



Analysis of genetic diversity of *Brassica rapa* var. *chinensis* using ISSR markers and development of SCAR marker specific for Fragrant Bok Choy, a product of geographic indication

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ABSTRACT. Non-heading Chinese cabbage [*Brassica rapa* var. *chinensis* (Linnaeus) Kitamura] is a popular vegetable and is also used as a medicinal plant in traditional Chinese medicine. Fragrant Bok Choy is a unique accession of non-heading Chinese cabbage and a product of geographic indication certified by the Ministry of Agriculture of China, which is noted for its rich aromatic flavor. However, transitional and overlapping morphological traits can make it difficult to distinguish this accession from other non-heading Chinese cabbages. This study aimed to develop a molecular method for efficient identification of Fragrant Bok Choy. Genetic diversity analysis, based on inter-simple sequence repeat molecular markers, was conducted for 11 non-heading Chinese cabbage accessions grown in the

Yangtze River Delta region. Genetic similarity coefficients between the 11 accessions ranged from 0.5455 to 0.8961, and the genetic distance ranged from 0.0755 to 0.4475. Cluster analysis divided the 11 accessions into two major groups. The primer ISSR-840 amplified a fragment specific for Fragrant Bok Choy. A pair of specific sequence-characterized amplified region (SCAR) primers based on this fragment amplified a target band in Fragrant Bok Choy individuals, but no band was detected in individuals of other accessions. In conclusion, this study has developed an efficient strategy for authentication of Fragrant Bok Choy. The SCAR marker described here will facilitate the conservation and utilization of this unique non-heading Chinese cabbage germplasm resource.

Key words: *Brassica rapa* var. *chinensis*, Fragrant Bok Choy; ISSR; SCAR; Genetic diversity

INTRODUCTION

Non-heading Chinese cabbage [*Brassica rapa* var. *chinensis* (Linnaeus) Kitamura] is an annual or biennial vegetable belonging to the family Brassicaceae. It was treated as an independent species and named *Brassica chinensis* L. in the Flora Reipublicae Popularis Sinicae (Editorial Board of the Flora of China, 1987), but was revised later as a variety of turnip, *Brassica rapa* Linnaeus, in the Flora of China (Wu and Raven, 2001).

Non-heading Chinese cabbage originated in China and is an important vegetable crop. It has a long history of cultivation and now includes numerous and complex germplasms. Recently, it has become globalized with gradual introduction and cultivation in Southeast Asia, Europe, and America (Hou, 2003). Historically, the identification of non-heading Chinese cabbage has been mainly based on morphological characteristics. For example, Cao and Li (1980, 1981, 1982) classified non-heading Chinese cabbage according to morphological traits, such as plant shape and stem and leaf characteristics, into the following six varieties: var. *communis* Tsen et Lee (var. *erecta* Mao); var. *rosularis* Tsen et Lee (var. *atrovirens* Mao); var. *tsaitai* Hort. (var. *purpurea* Mao); var. *tai-tsai* Hort.; var. *multiceps* Hort. (var. *nipponsinica* Hort.); and var. *utilis* Tsen et Lee (var. *oleifera* Makino). Liu et al. (1998) proposed that seven quantitative traits might serve as the basis for the identification of 14 accessions of non-heading Chinese cabbage, including plant height, extent of spread, number of leaves, and weight of individual plants. Cao and Cao (1994, 1995) used the morphology of leaves and the seed coat to identify non-heading Chinese cabbage varieties and also conducted a study on the genetic relationships based on chromosomal G-banding patterns (Cao et al., 1994). However, transitional and overlapping traits are present, which hamper identification based on morphological characteristics. For example, the seed husks of the accession Suzhouqing exhibit hole-shaped and long slit-shaped reticulate textures; the same types of reticulate texture have also been observed in two other accessions, Huangyacai and Xuekeqing (Cao et al., 1994). In addition, some morphological traits can be affected by external factors such as the growth period or environment and are not stable. For example, the leaves of Huangxinwu are round during the seedling stage, but become oval at the rosette stage (Cao and Cao, 1995). Therefore, it is virtually impossible to identify numerous varieties or accessions of non-heading Chinese cabbage solely from their morphological characteristics.

