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Genetic variations and evolutionary relationships among radishes (*Raphanus sativus* L.) with different flesh colors based on red pigment content, karyotype and simple sequence repeat analysis

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To determine the genetic diversity and evolutionary relationships among red radishes, 37 accessions with different flesh colors were analyzed in terms of the red pigment content, karyotypes, and simple sequence repeat markers. Red pigment content of red radish was 3.4 to 28.8% with an average of 15.62%. The karyotype formulas were 14 m (median) + 4 sm (submedian), 16 m + 2 sm, and 18 m for radishes with the same number of chromosomes. The number of alleles detected among the 86 simple sequence repeat primers was 2 to 15 in red-flesh radishes and 2 to 11 in white-flesh radishes. Clustering analysis separated the accessions into three clusters, with most accessions from the same region clustering together. The results indicated that (1) red radish is abundant in red radish, which is a valuable material in red pigment industry; (2) the white-flesh radish is an ancestor of the red-flesh radish, which should be considered a variety in *Raphanussativus*, and (3) a low level of genetic diversity exists among the 37 accessions. The available radish germplasms should be expanded by creating new hybrid or introducing genes from other crops.

Key words: Genetic diversity, karyotypes, *Raphanus sativus*, red pigment content, radish, simple sequence repeat.

INTRODUCTION

Radish (*Raphanus sativus* L.), belonging to the family Cruciferae and genus *Raphanus*, is an important commercial root vegetable, with a cultivation history of more than 2700 years. *R. sativus* (2n = 2x = 18) is normally a self-incompatible, insect-pollinated crop (Wang et al., 2015a). Cultivars have been developed and maintained as open-pollinated, out crossing populations (Zhang, 2006). Radish is thought to have first evolved in the Mediterranean region and has since become an important vegetable crop in China, where it is grown on

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Figure 1. Images of white radish (A), red radish with white flesh (B), green radish with red flesh (C), and red radish with red flesh (D).

areas encompassing 120 millionhectares (Wang and He, 2005; Cheng et al., 2013). There are numerous Chinese radish genetic resources possesses numerous, and the vegetable can be differentiated based on root flesh size, shape, and color, as well as by leaf differences (Wang and He, 2005). Chinese radishes are classified according to root skin color, which can be white, red,or green with white or red flesh (Wang et al., 2015b). In China, white radish cultivars are the most widely distributed (Figure 1A). Red radish with white flesh (Figure 1B) is commonly grown in southern China, green radish with red flesh (red-core radish) (Figure 1C) is grown mainly in the north, and red radish with red flesh (Figure 1D) is indigenous to the Fuling region (Chen et al., 2014).

Recently, reseachers have focused on red radish with red flesh because it contains large amounts of a natural red pigment widely used in foods, wine, and cosmetics (Ganapathi et al., 2009; Jing et al., 2012). Currently, it is unknown where or when people began to cultivate red radish with red flesh. It is believed to have originated in the Fuling district Chongqing China before the mid-18th century. Record of the plant first appeared in 1876 during the Qing Dynasty (Wang and He, 2005). Using random amplified polymorphic DNA (RAPD) markers, Ren et al. (2005) identified seed impurities in cultivars of red radish with red flesh. Lv et al. (2006) reported that the root is the main plant part that accumulates pigment and the root peel contains the highest amount of pigment among radish parts. Si et al. (2010) investigated different pigment extraction methods as well as the mechanisms of pigment formation in red radish. They concluded that 50% ethanol was the most efficient extraction agent for carmine radish pigment extraction. They also observed that red pigment content steadily increased from the seedling stage to the flowering stage up to a maximum of 3%, and then gradually decreased until the silique setting stage. Dong et al. (2013) reported that the red pigment of radish degraded considerably during heat treatment at 75 to 95°C in a temperature-dependent manner following a first-order reaction kinetic model. Qin et al. (2014) determined that plant height, fresh leaf weight, and root length significantly affected red radish root flesh yield. However, despite these studies, little is known about the genetic diversity of red radish with red flesh because of its specific distribution, while white radish has been better characterized (Jiang et al., 2012; Park et al., 2013; Wang et al., 2015b; Zhai et al., 2013). The red pigment of radish is natural, nutritious, and multi-functional, which suggests it may have practical uses to satisfy consumer demands for natural and safe products. Hence, it is necessary to use and protect the genetic resources available for the red-core radish. In this manuscript, we present our findings regarding red pigment content, karyotypes, and simple sequence repeat (SSR) markers of radishes with different flesh colors. The study objectives were to (i) estimate the red pigment content and potential utility of red radish, (ii) investigate the evolutionary relationships and genetic diversity among radishes with different flesh colors, and (iii) generate valuable information relevant to breeding for the improvement of red radish with red fresh.

MATERIALS AND METHODS

Plant materials

We used 37 accessions of four radish types collected from different regions in China. We analyzed 24 red radishes with red flesh, one green radish with red flesh, four red radishes with white flesh, and eight white radishes (Table 1). Of these, 25 radishes with red flesh (Codes 1-25) were used for red pigment analyses. Four red radishes with red flesh (Codes 2, 16, 22 and 24), one green radish with red flesh (Code 25), four red radishes with white flesh (Codes 26, 27, 28 and 29), and four white radishes with white flesh (Codes

Table 1. Characteristics of the 37 accessions used in this study.

