area had the same or similar genetic type.

Overall, 39 samples of germplasm, Luosheng No.1, Purple Sha and purple lettuce, were all located in the same clade (Table 13). Due to their closest genetic relationship and the small number of detected SSR loci, primer screening was limited, and it was not possible to completely separate the three. Luosheng No.1 and Purple Sha's plant type, leaf shape, leaf colour and other apparent traits were very similar. Possibly, the different seed companies trade names were not standardized, and the same variety might have different names. Luosheng No.1 and purple lettuce belonged to different sources. Although the phenotypic traits were similar, the degree of leaf fold and the distribution of purple leaves differed. Luo Sheng No. 1 is a type of edge purple, and the colour of the leaves is light purple, while purple lettuce is a gradient purple type, and the leaf colour is dark purple. According to the variety of packaging and the appearance, it was concluded that they are different species. The reason for their identification as the same species may be the limited amount of primer screening, or it may be that the variation between them does not occur at the DNA level but at the RNA level.

Most of the germplasm resources of the 39 experimental varieties were introduced from the United States, and their diversity was relatively low. Local breeds also had close genetic relationships with them. Therefore, it takes an especially long time to renew germplasm resources in purple leaf lettuce, and their development in the United States is comparatively mature. However, research into these species in China started relatively late, and thus, there is less variety. Therefore, it is urgent to develop and introduce new varieties (Pang *et al*., 2008).

Table 11. Statistics of genetic variation and heterozygosity of all loci.

S. No.	Locus	Sample size	na*	ne*	I*	Obs_Hom	Exp_Het*	Nei**	Ave_Het
1.	sml001	42	4	2.2161	1.0075	1	0.5621	0.5488	0
2.	smll022	42	5	3.2667	1.3269	1	0.7108	0.6939	0
3.	ksl115	46	3	2.1076	0.826	1	0.5372	0.5255	0
4.	ksl123	44	4	2.7816	1.1514	1	0.6554	0.6405	0
5.	ksl173	46	5	3.9774	1.4744	1	0.7652	0.7486	0
6.	sml013	44	4	2.6022	1.0825	1	0.63	0.6157	0
7.	ksl26	42	6	3.0414	1.4086	1	0.6876	0.6712	0
8.	ksl32	42	4	2.4298	1.0194	0.9524	0.6028	0.5884	0.0217
9.	ksl271	46	2	1.4593	0.4943	0.9565	0.3217	0.3147	0.0217
10.	ksl51	46	3	2.3937	0.9772	1	0.5952	0.5822	0
11.	ksl97	46	3	2.195	0.9184	1	0.5565	0.5444	0
12.	sh42	42	5	2.4098	1.1126	1	0.5993	0.585	0
13.	sh43	46	3	2.3937	0.9772	0.4783	0.5952	0.5822	0.2609
14.	sh66	46	4	2.8907	1.1929	1	0.6686	0.6541	0
15.	ksl92	46	5	2.7696	1.2051	1	0.6531	0.6389	0
16.	sml015	46	3	1.9236	0.7771	1	0.4908	0.4802	0
17.	sml026	44	2	1.198	0.3046	1	0.1691	0.1653	0
Mean		44	3.8235	2.4739	1.0151	0.964	0.5765	0.5635	0.0179
St. Dev			1.1311	0.6539	0.3004	0.1261	0.144	0.1407	0.063

Note: Na = Observed number of alleles; Ne = Effective number of alleles; I = Shannon's information; Ho = Observed heterozygosity; He = Expected heterozygosity; Nei** = Nei´s gene diversity index

Table 12. F statistics and gene flow of all loci.

S. No.	Locus	Sample Size	Fis	Fit	Fst	Nm*
1.	sml001	42	****	1	1	0
2.	smll022	42	****	1	1	0
3.	ksl115	46	****	1	1	0
4.	ksl123	44	****	1	1	0
5.	ksl173	46	****	1	1	0
6.	sml013	44	****	1	1	0
7.	ksl26	42	****	1	1	0
8.	ksl32	42	-1	0.9338	0.9669	0.0086
9.	ksl271	46	-1	0.8619	0.9309	0.0185
10.	ksl51	46	****	1	1	0
11.	ksl97	46	****	1	1	0
12.	sh42	42	****	1	1	0
13.	sh43	46	-1	0.1039	0.5519	0.2029
14.	sh66	46	****	1	1	0
15.	ksl92	46	****	1	1	0
16.	sml015	46	****	1	1	0
17.	sml026	44	****	1	1	0
Mean		44	-1	0.9369	0.9685	0.0081

Note: Nm* = Gene flow estimated from Fst = 0.25(1-Fst)/Fst

Wang *et al*., (2014) used TRAP markers to analyse the genetic relationship and genetic diversity of 47 purple leaf lettuce germplasms. The experimental results showed that 20 pairs of primers amplified polymorphic sites ranging from 15 to 53, with an average of 21.05, and the polymorphic site percentage (PPB) was 67.05%. The average Nei's gene diversity index was 0.2553, and the average Shannon's index was 0.3976. The level of genetic diversity was low. According to the cluster analysis, 47 germplasms were divided into two major groups. The phylogenetic relationships of the morphologically similar cultivars often clustered together. Because most of the test varieties are the same, they have certain reference values, and the article verifies the correctness of the research.