In recent years, molecular marker techniques have been developed and widely applied in the study of genetic relationships among plants, including identification of species, reconstruction of phylogenetic relationships, and gene mapping (Bornet and Branchard, 2004; Du et al., 2011; Shi et al., 2011; Moghaddam et al., 2012; Wu et al., 2012). Amplified fragment length polymorphisms (AFLPs) were used by Li et al. (2008) to analyze genetic diversity in 10 accessions of non-heading Chinese cabbage that had been grown in Zhejiang Province. They identified a specific molecular marker fingerprint to differentiate Youdonger, an accession cultured in Hangzhou City, from the other nine accessions. Han et al. (2007) used sequence-related amplified polymorphisms (SRAPs) for genetic diversity analysis of 64 non-heading Chinese cabbage accessions. The analysis showed that there was considerable variation within populations and that gene flow between populations was rare. Wang et al. (2008) analyzed 80 non-heading Chinese cabbage accessions and found that they could be distinguished using combinations of five simple sequence repeat (SSR) primers. Ma et al. (2012) analyzed 20 accessions of non-heading Chinese cabbage from China and Japan using SSR molecular markers. Their results suggested a relationship between genetic diversity and geographic area. Most of the studies described above focused on genetic diversity of germplasm; however, molecular markers, especially unique and specific markers for particular accessions, have not yet been developed.

Fragrant Bok Choy, a unique variety of non-heading Chinese cabbage, has a rich aromatic flavor. Fragrant Bok Choy from Wujiang in Jiangsu Province is certified as a product of geographic indication by the Ministry of Agriculture of China (ID: AGI2009-09-00190), which represents a product cultivated in a specific geographical location and possesses certain qualities. Fragrant Bok Choy has been cultivated in Wujiang region for more than 100 years. Although Fragrant Bok Choy has a distinctive flavor, which is often changed in response to the variation of the environments, it still displays high morphological similarities to other accessions of non-heading Chinese cabbage. Here, we describe the use of inter-simple sequence repeats (ISSRs) to authenticate 11 accessions of non-heading Chinese cabbage and to differentiate Fragrant Bok Choy at the molecular level.

MATERIAL AND METHODS

Plant materials

Fragrant Bok Choy and 10 other accessions of Chinese cabbage commonly cultivated in the middle and lower reaches of the Yangtze River were selected for the present study (Table 1). Mature and plump seeds of these accessions were planted in plug trays and routinely managed until the seeds grew into plants with more than five leaves.

Table 1. Accessions sampled in the present investigation.

Accession	Number of plants	Locality	Provenance
Heixinwu	10	Changzhou, Jiangsu	Hefei local accession
Zhongqibai	10	Changzhou, Jiangsu	Changzhou local accession
Suzhouqing	10	Suzhou, Jiangsu	Suzhou local accession
Wuyueman	10	Nantong, Jiangsu	Shanghai local accession
Gaogengbai	10	Xuyi, Jiangsu	Wuhu local accession
Aijiaohuang	10	Nanjing, Jiangsu	Nanjing local accession
Siyueman	10	Nantong, Jiangsu	Shanghai local accession
Huangxinwu	10	An'hui	Hefei local accession
Shanghaiqing	10	Shanghai	Shanghai local accession
Xiaobayee	10	Shanghai	Shanghai local accession
Fragrant Bok Choy	10	Suzhou, Jiangsu	Suzhou local accession

Extraction of total genomic DNA

Plants with good growth status were selected, and 100 mg young leaves were collected from healthy plants. The leaves were macerated using a tissue disrupter (TissueLyser LT, QIAGEN, Germany), and total DNA was isolated using the Easy Pure Plant Genomic DNA Kit (TransGen Biotechnology Co., Ltd., Beijing). Subsequently, the concentration and purity of the extracted DNA were examined using a nucleic acid and protein detector (Eppendorf, USA). After adjusting the DNA concentration to 20 ng/ μ L, the DNA samples were stored at -20°C for future use.