Code	Collection locale	Accessions	Characteristics			
			Leaf color	Leaf shape	Root skin color	Flesh color
1	Fuling, Chongqing	Inbred line	Dark green	Strip-shaped	Red	Red
2	Fuling, Chongqing	Inbred line	Green	Flower-shaped	Red	Red
3	Fuling, Chongqing	Inbred line	Green	Flower-shaped	Red	Red
4	Fuling, Chongqing	Cultivar	Green	Flower-shaped	Red	Red
5	Fuling, Chongqing	Inbred line	Green	Flower-shaped	Red	Red
6	Fuling, Chongqing	Inbred line	Green	Strip-shaped	Red	Red
7	Fuling, Chongqing	Cultivar	Green	Strip-shaped	Red	Red
8	Fuling, Chongqing	Hybrid	Green	Strip-shaped	Red	Red
9	Fuling, Chongqing	Inbred line	Green	Flower-shaped	Red	Red
10	Fuling, Chongqing	Inbred line	Green	Flower-shaped	Red	Red
11	Fuling, Chongqing	Inbred line	Dark green	Flower-shaped	Red	Red
12	Fuling, Chongqing	Inbred line	Green	Flower-shaped	Red	Red
13	Fuling, Chongqing	Inbred line	Light green	Flower-shaped	Red	Red
14	Fuling, Chongqing	Inbred line	Dark green	Strip-shaped	Red	Red
15	Fuling, Chongqing	Hybrid	Green	Strip-shaped	Red	Red
16	Fuling, Chongqing	Hybrid	Green	Strip-shaped	Red	Red
17	Fuling, Chongqing	Inbred line	Green	Strip-shaped	Red	Red
18	Fuling, Chongqing	Inbred line	Green	Flower-shaped	Red	Red
19	Fuling, Chongqing	Inbred line	Red	Flower-shaped	Red	Red
20	Fuling, Chongqing	Hybrid	Green	Flower-shaped	Red	Red
21	Fuling, Chongqing	Cultivar	Green	Flower-shaped	Red	Red
22	Fuling, Chongqing	Hybrid	Green	Flower-shaped	Red	Red
23	Tonghai, Yunnan	Hybrid	Green	Flower-shaped	Red	Red
24	Yutian, Hebei	Hybrid	Light green	Flower-shaped	Red	Red
25	Yutian, Hebei	Landrace	Dark green	Flower-shaped	Green	Red
26	Mianyang, Sichuan	Hybrid	Green	Strip-shaped	Red	White
27	Chengdu, Sichuan	Hybrid	Green	Strip-shaped	Red	White
28	Mingquan, Henan	Hybrid	Green	Flower-shaped	Red	White
29	Chengdu, Sichuan	Hybrid	Light green	Strip-shaped	Red	White
30	Chengdu, Sichuan	Hybrid	Green	Strip-shaped	White	White
31	Mianyang, Sichuan	Hybrid	Dark green	Strip-shaped	White	White
32	Mianyang, Sichuan	Hybrid	Light green	Strip-shaped	White	White
33	Mingquan, Henan	Hybrid	Dark green	Strip-shaped	White	White
34	Suzhou, Zhejiang	Hybrid	Dark green	Strip-shaped	White	White
35	Suzhou, Zhejiang	Hybrid	Dark green	Flower-shaped	White	White
36	Yangling, Shanxi	Hybrid	Dark green	Flower-shaped	White	White
37	Yutian, Hebei	Landrace	Dark green	Flower-shaped	White	White

30, 35, 36 and 37) were used to analyze the karyotype. All 37 accessions were used to evaluate the SSR markers.

Red pigment content measurements

A field trial was completed at the Research Institute for Agricultural Sciences in the Fuling district of Chongqing, China. We used a randomized complete block design with two replications. Each accession was planted in single rows of 10 plants, with 40 cm between rows and 30 cm between plants. Before bolting, three representative plants were sampled to measure red pigment content. For each sample, the skin and flesh were mixed and the root flesh was divided into two parts. One part was used to determine the water content and the other was used for juice extraction. After centrifuging the juice at 4,000×g for 10 min, the absorbance of the supernatant at 520 nm was determined by spectrophotometry. Using the standard curve method, the red pigment content of the juice and root flesh was determined (Si et al., 2010).

Karyotype analysis

More than 30 cells of each material used for karyotype were analyzed. The number of chromosomes of a cell was considered

Table 2. Red pigment content of 25 accessions.

Code	Red pigment content (‰)	Code	Red pigment content (‰)	Code	Red pigment content (‰)	Code	Red pigment content (‰)	Code	Red pigment content (‰)
1	19.2	6	10.6	11	11.3	16	10.9	21	12.9
2	28.8	7	17.6	12	13.9	17	15.1	22	14.1
3	18.9	8	17.4	13	18.2	18	8.3	23	6.4
4	11.2	9	21.9	14	19.2	19	21.2	24	18.7
5	16	10	23	15	14.8	20	17.4	25	3.4

accurate if 85% of the analyzed cells produced the same result. Mitotic preparations were obtained from root tips of germinating seeds. After pretreatment in 0.002 M 8-hydroxyquinoline for 3 h at room temperature, the material was fixed in an acetic acid-ethanol solution (1:3), stained using Feulgen's technique (Arano, 1965), and then flattened in a drop of 2% acetic orcein to release the chromosomes. For numerical characterization of the karyotype, the following parameters were calculated: Total chromosome length (short arm length + long arm length), relative chromosome length (chromosome length × 100 / total chromosome length), centromeric index (short arm length × 100 / chromosome length), arm ratio (∑q/p/n; where p and q are the mean lengths of the short and long arms of each homologous pair, respectively, and n is the number of homologs), and asymmetrical karyotype coefficient (Arano, 1965). Chromosome morphology was determined based on the centromeric index. The chromosomes were classified as median (m): 50-37.5 and submedian (sm): 37.5-25. Idiograms were constructed by organizing the chromosomes into groups according to their centromeric index (m, sm). They were arranged in order of decreasing length within each category, and finally numbered consecutively using the same scheme.

DNA extraction

Genomic DNA was extracted from approximately 2 g flesh leaves using the CTAB procedure (Doyle and Doyle, 1987). The purity and concentration of the extracted DNA were determined using a spectrophotometer (Shanghai AuCy Technology Instrument, Shanghai, China).

SSR amplification

Six hundred pairs of SSR primers were synthesized according to the published common primers of *Brassica* species (http://www.brassica.info). The primer pairs used to amplify genomic DNA of all accessions were selected based on their ability to generate stable and polymorphic products from the genomic DNA of five randomly selected cultivars. The SSR loci were amplified by a polymerase chain reaction (PCR). The final volume of the reaction solution was 15 µL, which contained 0.2 µM of each primer, 1 U Taq DNA polymerase, 0.15 mMdNTP, 1.5 mM $MgCl_2$, and 2.5 ngtemplate DNA. The PCR program used to amplify SSRs was as follows: 95°C for 5 min; 30 cycles of 95°C for 30 s, 65°C for 1 min, and 72°C for 1 min; and 72°C for 7 min. The PCR was performed in a Mastercycler PCR system (Eppendorf, Saxony, German). The amplification products were separated by 6% (w/v) denaturing polyacrylamide gel electrophoresis and visualized by silver staining.

