Genetic evolution of *Brassica* crops revealed with srap markers

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Abstract

To investigate the genetic evolution of *Brassica* crops, 16 accessions from 5 *Brassica* crops were analyzed by use of sequence-related amplified polymorphism (SRAP). In total 509 bands, there were 118 polymorphic bands. And the frequency of polymorphic bands was 23.18%. Twenty-eight specific bands were obtained and accounted for 5.50%. Eight specific bands were from A genome, it suggested that A genome had remote relationship with other *Brassica* genomes. There was one absent band in C genome. *B. napus*, *B. juncea* and *B. carinata* had 4, 1 and 2 specific bands, respectively. There were also some specific bands shared by two kinds of *Brassica* crops. The order of genetic distances (GD) among *Brassica* crops was as followings: $GD_{BBCC\text{-}AABB} > GD_{CC\text{-}AABB} > GD_{AA\text{-}CC} > GD_{AA\text{-}BBCC} = GD_{BBCC\text{-}AACC} > GD_{CC\text{-}BBCC} > GD_{AACC\text{-}AABB} > GD_{CC\text{-}AACC} > GD_{AA\text{-}AACC} > GD_{AA\text{-}AABB}$. Results demonstrated that A genome, B genome and C genome did not equally contribute to allotetraploid genomes. In the phylogenetic tree, 16 accessions were clearly clustered into *B. napus*, *B. oleracea*, *B. juncea* and *B. rapa* four groups, respectively. *B. napus* had closer relationship with *B. oleracea*. *B. juncea* had closer relationship with *B. rapa*. *B. napus* had relatively more remote relationship with *B. rapa. B. carinata* had the most remote relationship with other *Brassica* crops. The order of evolutionary time of *Brassica* crops was as followings: *B. carinata*>*B. campestris* >*B. oleracea* > *B. juncea* > *B. napus*.

Key words: *Brassica* crop, genome, genetic evolution, SRAP markers

Brassica are commercially important oilseed crops and horticultural crops worldwide. Based on the cytogenetic study, U. Nagahara [1] classified *Brassica* crops into three basic species (*B. rapa*, AA, 2n=20; *B. nigra*, BB, 2n=16; *B. oleracea*, CC, 2n=18) and three complex species (*B. juncea*, AABB, 2n=36; *B. napus*, AACC, 2n=38; *B. carinata*, BBCC, 2n=34) and founded the famous U-Triangle hypothesis to explain the relationship of the six *Brassica* species. Many scientists have confirmed U-Triangle hypothesis and the genetic relationships among A, B, and C genomes has been further studied. Röbbelen et al [2] pointed out that A, B and C genome originated from the same ancestor with the basic number six chromosomes according to the chromosomal shapes during meiosis. When Attia et al [3] studied the pairing of chromosome A, B and C, they found that the pairing of A and C was much easier than that of A to B and B to C. Therefore they drew conclusion that the relationship between A genome and C genome was closer than that between A genomeand B genome and that between B genome and C genome. After Lagercrantz et al [4] studied the evolution of *Brassica* and *Arabidopsis Thaliana* with RFLP, they found that *B. nigra* differentiated from *B. rapa* and *B. oleracea* before 20 million years ago, *Arabidopsis Thaliana* differentiated from *B. nigra* before 3.5 million years ago and *B. rapa* differentiated from *B. oleracea* before one million years ago.

Most of previous studies were limited in the research of A, B and C genomes with cytological methods. At present, the major *Brassica* vegetables in production include Chinese cabbage (*B. campestris* ssp. Pekinensis, AA, 2n=20), *B. campestris* ssp. Chinensis (AA, 2n=20), *B. oleracea* (CC, 2n=18), *B. juncea* var tumida (AABB, 2n=36) and so on. The main *Brassica* oilseed crops in production are *B. rapa*, *B. napus* and *B. juncea*. Some researchers have studied the origins and evolution of *Brassica* crops. Liu [5] pointed out that *Brassica* crops had multiple origins. Having studied the origins and evolution of *B. rapa* in China, He et al [6] confirmed that *B. campestris* var. oleifera originated earlier than *B. chinensis* var. oleifera and winter type *B. campestris* var. oleifera originated earlier than spring type *B.campestris* var. oleifera. However, there were no reports about genetic evolution of *Brassica* crops revealed with molecular markers by now. SRAP(sequence-related amplified polymorphism)has been used successfully in *B. oleracea, Arabidopsis thaliana*, *Prunus persica L, Buchloe datyloides, Cucurbita pepo and Cucurbita maxima* [7-11]. After Budak et al[12] compared SRAP with ISSR、SSR and RAPD in buffalo grass, they pointed out that SRAP was simple, polymorphic and the information revealed by SRAP was close to agronomic trait differences and historic evolutionary results. Five kinds of *Brassica* crops, such as *B. rapa, B. napus, B. oleracea, B. juncea* and *B. crinata*, are grown worldwide. The objectives of this study are to reveal genetic evolutionary relationship of *Brassica* crops with SRAP markers and to supply references for the genetics and breeding of *Brassica* crops.