Selection of ISSR primers

DNA samples were mixed to construct DNA pools for each of the 11 accessions of Chinese cabbage. ISSR primers reported in the literature were selected for the present study (Song et al., 2012). The primers were synthesized by Nanjing Realgene Bio-Technologies, Inc. Polymerase chain reaction (PCR) assays were performed using the BioMetra T1 PCR thermocycler. The PCR components and their final concentrations were as follows: 1.0 μ L DNA template (20 ng/ μ L), 10.0 μ L 2X reaction mix (containing 20 mM Tris-HCl, 100 mM KCl, 3 mM MgCl₂, 400 μ M dNTPs, and bromophenol blue), 1.5 μ L primer (10 mM), 0.4 μ L Taq DNA polymerase (2.5 U/ μ L), and double-distilled water to achieve a final volume of 20 μ L. The following PCR program was adopted to amplify the ISSR molecular markers: pre-denaturation at 94°C for 7 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 45 s (the annealing temperature varied for different primers), and elongation at 72°C for 1 min; and a final elongation at 72°C for 10 min. The PCR amplification products were subjected to electrophoresis on a 3% agarose gel (at 80 V) and stained with ethidium bromide. After electrophoresis for 1.5 h, the PCR products were examined and imaged using a gel documentation system. The 2000-bp DNA ladder (Guangzhou Dongsheng Biological Technology Co., Ltd.) was used as a molecular weight marker.

Genetic relationship analysis

ISSR primers that produced sharp bands with abundant polymorphic profiles were selected from the 22 ISSR primers. The selected primers were used to amplify the DNA pools of the 11 accessions of Chinese cabbage. Bands exhibiting genetic variation were identified and counted, and bands with the same migration rate were treated as homologous loci. The amplification results were transformed into dichotomous data (the results were quantized to 1 if one or more bands were produced and 0 if no bands were amplified) for further analysis. Using the POPGENE 32 software (Yeh et al., 2000), Nei's gene diversity index (H), the effective number of alleles (N_E), Shannon's information index (I), the genetic differentiation coefficient (G_{ST}), and gene flow (N_m) were calculated. The NTSYS2.10 software (Rohlf, 2004) was used to estimate genetic similarity coefficients and genetic distances between samples. Cluster analysis was performed based on the matrix of genetic similarity coefficients using the unweighted pair-group method with arithmetic mean (UPGMA), and a dendrogram was plotted.

Cloning and sequencing of specific ISSR-sequence-characterized amplified region (SCAR) bands

The primers that amplified specific bands in Fragrant Bok Choy were selected. The

specific bands were cut out of the gel, and the DNA was recovered. The recovered DNA fragments were ligated into the pMD19-T vector. The ligation products were transformed into *E. coli* DH5 α competent cells. Single colonies were picked and subjected to colony PCR. The clones producing the correct bands were submitted to Nanjing Realgene Bio-Technologies, Inc. for DNA sequencing. Based on the results of DNA sequence analysis, a pair of specific primers was designed and used in the subsequent SCAR analysis.

Verification of the ISSR-SCAR markers

The accession specificity was individually verified in the 110 samples from the 11 Chinese cabbage accessions using the synthesized SCAR primers. The components of the PCR system and their final concentrations were as follows: 1.0 μ L DNA template (20 ng/ μ L), 10.0 μ L 2X reaction mix (containing 20 mM Tris-HCl, 100 mM KCl, 3 mM MgCl₂, 400 μ M dNTPs, and bromophenol blue), 0.8 μ L each primer (10 mM), 0.4 μ L Taq DNA polymerase (2.5 U/ μ L), and double-distilled water to a final volume of 20 μ L. The following amplification program was performed: pre-denaturation at 94°C for 5 min; 30 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 45 s, and elongation at 72°C for 1 min; and a final elongation at 72°C for 8 min. The PCR amplification products were subjected to electrophoresis on a 1% agarose gel (at 80 V) and stained with ethidium bromide. After 0.5 h of electrophoresis, the PCR products were examined and imaged using a gel imaging system. A 2000-bp DNA ladder was used as a molecular weight marker.

RESULTS

Analysis of the results of ISSR-PCR amplification

Nine ISSR primers that amplified sharp, highly polymorphic, and reproducible bands were selected from 22 candidate ISSR primers. The DNAs of the 11 accessions of Chinese cabbage were amplified using the nine ISSR primers, which produced a total of 77 distinguishable bands. On average, each primer produced 8.6 bands. Among the 77 bands, 57 were polymorphic. Each primer amplified an average of 6.3 polymorphic bands. The proportion of polymorphic bands was 74.0% (Table 2). Among the nine primers, ISSR-840 produced the most bands (12 bands), whereas ISSR-810 generated the fewest (only five bands). ISSR-811 and ISSR-890 produced the highest proportion of polymorphic bands (90.0%).