Table 13. Fingerprints of all 39 purple lettuce tested varieties.

No.	Name	
1.	Red Wrinkle	0000100010001000000010000010000010000000101010001000000001010000010001000000010000
2.	Luo Sheng No.1	0000100010001000100010000010000010000000101010001000000001010000010001000000010001
3.	Purple Sha	0000100010001000100010000010000010000000101010001000000001010000010001000000010001
4.	S39	0010000010010000100000100010000010000000101001010001000000010101000010000000010001
5.	Crimson Rosa	0000100010001000100010000010000010000000101010001000000000010000010001000000100001
6.	Violet	0100000010010000100001000001000100000001000110001000000100011000010000010001001000
7.	Purple leaf lettuce	0100000010000100100000010010000000100001001100101000000001001000010000100000010001
8.	171	0000100010010000100001000000100100000001001010000100000100010100010001000000010001
9.	American purple leaf lettuce	0100001000010001000000100000100000010000101010001000000100001010000001000000010001
10.	Purple lettuce	0000100010001000100010000010000010000000101010001000000001010000010001000000010001
11.	Red No.1	0100000010001000100010000001000010000001001010001000000001011000010100000000010001
12.	Italian purple leaf lettuce	0100001000010001000000100000100000010000010101000100000010001101000000100000010001
13.	American purple lettuce	0100001000010000000001000001000000000001010100010000000000010100000010000010000 1
14.	W94	0100000000110000001100000000100010000001000100101000000000101010100000010000010001
15.	H4	0100000000101000001100000000101000000001001001000100010000010101000000100000010001
16.	H5	0000000000101000001100000010000010000000101001000100000001010001000000100001000 1
17.	H10	0100000000101000001100000000101000000001001001000100000000010101000000100001000 1
18.	Acorn lettuce	0100001000001001000000010000100001000000011001001000000100010101000000100001000 1
19.	MERLOT	0100000000001000100010000000000010000011001010001000000001011000010001000000010000
20.	Red salad bowl	0100001000001001000100000000101000000001001010001000001000011000010000010000010001
21.	RED SAILS	0001000010010000100010000001000100000001000110000100000100000000010001000001000 1
22.	YN-F	0010001000001001000000100000100100000000000100100010000000101010001000100000010001
23.	Fast growing purple leaf lettuce	0100001000010001000001000000100010000000101010001000000100001000100000001000100100
24.	Hong sheng	0000100010001000100010000010000010000000101010001000000001010000010001000000010001
25.	00127	0100000000110000001100000000100000000000000010010100000000010101010000001000001000 1
26.	P-J1	0000100100001001000000100000100010000000001101000010000010001010001000100000100001
27.	Erect lettuce No.3	0100000001001000010100000000100000100001000100110000100000000101000001000010000 1
28.	Caesar Duo	0100000001001000010100000000100000100001001001010000100000000101000001000010000 1
29.	PRIZEHEAD	0100001000100010000001000010000000010010010100010000001000010000100000001001000 1
30.	RED DEER TONGUE	0100000010001000100100000010000010000001001010010000100000000100100001000010000 1
31.	Speckled Butterhead	0000100010010000100001000000100100000001000101000100100000000100001000100100000 10
32.	ROXY PELLETED	0010000010001000100010000001000010000001001001000100000100000100010000100000 1
33.	CANASTA RRAT	0010001000001001000000100000100100000000001100100010000010001011000000100000010001
34.	YN-B	0100000010001000100001000000100010000001000101000100000001010100100000100000 1
35.	Purple Roma	0001000010010000100010000010000001000001001000100100000001010000100000100001000 1
36.	0047	0100001000001000010000100000100100000000001100100010000010001011000000100001000001
37.	RED LETTUCE	0100000001001000010100000100000000100001000101010000100000010101000001000010000 1
38.	L581	0000100010001000100010000010000010000000010101000100000000001000001000100000010001
39.	W31	0000000000001000100010000010000010000000101010010000000001010000010000100000010001