1 Materials and methods

1.1 Plant materials Four *B. rapa* accessions, two *B. oleracea* accessions, six *B. napus* accessions, three *B. juncea* accessions and one *B. carinata* accession were used in this study. All of these accessions were obtained from Institute of Oil

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Crops, Chinese Academy of Agricultural Sciences (Table 1).

Table 1 Accessions used in this study

Code	Accession	Species	Chromosome	Code	Accession	Species	Chromosome
1	Chunan native	*B. rapa*	AA	9	Shengli rapeseed	*B. napus*	AACC
2	Haiyan yellow seed	*B. rapa*	AA	10	Yuejin rapeseed	*B. napus*	AACC
3	Hexian colourful seed	*B. rapa*	AA	11	Huashuang No.4	*B. napus*	AACC
4	Suzhou white flower	*B. rapa*	AA	12	Zhongyou 821	*B. napus*	AACC
5	90017	*B. oleracea*	CC	13	Mianyang juncea	*B. juncea*	AABB
6	90059	*B. oleracea*	CC	14	Tayou 2	*B. juncea*	AABB
7	Oro	*B. napus*	AACC	15	Zangyou 9	*B. juncea*	AABB
8	Bronowski	*B. napus*	AACC	16	1B16-4	*B. cainata*	BBCC

1.2 Genomic DNA extraction All the accessions were grown in experimental field. At seedling stage, fresh leaves from ten plants of each accession were collected and stored in -20ºC. Total genomic DNA was extracted according to SDS method described by Li et al [13]. .

1.3 SRAP marker The SRAP primers were designed as described by Li et al [8] and synthesized by Shanghai Sangon Biological Technology Co. L td. The primer combinations used in this study were shown in Table 2. The primer combinations were prescreened on four different *B. napus* cultivars and those primers were excluded from this study if their banding patterns were difficult to score or if they failed to amplify consistently in all accessions. PCR reaction mixture (total volume 10 μ l) consisted of 1.0μl 10×PCR Buffer, 0.8μl $MgCl_2$ (25mM), 0.2μl dNTPs (10mM), 0.1μl *Taq* (5U/μl) polymerase (MBI), 1μl (50ng/μl) forward primer, 1μl (50ng/μl) reverse primer, 1μl (50ng/μl) DNA plate, and 4.9μl ddH_2O. The PCR reaction program was as followings: Firstly 1min at 95°C; then 5 cycles of 1min at 94ºC, 1 min at 35ºC and 1 min at 72ºC; followed by 35 cycles of 1 min at 94ºC, 1 min at 55ºC and 1 min at 72ºC. PCR reaction was carried out on Gene Amp9700 thermocycler.

Table 2 Sequences (5′-3′) of SRAP forward primers, reverse primers and primer combinations