Table 2. ISSR primers used in the analysis of the 11 accessions of non-heading Chinese cabbage.

Name of primer	Primer sequence (5' to 3')	No. of amplified bands	No. of polymorphic bands	Proportion of polymorphic loci (%)
ISSR-810	GAGAGAGAGAGAGAT	5	4	80.0
ISSR-811	GAGAGAGAGAGAGAC	10	9	90.0
ISSR-840	GAGAGAGAGAGAGAYT	12	10	83.3
ISSR-856	ACACACACACACACYA	7	3	42.9
ISSR-857	ACACACACACACACYG	6	5	83.3
ISSR-887	DVDTCTCTCTCTCTC	8	7	87.5
ISSR-888	BDBCACACACACACA	8	5	62.5
ISSR-889	DBDACACACACACAC	11	5	45.5
ISSR-890	VHVGTTGTGTGTGTGT	10	9	90.0
Total	-	77	57	74.0
Average	-	8.6	6.3	-

Genetic distance and cluster analysis based on ISSR molecular markers

The results of the genetic diversity index analysis showed that the mean N_E in the 11 Chinese cabbage accessions was 1.4008. The value of H was 0.245, which fell between that of selfing plants ($H = 0.086$, $I = 0.131$) and that of outcrossing plants ($H = 0.178$, $I = 0.301$) (Zhang and Yang, 2008). The value of I was 0.3741. In addition, the value of G_{ST} among the 11 Chinese cabbage accessions was 1, indicating that the genetic variation of Chinese cabbage occurred entirely between the accessions. The value of N_m was 0. In angiosperms, the level of N_m is divided into three grades: high, N_m equal to or larger than 1.0; moderate, N_m ranging from 0.250 to 0.99; and low, N_m ranging from 0.00 to 0.249 (Slatkin, 1981, 1985). Our results indicated that virtually no gene flow occurred between the accessions of Chinese cabbage.

Genetic distances and genetic similarity coefficients were calculated: genetic distances ranged from 0.0755 to 0.4475; genetic similarity coefficients ranged between 0.5455 and 0.8961. Based on the genetic similarity coefficients, a dendrogram was established using the UPGMA clustering method to reveal the genetic relationships among the 11 accessions (Figure 1). This analysis identified two main branches: Siyueman and Huangxinwu formed one independent branch; the remaining nine accessions were clustered into the second branch. In the latter branch, Fragrant Bok Choy was clearly independent of the other eight accessions. In contrast, Shanghaiqing displayed an extremely close genetic relationship with Wuyueman, Suzhouqing, and Gaogengbai.

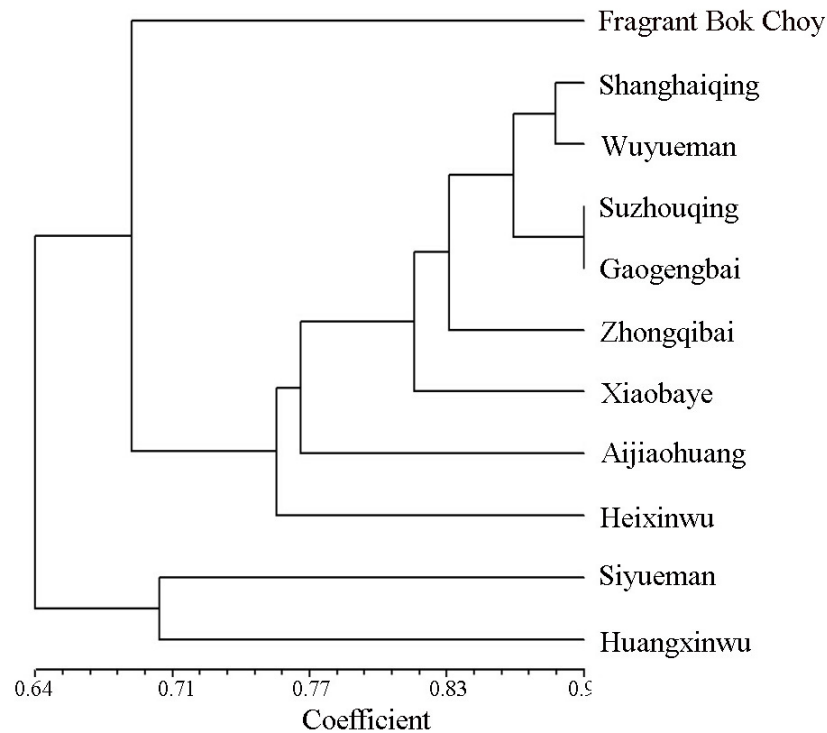


Figure 1. Dendrogram of 11 *Brassica rapa* var. *chinensis* accessions based on analysis using ISSR markers.