SSR data scoring and analysis

The SSR bands were scored as present (1) or absent (0), with each being treated as an independent character. Genetic diversity analysis was completed based on the scores. The statistical methods and formulas used are described following:

(1) Index of genetic similarity: $GS = 2N_{ij} / (N_i + N_j)$, where N_{ij} is the number of SSR alleles common to landraces *i* and *j*. N_i and N_j are the total number of SSR alleles observed for accessions *i* and *j*, respectively. Dendrograms were constructed using the unweighted pair-group method with arithmetic mean (UPGMA) clustering and the NTSYS-pc software, version 2.10.

(2) Mean number of alleles: $A = \sum_{i=1}^{n} A_i / n$, where A_i is the number of alleles at the *ith* allele.

(3) Effective allelic number: $A_e = \sum_{i=1}^{n} A_{ei} / n = \sum_{i=1}^{n} (1 / \sum_{j=1}^{m} q_j^2) / n$, where A_{ei} is the effective allelic number at the *ith* allele and q_j is the frequency of the *jth* allele.

(4) Shannon's index: $H_j = \sum p_i \log_2 p_i$, where p_i is the frequency of the presence or absence of a band in a locus for all individuals comprising an accession.

Equations 2 to 4 were computed using the POPGENE software, version 1.2 (Department of Renewable Resources, University of Alberta, Edmonton, Canada).

RESULTS

F-test for significant differences in red pigment content between accessions

F-test from SPSS software was used to identify significant differences in red pigment content between accessions. As shown in Table 2, the average red pigment content in red radish with red flesh was 16.12% (range: 3.4-28.8%). Significant differences were found among the 25 accessions (F = 0.6785, P = 0.0001).

Karyotypes of four radish types

The karyograms and idiograms of the four radish types are shown in Figures 2 and 3. The karyotype parameters used during analysis are summarized in Table 3. Karyotype analysis revealed a diploid chromosome number of 2n = 18, and 9 pairs of homologous chromosomes were observed (2n = 2x = 18) in all radishes

Figure 2. Karyograms of red radish with red flesh (A), green radish with red flesh (B), red radish with white flesh (C), and white radish (D).

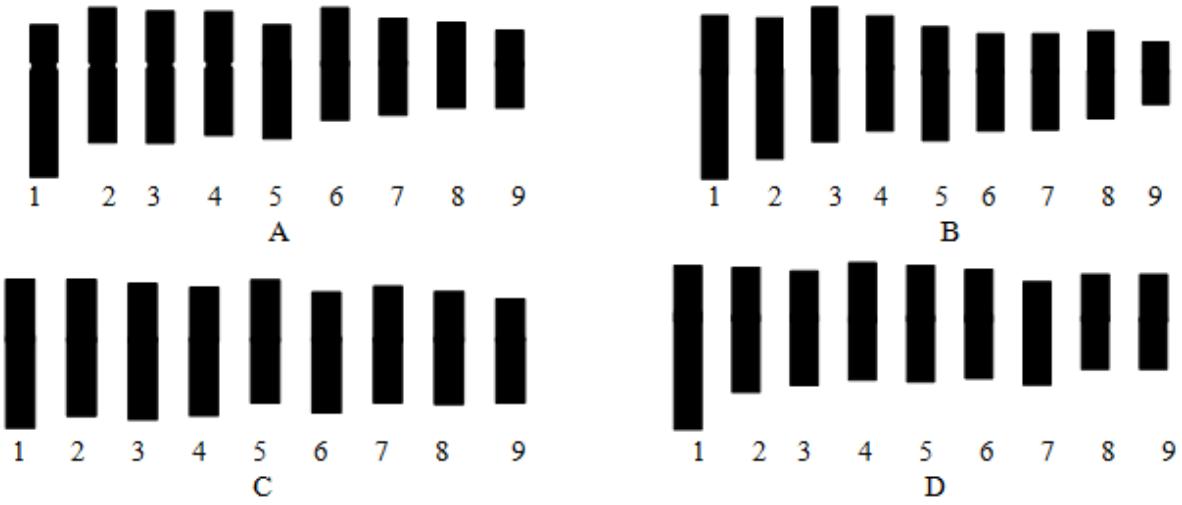

Figure 3. Idiograms of red radish with red flesh (A), green radish with red flesh (B), red radish with white flesh (C), and white radish (D).

studied.

The karyotype formula forred radish with red flesh was 14 m + 4 sm. Chromosomes 2-4 and 6-9 were the m-type, while chromosomes 1 and 5 were the sm-type, according to Levan's karyotype classification standards (Levan et al., 1964). The total chromosome length was 5.67-10.94 µm, with an average of 1.73 µm. The relative chromosome length was 7.65-14.77. The average ratio between the longest and shortest chromosomes was 1.93 (range: 1.01-2.86). Accordingly, the asymmetrical karyotype coefficient was 59.28, which was categorized as 2A based on Stebbin's classification of karyotype asymmetry (Stebbins, 1971).

The karyotype formula for green radish with red flesh was 16 m + 2 sm. Chromosome 1 was the sm-type, while the remaining chromosomes were the m-type. The total chromosome length was 5.23-13.84 µm, with an average of 9.44 µm. The relative chromosome length was 6.15-16.30. The average ratio between the longest and shortest chromosomes was 1.41 (range: 1.10-1.86). Accordingly, the asymmetrical karyotype coefficient was 58.61, which was categorized as 1B.

The karyotype formula forred radish with white flesh was 18 m (that is, all chromosomes were the m-type).The total chromosome length was 5.50-7.83 µm, with an average of 6.60 µm. The relative chromosome length was 9.26-13.19. The average ratio between the longest and shortest chromosomes was 1.42 (range: 1.05- 1.54). Accordingly, the asymmetrical karyotype coefficient was 57.38, which was categorized as 1A.