Forward primer	Forward primer	Reverse primer	Primer combination
Me1TGA GTCCAA ACCGGATA	Me13AGCGAGCAAGCCGGTGG	Em1 GAC TGCGTACGAATTATT	Em1-Me7, Em1-Me9
Me2TGAGTCCAA ACCGGAGC	Me14 GAGCGTCGAACCGGATG	Em2 GAC TGCGTACGAATT TGC	Em1-Me10, Em1-Me12
Me3 TGAGTCCAAACCGGATG	Me15 CAAATGTGAACCGGATA	Em3 GACTGCGTACGAATTGAC	Em1-Me1, Em1-Me15
Me4 TGAGTCCAAACCGGACA	Me16 GAGTATCAACCCGGATT	Em4 GACTGCGTACGAATT TGA	Em1-Me18, Em2-Me6
Me5 TGAGTCCAAACCGGGAT	Me17 GTACATAGAACCGGAGT	Em5 GACTGCGTACGAATTAAC	Em2-Me22, Em2-Me23
Me6 TGAGTCCAAACCGGGCT	Me18 TACGACGAATCCGGACT	Em6 GACTGCGTACGAATTGCA	Em3-Me4, Em3-Me6
Me7 TGAGTCCAAACCGGTAA	Me19 CACAGTCATGCCGGAAT	Em7 GACTGCGTACGAATTATG	Em3-Me7, Em3-Me14
Me8 TGAGTCCAAACCGGTGC	Me20 GACCAGTAA ACCGGATG	Em8 GACTGCGTACGAATTCTG	Em4-Me4, Em4-Me9
Me9 TTCAGGGTGGCCGGATG	Me21 CAGGACTAAACCGGATA	Em9 AGGCGGTTGTCAATTGAC	Em4-Me1, Em4-Me15
Me10TGGGGACAACCCGGCT	Me22 ATCAGTCGGACCGGATT	Em10 TGTGGTCCGCAAATTTAG	Em5-Me5, Em5-Me13
Me11CTGGCGAACTCCGGATG	Me23 GATTGCATCACCGGATG		Em5-Me16, Em6-Me1
Me12GGTGAACGCTCCGGAAG	Me24 CTTACTTAGACCGGAGT		Em6-Me12, Em8-Me17 Em10-Me19
			Em10-Me19

1.4 Electrophoresis The PCR products were fractionated on 6% polyacrylamide gels. Gels were 0.75 mm in thickness and 32 × 37 cm in dimension. Electrophoresis were carried out at 1500 V for 80 min. Fixing, silver staining and developing were as described by Lu G-Y et al [14].

1.5 Analysis of phylogenetic trees Presence or absence of SRAP fragments was coded as "1" or "0", respectively. Genetic similarity (GS) and genetic distance (GD) were calculated according to the method of Nei and Li[15] as following formula: $GS=2\times X_{12}/(X_1+X_2)$, GD = -ln (GS). X_{12} is the band number shared by both of the compared accessions. X_1 and X_2 are the band numbers of each accession of the two compared accessions, respectively. According to the bands revealed with SRAP markers, genetic distances between accessions were computed and generated two genetic distance matrixes. Phylogenetic tree was constructed with software MEGA 2.1 [16] based on the above genetic distance data. When MEGA software was used, evolution ratio was given as "1" and then calculated the genetic evolutionary time of every accession. Other data analysis was performed with SAS or Excel.

2 Results and Analysis

2.1 Polymorphic bands in *Brassica* After genomic DAN of 16 *Brassica* accessions were amplified with 26 SRAP primer combinations, total 509 bands with 118 polymorphic bands generated and polymorphic bands accounted for 23.18%. Meanwhile, 28 specific bands in some *Brassica* crops were obtained. And the specific bands accounted for 5.50%. There were 8 specific bands in A genome and one absent band both in C genome and *B. oleracea*(CC). *B. napus* had 4 specific bands and *B. juncea* had one specific band. There were two specific bands in *B. carinata*. *B. oleracea* and *B. napus* shared 7 specific bands, *B. rapa* and *B. napus* shared one specific band, *B. oleracea* and *B. carinata* shared one specific band. *B. napus* and *B. carinata* shared one specific band. *B. juncea* and *B. napus* shared one specific band (Table 3). Since A genome had the most specific bands, it suggested that A genome had more remote relationship with other *Brassica* genomes.

Table 3 Specific bands of *Brassica* species

Brassica species	No. of specific bands	Primer combinations producing specific bands
A genome	8	Em1-Me7, Em1-Me10, Em4-Me15, Em1-Me14, Em1-Me18, Em3-Me14, Em2-Me22
CC had no band	1	Em3-Me7
C genome had no band	1	Em3-Me4
AACC	4	Em1-Me9, Em1-Me12, Em4-Me14
AABB	1	Em1-Me7
BBCC had no band	2	Em1-Me12, Em2-Me23
CC and AACC	7	Em4-Me14, Em2-Me23, Em3-Me4, Em2-Me23, Em10-Me19
AA and AACC	1	Em2-Me23
CC and BBCC	1	Em1-Me10
AACC and BBCC	1	Em4-Me14
AABB and AACC	1	Em1-Me9