Screening for ISSR molecular markers related to the accession specificity of Fragrant Bok Choy

The DNA pools of the 11 accessions of Chinese cabbage were PCR amplified using 22 ISSR primers. After screening, a specific primer capable of identifying Fragrant Bok Choy, ISSR-840 (5'-GAGAGAGAGAGAGAGAYT-3') was obtained. ISSR-840 generated an amplification product of approximately 1500 bp only in Fragrant Bok Choy, indicating the presence of a single band unique to Fragrant Bok Choy (Figure 2).

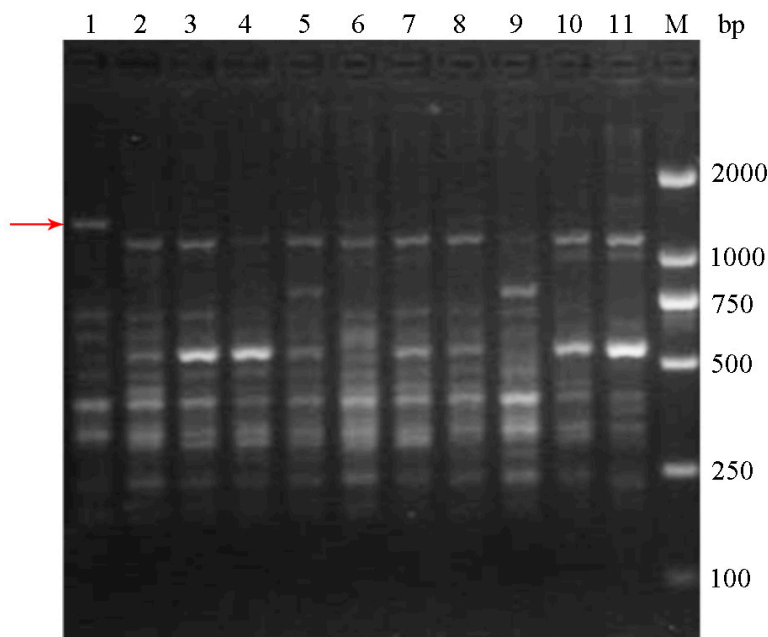


Figure 2. Agarose gel electrophoresis of the PCR products obtained using the primer ISSR-840 in 11 Chinese cabbage accessions. *Lanes 1-11* = Fragrant Bok Choy, Shanghaiqing, Xiaobaye, Heixinwu, Zhongqibai, Suzhouqing, Wuyueman, Gaogengbai, Aijiaohuang, Siyueman, and Huangxinwu, respectively. *Lane M* = DL2000 DNA marker; the red arrow indicates the specific band in Fragrant Bok Choy.

Sequencing and analysis of the Fragrant Bok Choy-specific fragment and design of the SCAR primers

The Fragrant Bok Choy-specific fragment amplified using primer ISSR-840 was cloned, transformed, and sequenced. A fragment of 1301 bp was isolated and its nucleotide sequence is shown in Figure 3. A pair of SCAR primers was designed (Figure 3) according to primer design principles: SCAR-F (5'-GAGAGATTGCTGAATAGTTACTG-3') and SCAR-R (5'-CTCCACGTGTTGTTGTTTAATTC-3'). The primers SCAR-F and SCAR-R corresponded to 11-33 and 1168-1191 nt, respectively, in the sequence of the Fragrant Bok Choy-specific fragment.