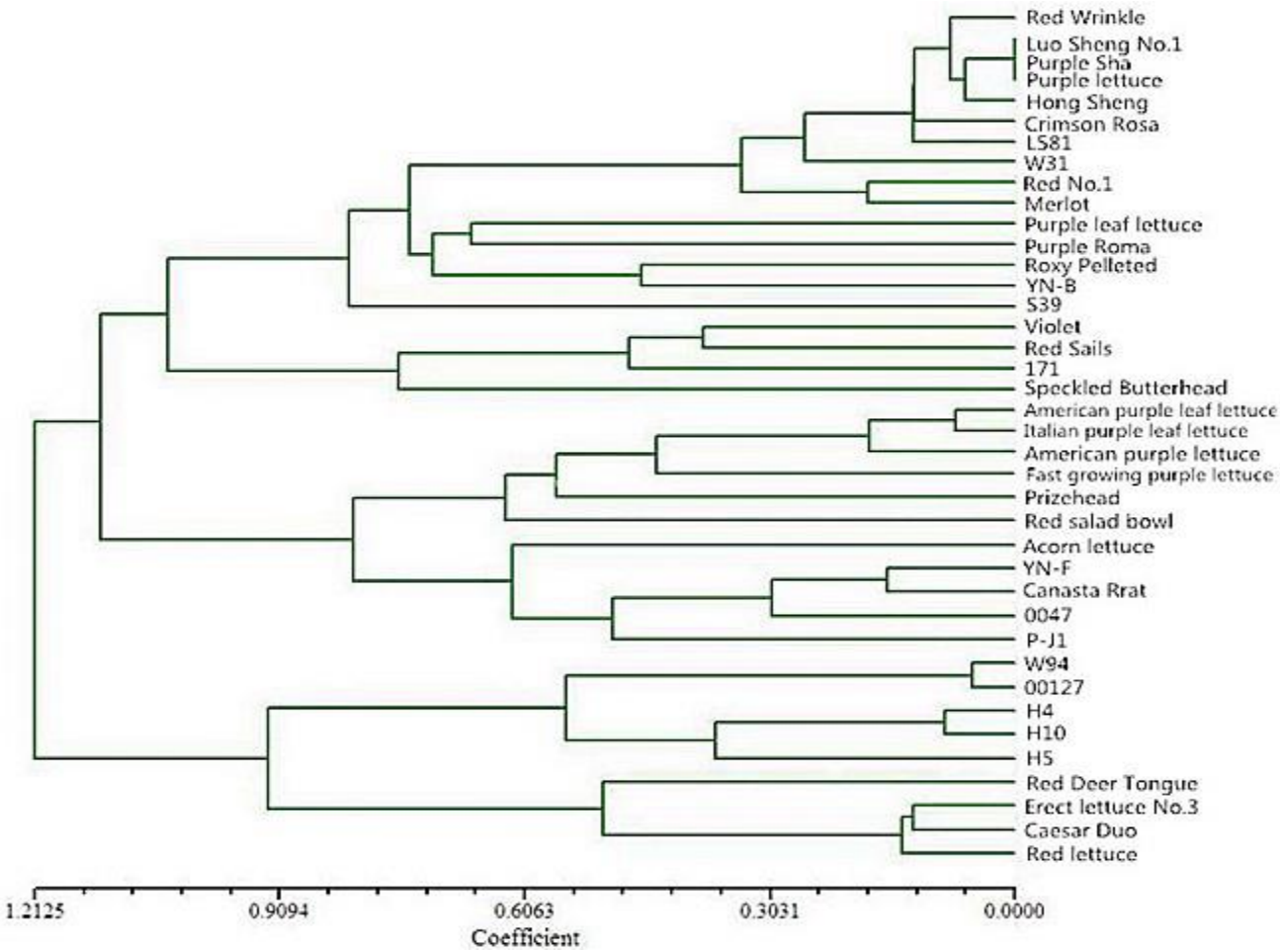

Fig. 4. Cluster analysis of 39 purple leaf lettuce samples. At similarity coefficient of 1.2125, all test varieties can be divided into two groups; each group includes two subclasses. In the first subgroup of Group I, purple leaf lettuce includes leaf fold and erect type; the second subtypes include leaf fold, ball, and erect lettuce. Group II consisted of type of leaf folds and erect lettuce, respectively. Each subgroup of each group includes at least two types of leaf lettuce, indicating that there is still high genetic variation among germplasms.

Conclusions

This study provides a basis for distinguishing lettuce germplasm resources at the molecular level and provides a theoretical basis for research into and utilization of these germplasm resources for Luosheng No.1, Purple Sha, purple lettuce varieties, pending the development of new primers to further improve future research.

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