The karyotype formula for white radish with white flesh was16 m + 2 sm. Chromosome 7 was the sm-type, while the remaining chromosomes were the m-type. The total chromosome length was 8.05 to 11.51 µm, with an average of 9.51 µm. The relative chromosome length was 9.35 to 13.44. The average ratio between the longest and

Table 3. Karyotype parameters for four radish types.

Accessions	TCL (μm)	RCL (%)	CI (%)	AR	Karyotype
Red radishes with red flesh (2n=18)	2.83 + 8.11 = 10.94	14.77	25.891	2.86	sm
	4.18 + 5.62 = 9.80	13.23	42.639	1.35	m
	3.83 + 5.67 = 9.50	12.83	40.337	1.48	m
	3.80 + 5.09 = 8.89	11.99	42.761	1.34	m
	2.83 + 5.36 = 8.19	11.05	34.595	1.89	sm
	4.00 + 4.06 = 8.06	10.88	49.669	1.01	m
	3.18 + 3.68 = 6.86	9.27	46.359	1.16	m
	3.00 + 3.17 = 6.17	8.33	48.676	1.05	m
	2.50 + 3.17 = 5.67	7.65	44.118	1.27	m
Green radishes with red flesh (2n=18)	4.84 + 9.00 = 13.84	16.30	34.987	1.86	sm
	4.51 + 7.33 = 11.85	13.95	38.098	1.62	m
	5.37 + 5.89 = 11.27	13.27	47.692	1.10	m
	4.34 + 5.34 = 9.67	11.39	44.831	1.23	m
	3.87 + 5.67 = 9.53	11.23	40.559	1.47	m
	3.17 + 5.00 = 8.17	9.62	38.776	1.58	m
	3.18 + 4.84 = 8.03	9.45	39.659	1.52	m
	3.38 + 3.96 = 7.34	8.64	46.094	1.17	m
	2.48 + 2.74 = 5.23	6.15	47.513	1.10	m
Red radishes with white flesh (2n=18)	3.17 + 4.67 = 7.83	13.19	40.426	1.47	m
	3.18 + 4.00 = 7.19	12.10	44.295	1.26	m
	2.97 + 4.17 = 7.14	12.03	41.624	1.40	m
	2.78 + 4.00 = 6.78	11.42	41.032	1.44	m
	3.17 + 3.33 = 6.50	10.95	48.744	1.05	m
	2.84 + 3.34 = 6.17	10.67	39.453	1.53	m
	2.50 + 3.84 = 6.34	10.39	45.950	1.18	m
	2.53 + 3.40 = 5.93	9.99	42.697	1.34	m
	2.17 + 3.33 = 5.50	9.26	39.394	1.54	m
White radishes (2n=18)	4.50 + 7.00 = 11.51	13.44	39.137	1.56	m
	4.34 + 6.24 = 10.58	12.36	41.002	1.44	m
	4.84 + 5.17 = 10.01	11.70	48.385	1.07	m
	4.50 + 5.33 = 9.84	11.49	45.781	1.18	m
	4.00 + 5.64 = 9.64	11.26	41.494	1.41	m
	4.17 + 5.04 = 9.21	10.76	45.241	1.21	m
	3.17 + 5.59 = 8.76	10.23	36.163	1.77	sm
	3.84 + 4.17 = 8.01	9.40	46.976	1.13	m
	3.78 + 4.27 = 8.05	9.35	47.918	1.09	m

TCL, Total chromosome lengths; RCL, relative chromosome length; CI, centromeric index; AR, arm ratio.

shortest chromosomes was 1.43 (range: 1.09 to 1.77). Accordingly, the asymmetrical karyotype coefficient was 56.61, which was categorized as 1A.

The karyotypes of the four radish types consisted mainly of m-type chromosomes. Submedian chromosomes were uncommon, comprising only two pairs in red radish with red flesh and one pair in green radish with red flesh. Satellites were not observed in any of the accessions. White radish had the longest mean chromosome length, followed in order by green radish with red flesh, red radish with red flesh, and red radish with white flesh. Similar karyotypes were also observed for radishes with the same flesh color.

Amplification products and genetic similarities in 37 accessions

Table 4 summarizes the SSR data. Eighty-six pairs of SSR primers produced 976 amplification products from 37 accessions, with 892 of these being polymorphic (91.39%). The mean number of alleles was 8.70 (range:

2-20), and the effective allelic number was 5.16 (range: 1.25-12.56). Shannon's index varied from 0.44 to 2.77, with an average of 1.76, indicating that genetic variation in the 37 accessions could be detected using SSR markers.

The genetic similarities in the SSR marker patterns of the 37 accessions ranged from 0.75 to 0.90, with an average of 0.84. More than 97% of accessions had a small genetic distance between them, with similarity coefficients greater than 0.80. Comparisons of accessions with the same flesh color revealed that radishes with red flesh and white flesh are genetically distant. Therefore, the accessions evaluated in this study could be classified as red- and white-flesh radish varieties.

Genetic diversity in red- and white-flesh radishes

The SSR markers used in this study were polymorphic among the 25 red-flesh radishes tested, with an average of 5.60 alleles per locus. The 12 white-flesh radishes were less polymorphic, with an average of 4.87 alleles per locus. The mean number of alleles detected by 86 SSR primers ranged from 2 to 15 in red-flesh radish and from 2 to 11 in white-flesh radish. The effective allelic number in red- and white-flesh radishes was 419 (64.66%) and 362 (75.10%), respectively. Shannon's index in red- and white-flesh radishes was 1.68 (range: 0.44-2.49) and 1.46 (range: 0.29-2.37), respectively. Among the red- and white-flesh radishes,97 and 91% of accessions had genetic similarity coefficients greater than 0.80, respectively. These results indicate a somewhat greater variation in red- than white-flesh radishes. The genetic similarity coefficient between red- and white-flesh radishes was 0.83, indicating a genetically close relationship between radishes with different flesh colors.