5'-GAGAGAGAGAGAGATGCTGAATAGTACTGTGTTGACAACATAGTCTTGAGACGAGTAGACTGAGTTT
TCTGCTGTGACCTCGTCTAAGGACGTCTCAGCTGGAGCAGACGATGAGGAGGCTCCATGACAGTAACCTCAA
TGTGCTGTGCGGAATCATCTCAGCCGGAACGCAGAAACCATTTTCATTATGCACCTCAGTAGATGGATTCGGC
GCTGCAGTAACCTGTTCCATCCAGACTGTTTCGATACTGTTTCTGTTACAGTTGCAGTCTCAATATCTTGAA
GCAGAGTCATCGAATGATATTTCTCAAACCTGGTGATATTTCTGGTGTGACAGAGACTCTTCCATTAGACTGATT
AGCGAATAGAAATGATTCTCACTAAAGACAACATCTCGAGAATGGAAGATGACTCTTTTCCAAAGTCATAACAC
AGCCAGCCCTCCTCTAGAAGGATAGCCTAAGAAGACACAACGCCGCTCTTAGGCGCAAATTTATCACCGACA
TGATTAATATTGTGAGCATAGGCGAGACGCAAACTCGTAGATGCCGAACCTGAGGCTCACGACCATATAATTT
TTCGAATGGAGTTTACCATCCGATACTGTGAAGGTGCCGTTTATAAGATATACGGCTGCTTTAACACATTCAC
CCAAACTCAATAGGAAGATGCGCTTGAAATCGCAGCGCCTTGCCACATTAATATAAGTATGTCTTTCGCTCC
ACCGCTGTGTTGTTGCGGTGTAAACACACAGAGTTTCATGATGGATACCATTCCAGAAAGAAACGGGTG
AGACAAACAAAATCCGTACCGTATCACTCTTATTGTCTTTATTTCTTAAAACTGTGTCTGCACAAGCGCCACA
AAATCTCATATAGCGGTGAGTTTATCTGGTAAAGATAAACCCAACTGCTCGTGTAGTGTCTCAATCGTC
AAAAAATATCGTACCACAGAAAGTGTGTACGATATGGACCCCAATACACAGTGAACATAAATCAAAAATGTC
ATCAACTTATGGAACTATCCGGGAAAGTTGGCGAGTTTGTTCGCACGAAAGCACACATCGCAGCTTTAAA
AAACACTATTACTAACCAGGAATTAACCAACACACGTTGGAGATGGGTGACCCAAACGGTTATGCCACA
AAACTGAGTCTCACGAACCTCGTATGATGATGTTACTTCTGAATCCACATAACCGGTAGAGTCTCTCTCT
CTCTC-3'

Figure 3. Nucleotide sequence of the Fragrant Bok Choy-specific fragment amplified using primer ISSR-840. The underlined sequences were used to design SCAR primers.

Verification of the ISSR-SCAR marker for Fragrant Bok Choy

The SCAR primers were verified using DNA from all 110 plants of the 11 Chinese cabbage accessions. A clear target band of 1181 bp was only amplified in the 10 Fragrant Bok Choy plants but not from plants of other accessions (Figure 4).

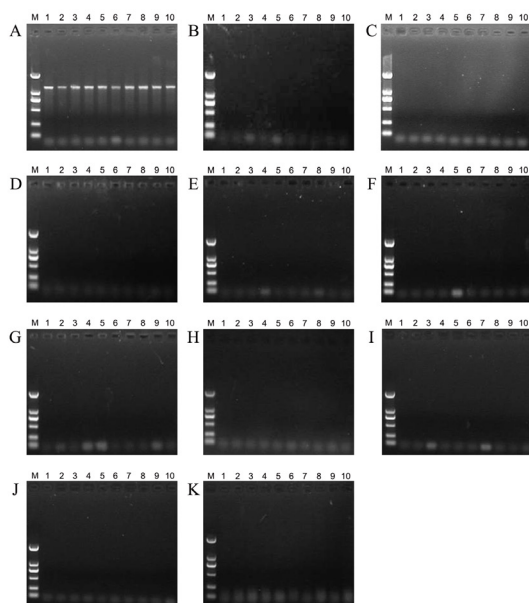


Figure 4. PCR verification of the Fragrant Bok Choy-specific ISSR-SCAR. Panels A to K show the results of PCR in the ten plants of each accession from Fragrant Bok Choy, Shanghaiqing, Xiaobaye, Heixinwu, Zhongqibai, Suzhouqing, Wuyueman, Gaogengbai, Aijiaohuang, Siyueman, and Huangxinwu, respectively. Lane M = DL2000 DNA marker. Lanes 1-10 = PCR products from each plant.