2.2 Genetic distance among *Brassica* species As shown in Table 4, the order of genetic distances among *Brassica* species was as followings: $GD_{BBCC-AABB} > GD_{CC-AABB} > GD_{AA-CC} > GD_{AA-BBCC} = GD_{BBCC-AACC} > GD_{CC-BBCC} > GD_{AACC-AABB} > GD_{CC-AACC} > GD_{AA-AACC} > GD_{AA-AABB}$. Since $GD_{CC-BBCC}$ (1.0120) was much larger than $GD_{CC-AACC}$ (0.5659), it indicated that the influence of B genome to C genome in *B. carinata* was much larger than that of A genome to C genome in *B. napus*. The genetic distance (0.5659) of *B. oleracea* to *B. napus* was slightly larger than that (0.5478) of *B. rapa* to *B. napus* showed that the influence of A genome to C genome in *B. napus* was slightly higher than C genome to A genome. Since the genetic distance (0.3390) between AA and *B. juncea* was the smallest, it showed that influence of B genome to A genome was the weakest in *B. juncea*.

Because genetic distance (1.1103) between *B. carinata* and *B. juncea* was larger than that (0.6419) between *B. napus* and *B. juncea*, it suggested that the influence of B genome to C genome was larger than the influence of A genome to C genome. Since genetic distance (1.0199) between BBCC and AACC was larger than that (0.6419) between *B. napus* and *B. juncea*, it indicated that the influence of C genome to B genome was much larger than that of A genome to B genome. Therefore when *Brassica* allotetraploids formed by *Brassica* haploids, A, B and C genome did not equally contribute to the genomes of allotetraploids.

2.3 The evolution of *Brassica* species Based on the genetic distances of *Brassica* accessions revealed by SRAP, phylogenetic tree was constructed with MEGA2.1 software (Fig.1). 16 accessions were clearly clustered into four groups, respectively. Six *B. napus* accessions (Oro, Bronowski, Shengli rapeseed, Yuejin rapeseed, Huashuang No. 4 and Zhongyou 821) were classified into group I, two *B. oleracea* accessions (90017 and 90059) were in group II, three *B. juncea* accessions (Mianyang juncea, Tayou 2 and Zangyou 9) were in group III and four *B. rapa* accessions (Chunan native, Haiyan yellow seed, Hexian colourful seed and Suzhou white flower) were in group IV. *B. carinata* accession 1B16-4 was not included into any group (Fig.1). In these evolutionary groups, *B. napus* (group I) had closer relationship with *B. oleracea* (group II), *B. juncea* (group III) had closer relationship with *B. rapa* (group IV). *B. napus* had relatively more remote relationship with *B.rapa*. *B. carinata* had the most remote relationship with all other *Brassica* crops.

Table 4 Genetic distance among *Brassica* species

Genetic Distance	Minimum GD*	Maximum GD	Mean GD
Between BBCC and AABB	0.9287	1.3157	1.1103
Between CC and AABB	0.8792	1.3166	1.0725
Between AA and CC	0.7885	1.2716	1.0692
Between AA and BBCC	0.8267	1.1486	1.0199
Between BBCC and AACC	1.1486	0.8267	1.0199
Between CC and BBCC	1.0055	1.0186	1.0120
Between AACC and AABB	0.5015	0.7528	0.6419
Between CC and AACC	0.4643	0.7340	0.5659
Between AA and AACC	0.3992	0.7230	0.5478
Between AA and AABB	0.2513	0.4308	0.3390