Cluster analysis

Cluster analysis based on the matrix of genetic similarities with the UPGMA clustering algorithm showed that all accessions could be classified into three clusters when the genetic similarity coefficient was 0.81 (Figure 4). Cluster I included six accessions, three red radishes with white flesh and three white radishes, accounting for 15.62% of all accessions. Cluster II consisted of three red radishes with red flesh, one red radish with white flesh, and five white radishes, accounting for 24.32% of all accessions. Cluster III was the largest with 21 red radishes with red flesh and one green radish with red flesh.

DISCUSSION

Utility of red radish with red flesh

Researchers have attempted to isolate natural pigments from plants (Goyeneche et al., 2015; Zhang et al., 2016), with a particular focus on the extraction of red pigment from radish because of its chemical stability and diverse uses(Ganapathi et al., 2009). In a study of 33 radish landraces from Oregon, USA, Giusti et al. (2008) reported that anthocyanin pigment content ranged from 0.393% to 1.85% in the skin of spring cultivar radishes and from 0.122 to 0.53% in the roots of red-fleshed winter cultivars. In the present study, we observed that red pigment was abundant in red radish with red flesh, with an average value of 15.62%. Hence, the elite germplasm of red radish with red flesh, mainly cultivated in the Fuling district of China, should be an ideal source from which to extract red pigment.

Evolutionary relationships among the four radish types

With respect to the radish karyotypes, the same number of chromosomes was observed in all four types. However, the karyotype formula varied (that is, 14 m + 4 sm, 16 m + 2 sm, and 18 m). We observed some changes in chromosome size, but chromosome morphology was relatively stable. All accessions had m- and sm-type chromosomes almost exclusively. Although the radish variants maintained karyotype uniformity, there were differences in chromosomal structure, such as in total chromosome length, relative chromosome length, ratio of the longest and shortest chromosomes, and arm ratio. Moreover, the chromosomal asymmetry index in red radish with red flesh was higher than in white radish. One of the chromosomal parameters most often used to determine evolutionary relationships in plants is chromosomal symmetry. Symmetrical karyotypes are widely considered to be more primitive than asymmetrical ones (Stebbins, 1971; Chen et al., 2011). Therefore, it is possible that red-flesh radish with asymmetrical karyotypes evolved from white-flesh radish with symmetrical karyotypes. This is consistent with the recorded history of cultivated radishes in China (Wang and He, 2005). White radish has been cultivated for more than 2,700 years, while red-flesh radish has been grown for only a little over 100 years (Wang and He, 2005).

Genetic diversity in radishes with different flesh colors

A high level of genetic diversity implies abundant germplasm variation, which may enable the selection of genes relevant to crop breeding for improved traits (Zhai et al., 2013). Rabbani et al. (1998) studied the diversity of 30 radishes in Pakistan and reported a high genetic variation. Using RAPD and amplified fragment length polymorphism (AFLP) markers, Kong et al. (2004, 2005, 2011) identified considerable diversity among 56 radish accessions from different countries and regions.

Table 4. Primers used to amplify DNA markers.