DISCUSSION

Genetic relationships among the 11 accessions of Chinese cabbage based on ISSR markers

The 11 Chinese cabbage accessions examined in the present study were all farm-raised. Domestication of the Chinese cabbage has involved strong artificial selection and the 11 accessions have limited regions that are suitable for growth. Both of these factors block efficient genetic exchange among the accessions. Therefore, genetic differentiation among Chinese cabbage accessions is extensive. Horticultural classification separates the 11 accessions into the var. *communis* group (which includes Shanghaiqing, Wuyueman, Gaogengbai, Suzhouqing, Zhongqibai, Aijiaohuang, and Siyueman) and the var. *tai-tsai* group (which includes Huangxinwu, Heixinwu, and Xiaobaye). The var. *tai-tsai* group has dark green leaves, and the plants have a prostrate or semi-prostrate morphology. Although Fragrant Bok Choy belongs to the var. *communis* group, the plants display a semi-prostrate morphology.

The analyses here showed that Fragrant Bok Choy was genetically independent of the other Chinese cabbage accessions. Similar results have been obtained from genetic diversity studies using SRAP markers (Han et al., 2007) and inter-SINE amplified polymorphism (ISAP) markers (Zhang et al., unpublished data). The three accessions, Shanghaiqing, Suzhouqing, and Wuyueman, which are all cultivated in Suzhou or Shanghai, exhibited a very close genetic relationship. Similar results were obtained using SSR (Wang et al., 2008; Ma et al., 2012), ISAP (Zhang et al., unpublished data), and SRAP markers (Han et al., 2007), indicating that the ancestors of the three accessions had similar genetic backgrounds. In the var. *tai-tsai* group, Huangxinwu displays a fully prostrate morphology, whereas Heixinwu and Xiaobaye have a semi-prostrate morphology. The present study indicates that Heixinwu and Xiaobaye are genetically closer to the var. *communis* group. However, in the dendrogram, Heixinwu and Xiaobaye were located in the outermost part of their branch. In addition, genetic relationships were established between these two accessions and Huangxinwu through Fragrant Bok Choy. The dendrogram analysis indicated that Heixinwu, Heixinwu, and Xiaobaye all had close phylogenetic relationships with Fragrant Bok Choy. The prostrate morphology of Chinese cabbage demonstrates that there is a significant correlation between this trait and the genetic relationship to the ancestor of Chinese cabbage. Huangxinwu displays a fully prostrate morphology, indicating that it is most phylogenetically distant from the other accessions. In addition, our results demonstrated a closer phylogenetic relationship between the ancestor of Fragrant Bok Choy and the ancestors of the var. *tai-tsai* group of Chinese cabbage.

Aijiaohuang and Wuyueman are two special accessions of Chinese cabbage. Aijiaohuang is a local accession from Nanjing and is the smallest of all the accessions of Chinese cabbage. Our analyses indicated that Aijiaohuang has a close phylogenetic relationship with Huangxinwu, Heixinwu, Xiaobaye, and Fragrant Bok Choy. The results further demonstrated that the prostrate trait of Chinese cabbage was present in ancestral species. SRAP (Han et al., 2007) and ISAP analyses have demonstrated similar results. Siyueman, an accession cultivated in Shanghai, is expected to have a close phylogenetic relationship to Wuyueman, an accession developed through selective breeding of Siyueman. However, studies using different molecular markers have demonstrated a variety of genetic relationships between Siyueman and other accessions of Chinese cabbage. SSR (Ma et al., 2012) and ISAP analyses also suggest a distant genetic relationship between Siyueman and Wuyueman.

ISSR-SCAR markers associated with Fragrant Bok Choy

The SCAR marker developed here was based on ISSR markers and was capable of uniquely identifying Fragrant Bok Choy with an efficiency of 100%. The PCR-based identification technique possesses a number of advantages over the traditional morphological methods and non-specific molecular markers, including high accuracy, high reproducibility, good stability, and good reliability. Fragrant Bok Choy is an agricultural product that is registered and protected by the Geographical Indications of Agricultural Products of China. The development of molecular markers for the specific identification of Fragrant Bok Choy provides a theoretical basis for identifying other agricultural products of geographical indications.

Conflicts of interest

The authors declare no conflict of interest.

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