*Note: GD=genetic distance

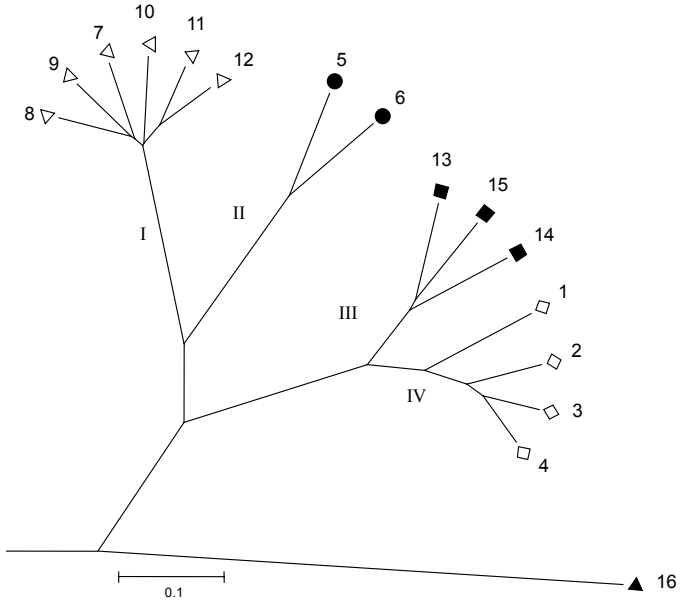

Fig.1 Phylogenetic tree of *Brassica* accessions constructed by SRAP markers(accession codes are the same as those in Table 1)

□:B. rapa ●: B. oleracea Δ:B. napus ■: B. juncea ▲: B. carinata

2.4 The evolutionary time of *Brassica* species Relative evolutionary time of *Brassica* species were calculated according to genetic distances among different *Brassica* species (Table 5). The maximum evolutionary time was the most suitable for identifying evolutionary history of *Brassica* species. Based on maximum evolutionary time, *B. carinata* had the longest evolutionary time, the second was *B. rapa*, *B. oleracea* was the third, and *B. juncea* was the forth. *B. napus* had the shortest evolutionary time. It suggested that *B. oleracea* was later than *B. rapa* in *Brassica* species evolutionary history. *B. napus* was later both than *B. rapa* and *B. oleracea*. *B. juncea* and *B. oleracea* had almost the same evolutionary time. Since *B. carinata* had the longest evolutionary time, it showed that *B. carinata* perhaps was relatively more ancient *Brassica* species.

3. Discussion

Brassica crops are commercially important oilseed crops and horticultural crops. It is necessary for researchers and breeders to investigate the genetic evolutionary relationship of *Brassica* species. In the past, scientists [2, 3, 17, 18] studied the relationships of *Brassica* species via cytological methods and judged their relationships by chromosome pairing. These experiments were qualitative analysis. In recent years, comparative genomics of *Brassica* crops developed fast. McGrath et al [19], Lydiate et al [20] and Cheung et al [21] studied *Brassica* genomes by molecular markers and found that different *Brassica* genomes were conservative. All these previous studies focused on the similarity of *Brassica* genomes. There were a few studies which focused on genetic evolution of *Brassica* genomes by quantitative analysis.

Table 5 Genetic evolutionary time of *Brassica* species

Species	Minimum ET*	Maximum ET	Mean ET
B. rapa	0.0543	0.1146	0.0740
B. oleracea	0.1038	0.1038	0.1038
B. napus	0.0596	0.0829	0.0697
B. juncea	0.0931	0.1045	0.0969
B. carinata	0.4981	0.4981	0.4981

*Note: ET = evolutionary time

In this study, a novel DNA marker system SRAP was used in the genetic evolution analysis of *Brassica* crops. The results demonstrated that SRAP was simple, polymorphic and effective marker system. It could completely separate different *Brassica* crops. 26 SRAP primer combinations generated 28 specific bands, which could distinguish *Brassica* crops from each other. These specific bands can be used to determine if the introgression of some *Brassica* genes occurs in inter-specific hybridizations. Genetic distances among *Brassica* crops varied. When *Brassica* allotetraploids formed by *Brassica* haploids, A, B and C genome did not equally contribute to the genomes of allotetraploids. In the evolutionary phylogenetic tree, *B. napus* had closer relationship with *B. oleracea*, *B. juncea* had closer relationship with *B. rapa*. *B. napus* had relatively more remote relationship with *B.rapa*. *B. carinata* had the most remote relationship with all other *Brassica* crops. These results were accordant with the order of maximum evolutionary times of *Brassica* species. These results will be available for genetics and breeding of *Brassica* crops. In this study, the *Brassica* species only included those *Brassica* crops that were used widely in agricultural production, which was the defect in this study. The materials in this study only included one *B. carinata* accession

and lacked of *B. nigra* accession because they were not used in production. In order to supply complete genetic evolutionary information of *Brassica* species, all three basic species and three complex species should be included in the further study.

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