S/N	Primer name	A	Ae	Hj	Base sequence (5' to 3')
1	sORA43	11	6.37	2.24	GCGCGTGTGGGATCAGAA/CTTCTCCACCGTCGATCG
2	sORA26	11	3.53	1.63	TGTTTACCTGTTGGAGAT/AACCCTAAGCATCTGCGA
3	sORA21b	11	2.15	1.02	TTCAGCACTAGCTCATGG/TCCTTCTCAGGCACTCTT
4	SSR Ol10-C10	6	3.21	1.34	AAGAAGGCGTAGAGATTGCC/GCAGATAAGATTCGAGTCCCC
5	SSR Ol10-D01	9	2.77	1.17	TCTCTGCCAAAAGCAAATAGC/CTTGGCTCTCTCTCACCACC
6	SSR Ol13-E08	10	7.65	2.22	TTCGCAACTCCTCCTAGAATC/AAGGTCTCACCACCGGAGTC
7	SSR Ol11-D12	15	9.57	2.49	CCTCCACCGCACTCAATTAC/TGGAGAAGTTTGGGACATTTTC
8	SSR Ra2-G05	11	7.73	2.26	GCCAACTTAATTGATGGGGTC/CCTCAATGTTCTCTCTCTCTCTCTC
9	SSR Ra2-G03	10	4.26	1.71	ACTTGTAATGCACTCGCACG/TGGAGATTATTCCGCTGTCC
10	SSR Ra2-G02	10	2.44	1.36	GGGTTATTTCACGCAACTCG/ACACAGGCGGGTTACATAGC
11	SSR Ni2-F11	5	4.43	1.72	AAAGGGTTTCAATTTCACGC/GGGAAACATACTCACCACGC
12	SSR Ni2-F07	11	4.01	1.73	ACAAACAAAGCCTCCCAACC/TCACACAACTTGTTCAATCTTGC
13	SSR Ra2-G04	10	11.50	2.59	AAAACGACGTCATATTGGGC/CGCTTCTTCTTCTCAGTCTCG
14	SSR Ra2-F11	9	4.08	1.50	TGAAACTAGGGTTTCCAGCC/CTTCACCATGGTTTTGTCCC
15	SSR Ni4-G09B	10	8.10	2.56	AAAAACTGGACCCAATTCC/GGTTAGGTCATAAACCCAAAGC
16	SSR Ol13-G05	13	5.45	1.89	GTGTGCAGGAAACGATGTTC/GGGAGTTTGAAGAGAAAGCG
17	SSR Ra1-H02	5	3.08	1.40	CGATTTGCTTTCCTCGAATC/CATGTCGCAATAATAGCATAAAGTG
18	SSR Ol10-B07	10	5.78	2.07	AATCAAGAAGCTGGACCACG/ACCCTGAAACCACTGTCACC
19	SSR Ol10-B02	13	8.32	2.28	CACGAACGCGAGAGAGAGAG/TGCATAAGCTCGAAGAGACG
20	BRMS-008	14	4.43	1.99	AGGACACCAGGCACCATATA/CATTGTTGTCTTGGGAGAGC
21	SSR Ni4-D08	13	3.99	1.80	AGAGATGCTAAAGTGGATCACC/CGGGATTTTGAAGACCTGC
22	SSR Ol10-D08	13	8.72	2.29	TCCGAACACTCTAAGTTAGCTCC/GAGCTGTATGTCTCCCGTGC
23	SSR Ol10-D11	12	6.43	2.07	GCATCATTGACCCTGAAACC/AACCTCCATTTGGTAAGCCC
24	SSR Ni2-A01	9	7.48	2.17	TGCTGCTACAGACAGTGTTGG/AAAGGCTACACACTCATGAAACC
25	SSR Na12-A07	15	5.87	1.94	TCAAAGCCATAAAGCAGGTG/CATCTTCAACACGCATACCG
26	SSR Ni4-C02	13	6.14	2.02	TCCCTTGTCTACTTGCGACC/ACCCTTGTTCCCTCATCTCC
27	SSR Ni4-C09	12	3.28	1.66	AGCATCAATCTTTTGCTCTGC/TGCACACAAACTCCTTCTCC
28	SSR Ni3-F01	11	5.25	1.77	AGCCGCTAAAGAGAAGGTCC/CGCTTTCAAGCTCTCTCCC
29	SSR Ni3-F02	11	1.25	0.45	TCCAACTCAATGGAAGAGG/ACCATTGAAACGTTGAACCC
30	SSR Ni3-G07	5	4.16	1.69	CACTCTCTCCGCCATTTTTC/CTTGAAGCGTTAAAGCCGAC
31	SSR Ni4-A06	8	7.32	2.19	ATCTTTGGCTTCACGATTGG/CCTTCTTCTTAGCATCTAACTCCC
32	SSR Ni2-C01	9	6.08	2.00	GAGTATGAGAGATGGGAATCCG/GACTGAGCAGCTTGGAGACC
33	SSR Ni2-D06	9	3.36	1.36	GGGGAAGAGAGAGAGAGAGAGAG/ATTTGTAGCCCTAGTGGCCC
34	SSR Ni2-F04	7	5.45	1.92	TTTCTTCTTAACCATCGGCG/TCTTCCTCTGCTTCTGGTGC
35	SSR Ni2-D08	12	3.57	1.51	TTTAGGGAAAGCGAATCTGG/ACAACAACCCATGTCTTCC
36	SSR Na10-B07	12	1.93	1.00	GCCTTAGATTAGATGGTCGCC/ACTTCAGCTCCGATTTGCC
37	SSR Ni3-D04	8	3.19	1.58	CACGTTTACTTCTCCAGCCC/GCCCATCAAGAAATGGAGAG
38	SSR Ni3-D09	8	2.97	1.31	GCTGATGACAAAGGGGGTA/AAAAGAGGACAAACAGCCCC
39	SSR Ni3-C08	11	3.57	1.46	CCCTAACACGGTGTCAACAG/GGCAGAATCATCGAGAGGTC
40	SSR Na10-A09	9	5.05	1.76	TCTTGAGCAAAGAAACTTGG/CAAACTGAGCCATACACAAAGG
41	SSR Na10-C01	9	2.58	1.39	TTTTGTCCCACTGGGTTTTC/GGAAACTAGGGTTTTCCCTTC
42	SSR Ra2-C07	9	6.25	2.01	ATTTCCGAATCGGGAGTTTC/ACTTGCAAACGCACACACAC
43	SSR Ra2-C03	11	8.95	2.27	AGACCGGTGTCATCATTATTATC/CCTCTCTGCAGAACTGCTCC
44	SSR Ra2-A05	10	5.54	1.80	GCTAGTTTACGCGGCGG/AAACGACATCGGCAAAGAAG
45	SSR Ra2-A04	12	5.41	1.76	AAAAACTCCTCTTCAACG/CCCAAAGTTAGGTTTTAATGTAATCTC
46	SSR Ni4-D10	14	6.74	2.12	ACATGCGAAAGGGATTTGAC/TGCAAGTGAACTCAAAACAAAAG
47	SSR Ni4-G04	15	12.56	2.77	GAGGCGCGTGGACTAACC/TTACACCCCATCCAAACTCC
48	SSR Ni2-G06	13	7.17	2.09	TGGATACGTCACTGTCACTGC/GAAACTCCGTCGCTATCTCG
49	SSR Ni2-F12	13	3.31	1.44	TGCACAAGAACGAAATGACC/ACGAATATCTCTCTCTCTCTCTC
50	SSR Ol10-F08	9	4.64	1.77	CTTGATCACGTCTAGAAAGAGC/TTGCTTTGGAACCCTAATCG
51	SSR Ol10-G06	18	2.57	1.41	GACAAGTTCCCTTGTAATGGC/TGTAATCATCACACATTTTGGG

Table 4. Contd.

52	SSR Ni4-A09	14	7.73	2.18	AAAGGGCGAAGAAGCAGC/TTTCTTCCATTTGACCGACC
53	SSR Ni4-A07	13	3.73	1.64	TTATCTGCTTGTCTTGGGGC/AGACACTCTCACCCCTCTGC
54	SSR Ra2-D09	7	3.52	1.41	TGCGCATAATAATATCTCGGG/ATTTGTCTCGGACAGATGC
55	SSR Ra2-E03	16	8.40	2.23	AGGTAGGCCCATCTCTCTCC/CCAAAACTTGCTCAAAACCC
56	SSR Ol11-C10	11	12.33	2.63	GGTTATTGGATCGAATGATGG/CGCTCTACCCTTATTGCAGC
57	SSR Ol11-C02	11	4.52	1.71	GCATTGCAATCTTGTTGGTC/CGTTTCCATACAGATCGTAAGAC
58	SSR Ol10-D09	2	1.73	0.44	GGATTTTCAAGACTCTTCGGG/TGCCAAGTTCGTAGTCTTGC
59	SSR Ol10-A05	11	9.71	2.43	TGTAATAACCCGACCCATCC/CTCTCTCGCTCTCTCGATCC
60	SSR Na10-C08	13	3.54	1.69	GTTTGGTTCAGAGGCAGAGG/CTATCGCTGCAGAAGAAGGG
61	SSR Na10-C03	12	3.15	1.37	TTGGGTGTCTTTGTTACCCC/ACCGAGAAGACTGATACGGG
62	SSR Ni4-A02	16	3.95	1.63	AGGACCACTGGGATACAAGC/ATTTGGAGCTGCGTACTTCG
63	SSR Ni2-G08	11	4.03	1.55	TCGACCAACAGAGAATGAAGAG/TTTCCCCATGAACACATTTC
64	SSR Ni2-F02	11	2.93	1.30	TGCAACGAAAAAGGATCAGC/TGCTAATTGAGCAATAGTGATTCC
65	BN12A	15	4.23	1.68	GCCGTTCTAGGGTTTGTGGGA/GAGGAAGTGAGAGCGGGAAATCA
66	BRMS-024	16	6.81	2.13	TGAATTGAAAGGCATAAGCA/CAGCCTCCACCACTTATTCT
67	FITO 066	18	7.73	2.18	AGCCCATTTACCTGCTGA/GAAAGACGATGCTTAGGGT
68	BRMS-025	14	3.92	1.54	TGAAAACAAGCGCTACATGTGG/CAAGCAAGCATGACAAGCAACA
69	BRMS-026	15	8.95	2.34	CCTATCCTCGGACTAATCAGAA/GTGCTTGATGAGTTTCACATTG
70	SSR Ra2-H10	18	5.68	1.82	GCGCGTGTAGGCTACGTC/CGGCCGCGGCAACTG
71	SSR Ra3-H10	16	5.25	1.77	TAATCGCGATCTGGATTCAC/ATCAGAACAGCGACGAGGTC
72	BN9A	18	3.75	1.57	GAGCCATCCCTAGCAAACAAG/CGTGGAAGCAAGTGAGATGAT
73	SSR Ni4-D12	12	1.64	0.72	ACCACCATCCACAGAGTTCC/GCAGGACAGACTGAAAGCG
74	SSR Ol10-H12	11	6.08	1.95	CTCCATTTCAGTGATTCTGAGG/TTGATTTGCTATCGGATCACC
75	FITO 099	9	3.28	1.42	ATTCCGTGGCTTATTTGTATG/TATCCATTCGGTTTGTATG
76	FITO 035	13	4.58	1.65	AAAGTCGTGGGAAGTATCGT/AGGTGTAAGGATGGTGGTAGT
77	SSR Ni4-F09	8	6.95	2.08	CTGTTATGCAAGGTCATCGC/TGTTCCAGGTGAAGAAACCG
78	SSR Ni4-F02	13	5.05	1.83	CACTCGGAGAGATAGAGAGAGAGAG/TGGTACGAAGAAGTGAAGAGAGAAG
79	SSR Ra2-C01	18	4.80	1.66	ATAGTAAGCGTCGCTCGTGG/AACCCTTTATGGGAAAACGG
80	SSR Ni4-E08	10	2.58	1.19	GATTTTGAGGAAGCGGAGG/CAAAGCACTGAGAGAGAGAGAGAG
81	SSR Ra2-H06	12	1.95	1.11	GAATTCAGAGGTATCTACACGGC/TAACAAAGACCCTGCGTTCC
82	FITO 156	8	4.40	1.65	TATGTGTGTGTGTGTGTGTGT/ATAACCTGACAACGAAGATTG
83	SSR Ol09-A06	8	6.08	1.98	TGTGTGAAAGCTTGAAACAG/TAGGATTTTTTTGTTCACCG
84	SSR Ni2-F06	17	5.25	1.91	AAGCTAAAAAGCCAAGCAAGG/CTTTTTCATCAAACCGCTCC
85	FITO 081	9	2.87	1.55	AACTAACTCGGGAAACAACC/GAATGTCCGTCAGAATACC
86	BRMS-020	6	3.25	1.50	AACAAGAGAAGGAGAGCCACCG/CGCTTATAAAATGGCAGTCGCA
Total		976	443.9		
Mean		11.34	5.16	1.76	

A, Mean number of alleles; Ae, Effective allelic number; Hj, Shannon's index.

Analyses using 12 RAPD primers resulted in the detection of 109 distinct amplification products, 72 of which (62.9%) were polymorphic (Kong et al., 2004). Liu et al. (2008) used 35 RAPD primers, 22 inter-SSR primers, and 17 sequence-related amplified polymorphism (SRAP) primer combinations to determine that the proportions of polymorphic amplification products were 85.44, 85.2 and 85.41%, respectively. The mean genetic similarity coefficients between pairs of genotypes were 0.781, 0.787 and 0.764, respectively. Cheng et al. (2013) investigated the genetic relationships among 30 radishes, in which the genetic similarity coefficient ranged from 0.60 to 0.87. Based on the expressed sequence tag SSR analysis by Wang et al. (2015a), a phylogenetic tree was constructed for 93 radish germplasms with a similarity coefficient between 0.61 and 0.83. The genetic variation of the 37 accessions used in our study could be detected with SSR markers. The genetic similarity coefficients based on the SSR marker patterns of the 37 accessions ranged from 0.75 to 0.90, with an average of 0.84. This result indicated a narrow genetic base and a close relationship among the germplasms of radishes with different flesh colors. This finding was expected because most accessions are inbred lines and hybrids from the

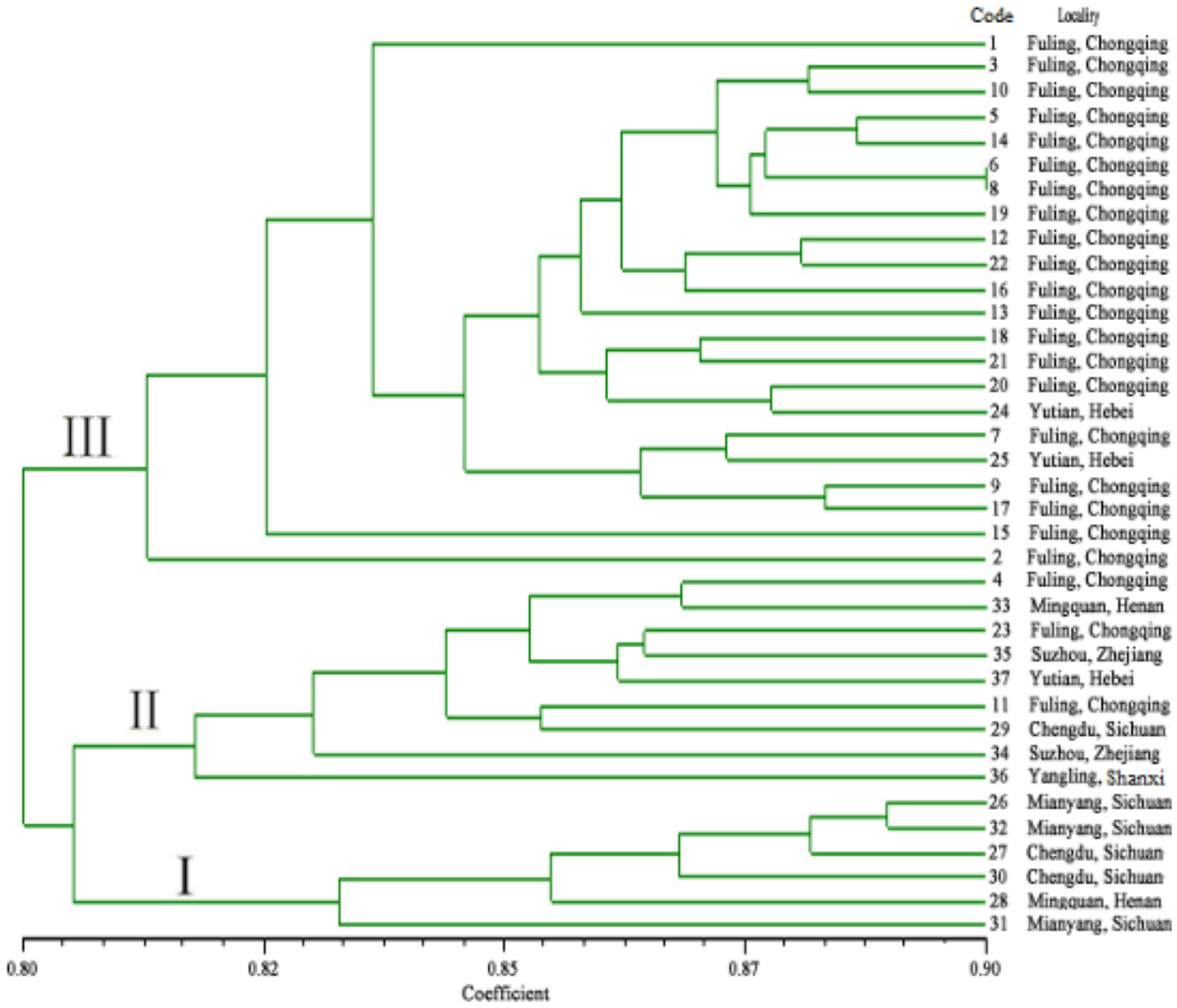

Figure 4. Dendrogram of 37 accessions constructed from SSR marker-based genetic similarities.

Fuling district of China and were selected mainly for the purpose of breeding. Accessions from adjacent areas were also selected, primarily for their well-developed, long, and fleshy roots for use as a vegetable. Consequently, artificial selection in radish breeding may have led to a low level of genetic diversity within the radish germplasms studied. Additionally, our findings represent important information regarding the genetic diversity of radishes with different flesh color, and can be used to support genetic resource management of red radish with red flesh.

Relationships between cluster results and radish flesh color

Chinese radishes have traditionally been classified into four groups according to root skin and flesh color (Jiang et al., 2012). Based on the effects of vernalization, cultivated radishes have been classified into four groups, 10 sub-groups, and 23 cultivars (Li et al., 1983). Using AFLP and RAPD markers, genetic diversity studies of radishes from Asia and Europe suggested the presence of abundant variation in radish germplasms, which could be clustered into four groups (Kong et al., 2004, 2005, 2011). Based on RAPD, inter-SSR, and SRAP marker data, Liu et al. (2008) clustered 35 radish cultivars into three major groups, which corresponded to their origins and main characteristics. Using target region amplification polymorphism markers, Cheng et al. (2013) clustered 30 radish genotypes into four groups, which were consistent with the groupings based on their resistance to turnip mosaic virus. With expressed sequence tag SSR markers, Jiang et al. (2012) grouped

32 radish accessions into three main clusters, which were mostly in agreement with the biological characterizations of the accessions. Additionally, Wang et al. (2015a) classified 93 radish germplasms into four groups. In this study, the results revealed a genetic distance between radishes with red flesh and white flesh and the accessions could be classified as red- and white-flesh radish varieties. However, some red- and white-flesh radishes could also be included in Cluster II. The results of our SSR analysis were consistent with those from previous studies that determined the molecular classifications are not fully in agreement with the traditional taxonomic classifications based on root skin and flesh color. The inconsistencies among morphological, karyomorphological, and molecular analyses are not surprising. High selection pressure during domestication may lead to accessions with similar genetic backgrounds evolving differently in terms of morphology. Natural hybridizations between radishes with similar genetic backgrounds occur frequently, resulting in intermediate or entirely new radish types. Furthermore, root skin and flesh color are controlled by multiple genes, which may result in genetically related radishes having different root skin and flesh color.

Conclusions

Red pigment content, karyotypes and SSR markers in 37 radish accessions with different flesh colors were analyzed. Our results indicated that red radish with red flesh contains abundant red pigment, with an average value of 15.62%, which makes it an ideal source of red pigment. Red-flesh radish with asymmetrical karyotypes may have evolved from white-flesh radish with symmetrical karyotypes. We confirmed the existence of a narrow genetic base and close relationship among germplasms of radishes with different flesh colors. Further study is needed to expand the available radish germplasms by creating new hybrids or introducing genes from other crops.

Conflict of Interests

The authors have not declared any conflict of interests.

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